Clinical Haemopoietic Implications Of Fucoidan Treatment

By

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DECLARATION

This is to certify that this thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information with due acknowledgment in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis.

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"هَذَا خَلْقُ اللَّه
فَأَرُونِي مَاذَا خَلَقَ الَّذِينَ مِن دُونِه
بَلِ الظَّالِمُونَ فِي ضَلَالٍ مُّبِينٍ"
لُقمان (11)

(This is the creation of Allah.
Now show me that which those (ye worship)
beside Him have created.
Nay, but the wrong doers are in error manifest)
[The Holy Quran 31:11]

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This dissertation is the culmination of an expedition that began in 1997 when I decided to start my Masters degree in the field of Haematology in Jordan. During that period I worked on the haemorheology of red blood cells. I used different natural preparations to protect the cell proteins and lipids from the oxidation processes caused by the free radical generating systems that I used. This work inspired me and gave me ideas towards the current work. Through my doctoral journey that started in September 2003, several people supported my efforts and championed my endeavour to investigate this new field.

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ABSTRACT

Haematopoiesis is a term that describes the formation of mature cellular blood components from haemopoietic progenitor stem cells (HPC). The majority of HPC reside in the bone marrow (BM) with a small number continually escaping into the circulation then re-homing back into the BM in a process called trafficking. Stromal cells in the BM constitutively express and secrete stromal cell derived factor (SDF-1). This highly conserved chemokine binds to heparin and to CXCR4 receptor acting as a chemo-attractant for $CXCR4^+$ cells; the system plays a role in regulating stem cell trafficking.

This study examined the clinical effects of ingesting fucoidan extracted from brown macro-algae (*Undaria pinnatifida*) *in vitro* and *in vivo* in a series of single blinded placebo-controlled clinical trials. Fucoidans comprise sulphated long branched chains of sugar, containing large amounts of fucose and galactose. Fucoidan is biologically active and is known to modulate coagulation, inflammation, cell proliferation and adhesion, tumorigenesis and resistance to viral infection. To study its clinical value an ELISA assay based on a novel antibody was established to quantify the level of the bio-available fucoidan in human plasma after oral doses. The consequences of ingesting fucoidan on healthy volunteers were investigated in detail by studying different biological and pathological parameters including liver and kidney functions.

This study established that daily ingestion of 3 g of different fucoidan extracts for 2 weeks is safe. To study the anticoagulant activity of fucoidan, haemostasis was examined closely *in vitro* and *in vivo*. Although, fucoidan is a highly potent anticoagulant *in vitro* there was limited activity when used orally. Fucoidan was found also to positively regulate the lipid profile by reducing cholesterol and triglyceride plasma levels. The effect of fucoidan on HSPC trafficking was tested. Ingestion of fucoidan increased the expression of CXCR4 on $CD34^+$ cells and increased the plasma level of SDF-1 and IFN-γ. A decrease in $CD4^+$ and $CD8^+$ cells was also observed in volunteers who ingested fucoidan. When either peripheral blood or cord blood $CD34^+$ cells were cultured *in vitro* in a cytokine expansion system CXCR4 on $CD34^+$ cells

was down-regulated. This study also showed that fucoidan slows down the $CD34^+$ cell cycle and interacts and binds with different cytokines (SCF, TPO, Flt-3 and SDF-1) and presents them to cells. In conclusion, this study showed that fucoidan has several clinical effects including effects on lipids regulation, haemostasis, immune system, haematopoiesis, trafficking of HSPC and related cytokines.

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PUBLICATIONS

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1. **Irhimeh MR**, Ko K, Fitton JH, Lowenthal RM, Nordon RE. Fucoidan down regulates the expression of CXCR4 *in vitro* on human hematopoietic $CD34^+$ cells. The 48^{th} American Society of Hematology Annual Meeting and Exposition, Orlando, FL USA. Abstract number 3384. *Blood* 2006; 108(11):966a.
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The following abbreviations have been used throughout this thesis:

°C	Degree Celsius
µg	Microgram
µL	Microlitres
1B1	A new novel MoAb (IgM isotype)
Ab	Antibody
AGAL	Australian Government Analytical Laboratories
ALB	Albumin
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AML	Acute myelogenous leukaemia
AP	Alkaline phosphatase
Baso	Basophil
BFU-E	Erythrocyte burst forming unit
BM	Bone marrow
BSA	Bovine serum albumin
BUN/Urea	Blood urea nitrogen
CB	Cord blood
CD	Cluster of differentiation antigen
CFCs	Colony forming cells
CFSE	5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester
CFU	Colony forming unit
CFU-GM	Colony forming unit-granulocyte-macrophage
Chol	Cholesterol
Cl^-	Chloride
CO_2	Carbon dioxide
Creat	Creatinine
CTCE-0021	SDF-1 peptide agonist
CY	Cyclophosphamide
DMB	1,9-dimethylmethylene blue
DMSO	Dimethyl sulfoxide
D-PBS	Dulbecco's phosphate buffered saline
ECM	Extracellular matrix
EDTA	Disodium ethylenediamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
Eos	Eosinophil
ESR	Erythrocyte sedimentation rate
FACS	Fluorescent activated cell sorting
FBC	Full blood count
FBS, FCS	Foetal bovine serum, foetal calf serum
FDA	Food and Drug Administration
FGF-1	Fibroblast growth factor-1
FITC	Fluorescein isothiocyanate

Flt-3	FMS-like tyrosine kinase-3
FSC	Forward scatter
g	Gram
G-CSF	Granulocyte colony stimulating factor
GF	Growth factor
GFS™	Galactofucan sulphate
GGT	Gamma-glutamyl transferase
GM-CSF	Granulocyte-macrophage colony stimulating factor
GvHD	Graft versus host disease
h	Hour
Hb	Haemoglobin
HCO_3	Bicarbonate
Hct	Haematocrit
HDL	High density lipoprotein cholesterol
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HOXB4	Homeobox B4 (a transcription factor)
HPC	Haematopoietic progenitor cell(s)
HRP	Horse-radish peroxidase
HSC	Haemopoietic stem cell(s)
HSCT	Haemopoietic stem cell transplantation
HSPC	Haemopoietic stem and progenitor cell(s)
HSV	Herpes simplex virus
HUCB	Human umbilical cord blood
i.p	Intraperitoneal
i.v	Intravenous
ICAM	Intercellular cell adhesion molecule
IFN-γ	Interferon gamma
IL	Interleukin
IMDM	Iscove's modified Dulbecco's medium
IU	International unit
K^+	Potassium
kD	Kilodalton
L	Litre
LDH	Lactate dehydrogenase
LDL	Low density lipoprotein
LFA-1	Lymphocyte function-associated antigen-1
LTC-IC	Long-term culture-initiating cell
LTR	Long term repopulating
Lymph	Lymphocyte
MACS	Magnetic activated cell sorting
MCH	Mean cell haemoglobin
MCV	Mean cell volume
mg	Milligram
MGDF	Megakaryocyte growth and development factor
MHC	Major histocompatibility complex
min	Minute
mL	Millilitre
mM	Millimolar
mm	Millimetre

MM	Multiple myeloma
mm^3	Cubic millimetre
mmol	Millimole
MMP-9	Matrix metalloproteinase-9
MNC	Mononuclear cell
MoAb	Monoclonal antibody
Mono	Monocyte
MPV	Mean platelet volume
MSC	Mesenchymal stem cells
MSE	Mean standard error
Na^+	Sodium
N-Cad	N-cadherin
Neut	Neutrophil
NHL	Non-Hodgkin's lymphoma
NK	Natural killer
nm	Nanometre
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NOD-SCID	Non-obese diabetic-severe combined immunodeficiency mice
NOS	Nitric oxide synthase
NSW	New South Wales
OPN	Osteopontin
p	Probability
PB	Peripheral Blood
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBSC	Peripheral blood stem cells
PBS-T	PBS Tween-20 0.1% (v/v)
PDW	Platelet distribution width
PI	Propidium iodide
Plat	Platelets
PMN	Polymorphonuclear cells
PNP	Pooled normal plasma
PoAb	Polyclonal antibody
PPP	Platelet poor plasma
PPR	Parathyroid hormone/ Parathyroid hormone- related protein receptor
PTH	Parathyroid hormone
PTHrP	Parathyroid hormone-related protein
RBC	Red blood cell(s)
RCC	Red cell count
RDW	Red cell distribution width
rhGH	Recombinant human growth hormone
RHH	Royal Hobart Hospital
rhPTH	Recombinant human parathyroid hormone
rhTPO	Recombinant human thrombopoietin
rHuSCF	Recombinant human stem cell factor
s.c	Subcutaneous
SCF	Stem cell factor
SCT	Stem cell transplant
SD	Standard deviation

SDF-1	Stromal derived factor-1
sec	Second
SEM	Standard error of the mean
SSC	Side scatter
TAS	Tasmania
TBIL	Total bilirubin
Tid	“tie in die”, three times daily
TNF	Tumour necrosis factor
TP	Total protein
TPO	Thrombopoietin
Trig	Triglyceride
U	Unit
UNSW	University of New South Wales
USA	United States of America
UTAS	University of Tasmania
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VIC	Victoria
VLA	Very late antigen
WBC	White blood cell
WCC	White cell count
xg	Times gravitational force

INTRODUCTION

This thesis concerns the uptake and clinical effects of ingested fucoidan on blood parameters, and the *in vitro* and *in vivo* effects of fucoidan on haemopoietic stem cells. Fucoidan is a naturally occurring fucose rich sulphated polysaccharide which is found in brown algae, sea urchins and sea cucumbers with the former being the main source. "Fucoidan" is a general term and encompasses a number of different preparations. The type of fucoidan, its sulphations, molecular weight and conformation of sugar residues varies with the species of seaweed (Berteau & Mulloy, 2003) and the extraction procedure. However, fucoidans consist of long branched chains of carbohydrates and include a substantial amount of fucose.

Fucoidan has a long history of use in experimental biology as a selectin blocker, and has a plethora of biological effects such as anticoagulant (Mourao & Pereira, 1999), anti-tumour (Maruyama *et al.*, 2003), anti-viral (Furusawa & Furusawa, 1989), anti complementary activity (Blondin *et al.*, 1994) and many others. More recently, fucoidan has been shown to induce a marked and prolonged stem cell release from the bone marrow in animal models (Sweeney *et al.*, 2000; Frenette & Weiss, 2000).

The use of whole seaweeds (brown algae) in the diet or as "medicine" seems to correlate with some of these activities. In countries where seaweed is a normal everyday part of the diet such as Japan and Korea, rates of cancer and HIV prevalence are lower (Shibata *et al.*, 2000). It has been proposed that high levels of seaweed ingestion, especially of brown algae (*Undaria pinnatifida*) may contribute to that low level of cancer and low HIV incidence (Cooper *et al*., 2002).

Seaweeds have been classified by the FDA as Generally Regarded as Safe (GRAS). It has not been proved clinically that ingesting large quantities of either dry seaweed or purified fucoidan by human is safe. In this study, one aim was to test the safety, taking into consideration the side effects that may arise. Ethics committee approval was sought from the appropriate bodies at the University of Tasmania (UTAS), the State of Tasmania (TAS).

Whilst fucoidans have known biological effects *in vivo* when ingested, there is little data on the amount of uptake. Indeed, it is a common assumption that this very high MW substance is not changed or absorbed in the intestine and no studies to date have reported on the detection of fucoidan in plasma after oral doses. Thus, a part of this thesis concerns a method for identifying uptake and measuring it especially after establishing the safety of treating human subjects with large quantities of fucoidan.
Few options were available taking into consideration that the method had to be quantitative and reproducible and able to be used in routine settings. Two methods in this study were successful in measuring the level of fucoidan in solutions, an antibody based method using ELISA and a colorimetric method using a dye.

The known effects of fucoidan on haemopoiesis have been induced by intravenous dosing. In this thesis, it was hypothesised that oral fucoidan has an effect on this system. Haemopoiesis is the process of formation of blood cellular components from haemopoietic and progenitor stem cells (HPC). These cells are the most undifferentiated precursor cell type in the haemopoietic system and are defined on the basis of their functional and phenotypic properties. They are pluripotent, capable of self-renewal and capable of giving rise to long-term haemopoietic reconstitution. Once they lose pluripotency they become lineage-committed with only limited potential for self-renewal (Berenson *et al.*, 1988 & 1991; Charbord, 1994).

The majority of HPC reside in the bone marrow (BM) with a small number continually escaping into the circulation then homing back into the BM in a process called “cell trafficking”. Stromal cells in the BM constitutively express and secrete stromal cell derived factor (SDF-1), which is a highly conserved chemokine, strongly basic, and binds to heparin. It acts as a chemo-attractant for $CXCR4^{+}$ cells, so playing a role in regulating stem cell trafficking.

Haemopoietic stem cell transplantation (HSCT) has become the standard of care for the treatment of many haematological malignancies, chemotherapy-sensitive relapsed acute leukaemias or lymphomas, multiple myeloma, aplastic anaemia and immunodeficiency states. A number of chemokines and cytokines have been tested in clinical trials and have been shown to enhance the trafficking of haemopoietic stem cells (HSC) into the peripheral blood (PB). This process of recruitment, termed stem

cell mobilisation, allows the collection of HSC via apheresis for both autologous and allogeneic transplantation (Cashen *et al.*, 2004).

Although the HSC normally reside in the BM, in recent years we have learned how to enhance trafficking of HSC into the PB in simple and safe ways. These HSC are capable of homing to the BM cavity and regenerating a full array of haemopoietic cell lineages in a timely fashion after ablative and non-myeloablative conditioning. This process mimics enhancement of the physiological release of HSC from the BM reservoir in response to stress signals during injury and inflammation. In the early 1980s the first autologous stem cell transplants using mobilised peripheral blood stem cell (PBSC) collected by apheresis were documented (Kessinger *et al.*, 1986; Juttner *et al.*, 1989). Initially, the mobilisation protocols used chemotherapy alone; however, since the discovery and clinical development of human granulocyte-colony-stimulating factor (G-CSF) (Welte *et al.*, 1985), cytokine mobilisation has become the standard of care. Currently, the unique cytokines approved by the United States Food and Drug Administration (FDA) for autologous and allogeneic stem cell mobilisation are G-CSF and granulocyte-macrophage colony-stimulating factor (GM-CSF). PBSC mobilisation and collection have been optimized in different clinical trials. Nevertheless, 14% of patients receiving standard mobilisation for the purpose of autologous donation and 4% of allogeneic donors still fail to mobilise (Moncada *et al.*, 2003). The pursuit of an enhanced understanding of HSC biology, the processes involved in HSC microenvironmental interactions, the ligands and receptors involved in HSC homing and mobilisation, with an emphasis on fucoidan induced HSC mobilisation, form the basis of this work.

Fucoidans are considered to have similarities to the (much smaller) mammalian molecule heparin sulphate, an anticoagulant that is in extensive clinical use intravenously (i.v). The potential risk of prion related diseases in mammals and the increasing requirement of antithrombotic compounds that can be delivered orally indicate that we need to look for a heparin alternative. To date, no studies have looked at the direct anticoagulant activities of fucoidan after oral treatment. This significant question remained to be answered.

It was found that fucoidan can increase the leucocytes and HPC in mice and monkeys (Frenette & Weiss, 2000). Its ingestion can lead to an increase in SDF-1 plasma level but a decrease in the BM. SDF-1 acts as chemoattractant for mature leucocytes and HPC which carry its receptor CXCR4 (Sweeney *et al*., 2002). Intravenous injection in rats of LMW fucoidan significantly increased the SDF-1 level in plasma (Luyt *et al*., 2003). Clinical trials have not demonstrated a definitive effect on the level of plasma SDF-1 in patients mobilised with G-CSF. Would ingesting fucoidan have the same effect on the SDF-1 plasma level?

Fucoidan is known to bind the lectin domain of P-selectin on platelets (Shibata *et al*., 2003). All fucoidan fractions of different molecular weights and different sulphate contents induced irreversible platelet aggregation in a dose-dependent manner. The low molecular weight fucoidan FF7/3 combines potent anticoagulant and fibrinolytic properties with only minor platelet activating effects (Durig *et al*., 1997).

Fucoidan extracts have been shown to have *in vitro* activity against herpes simplex virus (HSV). The extracts appear to inhibit HSV by blocking virus attachment and entry into the host cell (Cooper *et al*., 2002).

In animal models, ingestion of fucoidan has inhibitory effects on tumours, which appear to be associated with a rise in IFN-γ, IL-12, and stimulation of innate immunity (Maruyama *et al.*, 2003; Mavier *et al.*, 2004; Funahashi *et al.*, 2001). *In vitro* treatment of BM mononuclear cells (MNC) with IFN-γ can up-regulate the expression of CXCR4 on granulocyte precursors and monocytes (Funahashi *et al.*, 2001). On the other hand, nitric oxide synthase (NOS-2) (iNOS) is rapidly induced by IFN-γ and IL-12 to produce nitric oxide (NO); a recently identified biological signal molecule that plays an important role in vascular regulation, immune responses, and neural signal transduction (Liao *et al*., 1999; Huang *et al*., 2001; Chesler & Reiss, 2002). Therefore ingestion of fucoidan in humans may have a similar effect on IFN-γ and IL-12 to subsequently regulate the expression of CXCR4 and the production of NO.

Ex vivo haemopoietic cell expansion using soluble cytokines has been proposed as a technology for production of various haemopoietic lineages for cell-based therapies. Neutropenia and thrombocytopenia are significant toxicities that limit the efficacy of

cancer therapies. Large-scale production and administration of expanded mobilised peripheral blood stem cells has been shown to abrogate postmyeloablative cytopenia (Boiron *et al.,* 2006). In the future it may be possible to manufacture myeloid progenitors using HPC from alternate sources such as cord blood. Stromal layers, which are the current method for long-term maintenance or expansion of blood stem cells, are unsuitable for clinical trials. There would be considerable clinical interest in a synthetic matrix that was suitable for blood stem cell maintenance or expansion. Fucoidan preparations may provide an easily manufactured proteoglycan component of an artificial BM matrix, since it displays sulphate sugars that interact with heparin binding proteins, integrins, and selectins. The present studies were designed to directly examine the characterization of fucoidan effects on *in vitro* human $CD34^+$ cells proliferation and differentiation and to determine the interaction between fucoidan and cytokines in the expansion system.

Objectives and Aims:

In this study the safety and possible effects of ingesting fucoidan on human volunteers will be examined. This work will define the effects of ingestion of fucoidan on the haemopoietic systems and will study the interaction of fucoidan with different haemopoietic cytokines *in vivo* and *in vitro*. It will also study the kinetics of the mobilisation effect of HSC and will seek possible detection methods for the presence of fucoidan in plasma.

The specific aims of this study are:

1. To establish the safety and maximum tolerated dose of *Undaria pinnatifida*-derived fucoidan extracts in volunteers. The pre-treatment evaluation includes volunteers' history, physical examination and routine laboratory studies.
2. To study and report the clinical, biological and pathological changes after oral doses of fucoidan extracts by examining volunteers' general health, physical examination and routine laboratory studies.
3. To study the effect of ingesting fucoidan on the lipid profile.
4. To develop a method suitable for routine laboratory settings that can measure the level of fucoidan in blood circulation after oral administration.
5. To determine the effects of fucoidan on haemostasis by studying various coagulation tests.

6. To study the immune-system by examining any changes in the WBC and their subsets including B-cells and T-cells.
7. To examine the direct effect of oral fucoidan on the level of pro-inflammatory cytokines such as IFN-γ and IL-12.
8. To define the possible effects of fucoidan on HPC and HSC in BM and PB by studying the PB $CD34^+$ properties, receptors, trafficking and cytokines.
9. To study the direct effect of fucoidan and G-CSF on SDF-1 level in PB.
10. To examine the effects and refine the characterization of different fucoidan extracts on *in vitro* human $CD34^+$ cell proliferation and differentiation and to determine the interaction between fucoidan and cytokines in the expansion system.

CHAPTER ONE

LITERATURE REVIEW

Chapter contents:

1.1 Haemopoietic stem and progenitor cells (HSPC)

1.1.1 Historical background

In 1909, a Russian biologist, Alexander Maximow, claimed that a small number of cells circulate in the PB within the lymphocyte population that might be capable of reacquiring pluri-potentiality and he called these cells "gemeinsame stamzellen" (Maximow, 1909). The first clinical investigation in the field of HSCT occurred in the 1940's, when experiments using mice showed that protection of the spleen by lead shielding allowed animals to survive otherwise lethal total body irradiation (Jacobsen *et al.*, 1949).

Until the 1950's, few attempts were made to confirm this concept, until Ford and his colleagues established through a series of murine experiments that cellular repopulation is the mechanism by which spleen and other tissues bring about their therapeutic effect rather than through "humoral factors" (chemical factors) (Ford *et al.*, 1956).

After that, and for the first time, a French group in Paris reported long-term survival of an adult patient with acute lymphoblastic leukaemia who received BM transplants from several relatives after being subjected to whole-body irradiation and methyl-nitro-imidazolyl-mercaptopurine administration (Mathé *et al.*, 1963).

Until this point, it had been known that i.v injection of BM or spleen cells into lethally irradiated animals could lead to animal recovery. This recovery had been shown to be the result of repopulation of the damaged haemopoietic tissues by stem cells from the donor. At the same time circulating stem cells were under investigation but the PB as a source of stem cells was still considered inadequate to permanently reconstitute haemopoiesis (Micklem *et al.*, 1975).

Decades later, a glycoprotein (CD34) that is present on colony-forming cells and myeloblasts but is lost at the level of the promyelocyte was detected on the surface of immature haemopoietic cells (Krauss *et al.*, 1996). Culture assays were able to document the number of haemopoietic progenitor cells in any preparation. At different levels of maturation they were called Colony Forming Units (CFUs). The PB of healthy individuals was found to have a small number of $CD34^+$ cells, although again

at a much lower level than in human BM (Jansen *et al.*, 2005). Also, it was documented in a dog model that HSC obtained from the PB could permanently reconstitute irradiated animals, just like BM could (Calvo *et al.*, 1976). These discoveries, plus the use of haemopoietic cytokines such as G-CSF and combined with the development of other techniques such as cell culture assays and the quantification of stem cells has led to a complete paradigm shift in the world of stem cell transplantation.

1.1.2 Definition of HSC and HPC

Stem cells are defined as cells capable of unlimited self-renewal and with the ability to give rise to multiple tissue types (Thomson *et al.*, 1998). Both of these parameters are subject to wide interpretation and depend to some degree on whether the stem cell is present *in situ* (in its normal environment) or in an experimental setting [Figure 1.1]. There are three possibilities when stem cells divide: the first is self-renewal that is when stem cells divide and generate new stem cells; the second is to differentiate into mature blood cells; the third is to be destroyed through apoptosis. Some genes that are involved in these processes have been identified (Sorrentino, 2004).

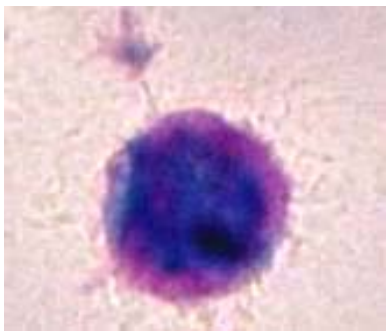


Figure 1.1: Haemopoietic stem cell. Stained with May-Grunwald-Giemsa at magnification of x1000

A haematopoietic stem and progenitor cell (HSPC) is a generic term to include both haematopoietic progenitor cells (HPC) and haematopoietic stem cells (HSC). This is because many properties are shared by these two cell populations. HSC and HPC are very similar, but the later term is used to describe cells which are more immature or undifferentiated and it is therefore less restrictive. Like HSC, HPC have a capacity for

self-renewal and differentiation, although these properties may be limited. For instance, both HSC and HPC share properties such as CAM expression, KIT expression, and trafficking in the blood and spleen.

HSC and HPC are found in BM and give rise to all the types of both the myeloid and lymphoid lineages. They are used in clinical transplantation protocols to treat a wide variety of diseases. The ability to increase the number of HSC or HPC either *in vivo* or *in vitro* or change their phenotypic properties would provide new treatment options.

However there are fundamental differences between the two. By definition, HSC are the only ones able to reconstitute the whole haematopoietic and immune systems for the life-time of the recipient following transplant. This functional definition is equivalent to what is also call long-term re-constitutive cells. Short-term re-constitutive cells reconstitute for 1 month and then crash. As they do not self-renew beyond this, they are not considered as HSC but multi-potent HPC. There are also all the possible HPCs including multi-potent, myeloid (such as CMP, GMP, MEP, colony-forming cells) and lymphoid progenitors (such as CLP).

The main functional properties of HSC as mentioned before are multi-potency and self-renewal. Multi-potency means an individual HSC have the ability to give rise to any of the end-stage blood cell types. During differentiation, daughter cells derived from HSC undertake a series of commitment decisions, retaining differentiation potential for some lineages while losing others. Intermediate cells become progressively more restricted in their lineage potential, until eventually lineage-committed end stage cells are generated. Self-renewal of HSC means some kinds of stem cells are thought to undertake asymmetric cell division, generating one daughter cell that remains a stem cell and one daughter cell that differentiates. For HSC, however, whether asymmetric cell division occurs during self-renewal is not known with certainty. It is instead possible that haematopoiesis occurs via symmetrical divisions, that sometimes give rise to two daughter HSC, and that at other times give rise to progeny that are committed to differentiate. The balance between self-renewal versus differentiation would therefore be regulated by the control of these two kinds of symmetrical cell division.

It is known that a small number of HSC can expand to generate a very large number of progeny HSC. This phenomenon is used in BM transplant when a small number of HSC reconstitute the hematopoietic system. This indicates that at least during BM transplant, symmetrical cell divisions that give two progeny HSC must occur, as expansion in HSC numbers seen during BM transplant cannot occur in any other way.

Stem cell self-renewal is thought to occur in the stem cell niche in the BM, and it is reasonable to assume that key signals present in this niche will be important in self-renewal. There is much interest in the environmental and molecular requirements for HSC self-renewal, as understanding the ability of HSC to replenish themselves will eventually allow the generation of expanded populations of HSC *ex vivo* that can be used therapeutically.

There are different types of stem cells. Embryonic stem (ES) cells, derived from the inner cell mass of mammalian embryos, have unlimited self-renewal properties and give rise to all embryonic tissue types *in vitro*, although unlimited self-renewal is not a property of cells of the inner cell mass *in situ*, where they differentiate into various tissues of the body and the ES cell phenotype is lost. These cells represent the ultimate in stem cells because of their abilities to be both self-renewing and multi-potent.

Adult stem cells, of which HSC are the best studied, give rise to a wide range of progenitor and mature cells within the confines of the haemopoietic system, and have self-renewal properties for the life of the organism. Umbilical cord blood has also been established as a clinical source of HSC.

HSC are relatively rare and are difficult to identify by morphology alone; they are thus frequently characterised by functional assays. Several methods exist to enrich HSPC, including fluorescence-activated cell sorting (FACS), immunomagnetic separation, and density-gradient centrifugation (Thomas *et al.*, 1999). The major cellular antigens which are used to characterise human HSPC and mice HSPC are shown in Table 1.1.

Table 1.1: The major common cellular antigens for both human and mice

Human HSPC cellular antigens	Mice HSPC cellular antigens
c-met	c-met
CD34	CD34
CD90	CD38
CD110	$CD90^{low}$
CD111	CD105
CD117	CD117
CD202	CD133
CD133	CD202
CD243	CDw338/ABCG2
Lineage ^{negative} (Lin^-)	Lineage ^{negative} (Lin^-)
	Sca-1

1.1.3 Characterisation of HSC in BM and microenvironment

BM has been traditionally envisioned as a 'home' of HSC (Ratajczak *et al.*, 2004b). HSC in BM are supported by an extracellular matrix (ECM) rich in fibronectin, collagens, and various proteoglycans (Nervi *et al.*, 2006) and cell-cell interactions with non-haemopoietic cells, which include osteoblasts, fibroblastic BM stromal cells and endothelial cells. These interactions form what is called the microenvironment or "niche" for HSC that is responsible for their localization to specific anatomical regions in the BM (Sorrentino, 2004). These niches play an important role in regulating HSC trafficking as well as in self-renewal, proliferation, and differentiation.

The BM endothelium is the first anchoring site for homing cells, presenting adhesion molecules and stimulating chemokines. Human and mouse BM share common structures, but species dependent differences can also be observed. The small blood vessels in both human and murine BM, the sinusoids in which trans-endothelial migration is thought to take place, are composed of specialized cell structures that regulate cell trafficking (Lapidot *et al.*, 2005). Different niches have been described by researchers such as "endosteal niche" where HSC are physically associated with osteoblasts at the endosteum of the BM (Petit *et al.*, 2002) and "endothelial niches" where HSC in the BM are closely associated with sinusoidal endothelial cells (Kiel *et al.*, 2005).

HSC are defined functionally by their ability to mediate long-term repopulation of all blood-cell lineages (known as long term repopulating (LTR) activity) and to form colony forming units in the spleen after transfer to lethally irradiated recipients.

All mouse LTR HSC are contained in the lineage-negative (Lin^-), stem-cell antigen1 ($SCA1^+$), KIT^+ subset (LSK subset) that comprises ~0.5% of BM (Morrison *et al.*, 1995). One hundred LSK cells are sufficient for multi-lineage LTR activity (To *et al.*, 1997). Additional markers have been identified which can further subdivide the LSK population into long-term HSC (LTR-HSC) and short-term HSC (STR-HSC), which have limited self-renewal activity. LTR activity is also enriched in the population of BM cells with low level staining by rhodamine 123 (Rho) (Endres *et al.*, 1996). In addition, functional adult LTR HSC can be isolated by their ability to actively efflux the DNA-binding dye Hoechst 33342. This characteristic is designated as the side-population (SP) ability (Lu *et al.*, 1997; Rosu-myles *et al.*, 2000) [Figure 1.2].

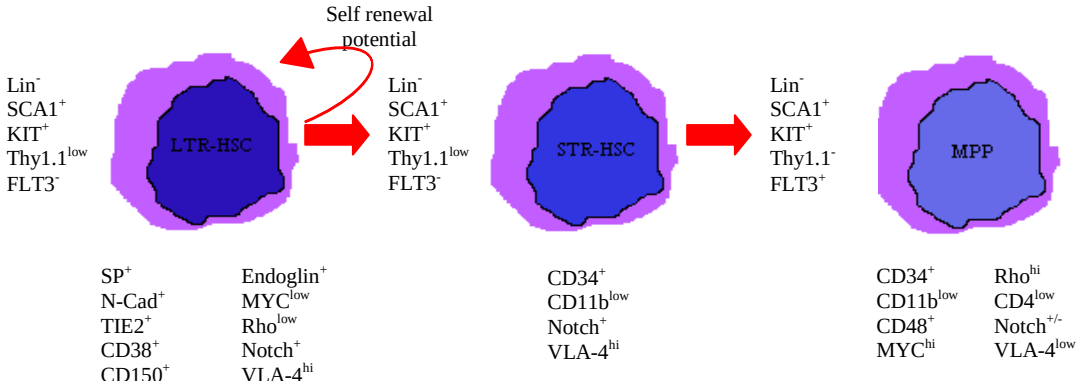


Figure 1.2: Phenotypic characteristics of mouse haemopoietic stem cells. Long term repopulating potential HSC (LTR-HSC), short term repopulating potential HSC (STR-HSC), multi-potential progenitor cells (MPP), side population (SP), N-Cadherin (N-Cad), tyrosine kinase receptor (TIE2), rhodamine 123 (Rho), FMS-related tyrosine kinase 3 (Flt3).

The majority of HSC reside in the BM with a small number continually escaping into the circulation then homing back into the BM. During foetal development, primitive HSC migrate through the circulation to a series of tissues until the bones are formed and the BM can finally be established (Mikkola *et al.*, 2005; Tavian & Peault, 2005).

These migrations involve both 'mobilisation' as well as 'homing' to newly formed haemopoietic tissues and final lodgement in specific niches where the cells are nurtured and provided with all the cellular interactions, cytokines and chemokines necessary for their self-renewal and function (Winkler & Levesque, 2006).

The effects of these supporting cells are mediated through specific molecular interactions. As an example, the HSC express Notch receptors, which are transmembrane signalling molecules known to inhibit differentiation in other systems. Notch-1 ligand (Jagged-1) is expressed by BM stromal cells, endothelial cells and osteoblasts. This indicates that cell contact might have a role in the regulation of the HSC population (Sorrentino, 2004).

The phenotypic properties of mobilised HSC with growth factors are different from HSC that reside in the BM under steady-state. Wright and his colleagues in 2002 showed that HSC are mobilised after the M-phase of the cell cycle, which explains the higher percentage of HSC in blood in the G_0 or G_1 phase of the cell cycle. At the same time circulating HSC display reduced expression of VLA-4 (Mohle *et al.*,1993; Watanabe *et al.*, 1997) and c-kit (Mohle *et al.*, 1993) on their cell surface.

Recently, BM HSC were reported to be able to 'trans-differentiate' into cells that express early heart, skeletal muscle, neural, liver or pancreatic cell markers besides being known as a 'home' of HSC (Ratajczak *et al.*, 2004b).

1.1.4 The bone marrow microenvironment

Bone marrow (or medulla ossea) is the soft tissue found in the hollow interior of bones. It is the place where most new blood cells are produced. Bone marrow contains two types of stem cells. HSC give rise to the three classes of blood cell that are found in the circulation, leucocytes, erythrocytes and thrombocytes. Mesenchymal stem cells are found arrayed around the central sinus in the BM. They have the capability to differentiate into osteoblasts, chondrocytes, myocytes, and many other types of cell. They also function as "gatekeeper" cells of the BM. The BM microenvironment is important for haemopoiesis. It is characterized by the presence of special stromal cells supported by the osteoblasts and surrounded by the ECM and cytokines.

1.1.4.1 Stromal cells

Stomal cells are required to facilitate the growth, development and survival of HSPC *in vivo* and *in vitro* (Bently, 1981). They synthesise ECM components and some cytokines. There are four different types of marrow stromal cells, the endothelial cell, the adventitial reticular cell, the macrophage and the adipocyte.

When mature blood cells are ready to join the circulation they must pass through the endothelial cells lining the sinus wall to enter the sinus lumen (Dexter, 1989). The adventitial reticular cells synthesise many components of the BM ECM plus G-CSF, GM-CSF, M-CSF, IL-1 and IL-11 *in vitro* (Brockbank *et al.*, 1986). Macrophages are considered as a source of GM-CSF, IL-1 Epo, TNF-α and IFN-γ, under steady state conditions (Rich, 1988). It has been reported the formation of adipocytes *in vitro* is required to support haemopoiesis (Riley & Gordon, 1987).

1.1.4.2 The extracellular matrix

The ECM consists of collagen types I, II, III, IV, laminin, fibronectin, vitronectin, haemonectin and thrombospondin (Verfaillie, 1998). It is secreted by the BM stromal cells and it provides support and cohesiveness for the marrow structure. The ECM also contains various glycosaminoglycans which include chondroitin, heparin, dermatan, keratan sulphates and hyaluronic acid, all of which are polymers capable of specifically retaining and delivering cytokines (Moore & Shapiro, 1994). It is believed now that ECM is capable of sequestering and compartmentalising certain colony forming cells in local areas and presenting them to HSC, thus creating a number of different haemopoietically inductive microenvironments.

1.1.5 Mobilisation of HSPC

Mobilised PB have replaced BM as the preferred source of haemopoietic rescue for patients undergoing high-dose chemo-radiotherapy because of improved neutrophil and platelet engraftment, increased safety and comfort for the donor, shortened hospital stay, greater disease-free survival in the recipient and lower overall cost. Worldwide, over 45,000 patients a year receive cellular support using mobilised HSC (To *et al.*, 1997; Korbling & Anderlini, 2001; Winkler & Levesque, 2006). However, the optimal method to mobilise and collect PB progenitor cells for haemopoietic rescue following autologous transplantation is unknown. In the steady state the

concentration of $CD34^+$ cells in BM is up to 100 times higher than in PB. The number of $CD34^+$ cells in the PB is only 1-5/mm^3 (Jansen *et al.*, 2005).

The use of PBSC in clinical autologous and allogeneic transplantation started in the mid 1980's and early 1990's, respectively. PB stem cells are usually collected from healthy donors by a leukapheresis procedure that can process large volumes of blood. One to four collection procedures are performed on consecutive days, and result in sufficient $CD34^+$ cells from most donors or patients for transplant. Typically, each leukapheresis procedure lasts 2-3 hours, and a total of 10-18 litres of blood are processed (2-3 x total blood volume) [Figure 1.3]. The blood vessels of many donors can be accessed through catheters into one or two antecubital veins (Jansen *et al.*, 2005).

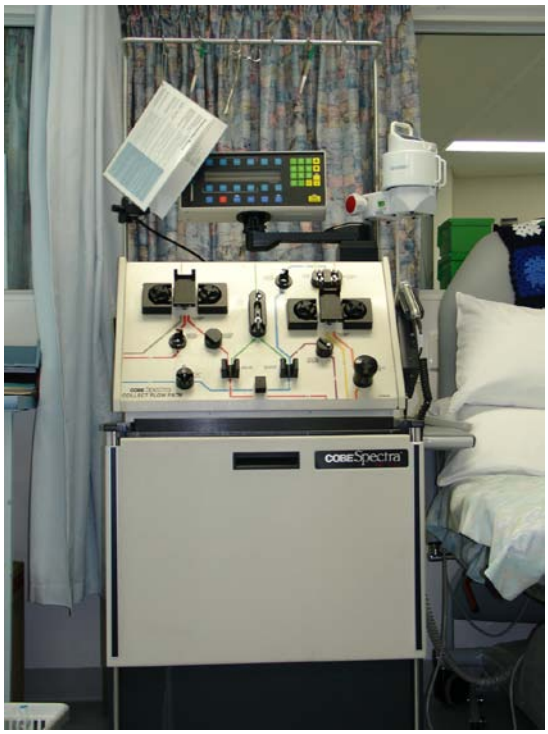


Figure 1.3: Leukapheresis machine. Stem cell transplantation unit, Royal Hobart Hospital.

It is now clear that mobilisation involves a perturbation of the SDF-1 chemotactic gradient in the BM (Lévesque *et al.*, 2003; Petit *et al.*, 2002), as well as a decrease in the responsiveness of mobilised HSPC to the SDF-1 chemotactic gradient as demonstrated in both humans (Lévesque *et al.*, 2003) and mice (Lévesque *et al.*, 2004).

Mobilisation of HSC is defined as the movement of these cells from their niches into the PB. Cells must migrate through the BM-blood barrier that separates the haemopoietic compartment from the circulation. BM venous sinuses are the sites of leucocyte egress from the haemopoietic compartment and represent the only complete barrier to the intravascular space. The sinus wall is a trilaminar structure composed of endothelial cells, a basement membrane, and a layer of adventitial cells. It is thought that mature-HSC egress through fenestrations that are called “diaphragmed fenestra”, which are located within the endothelial cell where luminal and abluminal membranes are fused (Nervi *et al.*, 2006).

1.1.5.1 Different mobilisation mechanisms

Currently, the unique cytokines approved by the FDA for autologous and allogeneic stem cell mobilisation are G-CSF and granulocyte-macrophage colony-stimulating factor (GM-CSF). PBSC mobilisation and collection have been optimised in different clinical trials. Nevertheless, 14% of patients receiving standard mobilisation for the purpose of autologous donation and 4% of allogeneic donors still fail to mobilise (Moncada *et al.*, 2003). The search for new mobilising agents and strategies is emerging. In the following sections different mobilisation agents and mechanisms will be described. However, none of them has significantly changed the clinical mobilisation practice and the search for new and better mechanisms is still required.

1.1.5.1.1 Chemotherapy

Mobilisation of HSPC from BM to PB using chemotherapy was the first mechanism employed. Marrow hypoplasia-producing chemotherapy such as that used for acute leukaemia was noted to result in a transient increase in the number of HSC in the PB. Mobilisation with chemotherapeutic agents such as cyclophosphamide occurs during the recovery phase following the chemotherapy-induced neutropenia, that is days 6-8 in mice, and days 10-14 in humans (Winkler & Levesque, 2006).

The first full haemopoietic reconstitution with autologous mobilised PB was documented in the 1980s (Kessinger *et al.*, 1986; Reiffers *et al.*, 1986; Juttner *et al.*, 1988). However, some patients, in particular patients with progressive disease, marrow infiltration, or prior exposure to alkylating agents or radiation therapy or after prolonged exposure to alkylating chemotherapy, failed to exhibit this response to chemotherapy and had an insufficient collection for transplantation. Even multiple leukapheresis procedures may fail to obtain sufficient numbers of $CD34^+$ cells to guarantee prompt and adequate haematological recovery (To *et al.*, 1990; Tricot *et al.*, 1995). Thus, the search for another mechanism to better mobilise HSC in those patients was still needed. The discovery that the administration of G-CSF during the recovery from chemotherapy increased the number of HSC to levels as much as 1,000 fold higher than in the blood before treatment (Socinski *et al.*, 1988; Gianni *et al.*, 1989) initiated the exploration of powerful mobilisation regimens using haemopoietic cytokines.

1.1.5.1.2 Cytokines such as G-CSF

Cytokines are small proteins that are usually secreted by white blood cells and act as chemical messengers between cells by binding to surface receptors thereby mediating the immune response and regulating inflammation and haemopoiesis. They signal via second messengers, often tyrosine kinases, to modify cell gene expression and cellular function such as proliferation, activation, and the secretion of effector molecules. Granulocyte colony-stimulating factor (G-CSF) is the most potent cytokine currently available for the mobilisation of HSPC.

The most common agent used to elicit HSC mobilisation is G-CSF alone or in combination with myelosuppressive chemotherapy (Winkler & Levesque, 2006). The use of G-CSF (Filgrastim) or even GM-CSF (Sargramostim) results in an increase in the number of circulating $CD34^+$ cells and CFU-GM (Jansen *et al.*, 2005).

G-CSF was initially tested in clinical trials for its ability to prevent or reduce severe neutropenia and its complications. Randomised trials demonstrated that G-CSF accelerates the recovery of neutrophil numbers and reduces the duration of severe neutropenia after chemotherapy (Ozer *et al.*, 2000). As a direct consequence of this accelerated neutrophil recovery, G-CSF administration also reduced the incidence of

inflammation of the oral mucosa, the duration of fever, the need for antibacterial and antifungal antibiotics and the duration of hospitalisation (Roberts, 2005). The benefits are more significant when more intensive chemotherapy is used and greatest after myeloablative chemo-radiotherapy and autologous BM transplantation. G-CSF is used routinely to support chemotherapy of moderate intensity in patients with curable malignancies including lymphoma, leukaemia and breast cancer.

While the acceleration of recovery of granulopoiesis was a predicted effect of G-CSF, the mobilisation of large numbers of progenitor and stem cells from the BM into the PB was entirely unexpected (Duhrsen *et al.,* 1988). It was discovered in the first Phase I studies of G-CSF that the numbers of multiple lineages of progenitor cells (myeloid, erythroid, megakaryocytic) in the blood were elevated dramatically (about 100-fold) 4–7 days after the beginning of G-CSF treatment. These blood stem cells proved easier to collect in large numbers than BM stem cells, and to reconstitute the haemopoietic system significantly faster than BM when infused following myeloablative chemoradiotherapy (Sheridan *et al.*, 1992). In particular, platelet recovery was accelerated by 5–10 days. Stem cell mobilisation is augmented further if G-CSF is administered after a myelosuppressive dose of chemotherapy, and for autologous stem cell transplantation this is now the most common method of obtaining a stem cell inoculum.

G-CSF-induced mobilisation is time and dose dependent, involving a rapid neutrophilia and a gradual increase in HSC numbers in the blood, peaking between 4-7 days of G-CSF administration. Usually, G-CSF is used at a daily dose of 10 µg/kg s.c for 4-5 days. This dose can increase the number of circulating $CD34^+$ cells up to 50-fold (Jansen *et al.,* 2005), which is usually required to achieve the mobilisation goal of 5×10^6 $CD34^+$ cells/kg of recipient body weight, a dose considered suitable for reproducible, rapid and consistent engraftment of both neutrophils and platelets (Henon *et al.,* 1992; Schmitz *et al.,* 1996). Successful haemopoietic reconstitution of a patient requires the transplantation of at least $2x10^6$ $CD34^+$ cells/kg (Demirer & Bensinger 1995; To *et al.,* 1997; Sezer *et al.,* 2000) and the transplantation of greater numbers of $CD34^+$ cells correlates with faster haemopoietic recovery and lower incidence of graft versus host disease (GvHD) (To *et al.*, 1986; Sheridan *et al.*, 1994; Brown *et al.*, 1997; Siena *et al.*, 2000).

Unfortunately the number of HSPC mobilised in response to G-CSF varies significantly (Villalon *et al.*, 2000; Roberts *et al.*, 1997) and a small proportion of normal donors (1-5%) fail to mobilise sufficient $CD34^+$ cells. Furthermore, following several cycles of high-dose chemotherapy and/or radiotherapy, up to 60% of patients will fail to mobilise sufficient $CD34^+$ cells for autologous transplantation (Demirer & Bensinger 1995; Bensinger *et al.*, 1995; Brown *et al.*, 1997; Villalon *et al.*, 2000).

The long-term safety profile of growth-factor therapy in normal individuals has not been established. G-CSF toxicity has been defined in studies of allogeneic donors (Anderlini *et al.*, 1996; Akizuki *et al.*, 2000). About 80% of healthy donors will develop bone pains and 50% will develop headaches (Jansen *et al.*, 2005). The pain begins after a single Filgrastim (G-CSF) injection and plateaus after two to three injections. Other side effects include fatigue, headache, and nausea. These complaints are generally tolerable, and few donors require a dose reduction or discontinuation of the drug. After five daily doses of G-CSF, serum chemistries such as lactate dehydrogenase (LDH), alkaline phosphatase (AP) and alanine aminotransferase (ALT) increase 2 to 4 fold, while the serum levels of potassium, blood urea nitrogen (BUN) and magnesium may show minimal declines during the treatment (Nervi *et al.*, 2006).

Although chemotherapy-based mobilisation “used only with patients” typically results in the collection of greater numbers of $CD34^+$ cells compared with G-CSF alone, it is also associated with greater morbidity due to complications with infection and has not clearly improved clinical outcomes following transplantation (Devine *et al.*, 2004).

Growth factors should be given with caution to people with a history of autoimmune diseases, because flares of the disease have been reported after Filgrastim administration (de Vries *et al.*, 1991). Two cases have been reported of spontaneous splenic rupture after G-CSF and there is one fatal case report that Filgrastim may precipitate severe sickle crisis in persons with sickle cell anaemia (Adler *et al.*, 2001). It also may induce a transient and reversible hypercoagulable state especially in donors with a history of a coagulation disorder, peripheral vascular disease, myocardial infarction, or stroke (Gutierrez-Delgado & Bensinger, 2001).

As mentioned earlier, a common side effect of G-CSF administration is bone pain (Vial & Descotes, 1995), which reflects the dramatic reduction in bone turn-over that occurs during G-CSF administration. Systemic administration of G-CSF rapidly inhibits osteoblast-mediated bone formation as well as increasing bone degradation by osteoclasts in both human and mouse. Osteocalcin, a bone matrix protein specifically produced by osteoblasts, is a good indicator of bone formation and osteoblast activity. In humans, osteocalcin concentration in the plasma drops during HSC mobilisation and this drop is significantly correlated with the number of CFU-GM mobilised in the PB (Takamatsu *et al.*, 1998).

Patients undergoing allogeneic HSC transplantation receive cells from normal donors. G-CSF administration to volunteer donors for patients undergoing allogeneic transplantation has been shown to be safe and effective, and G-CSF mobilised blood stem cells now have replaced BM as the standard source of HSC for allogeneic BM transplantation (Roberts, 2005).

Normal donors injected with G-CSF develop a marked neutrophilia within 4 h of injection, mobilise stem cells after 3–4 days (peak days 4–5), and sub-clinical spleen enlargement over 4–6 days (Grigg *et al.*, 1995, Stroncek *et al.*, 2003). Between normal individuals, a 3–10-fold variation in magnitude of these responses is observed (Grigg *et al.*, 1995), and spleenic rupture is a rare life-threatening side effect of G-CSF mobilisation (Falzetti *et al.*, 1999). Each of these responses occurs in mice injected with G-CSF (Roberts *et al.*, 1997a), and research over the last decade using genetically manipulated mice has elucidated the mechanism of stem cell mobilisation by G-CSF. In keeping with the early observation that progenitor cells of all lineages (including those not expressing G-CSFRs such as erythroid, megakaryocytic, lymphoid progenitors) are mobilised (Duhrsen *et al.*, 1988, Roberts *et al.*, 1997b), G-CSF-induced mobilisation is a trans effect (Liu *et al.*, 2000), dependent on the presence of G-CSFR-bearing neutrophils (Liu *et al.*, 1997, 2000).

In response to G-CSF, increased secretion of proteases by the expanding neutrophil mass within the bone marrow cleaves critical adhesion molecules and chemoattractant receptors which normally function to retain progenitor and stem cells within bone marrow micro-environmental niches (Levesque *et al.*, 2001, 2002, 2003, 2004). These

factors also serve to mobilise neutrophils (Semerad *et al.*, 2002), and the 2-3 day delay between neutrophil and stem cell mobilisation most likely reflects the requirement for concomitant G-CSF-driven stem and progenitor cell proliferation and expansion. Within the bone marrow, stem and progenitor cells are rapidly cycling after G-CSF stimulation. In contrast, circulating cells are almost always found to be in G_0 or early G_1 phase of the cell cycle (Roberts & Metcalf, 1995), suggesting either that mobilisation only occurs immediately after the completion of mitosis, or clearance mechanisms prevent cycling cells from remaining in the circulation after marrow release.

1.1.5.1.3 Chemotherapy plus cytokines

Combining chemotherapy with cytokine administration has been used to enhance the HSC mobilisation and reduce the contamination with malignant cells, but it has been associated with an increased risk of neutropenia, infections and costly hospitalisations (Ravagnani *et al.*, 1990; Bensinger *et al.*, 1995). Until today, there have been no appropriate clinical trials to investigate the use of growth factor (GF) alone or in combination with chemotherapy to mobilise stem cells in normal donors.

Previous strategies designed to improve $CD34^+$ cell yield following G-CSF-based PBSC mobilisation through combination with other haemopoietic cytokines have met with limited success because of lack of efficacy or increased toxicity. Novel strategies are required, particularly for those patients who have been heavily pre-treated and who are predicted to have poor stem-cell mobilisation with current approaches.

1.1.5.1.4 AMD-3100

AMD-3100 is a bicyclam molecule that was first described for its potent and selective inhibition of HIV type 1 and 2 replication through binding to the chemokine receptor CXCR4, used by T-tropic HIV for entry into $CD4^+$ cells. AMD-3100 also reversibly blocks the binding of CXCR4 with SDF-1 but has no effect on other cell surface chemokine receptors. Initial clinical trials of AMD-3100 evaluated its safety and efficacy in the treatment of patients with HIV-1 infection (Devine *et al.*, 2004). AMD-3100 pharmacokinetic studies indicate that the drug is rapidly absorbed after s.c injection and is eliminated from plasma in a biexponential manner (Lack *et al.*, 2005).

Broxmeyer and his colleagues (2001 & 2002) demonstrated a 40 fold increase in the mobilisation of haemopoietic progenitors within one hour of AMD-3100 injection in mice. In contrast, with G-CSF-based progenitor cell mobilisation, this effect did not appear to be mouse-strain dependent (Broxmeyer *et al.*, 2002). Furthermore, human $CD34^+$ cells mobilised from normal volunteers in response to a single dose of AMD-3100 were efficiently engrafted in non-obese diabetic-severe combined immunodeficiency (NOD-SCID) mice. AMD-3100 mobilised human $CD34^+$ were as effective as, or more effective than G-CSF mobilised human CD34 for the engraftment of NOD-SCID mice (Broxmeyer *et al.*, 2005).

In the first phase-I human clinical study, AMD-3100 was well tolerated at doses up to 80 µg/kg i.v or s.c in 12 healthy volunteers (Hendrix *et al.*, 2000). Another study with 26 volunteers demonstrated up to a 12-fold increase in $CD34^+$ cell mobilisation within 4 to 6 h of s.c AMD-3100 at well tolerated doses (Liles *et al.*, 2003). In another study, in patients with multiple myeloma (n = 7) and non-Hodgkin's lymphoma (n = 6) who received a single dose of AMD-3100 (160 or 240 µg/kg s.c), the total WBC and PB $CD34^+$ counts increased rapidly and significantly at both 4 and 6 h (Devine *et al.*, 2004). AMD-3100 when combined with G-CSF resulted in a synergistic increase in $CD34^+$ cell mobilisation within 6 hours of injection (Liles *et al.*, 2003). A single dose of 240 µg/kg of AMD-3100 yielded similar $CD34^+$ cells/kg after a single leukapheresis when compared to the same normal volunteers mobilised with G-CSF (10 µg/kg/day) for 5 days (Liles *et al.*, 2005).

In another recent study by Flomenberg and his group, multiple myeloma (MM) and non-Hodgkin's lymphoma (NHL) patients were randomly assigned to an initial mobilisation with G-CSF versus G-CSF plus AMD-3100. The combination of G-CSF plus AMD-3100 was a superior mobilising regimen regardless of with which regimen the patient was first mobilised. It resulted in a daily increase of over 50% in the number of $CD34^+$ cells collected compared to G-CSF only (Flomenberg *et al.*, 2005). The use of AMD-3100 alone as a mobilising agent in humans is still under investigation and long-term studies are underway to accrue more patients and to examine other endpoints such as GvHD, relapse free survival, long term stable engraftment and overall survival (Devine *et al.*, 2004).

1.1.5.1.5 CTCE-0021 (SDF-1 peptide agonist)

CTCE-0021 (cyclo(Lys20-Glu24)-sdf-(1-31)-NH2) is a novel cyclized CXCR4 agonist peptide (SDF-1α analogue) developed to stabilize the SDF-1α-helix in order to increase its bioactivity, and terminating the C-terminus as an amide to reduce its immunogenicity. This compound retains comparable CXCR4 receptor agonist activity. In mice, a single bolus administration of CTCE-0021 demonstrated a rapid dose-dependent mobilisation of HSC between 5 min and 4 h post-dosing, with an increase in WBC resulting from an increase in granulocytes within 5 min post-dosing that persisted for approximately 24 h. The mechanisms involved in this CXCR4 agonist peptide mobilisation remain unknown, but scientists suggest that CTCE-0021 mobilisation is associated with a down-regulation of CXCR4 on HSC, and an alteration in the plasma to marrow SDF-1 gradient (Fukuda *et al.*, 2005). CTCE-0021 is an efficient and rapid mobiliser of polymorphonuclear cells (PMN) and HSC when used alone and shows synergistic activity when used in combination with G-CSF.

1.1.5.1.6 Stem cell factor (SCF)

SCF is a glycoprotein cytokine that plays a key role in haemopoiesis, acting both as a positive and negative regulator, often in synergy with other cytokines. It is also known as Kit ligand and binds its receptor, Kit, on the target cells that have an intrinsic protein-tyrosine kinase domain in their cytoplasmic regions. Recombinant human SCF (rHuSCF) stimulates pre-lineage-committed HSC. Most clinical studies of SCF report the use of this agent together with other cytokines. Limited reports of SCF by itself are available however, and this cytokine appears to result in a dose-dependent six- to ten-fold mobilisation of CFU-GM (Morstyn *et al.*, 1994). When SCF was used in conjunction with G-CSF it enhanced the mobilisation of stem cells from lymphoma patients undergoing auto-stem cell transplant (SCT) (Moskowitz *et al.*, 1997). Recently, rHuSCF (20 µg/kg/day) when combined with G-CSF (10 µg/kg/day) was shown to enhance the mobilisation of HSC in heavily pre-treated patients who have failed a previous attempt with G-CSF alone (Dawson *et al.*, 2005). Due to occasional anaphylactoid reactions to SCF, including angioedema, urticaria, pruritus, and laryngospasm (Costa *et al.*, 1996), the FDA decided not to approve the agent for use as an agent to enhance autologous stem cell mobilisation in the United States. SCF is approved for use in Canada and New Zealand (Nervi *et al.*, 2006) and is used under special arrangements in Australia.

1.1.5.1.7 CXCL2 (Gro-β)

Gro-beta is a CXC chemokine expressed when induced by serum or GF and/or by a variety of inflammatory mediators, such as IL-1 and tumour necrosis factor (TNF), in monocytes, fibroblasts, melanocytes and epithelial cells and has biological activities related to specific binding to the CXCR2 receptor.

The human CXCR2 selective ligand SB-251353, a recombinant N-terminal 4-amino acid truncated form of the human chemokine Gro, specifically binds only to CXCR2 and with greater potency than full-length Gro-β. It induces rapid mobilisation of HSC in mice and monkeys and synergizes with G-CSF (Hepburn *et al.*, 2001; King *et al.*, 2001). Hepburn and his colleagues suggested that binding of SB-251353 with hepatic sinusoids and connective tissue in the dermis possibly resulted from its interaction with heparin sulphate proteoglycan (Hepburn *et al.*, 2001). Administration of this ligand i.v is associated with a leukopenia within 5 min followed by a period of neutrophilia 30-45 min later. The combination of SB-251353 with G-CSF resulted in an augmented stem cell mobilisation compared with the use of G-CSF alone. The mechanism of action of SB-251353-induced HSPC mobilisation appears similar to IL-8, which involves up-regulation of matrix metalloproteinase-9 (MMP-9) activity (King *et al.*, 2001).

1.1.5.1.8 Interleukin-8 (IL-8)

IL-8 is an 8.9 kD protein with 77 amino acid residues. It belongs to a family of CXC chemo-attractant cytokines involved in chemotaxis and activation of neutrophils (Laterveer *et al.*, 1995). It is produced by a variety of cells including monocytes, neutrophils, fibroblasts, and endothelial cells, induced by pro-inflammatory cytokines such as TNF-α, IL-1, IL-2, IL-3, and GM-CSF. It promotes neutrophil chemotaxis and degranulation (Nervi *et al.*, 2006). It induces rapid mobilisation of progenitor cells and pluri-potent stem cells that are able to rescue lethally irradiated mice and that are able to completely and permanently repopulate host haemopoietic tissues (Laterveer *et al.*, 1995). Pre-treatment with an inhibitory anti-gelatinase-B Ab inhibited mobilisation of HSC, indicating that MMP-9 is involved as a mediator of IL-8-induced mobilisation of HSC.

Neutrophils are indispensable for IL-8-induced HSC mobilisation. This process is abolished in mice that are rendered neutropenic after administration of a depleting anti-GR-1 Ab, and is restored upon the infusion of purified neutrophils (Pruijt *et al.*, 2002). Also, neutralizing Abs against the β2 integrins lymphocyte function-associated molecule 1 (LFA-1) and Mac-1 (CD11b) prevented IL-8-induced HSPC mobilisation (Pruijt *et al.*, 1998).

1.1.5.1.9 Recombinant human growth hormone (rhGH)

GH is a polypeptide hormone synthesised and secreted by the anterior pituitary gland and which stimulates growth and cell reproduction in humans and other vertebrate animals. It is considered as a pleiotropic cytokine targeting a variety of non-haemopoietic and haemopoietic cells by binding to its specific receptor (Kopchick & Andry, 2000). RhGH increases colony formation by HSC (CFU-GM and erythrocyte burst forming unit (BFU-E)) *in vitro* (Merchav *et al.*, 1988) and induces significant HSC mobilisation in mice (Carlo-Stella *et al.*, 2004a). In humans, 16 patients with relapsed or refractory haematological malignancies who had failed a first mobilisation attempt with chemotherapy plus G-CSF have been remobilised with chemotherapy plus G-CSF plus rhGH. This combination resulted in efficient mobilisation and collection of $CD34^+$ cells with maintained functional properties. The exact mechanism by which rhGH restores stem cell mobilisation capacity in heavily pre-treated patients with relapsed or refractory haematological malignancies is not clear, but is probably related to the expansion of HSPC which become susceptible for release upon a subsequent or concomitant stimulus, such as G-CSF (Carol-Stella *et al.*, 2004b).

1.1.5.1.10 Recombinant human parathyroid hormone (rhPTH)

It is known that osteoblasts are activated by parathyroid hormone (PTH) or the locally produced PTH-related protein (PTHrP) through the PTH/ PTHrP receptor (PPR), and they produce haemopoietic growth factors (Taichman & Emerson, 1994; Taichman *et al.*, 1996; Taichman *et al.*, 2001). HSC derive regulatory information from bone, accounting for the localization of haemopoiesis in BM. It has been shown that PTH/PTHrP receptor-stimulated osteoblastic cells that are increased in number produce high levels of the Notch ligand, Jagged-1, and support an increase in the number of HSC with evidence of Notch1 activation *in vivo*. Furthermore, ligand-dependent activation of PTH/PTHrP receptors with PTH increased the number of

osteoblasts in stromal cultures, and augmented *ex vivo* primitive haemopoietic cell growth that was abrogated by gamma-secretase inhibition of Notch activation. An increase in the number of stem cells was observed in wild-type animals after PTH injection, and survival after BM transplantation was markedly improved. Therefore, osteoblastic cells are a regulatory component of the HSC niche *in vivo* that influences stem cell function (Calvi *et al.*, 2003).

1.1.5.1.11 Pegfilgrastim (pegylated G-CSF)

Pegfilgrastim is a covalent conjugate of recombinant methionyl human G-CSF (Filgrastim) and monomethoxypolyethylene glycol. Filgrastim is obtained from the bacterial fermentation of *Escherichia coli* transformed with a genetically engineered plasmid containing the human G-CSF gene. A 20 kD monomethoxypolyethylene glycol molecule is covalently bound to the N-terminal methionyl residue of Filgrastim to make Pegfilgrastim (average molecular weight 39 kD).

Pegylated G-CSF has a prolonged half life and was approved by the FDA in the USA to prevent prolonged neutropenia following chemotherapy for non-haematological malignancies. The administration of a single dose of 30-300 µg/kg Pegfilgrastim resulted in a significant mobilisation of $CD34^+$ cells in healthy donors (Molineux *et al.*, 1999). Current trials are underway to determine the relative efficacy of pegylated G-CSF as a mobilising agent both for patients undergoing autologous stem cell transplantation and for normal sibling donors who are donating stem cells for HLA-matched allo-SCT.

1.1.5.1.12 Thrombopoietin (TPO/MGDF)

The term "thrombopoietin" was first used by Kelemen in 1958 to describe the humoral substance responsible for increasing platelet production after the onset of thrombocytopenia (Kelemen *et al.*, 1958). Thrombopoietin (TPO), also referred to as c-MpI ligand, mpl ligand, megapoietin, and megakaryocyte growth and development factor (MGDF), is the most potent cytokine that physiologically regulates megakaryocytopoiesis (Wendling *et al.*, 1998). TPO is a hormone constitutively produced by the liver and kidneys. Plasma levels of TPO are regulated through receptor-mediated uptake, internalization and catabolism.

Of the haemopoietic GF, thrombopoietin has the longest half-life (i.e. 30 h). PEGylation of thrombopoietin further increases the plasma half-life by 10 fold. Following systemic administration, the platelet count begins to increase after 3-5 days. This is because thrombopoietin acts by stimulating the production and maturation of megakaryocytes (Kaushansky, 1998). The most common adverse events were disturbances of the gastrointestinal system, and arthralgia. In therapeutic doses TPO has no effect on platelet function.

TPO has pleiotropic effects on haemopoiesis. Some studies have showed that it also induces mobilisation of $CD34^+$ and it synergizes with G-CSF to enhance stem cell mobilisation (Kuter, 1996). In patients undergoing autologous transplantation using BM stem cells for breast cancer, administration of PEG-rhTPO accelerated the increase in the platelet count and permitted a 48% reduction in the use of platelet transfusions as compared with placebo (Beveridge *et al.*, 1997). Currently no thrombopoietins have been approved by the FDA or by Australian authorities.

1.1.5.2 The role of neutrophils and proteases in mobilisation

Mobilisation of HSPC requires a normal number of functional neutrophils (Liu *et al.*, 2000; Winkler & Levesque, 2006). Mice do not mobilise during the neutropenic phase caused by the injection of moAbs that bind to the mouse granulocyte antigen Ly-6G (Gr-1) (Pruijt *et al.*, 2002; Pelus *et al.*, 2004), but mobilisation can be restored following the infusion of purified mature neutrophils (Pruijt *et al.*, 2002). Mice knocked-out for G-CSF receptor ($G\text{-}CSFR^{-/-}$) are also neutropenic and do not mobilise (Liu *et al.*, 2000). Mobilisation with G-CSF activates neutrophil expression of FcγRI/CD64 (Kerst *et al.*, 1993), CD11b, FcγRIII/CD16 as well as the increased release of lactoferrin (de Haas *et al.*, 1994) and neutrophil elastase (de Haas *et al.*, 1994; Lévesque *et al.*, 2001) Thus, it is possible that neutrophil progenitors may be activated and degranulated directly in the BM stroma during mobilisation prior to their migration into the PB. Mobilisation induced by G-CSF or cyclophosphamide (CY) releases neutrophil elastase (NE) and cathepsin-G (CG) in large amounts in a proteolytically active form in the BM stroma during mobilisation induced by G-CSF or CY (Lévesque *et al.*, 2001; Lévesque *et al.*, 2002) or Groβ/CXCL2 (Pelus *et al.*, 2004), as is MMP-9/gelatinase B. Once released into the BM environment, these proteases can cleave and inactivate a number of proteins essential to the retention of

HSPC within the BM, particularly the cell adhesion molecule VCAM-1/CD106, the chemokine SDF-1, its receptor CXCR4 (in humans) and the receptor c-KIT, events that can all individually trigger HSPC mobilisation (Winkler & Levesque, 2006). Moreover, expression of serpina1 and serpina2, naturally occurring inhibitors of these proteases, is markedly reduced after G-CSF treatment (Winkler *et al.*, 2005). Consequently the role of NE and CG in HSC mobilisation remains unclear.

Neutrophilia always precedes the HSPC mobilisation induced by physical exercise, adrenocorticotropic hormone (Barrett *et al.*, 1978), endotoxin (Cline & Golde, 1977), sulphated polysaccharides and polyanions (van der Ham *et al.*, 1977), myelosuppressive chemotherapy (To *et al.*, 1984; To *et al.*, 1989), chemokines (Pruijt *et al.*, 1999; King *et al.*, 2001), or haemopoietic growth factors (Molineux *et al.*, 1990; Sato *et al.*, 1994; Glaspy *et al.*, 1997; Molineux *et al.*, 1991; Molineux *et al.*, 1997; Torii *et al.*, 1998) and the degree of neutrophilia is predictive of the level of mobilisation (Krieger *et al.*, 1999; Roberts *et al.*, 1997).

VCAM-1, which is expressed on BM stromal cells, is a substrate of the two neutrophil serine-proteases, NE and CG (Lévesque *et al.*, 2001; Lévesque *et al.*, 2002; Lévesque *et al.*, 2004). It is essential for homing of HSPC to the BM during development and following transplantation (Winkler & Levesque, 2006). During mobilisation, active NE and CG (sole regulators of VCAM-1 levels) in the extracellular fluid of the BM directly cleave VCAM-1 removing the integrin-binding domain from the surface of BM stromal cells. This increases the concentration of soluble VCAM-1 fragments and NE in the blood of mobilised patients. Any inhibition of the interaction between VCAM-1 and α4-integrins particularly α4β1 (VLA-4) expressed by HSPC leads to strong mobilisation of HSPC within the BM (Lévesque *et al.*, 2001; Lévesque *et al.*, 2002).

Several studies suggest an important role of the tyrosine-kinase receptor c-KIT/CD117 in mobilisation. Studies suggest c-KIT plays an important role in the retention of HSPC within the BM and their mobilisation, since PB HSPC express less c-KIT than steady-state BM HSPC in human and mouse (Winkler & Levesque, 2006). c-KIT tyrosine-kinase activates VLA-4 and VLA-5-mediated adhesion to VCAM-1 and fibronectin (Winkler & Levesque, 2006) and HSPC motility (Kim & Broxmeyer,

1998). c-KIT is cleaved by the neutrophil proteases NE, CG and MMP-9 and therefore that proteolytic cleavage may be in part responsible for reduced c-KIT expression on mobilised human and mouse HSPC (Lévesque *et al.*, 2003). Since c-KIT and CXCR4 are strong activators of VLA-4 avidity for its ligands, the cleavage of c-KIT and CXCR4 may also be responsible for the reduced capacity of VLA-4 on the surface of mobilised blood HSPC to bind to VCAM-1 (Lichterfeld *et al.*, 2000).

Proteases are expressed by neutrophils in a form that must be 'activated' to gain function. Active proteases are generally either retained within specific cell granules (to be released only upon cell activation/degranulation) or attached to the cell surface. Triggering the expansion and/or activation and/or degranulation of neutrophils within the BM is a common step in the majority of mobilising protocols. This suggests that mobilisation involves a shift in the proteolytic balance between protease release and sequestration by inhibitors (Winkler & Levesque, 2006).

On the other hand, neutrophil activation is controlled by a group of protease inhibitors such as the serine-protease inhibitors (serpins) of the 'Clade A' group. They are the main inhibitors of neutrophil serine-protease activity within BM extravascular compartments. There are two serpins, serpina1 and serpina3 that inhibit NE and CG activity (serpina1 has a preference for NE and serpina3 for CG).

It has been reported that the levels of serpins drop dramatically within the BM during mobilisation induced by either G-CSF or CY, boosting the levels of active neutrophil serine-proteases with concomitant cleavage and inactivation of molecules essential for the retention of HSPC (Winkler *et al.*, 2005). Recent data suggest that maintaining proteolytic balance is an essential component in the regulation of homeostasis within the BM microenvironment and in the trafficking of HSPC, and the degree of mobilisation in humans is significantly correlated with blood concentrations of NE and MMP-9 (Lévesque *et al.*, 2001). However it appears that protease activity is not the only mechanism that plays a role in HSPC mobilisation. For example, recently it has been deduced from a mouse model that an array of non-specific inflammatory/immune responses may play a role in HSPC mobilisation (Velders *et al.*, 2004). G-CSF activates the complement system, critical in inflammation and

innate immunity, possibly as a result of local neutrophil activation, which seems also to be involved in BM retention and mobilisation of HSPC (Ratajczak *et al.*, 2004a).

Administration of G-CSF results in an increase in blood concentration of soluble urokinase-type plasminogen activator (suPAR), up-regulation of uPAR on $CD33^+$ myeloid and $CD14^+$ monocytic cells and release of a cleaved form of suPAR that inhibits $CD34^+$ cell migration in response to SDF-1, suggesting that uPAR shedding may have an important role in HSPC mobilisation (Selleri *et al.*, 2005).

1.1.6 Homing of HSPC

Homing is the first process, measured in hours and no longer than 1-2 days, in which circulating HSPC actively cross the blood/BM endothelium barrier and lodge at least transiently in the BM compartment by activation of adhesion interactions prior to their proliferation. Homing is a rapid process, which can lead to transient retention and does not require cell division, while engraftment does (Lapidot, 2005) [Figure 1.4 and 1.5]. The mechanism can be described in three steps.

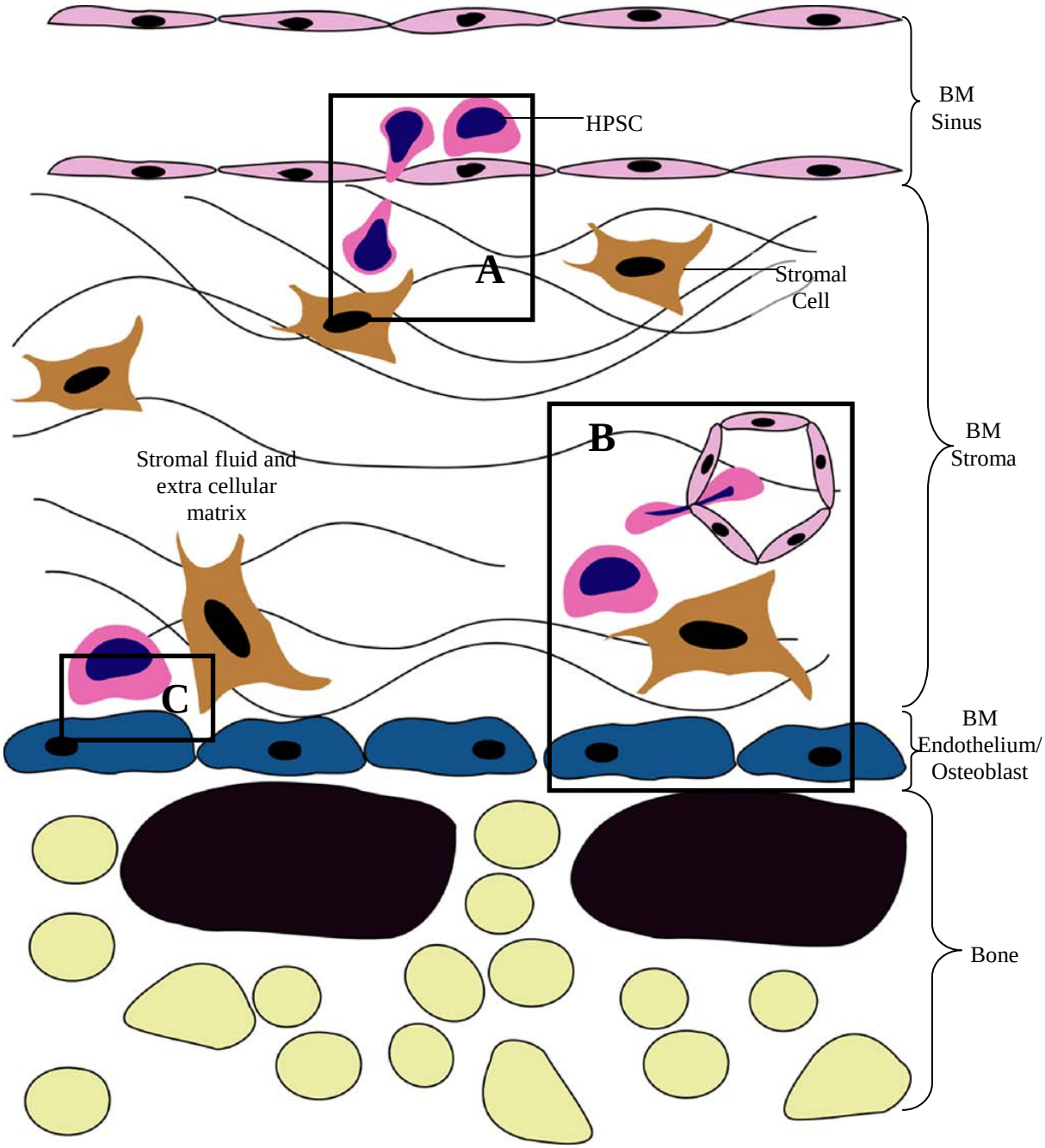


Figure 1.4: General schematic diagram for the process of HSC homing from the PB until they are lodged into their specific niches in the BM. (A) the process of homing, (B) migration through BM stroma and (C) HSPC-endosteal specialised niche synapse.

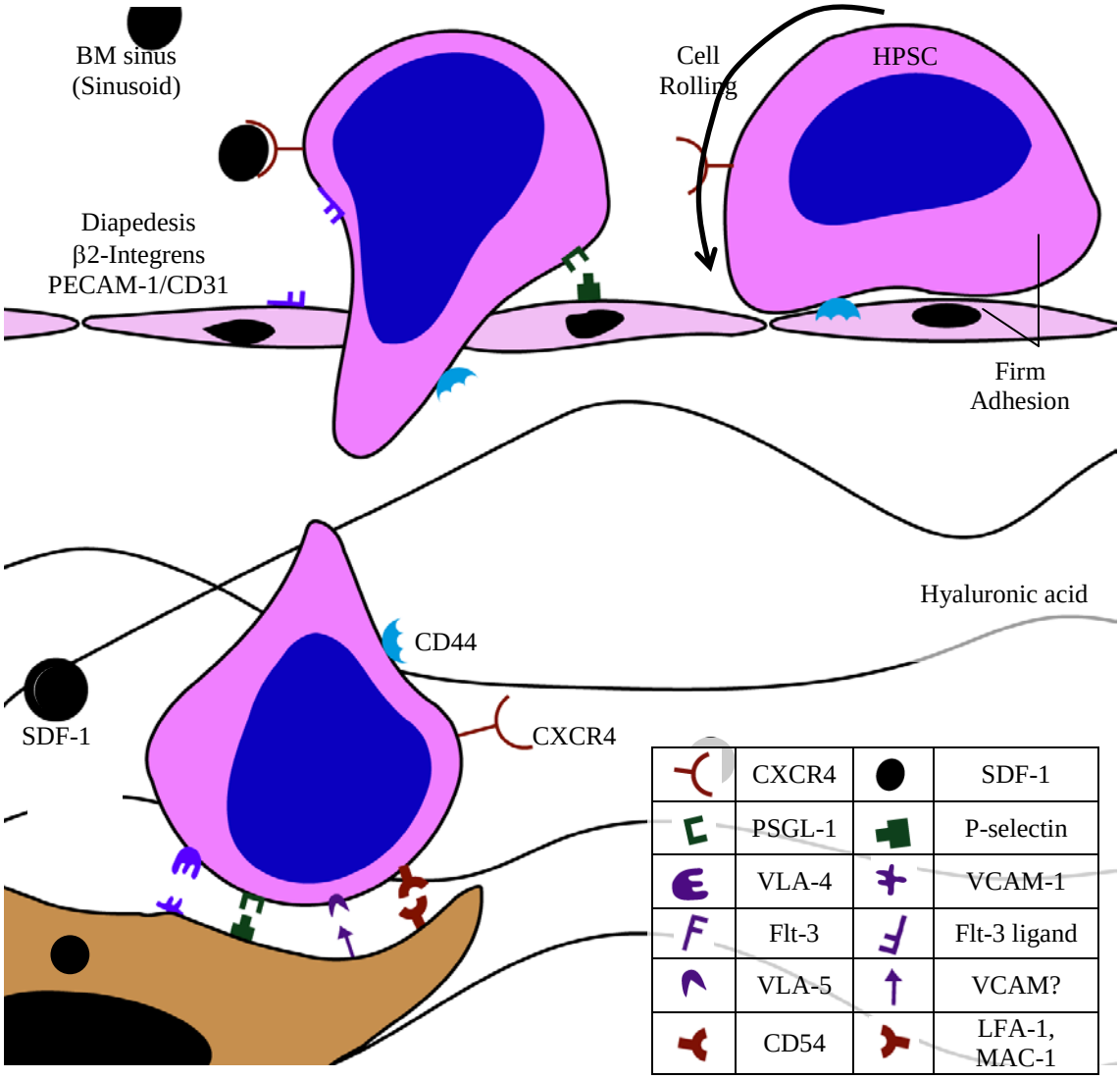


Figure 1.5: Schematic diagram showing the process of HSC homing.

1.1.6.1 Extravasation of HSPC through BM sinuses

Extravasation refers to the movement of the HSPC from blood capillaries to the BM tissue [Figure 1.6]. It requires the presence of both selectins and integrins and it begins with the rolling and tethering of circulating HSPC, which express selectin receptors, upon the BM endothelium (Frenette *et al.*, 1998; Lévesque *et al.*, 1999; Dimitroff *et al.*, 2001; Mazo *et al.*, 1998; Katayama *et al.*, 2005; Winkler *et al.*, 2004). The BM endothelium itself expresses the adhesion molecules P-selectin, E-selectin (CD62P and CD62E), the α4 integrin ligand and the vascular cell adhesion molecule-1 (VCAM-1/CD106) (Schweitzer *et al.*, 1996; Sipkins *et al.*, 2005). This was proved when mice knocked-out for both P- and E-selectin genes, halved the degree of HSPC homing (Frenette *et al.*, 1998).

The firm attachment of HSPC and keeping them in place requires both E-selectin-mediated and α4 integrin-mediated interactions. HSPC express selectin receptors such as PSGL-1/CD162 (which binds to both P- and E-selectin) and a glycoform of CD44 (which binds E-selectin), and two α4 integrins; α4β1/VLA-4 (Arroyo *et al.*, 2000; Vermeulen *et al.*, 1998; Zanjani *et al.*, 1999; Katayama *et al.*, 2004) and α4β7 (Katayama *et al.*, 2004) with the respective ligands VCAM-1/CD106 and the mucosal addressin cell adhesion molecule-1 (MadCAM-1) expressed on BM stroma.

The importance of both integrins and selectins in the process of extravasation was proven when homing of HSPC was shown to be reduced up to half following blocking of donor HSPC α4-integrins. It was further reduced to 90% if the blocked cells were infused into E-selectin knockout mice (Frenette *et al.*, 1998; Katayama *et al.*, 2003; Bonig & Priestley, 2005).

α4β1 integrin is activated *in vitro* by the SDF-1 (Hidalgo *et al.*, 2001; Peled *et al.*, 2000; Wright *et al.*, 2002) and a range of haemopoietic growth factors including KIT ligand, GM-CSF, G-CSF (Lévesque *et al.*, 1996; Lévesque *et al.*, 1995), thrombopoietin (Cui *et al.*, 1997), Flt-3 ligand and hepatocyte growth factor/c-met ligand (Weimar *et al.*, 1998; Solanilla *et al.*, 2003). Mice deficient for either SDF-1 or CXCR4 die perinatally with dramatically reduced BM lymphopoiesis and myelopoiesis (Ma *et al.*, 1998; Zou *et al.*, 1998) and defective colonization of the BM

by HSC coming from the foetal liver (Ara *et al.*, 2003). The SDF-1/CXCR4 chemotactic axis plays a critical role in BM colonization and homing during ontogeny. After the establishment of a firm adherence between the HSPC and the endothelial cells of the sinusoid, a complex series of interactions between the HSPC and the endothelium leads to diapedesis of the HSPC and the endothelial cells. This particular step is the least explored and understood in HSPC homing. However, it is believed to involve heterotypic adhesion molecules such as β2 integrins and homotypic cell adhesion molecules such as platelet-endothelial cell adhesion molecule-1 (PECAM-1/CD31) (Yong *et al.*, 1998).

1.1.6.2 Migration of HSPC through the BM stroma

The HSPC migrate through the BM stroma, after passing the endothelial sinus, by interacting with components of the surrounding extracellular matrix (ECM) such as hyaluronic acid, laminins, collagens and fibronectin via CD44 (Peach *et al.*, 1993) and β1 integrins (Simmons *et al.*, 1997). The migratory direction is maintained by the SDF-1 gradient produced by BM stromal cells, where HSPC and HSC follow different routes depending on their maturation stage. The committed HSPC distribute in an apparent random manner within the central marrow and the true HSC preferentially home in proximity to the bone-BM interface or endosteum (Lord *et al.*, 1975; Mason *et al.*, 1989; Nilsson *et al.*, 2001) and in the proximity of BM endothelial sinuses (Kiel *et al.*, 2005). The preferential lodgement of HSPC at the endosteum is likely to be due to osteoblasts, which have been recently identified as a critical component of the HSC niche (Zhang *et al.*, 2003; Calvi *et al.*, 2003; Visnjic *et al.*, 2004) [Figure 1.6].

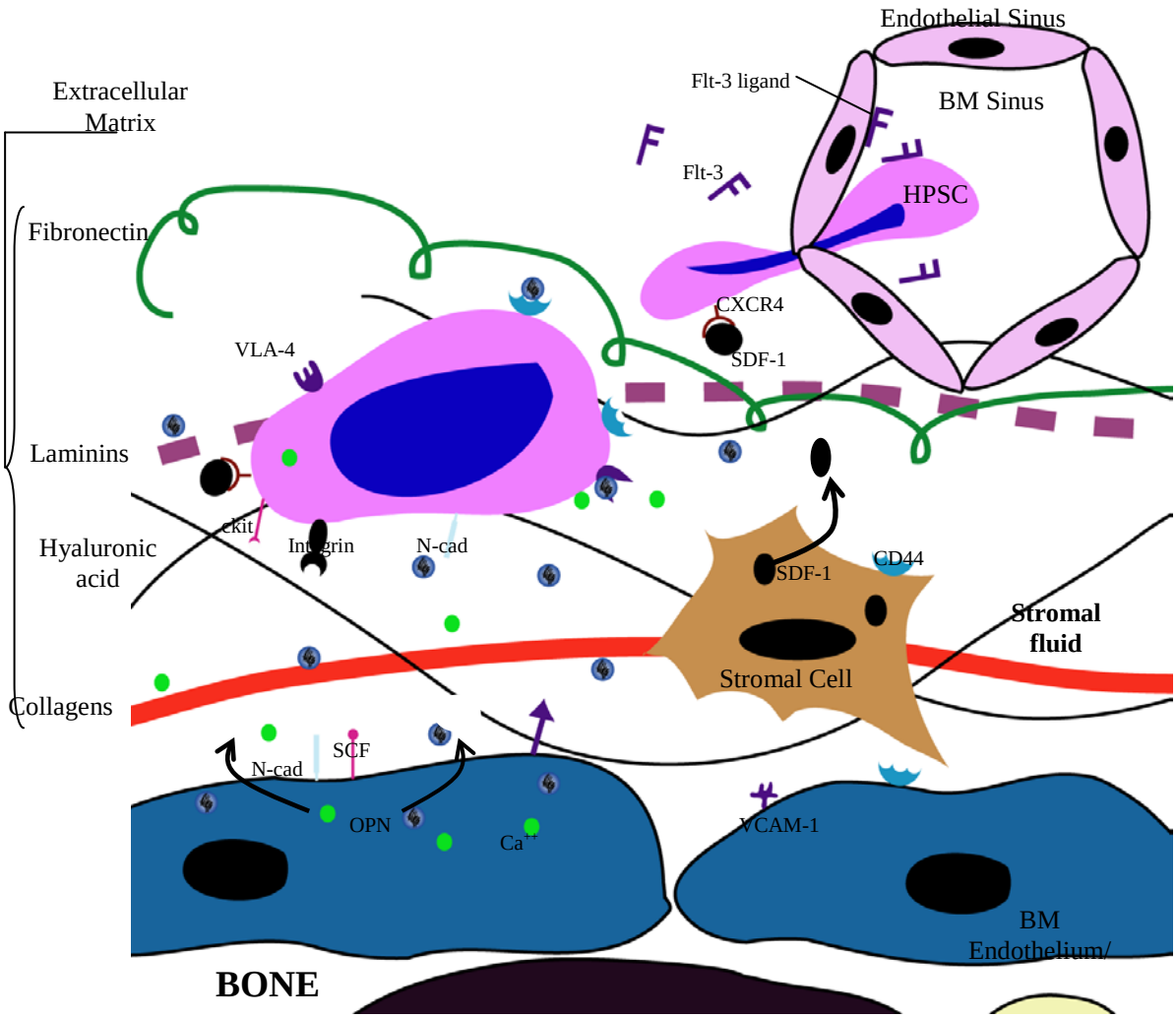


Figure 1.6: Schematic diagram showing the process of HSC extravasation and migration through BM stroma.

1.1.6.3 Lodgement of HSPC into specific niches

HSPC will lodge into the endosteal niche which is a site of bone turnover with concomitant bone formation and bone degradation that releases soluble Ca^{2+} in the BM stromal fluid (Winkler & Levesque, 2006). This process is driven by the essential calcium gradient (Adams *et al.,* 2006) and an array of adhesive interactions mediated by osteoblast-produced ECM proteins such as osteopontin (OPN, a bone matrix protein that mediates cell adhesion via CD44 and integrins), N-cadherin (N-Cad, which mediates homotypic calcium-dependent adhesion and is expressed by both osteoblasts and HSC), transmembrane KIT ligand and the polysaccharide hyaluronic acid (Winkler & Levesque, 2006).

Adhesive interactions mediated by OPN, hyaluronic acid and N-cad also initiate signalling events in HSPC that all delay their proliferation whereas a c-KIT-mediated interaction promotes proliferation. Thus, these adhesive interactions not only mediate tight adhesion to the haemopoietic niche but are also likely to regulate their self-renewal *in vivo* (Winkler & Levesque, 2006) [Figure 1.7].

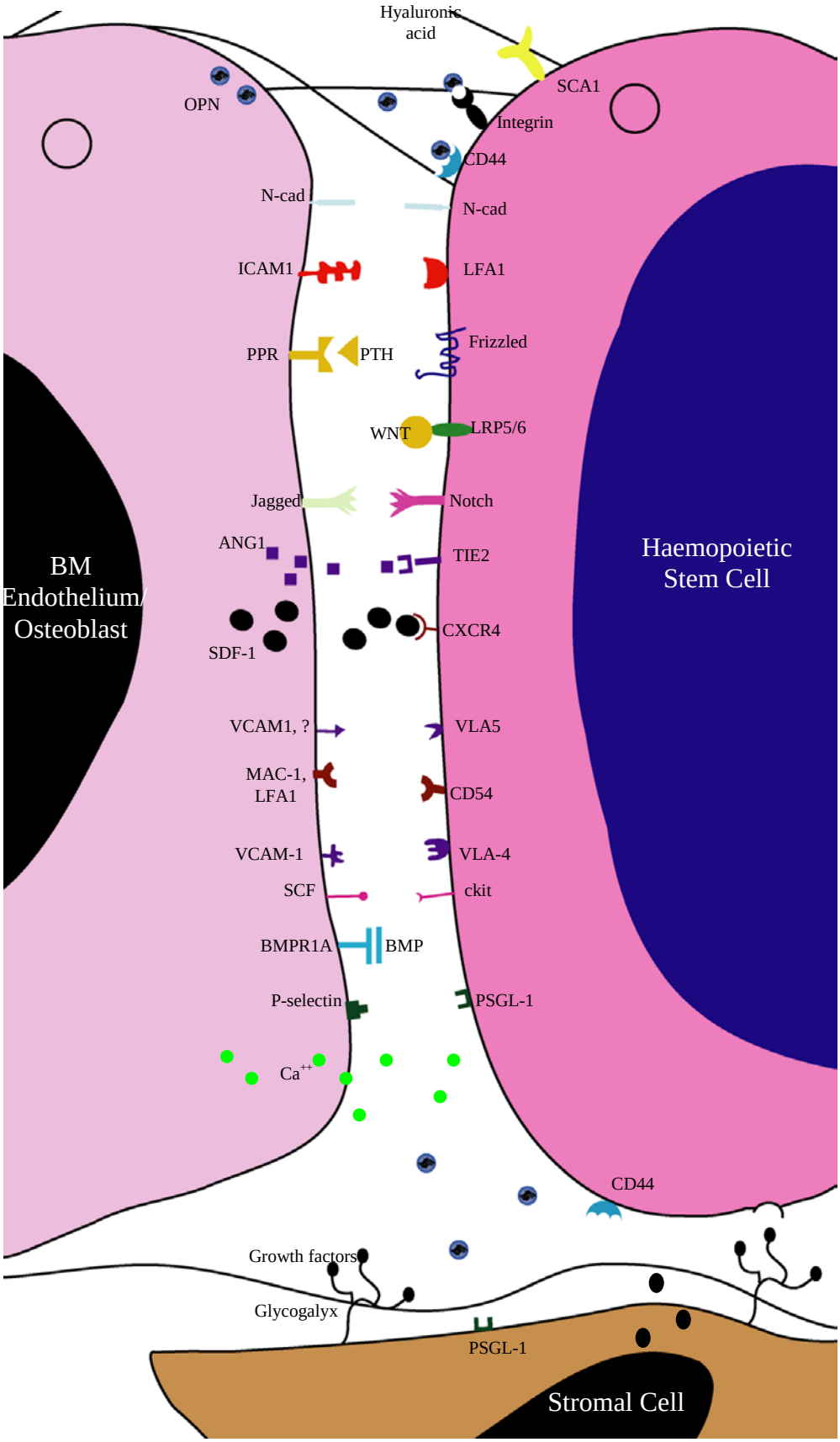


Figure 1.7: Schematic diagram showing the synapse between HSPC and osteoblast specialised niche cell.

1.1.7 *In vitro* expansion of HSPC

All mature cells in the blood and immune system are derived, and can be generated from, single haemopoietic stem cells. Because HSPC have the ability to reconstitute the whole haemopoietic system and even some other tissues in the body, the development of transplantation strategies have become widely spread. Although the first transplant attempts were carried out using BM cells, we now know that cells from umbilical cord or PB can also be used.

There are several types of HSPC transplantation. If cells are collected from the patient, it is an autologous transplant. Use of cells derived from another person may be syngeneic (i.e. the donor is an identical twin) or allogeneic. Allogeneic donors may be matched or partially mismatched siblings or other family members, or unrelated. In the latter case the donor is usually identified through the international Bone Marrow Donor Registry.

For expansion, scientists culture cells in a base liquid medium containing bovine serum albumin (BSA), human insulin, human transferrin, and a reducing agent such as 2-mercaptoethanol. Cytokines, or specific GFs, appropriate to the culture of a specific HSC, are also added. The goal of an expansion approach, as its name suggests, is to increase the number of HSC with minimal differentiation.

Recently, studies of the regulation of HSC expansion at the molecular level have focused on genes which can be divided into three main categories: genes that encode transcription factors such as homeobox B4 (HOXB4); genes that encode signalling molecules such as haemopoietic cytokines, the WNT-protein family and the Notch family of receptors; and genes that regulate the cell cycle, such as p18 and p21. There is evidence that modulating the expression of genes of these categories can be used to achieve expansion of mouse HSC (Sorrentino, 2004). There is less information regarding the human HSC, especially because HSC assay systems are not as well defined for human as for mouse HSC.

The first attempt to amplify HSC focused on haemopoietic cytokines. For example, human HSC are induced to proliferate by exposure to Flt-3 and TPO (Petzer *et al.*, 1996). However, exhaustive studies using combinations of multiple cytokines have

not yet identified conditions that result in more than a fourfold increase in HSC numbers (Sorrentino, 2004).

In mice, HSC can be stimulated to under go self-renewal by exposure to IL-3, IL-6 and SCF. Use of vascular endothelial growth factor (VEGF) to modulate HSC function *in vitro* is difficult because VEGF needs to interact with its intracellular ligand. Fibroblast growth factor-1 (FGF-1) also promotes long-term growth of HSC in culture, but it is not clear whether FGF-1 functions mainly as a survival factor or induces proliferation (Sorrentino, 2004).

Expansion of mouse HSC has been achieved by modulation of the Notch-1 signalling pathway. Constitutive expression of activated Notch-1 HSC resulted in immortalized, cytokine-dependent cell lines that could reconstitute irradiated mice (Varnum-Finney, 2000).

The soluble form of human Jagged-1 is a possible new growth factor for HSC and mediates their expansion when added to the liquid culture, providing an opportunity for the clinical utility of Notch ligands in the expansion of primitive cells capable of haemopoietic reconstitution (Karanu, 2000). However, this approach could be limited because Notch-1 could favour lymphoid differentiation compared to myeloid differentiation (Stier *et al.*, 2002).

Antonchuk and colleagues showed that mouse BM HSC can be expanded 1,000-fold when transduced with the transcription factor HOXB4.5 (Antonchuk *et al.*, 2002). Researchers in George Daley's laboratory injected HSC expressing HOXB4 into irradiated adult mice and showed that the donor cells differentiated into lymphoid and myeloid cells (Kyba *et al.*, 2002). These findings suggest that transduction of HSC with HOXB4 could serve as a more efficient method for growing the large quantities of HSC necessary for therapeutic applications.

However, whether it is safe to use HOXB4 expression for the expansion of HSC is an open question. Vectors that express other HOX genes, such as HOXB8 or HOXA10, can cause leukaemia in mouse models (Perkins & Cory, 1993; Thorsteinsdottir *et al.*, 1997) by inducing conditional immortalization and by altering normal differentiation.

Although there have been no cases of leukaemic transformation when using HOXB4 in similar assays, it is possible that transformation could occur with a low incidence or a long latency period. Another concern is that high levels of HOXB4 expression, achieved by using adenoviral vectors or retroviral vectors with strong promoters, have been associated with abnormalities in myeloid differentiation (Brun *et al.*, 2003; Schiedlmeier *et al.*, 2003). Therefore, the use of a HOXB4 gene that can be regulated, or the direct use of a recombinant protein, might be necessary to minimize the risk of inadvertent effects.

1.1.8 Engraftment and repopulation

Short-term engraftment ranging from weeks to a few months is initiated by differentiating progenitors. Durable long-term multilineage engraftment (many months in mice, and years in patients) is carried out by stem cells following their unique homing to their specialized niches. HSC require the BM microenvironment, which regulates their migration, proliferation, and differentiation in order to maintain active haemopoiesis throughout life. (Lapidot *et al.*, 2005) Engraftment does require cell division. Many cell types, including long-term repopulating human $CD34^+/CD38^-$ stem cells, short-term repopulating $CD34^+/CD38^+$ progenitors, and mature, specialized T-cells and neutrophils, can home to the BM. But it is only the stem cells which can home to their endosteal niches that can initiate long-term repopulation (Lapidot *et al.*, 2005).

1.2 Haemopoietic cytokines and their receptors

Haemopoietic cytokines are signalling molecules that induce proliferation at various stages of haemopoietic development. Many of these cytokines are locally produced in the BM microenvironment by stromal cells (Sorrentino, 2004). Chemokines are classified into two main subfamilies, based on the relative position of the two first cysteine residues CC and CXC chemokines (Amara *et al.*, 1999). Since the 1980s the rapid discovery of new haemopoietic cytokines has enabled scientists to test them in HSC culture (Sorrentino, 2004).

1.2.1 SDF-1 (CXCL12)

SDF-1, which is also called pre-B cell-stimulating factor, is a CXC chemokine which was originally purified from BM cell supernatants (Devine *et al.*, 2004). There are two variants of SDF-1 (α and β), which are generated by alternative splicing from a unique SDF-1 gene (Tashiro *et al.*, 1993; Amara *et al.*, 1999), on chromosome 10 (Shirozu *et al.*, 1995). The alpha form is the most abundant. Human and murine SDF-1α proteins differ by a single residue at position 18 (valine to isoleucine in the murine protein) (Tashiro *et al.*, 1993; Amara *et al.*, 1999).

SDF-1 also has a higher degree of inter-species conservation than other chemokines, with 99% identity between the human and the mouse sequences (Tashiro *et al.*, 1993; Shirozu *et al.*, 1995). SDF-1 is a highly basic (Tashiro *et al.*, 1993), heparin-binding protein with no glycosylation site in its sequence (Bleul *et al.*, 1996).

SDF-1 binds to and activates the CXCR4 receptor (Oberlin *et al.*, 1996; Crump *et al.*, 1997). The receptor activation involves dimerisation, interaction with Gαi (alpha subunit of inhibitory G protein), phosphorylation by JAK2/JAK3 kinase (janus kinase, non-receptor protein tyrosine kinase) and phosphorylation of signal transducers and activators of transcription (STAT) factors (Vila-Coro *et al.*, 1999).

SDF-1 is constitutively expressed by several non-haemopoietic BM stromal cells, especially osteoblasts, and is present in many other tissues (Winkler & Levesque, 2006; Semerad *et al.*, 2005). It is a potent chemoattractant for lymphocytes and monocytes, and it enhances B-cell proliferation (Tashiro *et al.*, 1993; Nagasawa *et al.*, 1994; Bleul *et al.*, 1996), leading to the suggestion that it may be an agent of immune

surveillance rather than a mediator of the inflammatory response (Bleul *et al.*, 1996). It appears to play a role in trafficking or homing of lymphocytes and HSC (Aiuti *et al.*, 1997) and in the chemoattraction towards the BM of other $CXCR4^+$ cells (Ratajczak *et al.*, 2004b).

1.2.2 CXCR4

CXCR4 (fusin or LESTR or HUMSTR) is a member of the large family of seven transmembrane domain receptors coupled to heterotrimeric G_1 proteins. Its size is 352 amino acids; 39,745 Dalton. A CXC chemokine has a single amino acid between the first two cysteines of the characteristic four cysteine motif. It is expressed by HSPC as well as a number of mature leucocytes (Winkler & Levesque, 2006). Binding with its only known ligand, SDF-1, results in activation of multiple signal transduction pathways, ultimately triggering chemotaxis (Bleul *et al.*, 1996; Oberlin *et al.*, 1996; Devine *et al.*, 2004). Furthermore, the interaction between SDF-1α and CXCR4 appears to be unique and non-promiscuous. SDF-1α stimulates intracellular calcium flux and chemotaxis in monocytes, T-lymphocytes, and neutrophils, a characteristic shared with other CXC chemokines (Amara *et al.*, 1999).

HIV and related viruses require co-receptors, in addition to T-cell antigen T4 (CD4), to infect target cells. CXCR4 acts as a co-receptor for the T-cell tropic HIV-1 and -2 viral entries into cells. The amino-terminal domain and the second extracellular loop of CXCR4 serve as HIV binding sites (Feng *et al.*, 1996; Endres *et al.*, 1996). A moAb (clone 12G5) to the CXCR4 structure was developed by Enders and his colleagues in 1996. By flow cytometry, clone 12G5 reacts with CXCR4 expressed on a variety of human cell lines, including Sup-T1, Hut-78, Molt4, CEMss, Daudi and Hela, as well as PB lymphocytes. Clone 12G5 can also neutralize infection and inhibit syncytium formation induced by the HIV virus (Endres *et al.*, 1996). The epitope for the 12G5 moAb has been mapped to a region of the CXCR4 receptor that includes extracellular loops 1 and 2 (extracellular loop 1 plays a more critical role in the overall epitope structure) (Lu *et al.*, 1997).

CXCR4 is also required for the infection by dual-tropic strains of HIV-1 and mediates CD4 independent infection by HIV-2. SDF-1 can prevent infection by T-tropic HIV-1 by blocking CXCR4. CXCR4 associates with the surface CD4-gp120 complex before

HIV enters target cells. CXCR4 messenger RNA levels correlate with HIV-1 permissiveness in diverse human cell types (Doranz *et al.*, 1996; Endres *et al.*, 1996).

CXCR4 is also involved in haemopoiesis and in cardiac ventricular septum formation. It plays an essential role in the vascularization of the gastrointestinal tract, probably by regulating vascular branching and/or remodelling processes in endothelial cells. It could be involved in cerebellar development and hippocampal-neuron survival. It also mediates migration of resting leucocytes and haemopoietic progenitors in response to SDF-1. It also plays a role of receptor for SDF-1 in directing primordial germ cell migration (Tachibana *et al.*, 1998).

1.2.3 SDF-1 and CXCR4 interaction and disruption

SDF-1/CXCR4 interactions and signalling have been implicated as a principal axis regulating retention, migration, and mobilisation of HSC during steady-state homeostasis and injury. It has been shown that enriched human primitive, normal human $CD34^+/CD38^+$ severe combined immunodeficient (SCID) repopulating cells (SRCs) homing to the BM require functional interactions between human CXCR4 and murine SDF-1, which is presented on the vasculature endothelium and various BM stromal cells. Repopulation by SRCs and leukaemia initiating cell (SLIC) function (both homing and development) are also CXCR4 dependent (Lapidot *et al.*, 2005).

The SDF-1 was first identified as an attractant to immature haemopoietic cells, expressing the primitive blood cell marker CD34, and it has been suggested to play a role in chemically induced mobilisation of human HSC (Rosu-Myles *et al.*, 2000).

Disruption of this interaction either by administration of SDF-1 or CXCR4 antagonists, or adenovirus-mediated over-expression of SDF-1 in the liver, leads to mobilisation in mice (Shen *et al.*, 2001; Broxmeyer *et al.*, 2005; Hattori *et al.*, 2001).

Studies of SDF-1 or CXCR4 deficient mice have established that the genes for both SDF-1 and CXCR4 are necessary for the normal migration of HSC from the foetal liver to the BM and in the efficient retention of myeloid precursors in the adult BM (Kawabata *et al.*, 1999; Ma *et al.*, 1999).

Genetic evidence for the role of SDF-1 and CXCR4 in the regulation of murine HSC has been examined by using SDF-1$^{-/-}$ and CXCR4$^{-/-}$ knockout mice. Null mutations of either ligand or receptor are lethal during embryonic development; however, transplantation of CXCR4$^{-/-}$ foetal liver stem cells into normal recipients permits BM engraftment with eventual defects in both myelopoiesis and B-lymphopoiesis (Rosu-Myles *et al.*, 2000).

According to the Ratajczak group findings, the BM provides not only a home for HSC but also a 'hideout' for already differentiated CXCR4^{+} tissue-committed stem/progenitor cells that follow an SDF-1 gradient, which could be mobilised into PB and subsequently take part in organ/tissue regeneration (Ratajczak *et al.*, 2004b). Elevation of SDF-1 levels in the blood by administration of SDF-1 or by injection of an adenoviral vector expressing SDF-1 is associated with a significant mobilisation of HSC into the blood (Kawabata *et al.*, 1999; Ma *et al.*, 1999).

During G-CSF-induced mobilisation, SDF-1 levels in the BM fall sharply. This is a critical step in HSPC mobilisation (Petit *et al.*, 2002; Semerad *et al.*, 2002; Levesque *et al.*, 2003). The magnitude of the decrease in SDF-1 expression correlates well with the magnitude of HSC mobilisation (Semerad *et al.*, 2005). Also, there is evidence that CXCR4 on HSC may be proteolytically inactivated during G-CSF treatment further disrupting SDF-1/CXCR4 signalling (Levesque *et al.*, 2003).

The mechanisms that regulate SDF-1 expression in the BM are controversial. As mentioned earlier, there is a drop in SDF-1 levels in the BM during mobilisation that coincides with the peak of proteolytic activity. Both SDF-1 and CXCR4 are substrates of the neutrophil proteases NE, CG and MMP-9, and reports suggest that the drop in SDF-1 is due at least in part to proteolytic cleavage, providing regulation for SDF-1 (Petit *et al.*, 2002; Levesque *et al.*, 2003). However, mice genetically lacking NE and CG display normal G-CSF-induced HSPC mobilisation, and the expected decrease in BM SDF-1 protein was observed (Levesque *et al.*, 2004). Thus, the G-CSF-induced decrease in SDF-1 protein expression in the BM does not require these proteases. Recently, it has been shown that SDF-1 mRNA expression decreases sharply during G-CSF induced HSC mobilisation and correlates well with SDF-1 protein expression (Semerad *et al.*, 2005). These data suggest that G-CSF regulates SDF-1 expression in

the BM primarily at the mRNA level. A potential mechanism for this is suggested by the observation that G-CSF treatment potently suppresses osteoblasts, the major source of SDF-1 in the BM (Semerad *et al.*, 2005).

During mobilisation, the neutrophil proteases NE, CG, MMP-9 and CD26 (a transmembrane dipeptidylpeptidase expressed on the surface of myeloid cells and BM HSPC) all cleave the N-terminal domain of CXCR4 on HSPC. This leads to loss in the chemotactic response of mobilised HSPC (Lévesque *et al.*, 2003). Therefore, both SDF-1 and CXCR4 on HSPC are inactivated by proteolytic cleavage.

SDF-1 and its receptor CXCR4 are expressed in oval cells during hepatic regeneration. It is also suggested that SDF-1 stimulates the proliferation of these precursor cells through an autocrine/paracrine pathway. Treatment of rats with fucoidan markedly decreased oval cell accumulation. This sulphated polysaccharide binds SDF-1, releases the chemokine from cell membrane-associated heparin sulphate protoglycans and could therefore inhibit its biological activity (Mavier *et al.*, 2004).

1.2.4 CXCR4 and tumour

Several paediatric tumours metastasizing to the BM such as rhabdomyosarcoma, neuroblastoma, nephroblastoma, hepatoblastoma and retinoblastoma express functional CXCR4 on their surface and follow an SDF-1 gradient. These tumours derive from early muscle, neural, kidney, liver and retinal pigment epithelial cells, respectively, which are also $CXCR4^+$. Hence it seems likely that the CXCR4/SDF-1 axis plays an essential role in the chemoattraction/retention in BM not only of $CXCR4^+$ tissue-committed stem/progenitor cells but also of $CXCR4^+$ tumour cells (Ratajczak *et al.*, 2004b).

1.2.5 Interleukin-12 (IL-12)

IL-12, also known as natural killer cell stimulatory factor (NKSF) or cytotoxic lymphocyte maturation factor (CLMF), is a pleiotropic cytokine originally identified in the medium of cultured EBV-transformed RPMI-8866 cells. IL-12 is a 75 kD glycoprotein heterodimer composed of two genetically unrelated subunits linked by a disulphide bond. The smaller subunit (p35) has homology to IL-6 and G-CSF while the larger subunit (p40) demonstrates similarity to the soluble receptor for IL-6,

leading to the suggestion that IL-12 might have evolved from a cytokine/soluble receptor complex (Kobayashi *et al.*, 1989; Stern *et al.*, 1990; Gubler *et al.*, 1991; Gearing & Cosman, 1991; Merberg *et al.*, 1992).

IL-12 apparently shows species specificity, with human IL-12 showing minimal activity in the mouse system (Gubler *et al.*, 1991; Schoenhaut *et al.*, 1992). Each subunit of IL-12 apparently arises from a single copy gene. The mRNA transcription of the subunits is closely coordinated, although an excess of the larger subunit has been shown to be produced by B cells in addition to active IL-12 (Kobayashi *et al.*, 1989; Gubler *et al.*, 1991). Expression of p35 is reported to be enhanced by simultaneous expression of p40. IL-12 activity cannot be demonstrated in the absence of either chain (Gubler *et al.*, 1991; Wolf *et al.*, 1991). As suggested by their names, p35 has a native molecular weight of 35 kD while p40 has a native molecular weight of 40 kD. In humans, p35 is 197 amino acid residues in length with a predicted molecular weight of 22.5 kD. This subunit possesses seven cysteines plus three potential N-linked glycosylation sites and the molecule is believed to be heavily glycosylated. The p40 subunit is 306 amino acid residues in length with a predicted molecular weight of 34.7 kD. The molecule contains ten cysteine residues and four potential N-linked glycosylation sites (Gubler *et al.*, 1991). It is not clear what separate functions can be attributed to p35 and p40. Preliminary evidence suggests, however, that p40 is involved in receptor binding and p35 is important for signal transduction (Ling *et al.*, 1995).

A unique, high affinity receptor for IL-12 (IL-12R) has been characterized from PHA-stimulated human PB mononuclear cells (Chizzonite *et al.*, 1992). Cross-linking studies also suggested an association with a second protein of approximately 85 kD. IL-12 receptor has also been reported to be present on PHA- or IL-2-stimulated $CD4^+$, $CD8^+$, and $CD56^+$ cells and on one T cell and one NK cell line (Chizzonite *et al.*, 1992; Desai *et al.*, 1992).

IL-12 is produced by macrophages and B lymphocytes and has been shown to have multiple effects on T-cells and NK cells (D'Andrea *et al.*, 1992; Chan *et al.*, 1991). These include inducing production of IFN-γ and TNF by resting and activated T and NK cells, synergizing with other IFN-γ inducers at both the transcriptional and post-

transcriptional levels to induce IFN-γ gene expression, enhancing the cytotoxic activity of resting NK and T cells, inducing and synergizing with IL-2 in the generation of lymphokine-activated killer (LAK) cells, acting as a co-mitogen to stimulate proliferation of resting T-cells, and inducing proliferation of activated T and NK cells (D'Andrea *et al.*, 1992).

Evidence indicates that IL-12, produced by macrophages in response to infectious agents, is a central mediator of the cell-mediated immune response by its actions on the development, proliferation, and activities of TH1 cells. These activities of IL-12 are antagonized by IL-4 and IL-10, factors associated with the development of uncommitted T-helper cells into TH2 cells and in the mediation of the humoral immune response. In its role as the initiator of cell-mediated immunity, it has been suggested that IL-12 has therapeutic potential as a stimulator of cell-mediated immune responses to microbial pathogens, metastatic cancers, and viral infections such as HIV (Locksley, 1993; Trinchieri, 1993; Hall, 1994; Hsieh *et al.*, 1993; Scott, 1993).

Ingestion of algae such as *Undaria*, and fucoidan fractions, has inhibitory effects on tumors, which appear to be associated with a rise in IL-12, IFN-γ, and a stimulation of innate immunity (Funahashi *et al.*, 2001; Maruyama *et al.*, 2003). *In vitro* treatment of BM MNCs with IFN-γ can up-regulate the expression of CXCR4 granulocyte precursors and monocytes (Lee *et al.*, 1999). Despite the evidence for biological effects via oral ingestion of fucoidan in tumour models, and the marked mobilizing effects on stem cells via intra-venous fucoidan, there are no reports to date of clinical effects of oral fucoidan with regard to stem cell modulation. The effects of orally ingested *Undaria* fucoidan (GFS^{TM}) on the peripheral blood stem cell expression of CXCR4, and associated cytokines SDF-1, IL-12 and IFN- γ is not examined. It will be of interest to examine the relationship and possible interaction of fucoidan with these cytokines.

1.2.6 Interferon-gamma (IFN-γ)

IFN-γ is a multifunctional protein first observed as an antiviral activity in cultures of Sindbis virus-infected human leucocytes stimulated by PHA. It is a pro-inflammatory cytokine produced by T lymphocytes and NK cells; IFN-γ is now known to be both an inhibitor of viral replication and a regulator of numerous immunological functions.

IFN-γ influences the class of Ab produced by B-cells; it up-regulates class-I and class-II major histocompatibility (MHC) complex antigens and increases the efficiency of macrophage-mediated killing of intracellular parasites (Ijzermans & Marquet, 1989; Mogensen & Virelizier, 1987; Chesler & Reiss, 2002). Most of the activities attributed to IFN-γ are believed to be mediated by IFN-γ-induced proteins. The appearance of such proteins is a consequence of IFN-γ binding to a specific receptor that is distinct from the receptor for IFN-α and β (Grossberg *et al.*, 1989). Human IFN-γ is reported to be active only on human and non-human primate cells (Adolf, 1985).

Human IFN-γ is a 143 amino acid residue, 20 or 25 kD glycoprotein that demonstrates little sequence homology to IFN-α or -β (DeGrado *et al.*, 1982; Rinderknecht *et al.*, 1984). Naturally occurring IFN-γ is found as either of two molecular weight species, differing in the degree of glycosylation. The 25 kD species is glycosylated at both potential N-linked glycosylation sites on the molecule, Asn 25 and 97, while the 20 kD species is glycosylated only at asparagine 97 (Yip *et al.*, 1982; Zoon, 1987). In neither case is glycosylation required for biological activity (Kelker *et al.*, 1983; Arakawa *et al.*, 1986). Two allelic variants of IFN-γ have been described, differing by the presence of an arginine or a glutamine at position 137 (Gray & Goeddel, 1982; Gray *et al.*, 1982). Although the cDNA encoding for IFN-γ predicts a protein of 146 amino acid residues, the form secreted by mammalian cells shows a truncation of three amino acid residues from the N-terminus and the conversion of the fourth residue from glutamic acid to pyroglutamate (Rinderknecht *et al.*, 1984). The secreted form of IFN-γ has no potential for the formation of disulphide bridges (Zoon, 1987). Human IFN-γ apparently exists as a head-to-tail dimer in solution with the C-terminus of one monomer aligned with the N-terminus of the other monomer (Ealick *et al.*, 1991; Lunn *et al.*, 1992).

A receptor for IFN-γ has been identified and its gene localized to chromosome 6 (Rashidbaigi *et al.*, 1986; Pfizenmaier *et al.*, 1988). Apparently the product of a single gene, the receptor is a single chain 90 kD glycoprotein that shows a high degree of species-specific binding of IFN-γ (Aguet *et al.*, 1988; Fischer *et al.*, 1988; Calderon *et al.*, 1988; Hershey & Schreiber, 1989). The cDNA for the receptor encodes a polypeptide with a 17 amino acid residue signal peptide, a 228 residue extracellular domain, a 21 residue transmembrane domain, and a 223 residue intracellular domain.

The predicted sequence shows the potential for extensive N- and O-linked glycosylation of the receptor (Aguet *et al.*, 1988). A soluble form of the IFN-γ receptor has been found in human urine under normal physiological conditions (Novick *et al.*, 1989). Evidence indicates that at least one additional receptor component, tentatively designated IFN-γ R β chain, is required for signal transduction following binding of IFN-γ (Hemmi *et al.*, 1994; Johnson *et al.*, 1994; Darnell *et al.*, 1994; Soh *et al.*, 1994). Binding of IFN-γ to its receptor apparently induces dimerisation of the receptor and activation of the tyrosine kinases JAK1 and JAK2. The IFN-γ receptor and an additional protein, STAT91 (or GAF), are subsequently phosphorylated. Phosphorylated STAT91 dimerises and, possibly in combination with another protein, enters the cell nucleus where the complex binds to distinct sites within the promoters of IFN-γ responsive genes, e.g., the gamma interferon activation site (GAS) and the interferon-stimulated response element (ISRE) (Williams, 1991; Shuai *et al.*, 1993; Mirkovitch *et al.*, 1992; Decker *et al.*, 1991; Sadowski *et al.*, 1993). This binding induces the expression of at least 20 distinct proteins, 12 of which are unique to IFN-γ stimulation (Weil *et al.*, 1983; Harris *et al.*, 1992).

Functionally, IFN-γ produces a variety of effects. Produced by $CD8^+$, NK, γδ, and TH1 T-helper cells, IFN-γ has documented antiviral, antiprotozoal and immuno-modulatory effects on cell proliferation and apoptosis, as well as the stimulation and repression of a variety of genes (Paliard *et al.*, 1988; Christmas, 1992; Billiau & Dijkmans, 1990; Locksley & Scott, 1991). The antiprotozoal activity of IFN-γ against *Toxoplasma* and *Chlamydia* is believed to result from indoleamine 2,3-dioxygenase activity, an enzyme induced by IFN-γ (Sen & Lengyel, 1992). The immuno-modulatory effects of IFN-γ are extensive and diverse. In monocyte/macrophages, the activities of IFN-γ include: increasing the expression of class I and II MHC antigens; increasing the production of IL-1, platelet-activating factor, H_2O_2, and pterin; protection of monocytes against LAK cell-mediated lysis; down regulation of IL-8 mRNA expression that is up-regulated by IL-2; and, with lipopolysaccharide, induction of NO production (Gusella *et al.*, 1993; Bulut *et al.*, 1993). IFN-γ has also been demonstrated to be chemotactic for monocytes but not neutrophils (Issekutz & Issekutz, 1993). IFN-γ selectively enhances both IgG2a secretion by LPS-stimulated B-cells and IgG3 secretion in T-cell independent type 2 antigen-mediated B cell activation (Snapper *et al.*, 1988; Snapper *et al.*, 1992). It has also been reported to

induce its own expression (Halloran *et al.*, 1992). Finally, IFN-γ has been shown to up regulate ICAM-1, (but not E-selectin or VCAM-1) expression on endothelial cells (Thornhill *et al.*, 1993).

In animal models, ingestion of marine macro-algae or fucoidan fractions has inhibitory effects on tumours, and appears to be associated with a rise in IFN-γ, IL-12, and the stimulation of innate immunity (Maruyama *et al.*, 2003; Mavier *et al.*, 2004; Funahashi *et al.*, 2001). *In vitro* treatment of bone marrow mononuclear cells with IFN-γ can up-regulate the expression of CXCR4 granulocyte precursors and monocytes (Funahashi *et al.*, 2001).

1.2.7 Nitric oxide (NO)

Nitric oxide (NO) is a recently identified biological signal molecule that plays an important role in vascular regulation, immune response, and neural signal transduction. NO maintains blood pressure by dilating blood vessels. It is produced through metabolism of the amino acid arginine in many types of cells. Bioavailability of NO in plasma is affected particularly by RBC availability and membrane status. RBC membrane and cytoskeleton-associated NO-inert proteins provide a barrier for the NO diffusion and thus account for the reduction in the NO uptake rate of RBC's (Liao *et al.*, 1999; Huang *et al.*, 2001).

NO and its derivatives have two facets. They can modify protein structure and function and damage DNA, but can also protect from cytotoxicity. NO is reported to have several important effects in the control of neoplasia. NO inhibits growth and induces differentiation and apoptosis in acute myeloid leukaemia cells (Hussain *et al.*, 2003).

NO is a chain-breaking antioxidant. It reduces oxygen consumption during free radical-mediated lipid peroxidation, and is generated in human macrophages having an anti-leukaemia activity. Nitric oxide synthase (NOS-2) (iNOS) is not expressed constitutively but is rapidly induced by IFN-γ, TNF-α, IL-12 and LPS. It has been found that IFN-γ can also initiate antiviral responses (David & Shoshkes Reiss, 2002).

1.3 Seaweed (Algae)

Algae are group of organisms that vary in size from microscopic such as picoplankton with a diameter of less than 2 µm to brown seaweeds which may extend to 60 meter long such as kelp in the kelp forests of the Pacific Ocean. Their only common characteristic is their photosynthetic ability. They are grouped into seven main taxa largely on the bases of their colour: (1) Chlorophyta (green algae), (2) Charophyta (stoneworts), (3) Euglenophyta (uglenas), (4) Chrysophyta (golden brown, yellow green algae and diatoms), (5) Phaeophyta (brown algae), (6) Pyrrophyta (dinoflagellates), and (7) Rhodophyta (red algae). Their morphology is simple and not well developed as in land plants (Witvrouw & De Clercq, 1997; Kiple & Ornelas, 2000).

Algae are eaten by many freshwater and marine animals as well as by several terrestrial domesticated animals such as sheep, cattle, and two species of primates: *Macaca fuscata* in Japan (Izawa & Nishida, 1963) and *Homo sapiens*. The human consumption of algae, or phycophagy, has developed thousands of years ago. In terms of quantity and variety of species of algae eaten, phycophagy is, and has been, most prevalent among the coastal peoples of Southeast Asia, such as the ancient and modern Chinese, Japanese, Koreans, Filipinos, and Hawaiians (Kiple & Ornelas, 2000).

Avicenna (Ibn-Sina) in the tenth century writing his book “Al-Qanoun Fil Tibb”, meaning “The Canon of Medicine”, described algae of different species as having value in the treatment of certain conditions. He classified the algae as riverine (freshwater), terrestrial, or marine. The marine were much more powerful as constringent and contracting agents. The rock algae, which grow on rocks, (Gallienus) he claimed could be used as drying and cooling agents. However, Avicenna did not agree with Discorides when he said that rock algae may prevent bleeding. Avicenna suggested that marine algae could be used in the treatment of tumours, vesicles, gout and joint pain (Avicenna, 1593).

1.3.1 *Undaria pinnatifida*

Undaria pinnatifida is one of the brown macro algae, originally endemic to Japan. Introduction to Tasmanian waters is suspected to have occurred via ballast water

discharged from ships transporting woodchips from the mill at Triabunna. A specimen was first found and identified in 1988 by a local marine botanist sifting through algae washed up on a beach at Rheban on Tasmania's east coast [Figure 1.8].

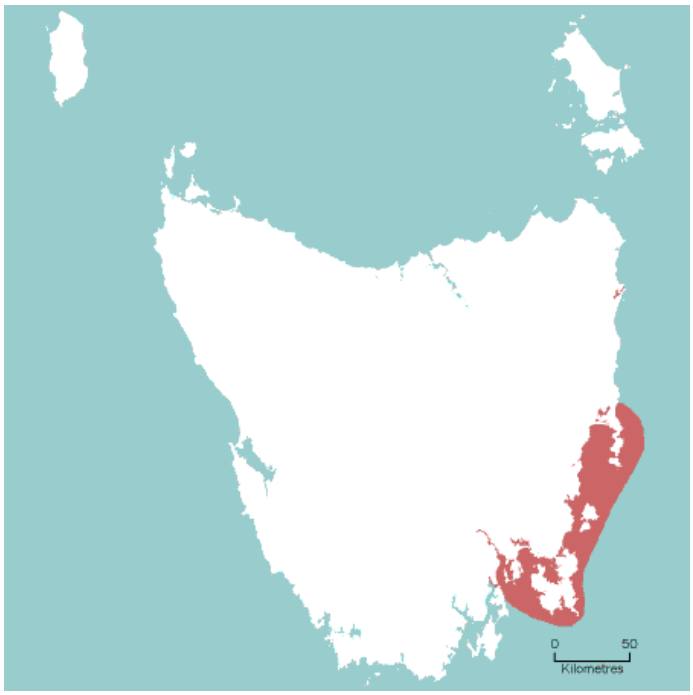


Figure 1.8: Map of Tasmania showing the east coast where *Undaria* is growing. *Undaria* is distributed on the East coast form Great Oyster Bay to the D'Entrecasteaux Channel. There is also an isolated population in Georges Bay (St Helens).

A subsequent survey estimated at least 400 tonnes of the algae were present, but the area of infestation was limited to the Triabunna-Rheban region (10 km of coast). A 1994 distribution survey indicated the infestation had spread to over 80 km of coast, from Coles Bay in the north and south to the Narrows (Marion Bay) (CSIRO).

Brown algae (Phaeophyceae) consist mainly of water (90%). Polysaccharides are major components and comprise alginates, cellulose, and sulphated polysaccharides such as fucoidans. There is considerable experimental evidence that brown seaweed and extracts from it including fucoidan-rich extracts, have significant biological activity.

This brown alga can grow to 2 m in length at maturity. It has an obvious central stem to 10cm wide that extends for the length of the plant. The blade may be up to 1m wide and extends from the tip of the plant for half to three-quarters the length of the plant [Figure 1.9]. *Undaria* looks similar to the native algae *Ecklonia* which is in the same family. However, *Undaria* is clearly identified by the sporophyll (the reproductive segment) which has the appearance of a convoluted or 'frilly' mass of tissue attached to the lower quarter to half of the stem. The *Ecklonia* stem is bare (CSIRO). The life cycle of the *Undaria pinnatifida* is shown in [Figure 1.9].

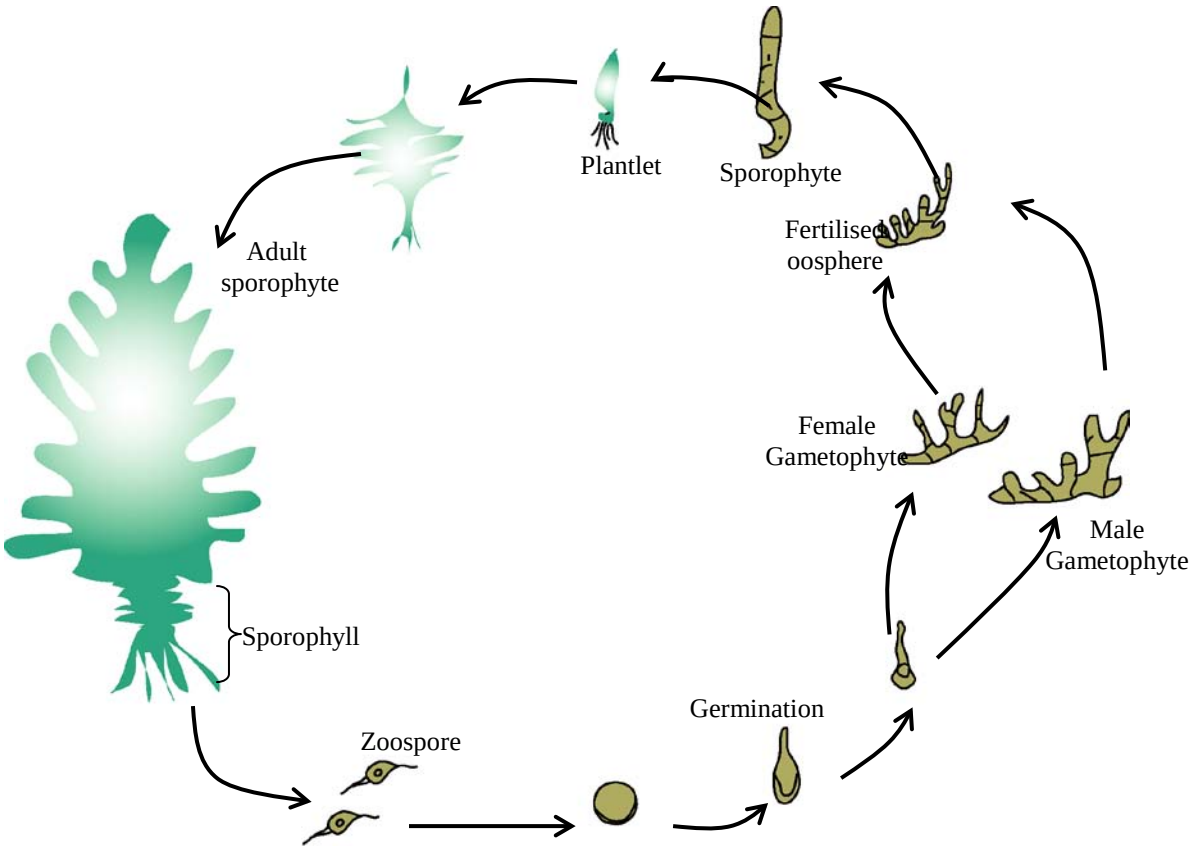


Figure 1.9: Schematic diagram showing *Undaria* life cycle.

Red and brown seaweed, including *Undaria pinnatifida*, have been classified by the United States FDA as GRAS (Generally Regarded as Safe) [Figure 1.10]. It is categorised as a food seasoning or flavouring. Seaweed is part of a normal Japanese and Korean diet, and estimates of intake are 5-7 grams per day. In addition, *Undaria* seaweed is regularly eaten at Japanese restaurants in Australia and it is common in the Japanese diet as wakame and kombu (Shibata *et al.*, 2000). In South Korea, a unique

nutrition transition has occurred, the consumption of seaweed increased from 0.8 g per day in 1969 to 6.6 g in 1995. Despite the huge increase in seaweed consumption in South Korea, the amount and rate of increase in fat intake have remained low which also has a relatively low prevalence of obesity compared with other Asian countries with similar or much lower incomes. South Korea's low fat intake may be part of the reason for the lower prevalence of obesity in South Korea than in many other Asian countries. There may be a correlation between the increased consumption of seaweed and the unexpectedly low obesity level in South Korea (Kim *et al.*, 2000).

Figure 1.10: *Undaria pinnatifida* harvested from the east coast of Tasmania by Marinova.

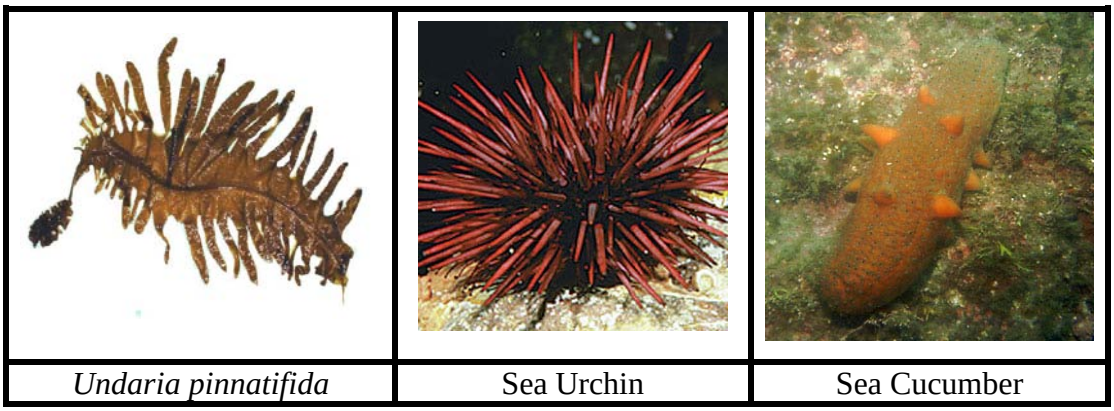
1.3.2 *Undaria* fucoidan

Sulphated fucans constitute a class of polysaccharides first isolated in 1913 from marine brown algae. Thirty five years later, evidence was published showing that fucans also occur in marine invertebrates. Fucoidans are the major brown seaweed sulphated polysaccharides. They are composed of α-(1→3) and α-(1→4) fucosyl units mostly sulphated at positions 2 and 3 (with branching sulphate or additional monosaccharides at free positions) and they have high molecular weights (Haroun-Bouhedja *et al.*, 2000). Fucoidans are considered to have similarities to the (much smaller) mammalian molecule heparan sulphate.

Algal fucoidans are present in several Orders, mainly Fucales and Laminariales but also in Chordariales, Dictyotales, Dictyosiphonales, Ectocarpales, and Scytosiphonales. Fucoidans seem to be absent from green algae (Chlorophyceae), red algae (Rhodophyceae), and golden algae (Xanthophyceae) and from freshwater algae and terrestrial plants. The only other sources of sulphated fucan known to date are marine invertebrates (egg jelly coat of many species of sea urchin and from the body wall of another type of marine echinoderm) (Berteau & Mulloy, 2003) [Figure 1.11].

The general sulphated polysaccharides of the Phylum Phaeophyta are called fucans. These include the compounds fucoidin, fucoidan, ascophyllan, sargassan and glucuronoxylofucan. They comprise families of polydisperse heteromolecules based on L-fucose, D-xylose, D-glucuronic acid, D-mannose and D-galactose (Shanmugam & Mody, 2000).

The fucans of brown algae, often called fucoidans, have been known for some time to have biological activity as modulators of coagulation, as have other algal polysaccharides (Berteau & Mulloy, 2003). For example, *Fucus vesiculosus* fucoidan has a specific anticoagulant activity of 9 -13 U/mg, as assayed by the activated partial thromboplastin time as compared with 167 U/mg for heparin. A fucoidan from *Laminaria brasiliensis* has a higher specific activity (30 U/mg); though its sulphate content is lower (Berteau & Mulloy, 2003).



Undaria pinnatifida	Sea Urchin	Sea Cucumber

Figure 1.11: Natural sources of fucoidan.

1.3.3 Structural comparison of different sulphated polysaccharides

1.3.3.1 Fucoidan structure

Fucoidans consist of long branched chains of carbohydrates and include a substantial amount of fucose. The type of fucoidan, its sulphation, molecular weight and conformation of sugar residues varies with the species of seaweed. Fucoidans are considered to have similarities to the (much smaller) mammalian molecule heparin, an anticoagulant that is in extensive clinical use parenterally. Seaweed also contains lipid soluble components and peptides, which may or may not be responsible for the reported therapeutic effects (Fitton, 2003). Fucoidan was shown previously in the literature with varying chemical structures (Gonzalez-Canga *et al.*, 2004; Bilan *et al.*, 2002). A general structure for the fucoidan used in this study is shown in Figure 1.12.

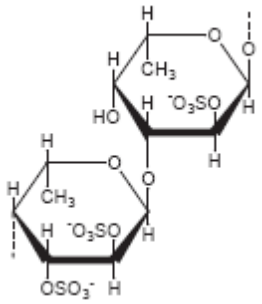


Figure 1.12: Fucoidan chemical structure; the disaccharide repeating unit [4)-α-L-Fuc*p*(2,3di-OSO_3^-)-(1→3)-α-LFuc*p*(2OSO_3^-)-(1] of a fraction of *A. nodosum* fucoidan representing the most abundant structural feature of fucoidans from both *A. nodosum* and *F. vesiculosus* (Chevolot *et al*., 1999 & 2001).

1.3.3.2 Heparan sulphate

Heparan sulphate is a sulphated polysaccharide that is found on the surface of most cells as part of the proteoglycan structures [Figure 1.13]. Heparan sulphate is also present in the ECM. The polysaccharide mediates the interactions between numbers of different proteins. Heparan sulphate consists of alternating hexuronate and glucosamine units. The hexuronate can be either D-glucuronate (GlcA) or L-iduronate (IdoA). The amine of the glucosamine is usually acetylated (GlcNAc) or sulphated (GlcNSO3), but it may also be un-substituted. Within the N-sulphated domains of heparan sulphate, 2-hydroxyl groups of GlcA and IdoA, and 6-hydroxyl groups of

GlcNSO3 may be sulphated. Sometimes there are also 3-O-sulphate groups present on the GlcNSO3 units. There is evidence for a temporally and spatially controlled expression of heparan sulphate epitopes within different organs and tissues (Lindahl *et al.*, 1998; Esko & Lindahl, 2001).

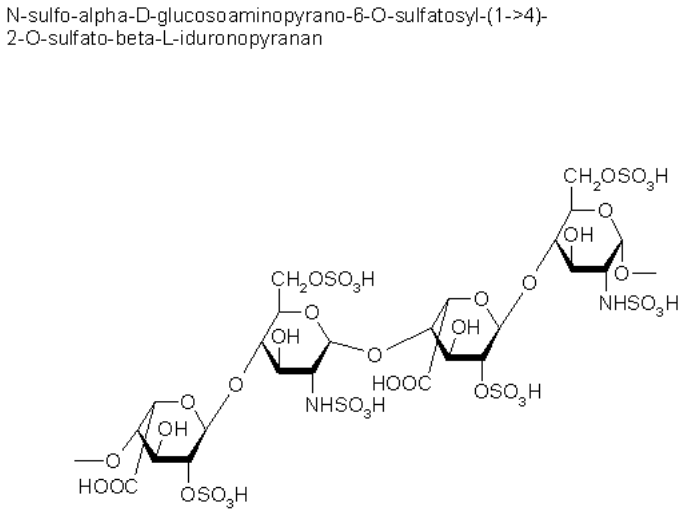


Figure 1.13: Heparan sulphate chemical structure.

1.3.3.3 Heparin

Heparin ($C_{12}H_{19}NO_{20}S_3$) was discovered in 1916 and was originally isolated from canine liver cells hence its name (*hepar* or "ηπαρ" is Greek for "liver"). It is a highly sulphated glycosaminoglycan widely used as an injectable anticoagulant. Pharmaceutical grade heparin is commonly derived from the mucosal tissues of slaughtered meat animals such as porcine or bovine intestine [Figure 1.14].

In contrast, heparan sulphate is made by virtually all cells. It also can contain anticoagulant activity, but the crude preparations have much less activity than heparin. During biosynthesis, heparin undergoes more extensive sulphation and uronic acid epimerization, such that more than 85% of the GlcNAc residues are N-deacetylated and N-sulphated and more than 70% of the uronic acid is converted to IdoA. Another way to distinguish heparin from heparan sulphate is by the susceptibility to bacterial (*Flavobacterium*) heparin lysases (Varki *et al.*, 1999). The major characteristic differences between heparan sulphate and heparin are shown in Table 1.2.

Table 1.2: The major characteristic differences between heparan sulphate and heparin.

Characteristic	Heparan sulphate	Heparin
Soluble in 2M potassium acetate (pH 5.7, 4°C)	yes	no
Size (kD)	10-70	3-40 average 12-15
Sulphate/hexosamine	0.8-1.8	1.8-2.4
GlcN N-sulphates	40-60%	≥85%
IdoA content	30-50%	≥70%
Binding to antithrombin	0-0.3%	~30%
Site of synthesis	Virtually all cells	Basophils and mast cells

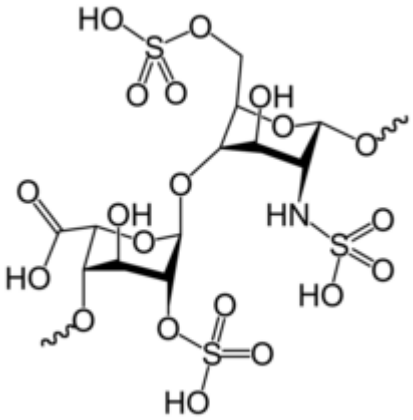


Figure 1.14: Heparin chemical structure; one repeating unit.

1.3.3.4 Hyaluronic acid

Hyaluronic acid (also called hyaluronan or hyaluronate) is a long non-sulphated glycosaminoglycan produced by hyaluronic acid synthetases-1, -2 and -3 expressed on the HSC plasma membrane (Nilsson *et al.*, 2003) [Figure 1.15]. It is naturally found in many tissues of the body such as skin, cartilage and in the vitreous humour. The chemical structure of hyaluronan was determined in the 1950s in the laboratory of Karl Meyer. It was found to mediate cell adhesion via CD44 (Peach *et al.*, 1993) as

expressed by BM stromal cells (Charbord *et al.*, 2002) and osteoblasts (Nakamura *et al.*, 1995). Hyaluronic acid is degraded by a family of enzymes called hyaluronidases. Degradation of cell surface hyaluronic acid by *ex vivo* treatment of HSC with hyaluronidase results in a 40% decrease in HSC lodgement at the endosteum (Nilsson *et al.*, 2003).

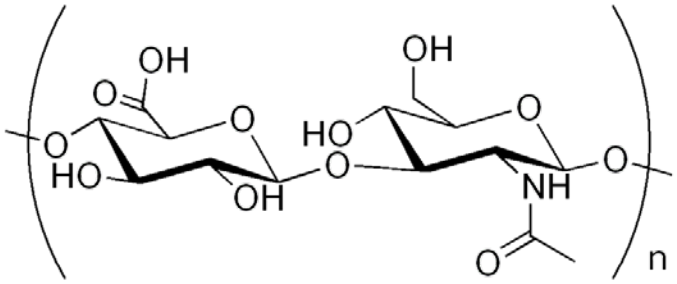


Figure 1.15: Chemical structure of hyaluronic acid.

1.3.4 Physiological properties of fucoidan

Ingesting 3.6 g per day of *Undaria* (wakame i.e. leaf) for 4 weeks resulted in a 14 mmHg drop in systolic blood pressure in Asian hypertensive patients (Nakano *et al.*, 1998). The relative longevity and health of Okinawan Japanese populations has been attributed in part to dietary algae. These studies compared Okinawan descendants living in Brazil with Okinawans remaining at home. The former have a higher risk of cardiovascular and other diseases. As a dietary intervention study, 3 g of DHA, 5g of seaweed (wakame, presumably leaf) powder and 50 mg of isoflavonoids from soy bean were given daily to high-risk immigrants in Brazil for 10 weeks. They were proven to reduce blood pressure, cholesterol level, to suppress the urinary markers of bone resorption or to attenuate a tendency to diabetes (Yamori *et al.*, 2001)

In another study, four male university students were given test diets including zero, 10, 20, and 40 g of wakame (leaf) for 5 days of each diet. The digestibility of the wakame was assessed and was found to vary according to the contribution it made to the total diet. Overall, carbohydrate digestibility was 55.8%. No side effects were noted (Yamada *et al.*, 1991).

A fucoidan extracted from *Laminaria brasiliensis* had a higher specific anticoagulant activity (30 U/mg) than the one from *Fucus*; although its sulphate content is lower (Berteau & Mulloy, 2003). Highly negatively charged polymers (polyanions) such as dextran sulphate, polymethacrylic acid (van der Ham *et al.*, 1977), sulphated fucoidan, sulphogalactosylceramide (Frenette & Weiss, 2000), or defibrotide (Carlo-Stella *et al.*, 2004a) induce rapid mobilisation of HSC, within the first two hours of administration. Apart from sulphated fucoidan, the mechanism of action of mobilising polyanions is unclear. Since fucoidans are polymers of sulphated fucose (Patankar *et al.*, 1993), they were initially believed to act primarily as inhibitors of selectin-mediated interactions between HSPC and BM endothelial cells (Frenette & Weiss, 2000). However, the primary mechanism of action may involve the release into the blood of large amounts of chemokines and cytokines, particularly SDF-1 and KIT ligand, sequestered in heparan sulphates and glycosaminoglycans that decorate the surface of BM endothelial cells, stromal cells and ECM (Sweeney *et al.*, 2000). Although fucoidans synergize with G-CSF to increase mobilisation 11 times over G-CSF alone in primates, to date no clinical trials in patients have been reported (Winkler & Levesque, 2006). Intravenous injection of low molecular weight fucoidan into rats significantly increased the SDF-1 level from 1.2 (± 0.1) to 6.5 (± 0.35) ng/ml in plasma (Luyt *et al.*, 2003).

There has been great interest for many years in the physiological and pharmacological effects of dietary fibre. Because of the diverse effects on various metabolic pathways, it is possible that dietary fibre also influences immune function as a consequence of changes in mucosal structure and gut microflora or it may influence the intestinal absorption of different compounds such as lipids. The physiological functions of dietary fibre depend at least on its physical characteristics. Lim and his colleagues showed that dietary fibre plays an important role in typical immune indices such as T-cell population, cytokine production and immunoglobulin production in rat mesenteric lymph node lymphocytes (Lim *et al.*, 1997).

Whole seaweed has a high total dietary fibre content that is between 32% -50% of dry matter. There is a range of soluble and insoluble fibres. Among the insoluble fibres, there is a low percentage of cellulose and Floridean starch. The soluble fibre fraction accounts for 51% to 56% of total fibres in green (Chlorophyta) and red algae

(Rhodophyta) and for 67% to 87% in brown algae (Phaeophyta). Soluble fibres are generally associated with hydration performance, *i.e.* absorption, retention and swelling, which affect the food bolus's passage through the stomach and small intestine, and can have cholesterol-lowering and hypoglycaemic effects or immune system activator (McDermid *et al.*, 2005).

The low molecular weight fucoidan FF7/3 combines potent anticoagulant and fibrinolytic properties with apparently only minor platelet activating effects. Platelet activation was subsequently studied by a standard aggregation assay and flow cytometric determination of the activation dependent platelet-surface markers CD62p (P-selectin, GMP-140) and CD63 (GP53). All the fucoidan fractions induced irreversible platelet aggregation in a dose-dependent manner. Comparing fractions of identical molecular weight (100 kD), the low sulphate content fucoidan FF5 (S = 7.6%) exerted a significantly greater effect than the highly sulphated fucoidan FF7 (S = 10.2%) over the whole concentration range (n = 5, $p< 0.05$). Among fractions of identical sulphate content, fucoidan-induced platelet aggregation was also found to depend on the molecular weight of the fucoidan (Durig et al., 1997).

Treatment with low molecular weight heparin has been observed to confer a lower mortality rate on cancer patients (Borsig *et al.*, 2001). It has been found that non-anticoagulant species of heparin and various sulphated polysaccharides, such as fucoidan, pentosan sulphate, carrageenan-λ, laminaran sulphate, that inhibit experimental metastasis also inhibit tumour cell heparanase while other polymers (e.g., chondroitin sulphate, carrageenan-κ, hyaluronic acid) have little or no effect on both parameters (Vlodavsky & Friedmann, 2001). It have been observed that heparin species containing more than ten sugar units and having sulphate groups at both the N and the O positions are the most potent both at inhibiting heparanase activity and at blocking experimental metastasis. While O-desulphation abolished the inhibitory effect of heparin, replacement of N-sulphates by N-acetyl or N-hexanoyl groups had only a small effect on the inhibitory activity (Vlodavsky & Friedmann, 2001). Potent inhibition of heparanase activity and tumour metastasis has also been demonstrated with other heparin-mimicking compounds and polyanionic molecules; although an effect on selectin-mediated cell adhesion cannot be excluded as the mechanism for these beneficial effects (Borsig *et al.*, 2001).

Parish and his colleagues have initiated a comprehensive screening program to identify sulphated oligosaccharides that can inhibit tumour metastasis and promote tumor regression by their effects on heparanase activity and angiogenic growth factor action. Oligosaccharide chain length and degree of sulphation emerged as more important parameters than the sugar composition and type of linkage in this study. With increasing sulphation there was a steady increase in the ability of maltohexaose to inhibit both heparanase activity and experimental metastasis (Parish *et al.*, 1999). Phosphomannopentaose sulphate (PI-88) and maltohexaose sulphate were comparable to heparin in their inhibitory activity (IC_{50} 1-2 μg/ml). Continuous administration of PI-88 inhibits growth, vascularity and lymph node metastasis of mammary adenocarcinoma tumors in rats. This compound is being evaluated in a multicenter phase II clinical trial (Parish *et al.*, 1999).

Competitive inhibition of heparanase by PI-88 and other sulphated oligosaccharides and modified heparin derivatives may also be applied to suppress autoimmune and chronic inflammatory diseases. However, the pleiotropic effects and interactions of such heparin/HS mimetics with heparin-binding proteins might elicit undesirable effects. Random, high-throughput screening of chemical libraries, preparation of neutralizing antibodies, and rational design of compounds that block the heparanase active site or ligand-binding domain are among the other approaches applied to develop effective heparanase inhibitors. Natural endogenous heparanase inhibitors may also be identified.

1.3.5 Urinary sulphated glycosaminoglycan

Toyoda and his colleagues established a new sensitive high performance liquid chromatography method for the determination of unsaturated disaccharides produced from heparin and heparan sulphate. This method was applied to the analysis of normal human urine. It revealed that the concentration of normal human urinary heparan sulphate is 1.53 mg/mg creatinine (Toyoda *et al.*, 1997).

Measurement of glycosaminoglycan content in urine is generally used as a screening procedure for mucopolysaccharidosis. A new direct method was established for quantifying excessive urinary sulphated glycosaminoglycan using the specific binding of 1,9-dimethylmethylene blue (DMB). This method can measure sulphated

glycosaminoglycan in very small specimens of urine without cumbersome separation techniques. This method has been used in diagnosing the mucopolysaccharidosis storage disease during early childhood (Whitley *et al.*, 1989; De Jong *et al.*, 1989).

1.3.6 Fucoidan antiviral activity

The inhibitory effects of polysaccharides from marine algae on viral replication were reported almost four decades ago. In 1958, Gerber and Adams reported that algal polysaccharides exhibited antiviral activity towards mumps virus and influenza B virus. In 1974, Deig and his colleagues (Deig *et al.*, 1974) associated the inhibition of herpes simplex and other viruses with polysaccharides fractions from extracts of ten red algae; similar observations were made by Richards and his colleagues (Richards *et al.*, 1978).

Soon after the role of CD4 as a receptor for HIV-1 was appreciated, it became apparent that CD4 alone is not sufficient to mediate HIV-1 entry. The chemokines which blocked the HIV-1 infection of T-cells were the first hint on the elusive co-receptors. The CCR5 was found to mediate HIV-1 entry into macrophages and primary T-cells, whereas CXCR4 was shown to allow HIV-1 entry into primary T-cells and T-cell lines (Pierson *et al.*, 2004).

While R5-viruses are transmitted between individuals, X4-tropic HIV-1 variants arise in about 50% of the patients at later stages of the infection and are associated with more rapid progression to AIDS. Therefore blocking utilisation of CXCR4 by HIV may be an important therapeutic target. Several small molecule compounds which antagonize CXCR4 and block X4-tropic HIV-1 at nanomolar concentrations have been described. These compounds are positively charged and specifically interact with the negatively charged surface of CXCR4. CXCR4 inhibitors like KRH-1636, AMD3100 or AMD070 strongly block replication of a variety X4-tropic viruses *in vitro*, and in the case of AMD3100, reduction of viral load has been observed upon treatment of a HIV infected patient harbouring only X4-tropic viruses (Pierson *et al.*, 2004).

However, CXCR4 and its ligand SDF-1 are critical for haematopoiesis, cardiac function and cerebellar development, suggesting that targeting of CXCR4 by small

molecule inhibitors without interfering with its natural function might be difficult. Other molecules such as polyanions (fucoidan) may be useful. Indeed, in preclinical tests AMD3100 caused significant cardiac problems, underlining that the development of compounds that block CXCR4 interactions with HIV-1, but not with natural ligands, is warranted (Pierson *et al.*, 2004).

In 1987 Nakashima and his co-workers reported inhibition of HIV reverse transcriptase by sulphated polysaccharides from the red alga *Schizymenia pacifica*. More recently, a complex sulphated polysaccharide fucoidan from alga *Fucus vesiculosus* was found to inhibit HIV *in vitro*. This activity may have been due to a direct interaction of the polysaccharide with the HIV binding site of the target cell (Witvrouw & De Clercq, 1997).

Undaria pinnatifida extract showed anti-leukaemic activity against Rauscher murine retrovirus-induced erythroleukaemia, it inhibited the reverse transcriptase of HIV, and suppressed the replication of HIV (Furusawa & Furusawa, 1989).

1.3.7 Fucoidan anti-tumour activity

Fucoidan has the ability to suppress the growth of implanted sarcoma 180 cells in mice and increases the splenic NK cell activity. The anti-tumour effect of fucoidan appears to be mediated by IFN-γ activated NK cells. *Undaria pinnatifida* powder can inhibit intestinal tumourigenesis induced by 1,2-dimethylhydrazine, it significantly enhances the cytolytic activity of NK cells and increases the amount of IFN-γ produced by T-cells up to about two-fold, compared with non-treated mice. Fucoidan was found to affect T-cell- and macrophage-mediated immune responses, namely the production of IL-2, IL-12 and IFN-γ (Maruyama *et al.*, 2003). Fucoidan can inhibit markedly the growth of Ehrlich ascites carcinoma in mice; it activates the reticuloendothelial system and enhances the phagocytic activity (Itoh *et al.*, 1993).

1.3.8 Fucoidan anticoagulation effect

So far, about 150 species representing three major divisions of marine algae, Rhodophyta (red algae), Phaeophyta (brown algae) and Chlorophyta (green algae), have been reported to have blood anticoagulant activities (Shanmugam & Mody,

2000). Presence of anticoagulant activity in brown algae was first reported in 1941, where *Laminaria* showed anticoagulant effects, its active compound being located in the hold-fasts (Kimura *et al.*, 1941).

Seaweed-derived heparin-like substances have been studied extensively over the last 60 years. In 1936, Chargaff and his colleagues made the first report on marine algal extracts possessing blood anticoagulant properties. An extract of a red alga *Iridae laminarioides* demonstrated anticoagulant activity. This material, a 'galactan sulphuric acid ester', was shown to possess 30 U/mg of heparin equivalence. Subsequent studies described similar anticoagulant properties in agar and carrageenan (Elsner *et al.*, 1937; Elsner & Hoppe-Seyler's, 1938). Later, another study showed that a potent anticoagulant was found in a highly sulphated galactosan isolated from seaweed (Houck *et al.*, 1957). There is a greater incidence of anticoagulant activity in extracts from the brown algae compared to red and green algae (McLellan & Jurd, 1992).

Fucoidan extracts from different brown seaweed cell walls exhibit anticoagulant properties. The chemical degradation of a crude extract from *Pelvetia canaliculata* with a molecular weight of 20,000 ± 5,000 kD revealed a potent antithrombin activity when studied using antithrombin III-depleted plasma or in the presence of purified heparin cofactor II. In contrast, no anti-factor Xa activity was detected in the presence of the degraded fucoidan, under the same conditions (Colliec *et al.*, 1991).

The aPTT is usually used to evaluate coagulation alterations in the intrinsic pathway and will also detect severe functional changes in factors II, V, X, or fibrinogen. The aPTT has also been widely advocated as a means to monitor the effectiveness of heparin therapy. In patients receiving oral anticoagulants, the circulating levels of factors II, VII, IX, and X are depressed; therefore the aPTT can be expected to be prolonged.

In preclinical studies, a fucoidan from *Laminaria brasiliensis* has a higher specific activity (30 U/mg) although its sulphate content is lower (Mourao & Pereira, 1999). The high potency of heparin depends on a specific pentasaccharide sequence with high affinity for the serine protease inhibitor antithrombin, a sequence that is absent from fucoidan. However, purified fucoidan fractions (from *Ecklonia kurome* (Nishino

et al., 1999), *Ascophyllum nodosum* (Millet *et al.*, 1999), and *Pelvetia caniculata* (Colliec *et al.*, 1994) have activity mediated both by antithrombin and by another plasma serine protease inhibitor (serpin), heparin cofactor II (HCII) (Colwell *et al.*, 1999). Heparin interacts with HCII by means of its regular repeating unit, not the antithrombin-binding sequence. These serpins act against several of the coagulation system proteases, including thrombin, Factor Xa, and Factor IXa (Mauray *et al.*, 1998). All of these factors may be involved in the ability of fucoidan to prevent venous thrombosis (Millet *et al.*, 1999). The release of the tissue factor pathway inhibitor from endothelium, which is stimulated by fucoidan more potently than by heparin, may also have an antithrombotic effect (Giraux *et al.*, 1998).

The anticoagulant and antithrombotic activities of fucoidan fractions from *Ascophyllum nodosum* increase with increasing molecular weight and sulphate content. However, fractions in which the native pattern of sulphation was intact were more potent than fractions of equivalent molecular weight and overall degree of sulphation in which this pattern had been disrupted by partial de-sulphation (Boisson-Vidal *et al.*, 2000). Fucoidans may also promote fibrinolysis by potentiating plasminogen activators (Nishino *et al.*, 2000). The predominant pattern of sulphation in *Ascophyllum nodosum* fucoidan is the tri-sulphated disaccharide motif, similar to that found in heparin, which also has a tri-sulphated disaccharide repeat unit. A heavily sulphated substituted disaccharide is also the repeat unit of a highly anticoagulant galactan isolated from red algae (Farias *et al.*, 2000).

Correlation of the sulphation patterns of less highly sulphated fucans and galactans with their anticoagulant activities (Mourao & Pereira, 1999; Pereira *et al.*, 2002) has used invertebrate fucans. The structural homogeneity of these compounds allows clear conclusions to be drawn. For example, a 3-linked, regularly 2-O-sulphated galactan has anticoagulant activity which is lacking in the corresponding 3-linked, 2-O-sulphated fucan or a 4-linked, regularly 3-O-sulphated galactan. This study established definitively that regular, linear sulphated fucans express anticoagulant activity which is not simply a function of charge density but is critically dependent on the exact structure of the polysaccharide.

1.4 Factorial experimental design and analysis

The main effect of a factor (such as a cytokine) is defined to be the change in response (e.g., cell number) produced by a change in the level of the factor. If the main effects are independent, they will be additive; however, if a factor interacts with a second factor, the response will possibly depend on the level of this second factor (and vice versa). A positive interaction is defined as a greater than additive response, whereas a negative interaction results in a less than additive response.

A two-level, full factorial experiment measures the response and effect of all factor combinations. The main effect of factor A is calculated from the difference between the averages of responses that include factor A and those that do not include factor A. The interaction effect AB is defined as the average difference between the effect of A at the high level of B and the effect of A at the low level of B.

The main and interaction effects were calculated using contrasts. These were defined for k factors using the algebraic formula,

$$Contrast_{AB\ldots K} = (a \pm 1)(b \pm 1)\ldots(k \pm 1)$$

where, by convention, lowercase letters denote the sum of the response of replicate experiments. The sign in each set of parentheses was negative if the factor was present in the effect and positive if the factor was absent. For example, the contrasts generated for factors A and B were

$$Contrast_A = ab + a - b - (1)$$
$$Contrast_B = ab + b - a - (1)$$
$$Contrast_{AB} = ab - a - b + (1)$$

where (1) denoted the response when no factors were present. Effects were estimated from contrasts by

$$Effect_{AB\ldots K} = \frac{2}{2n^k} contrast_{AB\ldots K}$$

where n is the number of replicate experiments and k the number of factors.

Because these contrasts are orthogonal, the total sum of squares, SS_{total}, can be partitioned into the sum of squares of main and interaction effects, $SS_{AB...K}$, and the error sum of squares, SS_{error}.

$$SS_{total} = \sum_{AB...K} SS_{AB...K} + SS_{error}$$

The error sum of squares is found from the difference between the total sum of square and those of the effects. The F statistic and its distribution were used to test the hypothesis that there were no treatment effects,

$$F = \frac{SS_{AB...K}}{S^2}$$

where each effect has a single degree of freedom (numerator) and the mean squared error $s^2 = SS_{error}/2^k(n-1)$ has $2^k(n-1)$ degrees of freedom (denominator). The null hypothesis (no effect) was rejected if the probability of the effect was less than 0.05 (Montgomery, 1997).

A fractional factorial design was initially employed to screen for possible interactions. The sparsity of effects principle asserts that when there are several variables, the system or process is likely to be driven primarily by some of the main effects and low-order interactions. A fractional design confounds high-order interactions with the main effects or two-factor effects to reduce the number of experimental runs. A resolution $_{IV}$ design was used.

1.4.1 Factorial analysis of cytokine interactions

The majority of cytokine-mediated expansion experiments involve the study of two or more factors (cytokines) and for this reason, factorial designs are the most efficient type of experiments. Factorial designs are widely used in experiments involving several factors where it is necessary to study the joint effect of the factors on a response. The effect of a factor is defined to be the change in response produced by a change in the level of the factor (Montgomery, 1997).

Factorial design analysis is a novel approach because it enables one to define which growth factors are likely to have an effect and to design experiments using a subset (fractional) or all (full) of the possible combinations of the growth factors. It also enables one to determine the independent contribution of each growth factor (or combination of factors) by calculating the "contrasts" and determine the significance of the effects by analysis of variance.

The most important factorial designs are those that involve k factors (*i.e.* different growth factors and cytokines in this model), each at two levels, where the levels can be either qualitative or quantitative. A complete replicate of such a design requires $2x2x2x\ldots x2=2^k$ observations. This type of factorial is known as a 2^K factorial design. The 2^k factorial design is useful in the early stages of experimental work when there are numerous factors to be investigated. They provide the smallest number of experimental runs with which k factors can be studied in a full factorial design. As there are only two levels for each factor it is assumed that the response is approximately linear over the range of the factor levels chosen (Montgomery, 1997).

In 1996, Petzer and his colleagues used a 2^5 factorial analysis design to investigate the relative roles of six cytokines in stimulating long-term culture-initiating cell (LTC-IC) amplification of primitive ($CD34^+38^-$) human BM cells. LTC-IC assays were performed on each of the 32 possible combinations of IL-3, IL-6, SCF, Flt-3-L, nerve growth factor β (NGF-β) and G-CSF both on the initial $CD34^+38^-$ cells and on the cells harvested after a ten day culture period. The amplification of LTC-ICs was then evaluated by multi-parameter analysis of variance of the normalised data on all of the factors tested. This type of analysis therefore allowed them to identify as significant the individual or interactive effects if the result of any particular factor or combination of factors was not explained by the variation accounted for by the normal probability distribution resulting from the combined data from all groups. They demonstrated that IL-3, SC and Flt-3-L were both necessary and sufficient to obtain an approximately 30-fold amplification of the input LTC-IC population. Additional studies using another 10 cytokines demonstrated that MGDF was also capable of significantly increasing LTC-IC numbers both alone and in combination with the six previously mentioned cytokines.

Further studies by the same group investigated the dependence of LTC-IC expansion on the relative concentration of IL-3, SCF and Flt-3-L by incorporating a 2^3 orthogonal factorial analysis design. Each of the eight possible cytokine combinations were tested on $CD34^+38^-$ BM cells at either 2 or 60 ng/mL for IL-3 and 10 or 300 ng/ml for both SCF and Flt-3-L. They demonstrated that the greatest expansion of LTC-ICs occurred when all three cytokines were present at the higher concentration. It was also known that LTC-IC expansion was primarily and significantly dependent on the presence of Flt-3-L at a high concentration (300 ng/mL). This demonstrated that high numbers of LTC-ICs can be obtained when the concentration(s) of IL-3, SCF, or both are reduced, as long as the Flt-3-L concentration remains high. They also observed that high levels of IL-3, when combined with SCF and Flt-3-L at low levels, had a direct and negative effect on LTC-IC expansion. However, this negative effect could be overcome when the concentration of either SCF or Flt-3 was sufficiently increased (Zandstra *et al.*, 1997).

CHAPTER TWO

HUMAN SUBJECTS, GENERAL MATERIALS AND METHODS

Chapter objectives:

Describe the general methodology that has been used and applied during the clinical trials and the experimental laboratory work

Chapter contents:

2.1 Ethics approvals

This project was carried out according to the National Statement on Ethical Conduct in Research Involving Humans (June, 1999) produced by the National Health and Medical Research Council of Australia and according to the Helsinki declaration.

Ethics approvals were sought from the appropriate ethics committees in each institution according to the state regulations. The clinical trials which were carried out in Tasmania were approved by the Southern Tasmania Health & Medical Human Research Ethics Committee (STH&MHREC) and the Royal Hobart Hospital Research Ethics Committee. The ethics application was approved on the 17th December 2003 and the approval ethics reference number is H0007673: effects of natural edible brown seaweed *Undaria pinnatifida* extract (GFS^{TM}) on mobilisation of human bone marrow stem cells and immune system activation. Approvals from the Pathology Department and Pharmacy Department at the Royal Hobart Hospital (RHH) for assistance were also given. The volunteer information sheet and consent form are shown in Appendix-1 and -2.

The use of human PB or CB CD34^{+} cells for study of expansion processes was approved by the human ethics committees at University of New South Wales, Sydney Eastern Area and Sydney Area Health Service and the Peter MacCallum Research Centre. The UNSW human ethics approval numbers are 03104: *In vitro* growth of umbilical cord blood stem cells and 04006: *in vitro* growth of adult mobilised peripheral blood stem cells. The volunteer information sheet and consent form are shown in Appendix-3.

2.2 Human subjects

Healthy human volunteers of either sex were chosen after advertising around the university hospital campus. Informed consent was obtained from all volunteers after human ethics approval was obtained from the STH&MHREC. All volunteers were recruited in the study according to the study design described in section 2.3.

2.3 Study design

The trial is a single blinded, analytical, experimental, clinical phase I/II trial using a single dose (3 g daily) of either guar gum fibre (Benefiber; Novartis Pty. Ltd., VIC Australia) as a placebo control group, whole seaweed containing naturally 10% of the possible active ingredient (fucoidan) (Marinova Pty. Ltd., Hobart, TAS Australia), or seaweed extract containing 75% fucoidan that has 75% fucoidan w/w (Marinova Pty. Ltd., Hobart, TAS Australia). The capsules were taken daily at the same time. During the study time, volunteers were asked not to eat any sort of seafood or food containing seaweed derived products and they did not take any drugs or food supplements. Blood samples from the three groups were collected as described later.

2.3.1 Population and setting

2.3.1.1 Target population and eligibility criteria

- Normal healthy adults (males and females)
- Written informed consent; age >18 years
- Able to comprehend and comply with study requirements
- Non-smokers
- No history of thyroid abnormalities
- No sulphur allergy
- No active treatment in the 28 days prior to study entry including contraceptive pills, high doses of Alcohol (≥2 glasses per week), seafood or food containing seaweed derived products, over counter medicines, or complimentary therapies.
- Normal organ function including LFT, U+E, and physiological examination
- Local recruitment by advertising around RHH, Medical School, University main campus in Sandy Bay, and fitness clubs around the city of Hobart.

2.3.1.2 Exclusion criteria

- Development of thyroid function abnormalities
- Pregnancy
- Volunteers who will not be able to swallow the study capsules for whatever reason.
- Volunteer request

Prior to enrolment of any volunteer, the study was submitted to the RHH Research Advisory Committee and Southern Tasmania Health and Medical Human Research Ethics Committee to obtain approvals. The study was later registered in the Australian Clinical Trials Registry (ACTR). The ACTR number is ACTRN12605000021673.

The trial protocol was publicised to the RHH Pathology Unit, Core Lab facility, and Outpatient Pathology Clinic nursing staff at the RHH. When the researcher felt that the volunteer was suitable for inclusion in the trial, the inclusion and exclusion criteria were assessed, and a determination made whether the volunteer could be enrolled into the trial. Subjects were followed up within the Discipline of Medicine by the researcher and if needed at the Outpatient Pathology Department at the RHH.

2.3.1.3 Number of volunteers

It was predicted that a total of 200 volunteers (50 volunteers in 4 sets where each set is divided into 3 groups; 10 volunteers in the placebo control, 10 in the 10% fucoidan and 30 in the 75% fucoidan) may be sufficient to produce significant results. The sample size was kept minimal to reduce the cost of the study. No changes in the outcomes or toxicity were predicted, based on the literature, in the placebo and 10% fucoidan groups (whole seaweed which is equivalent to the average daily consumption of seaweed in Japan and Korea) therefore, the sample size in these two groups were set to be smaller than the 75% fucoidan group. This policy allowed more volunteers to be recruited in the 75% fucoidan group to increase the statistical power. This was done as one of the main aims was to compare the outcome at baseline with the outcome after treatment within the 75% fucoidan group itself. At the end of the study a total of 162 volunteers had been recruited in all different study sets as shown below and in each chapter.

2.3.2 Study scheme

Volunteers ingested nine capsules (333 mg each) three times daily for 12 days of either:

- Guar gum placebo fibres,
- Whole seaweed containing 10% GFS^{TM} or,
- Seaweed extract containing 75% GFS^{TM}

Capsules were dispensed through the RHH Pharmacy for a maximum of 12 days or by the researcher at the Clinical School, Haematology Department.

Treatment was continued for 12 days or until one of the following criteria applied:

- Intercurrent illness that prevented further administration of treatment,
- Unacceptable adverse event(s),
- Volunteer decision to withdraw from the study, or
- General or specific changes in the volunteer's condition rendered the volunteer unacceptable for further treatment in the judgment of the investigator.

2.3.3 Drug administration and compliance

The drug was administered either before or after food depending on the volunteer's preference. The volunteers self-administered the doses of medication per schedule. Volunteer compliance, in light of the large number of capsules taken daily was documented because decreased compliance might interfere with interpretation of results and make assessment of toxicity difficult.

Vial counts were used to monitor compliance as well as volunteer's calendars. Volunteers were requested to record any partial doses. If a volunteer missed or vomited a dose, that dose was not repeated, but the volunteer resumed his/her schedule for subsequent doses.

Compliance was scored according to; volunteers have to take 12 days of treatment, ingest ≥90% of the intended dose, and not miss >1 dose/day to be considered fully evaluable as compliant.

Treatment dispensed was recorded on the hospital pharmacy computing system and/or on the investigator computing system, and batches dispensed were recorded manually.

2.3.4 Outcomes and measures

- **Primary end point of phase I/II:**

  Determine and characterize toxicities of the study drug, fucoidan, and identify side effects.

- **Secondary endpoint of phase I/II:**

  To test the bio-availability of the investigational drug in plasma and urine, test its effectiveness and to further evaluate its safety,

  Assess the pharmacokinetic properties and the pharmacodynamic properties of the investigational agent,

  Test the changes in different blood chemicals that the active agent may change.

- **Tertiary end point of phase I/II:**

  To assess the changes in the coagulation system and stem cell mobilization associated with active treatment.

  Assess the characteristics of the mobilized stem cells and the mobilization/homing system.

2.3.5 Study procedures

Pre-treatment evaluation with

- History
- Physical examination
- Routine laboratory studies were performed and the following pre-treatment chemistry tests were measured in blood samples:
  - Complete Blood Count (CBC)
  - Glucose/Insulin
  - ESR
  - Liver function test (LFT)
  - Kidney function test (U+E)
  - Thyroid Hormone Tests
  - Lipid profile
  - Electrolytes, BUN, creatinine

Set number 1:

The pre-treatment blood tests were measured at baseline and then every 4 days thereafter in a set of 42 volunteers. The 42 volunteers are grouped as follows:

- 6 subjects in the placebo treatment,
- 6 subjects in the 10% fucoidan and
- 30 subjects in the 75% fucoidan.

The data and results of this set are presented in Chapter 3. Subjects were reviewed every 4 days until the end of the study. At each review, the physical examination was repeated, blood samples were drawn and volunteers were questioned about expected toxicities which were recorded. When recruiting and testing volunteers in this set were finished no toxicity was reported and 25/30 of the previously tested volunteers in the highest treated active group (75% fucoidan group) passed the given dose (3 g per day for 12 days). Therefore, another 3 sets of volunteers were recruited for other tests.

Set number 2:

A total of 52 volunteers were recruited in the second set to assess the GFS^{TM} concentration in plasma in 3 groups, the data and results of this set are presented in Chapter 4.

- 6 subjects in the placebo treatment,
- 6 subjects in the 10% fucoidan and
- 40 subjects in the 75% fucoidan.

Set number 3:

A total of 31 volunteers were recruited in the third set to assess the coagulation changes in volunteers' blood samples, the data and results of this set are presented in Chapter 5.

- 6 subjects in the placebo treatment and
- 25 subjects in the 75% fucoidan

Set number 4:

The fourth set was to study stem cell mobilization, Interferon-gamma (IFN-γ), Interleukin-12 (IL-12), and Stromal Derived Factor (SDF-1). The data and results of this set are presented in Chapter 6.

A total of 37 volunteers were recruited in 3 groups:

- 6 subjects in the placebo treatment,
- 6 subjects in the 10% fucoidan and
- 40 subjects in the 75% fucoidan.

2.3.6 Statistical considerations

The volunteers were allocated by the researcher to the treatment groups (placebo, 10% fucoidan or 75% fucoidan) unequally but gender and age were taken into consideration so each group would have a balanced ratio of males versus females and young versus old. The unequal allocation method of participants to treatment and control groups is more appropriate in this study to reduce the study cost since the intervention is expensive. Unequal numbers of volunteers were recruited in the treatment groups. More volunteers were recruited in the 75% fucoidan group. This was mentioned and explained earlier in section 2.3.1.3.

2.3.7 Accrual rate and feasibility

Accrual rate of 2-3 volunteers per week would be expected and enough. The predicted sample size for this study is relatively small and the accrual rate achievable.

2.4 Blood samples collection

Venous blood from the antecubital vein was collected from all volunteers for different tests for the clinical trials at the Royal Hobart Hospital. The evacuated blood collection tubes are shown in Table 2.1. For some special tests which are described later in each chapter, plasma fractions were collected and stored in aliquots at80°C within 30 min of collection for later analysis.

Table 2.1: The evacuated blood collection tubes and anticoagulants used for different pathology tests.

Anticoagulant	Tests	Tube stopper colour code
EDTA K3	CBC, flow cytometry,	Purple
Sodium citrate (0.105 mol/mL)	ESR	Dark grey (ESR tube)
Serum separator and clot activator	LFT, U+E,	Red
Sodium citrate (3.8%)	Coagulation, fucoidan detection	Blue

2.5 Preparation of study capsules

Three different types of capsules were prepared using gelatinous capsules of size ‘00’ under aseptic conditions in a laminar flow cabinet. A special encapsulater was used in the filling process. Each capsule contained 0.33 g of the designated treatment (guar gum, whole seaweed or 75% fucoidan). These capsules were carefully weighed, and conformed to the 0.33 g standard [Figure 2.1].

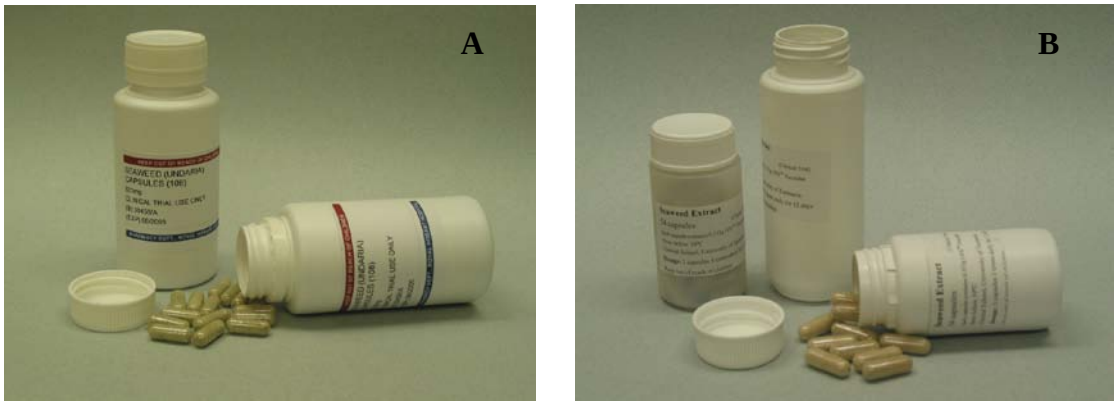


Figure 2.1: Seaweed capsules used in clinical trials. (A) Whole *Undaria pinnatifida*. (B) 75% fucoidan.

The guar gum is a dietary fibre that is called also guaran and which is extracted from the seed of the leguminous shrub *Cyamopsis tetragonoloba*, where it acts as a food and water store. It is a natural gum and edible thickening agent. The molecular weight is around 220,000 ± 20,000 Daltons and it is a water soluble non-sulphated linear galactomannan. It has a (1→4)-linked β-D-mannopyranose backbone with branch points from each 6-position linked to a α-D-galactose (*i.e.* 1→6-linked-α-D-galactopyranose). There are between 1.5 - 2 mannose residues for every galactose residue [Figure 2.2]. Guar gum was used in this study as a control substance.

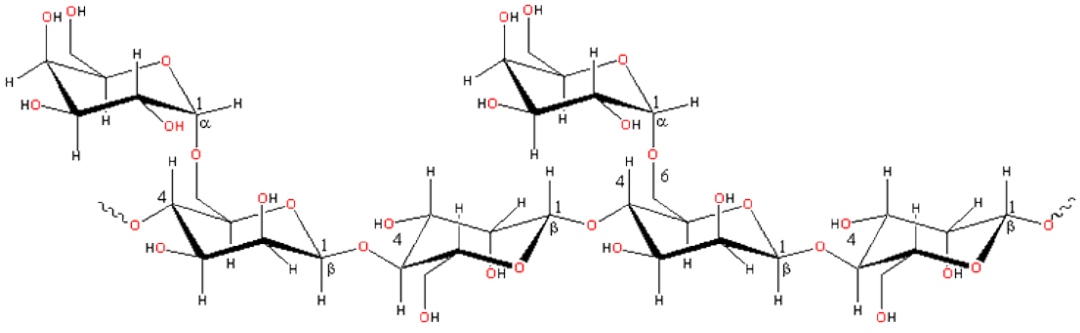


Figure 2.2: Guar gum chemical structure. Adapted from http://www.lsbu.ac.uk/water/hygua.html (accessed in Nov 2006).

The whole seaweed powder that contains 10% w/w fucoidan and the fucoidan extract with 75% fucoidan w/w, which are used in this study, are derived from Tasmanian *Undaria pinnatifida* obtained from Marinova Pty. Ltd., Hobart, TAS Australia. Some chemical characteristics of the polysaccharides were described before and are shown in Table 2.2 (Martha *et al.*, 1976; Gonzalez-Canga *et al.*, 2004).

Table 2.2: General characteristics of the polysaccharides administered orally to volunteers in the clinical trials.

Polysaccharide	Sugar type	Mean MW (kD)	Type of linkage	% SO_4
Guar gum	Galactomannan	~220 ± 20	Backbone: (1→4) -β-D-mannopyranose Branches: 1→6-linked-α-D-galactopyranose	Zero
Fucoidan	Galactofucan sulphate	~713	L-Fucose linked 1→3 and 1→4, and C-2 or C-4 is sulphated	23

For the *in vitro* work a further three different fucoidans have been used. They are described in Table 2.3.

Table 2.3: Properties of fucoidan fractions used in the study.

Fucoidan fraction	Purity (%)	Sulphation (%)	Acetylation (%)
75% GFS™	~75	23	35
NOV1-GFS (N1)	~100	22.2	44
NOV6-GFS (N2)	~100	4.5	23
DP-GFS-001 (N3)	~100	32	Zero

2.6 Harvest of *Undaria pinnatifida* and the extraction procedure of fucoidan

(A) A diver harvesting seaweed, with a handful of mature *Undaria* underwater. You can see the spore bodies, and the leafy parts of the plant, and their size relationship to him.

(B) A close up view of stalks of young *Undaria*, prior to the development of the spore body. The spore body will develop just below the leafy section, on the 'stem'.

(C) Wet spores in a bin, prior to being racked for drying. These large spores are about 30 cm or more in length.

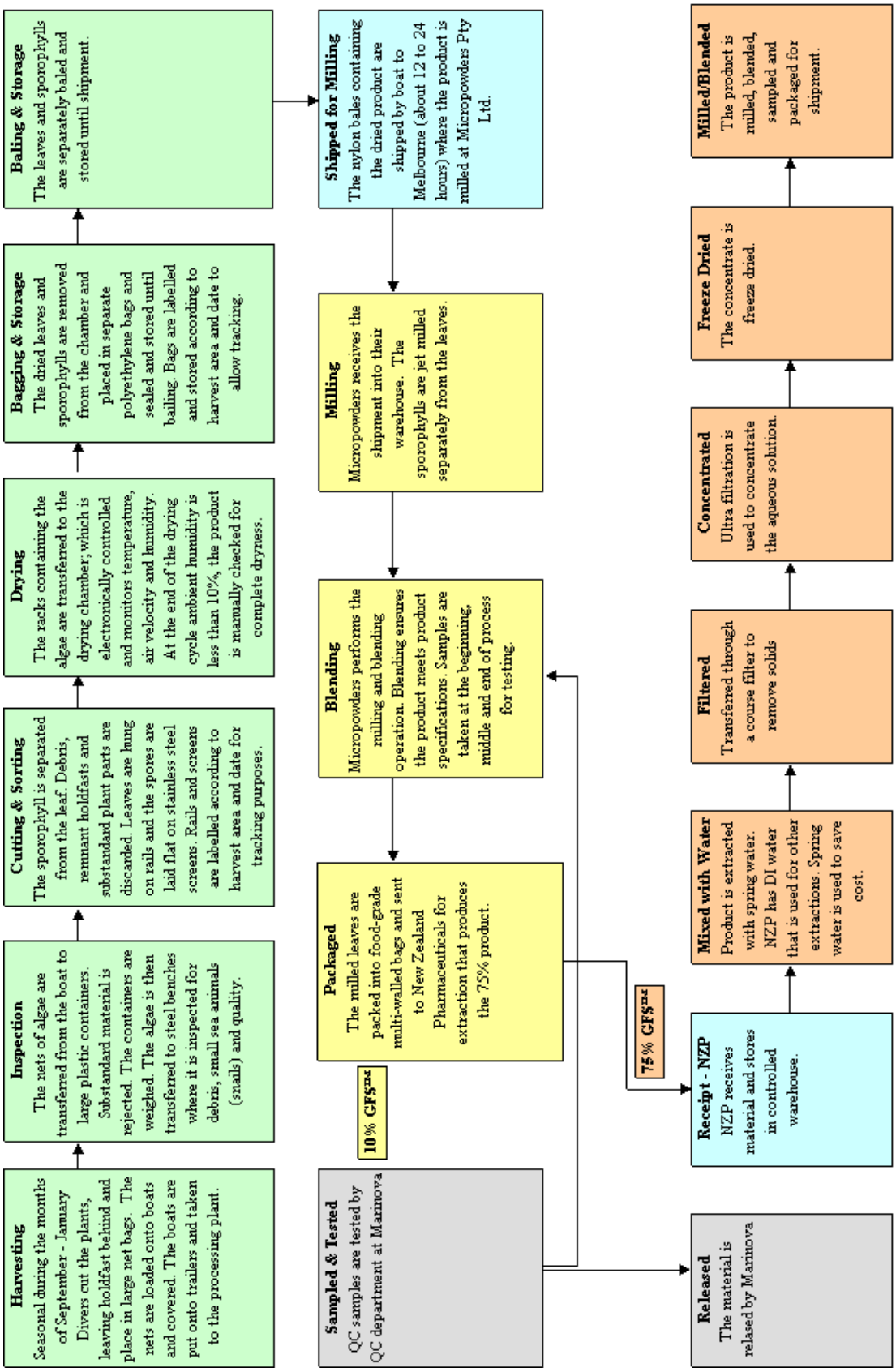
(D) Onsite *Undaria* sorting, separation and quality control.

(E) Racked *Undaria* plants. The height of the stand is about 2m. The plants are draped over poles.

(F) The *Undaria* spores on drying racks, having been separated from the leaves. They are about to be placed into the gas fired drying chamber behind.

Figure 2.3: Pictures showing the process of Undaria harvest, processing, and drying at Marinova's factory in Triabunna, TAS.

Undaria pinnatifida was harvested and prepared according to the following flow chart



2.7 Assay data for different seaweed extracts

2.7.1 Assay data for 10% GFS™ fucoidan

A typical seaweed sample taken randomly from November 2003 batch assay indicates that galactofucan sulphate (GFS™), fucoidan, is present at a minimum of 10%. This analysis that is shown in Table 2.4 was conducted in accordance with the methods of Stevenson and Furneaux (1991). Table 2.5 shows the microbiological assay data conducted on 10% GFS™ samples collected randomly from the November 2003 stock.

Table 2.4: Sugar analysis of a typical whole seaweed sample (10% GFS™ fucoidan) collected and analysed in November 2003.

Test (%)	Specification
Fucose	3.50
Galactose	2.77
Mannose	15.01
Galactose + Fucose	6.27
Sulphate*	4.24
GFS™ bound protein**	0.32
GFS™	10.83

* Assay result assumes sulphation at ratio of 1.21 sulphates per fucose residue, as obtained for water extract.

** Assumes GFS™ bound protein at ratio of 0.09 protein per fucose residue, as obtained for water extract.

Table 2.5: Microbiological data on historical stock illustrates stability of 10% GFS™ over three years. Two samples were assayed from each bag, chosen randomly from stock held in January 2003.*

Test (%)	Specification (CFU/g)
Standard Plate Count	20
Coliforms Count	<20
E. coli	<20
Coag. Positive Staph	<100
Salmonella	ND/10g
Moulds	<100
V. parahaemolyticus	ND/10g
Yeast	<100

* All tests were conducted by the Australian Government Analytical Laboratories (AGAL), Melbourne, VIC Australia, a NATA accredited facility.

Different tests were conducted to assay for the presence of different pesticides on a randomly chosen 10% GFS^{TM} samples. The assay results are shown in Table 2.6 and it indicates the absence of all listed pesticides at a detection limit of 0.05 mg/kg whole seaweed.

Table 2.6: Pesticides assay conducted to detect the pesticide residues in randomly chosen seaweed samples. Assays tested for the presence of organophosphates, organochlorins and polychlorinated biphenols. The detectable limit is 0.05 mg/kg seaweed.*

Azinphos Ethyl	Parathion	cis Chlordane	Hexachlorobenzene (HCB)
Azinphos methyl	Parathion methyl	trans Chlordane	DicofolPCB1254 PCB 1260
Bromophos ethyl	Trichlorfon	Oxychlordane	Endosulfan sulphate
Carbophenthion	Vamidothion	Total Chlordane	Total Endosulfan
cis Chlorfenvinphos	Demeton-S-methyl	Aldrin	Methoxychlor
trans Chlorfenvinphos	Dimethoate	Endrin	DDD
Total Chlorfenvinphos	Methacrifos	Dieldrin	DDE
Chlorpyriphos	Mevinphos	alpha BHC	DDT
Chlorpyriphos methyl	Omethoate	beta BHC	Total DDT analogs
Diazinon	Phosalone	delta BHC	Methamidophos
Dichlorvos	Phosmet	gamma BHC (Lindane)	Methidathion
Ethion	Pirimiphos methyl	Total BHC	Monocrotophos
Fenamiphos	Profenofos	Heptachlor	beta Endosulfan
Fenchlorphos	Temephos	Heptachlor epoxide	
Fenitrothion	Triazophos	Total Heptachlor	Malathion (Maldison)
Fenthion	Xathion	alpha Endosulfan	

* All tests were conducted by Australian Government Analytical Laboratories (AGAL), Melbourne, VIC Australia, a NATA accredited facility.

The general properties, handling, storage, safety, health hazards and different metals assay of the whole seaweed (10% GFS^{TM}) are listed in Table 2.7.

Table 2.7: Metals assay and other different properties for randomly chosen samples of 10% GFS™ fucoidan have been tested at AGAL.

Heavy metals	**Assay**
Lead	Typically less than 1μg/g, not more than 1μg/g
Arsenic	Inorganic arsenic typically less than 1μg/g, not more than 1μg/g
Organic arsenic	Typically <40μg/g
Mercury	Typically <0.01μg/g, not more than 0.05μg/g
Other metals	
Iodine	Typically circa 55μg/g, not more than 100μg
Sodium	Typically circa 40mg/g, not more than 50mg/g
Potassium	Typically circa 90mg/g, not more than 110mg/g
Calcium	Typically <11mg/g
Magnesium	Typically <7mg/g
General	
Stability	When stored <25C in dark below 10% humidity, stability of GFS™ at least three years
Notional shelf life	At least 2 years under correct storage conditions
Foreign matter (insect fragments, etc.)	No foreign matter present At least to US FDA guidelines
Safety and storage	
Flammability	Not flammable. Will char if burned
Stability of GFS™	Stable with respect to GFS™ content over at least three years if stored below25°C in dark, at less than 10% humidity
Stability- organoleptic properties	No apparent changes in colour, taste, and smell over three years when stored as above
Swelling	Attains approximately ten times dry weight when placed in water. Forms non-cohesive slurry
Storage conditions	Less than 25°C, dark sealed packaging, moisture less than 10%, preferably less than 7%
Health hazards	
Contraindications	Persons with iodine sensitivity should avoid this
Threshold limit	Can be ingested without side effects
Effect of exposure	Nil, material is non-toxic
Emergency and first aid	Nil, wash material away, non-toxic
Handling	
Protective clothing	Not required for personal safety
Contact with skin	Non-hazardous, non-toxic. Rinse material off
Respiratory precautions	As with all powders, wear dust protection if handling. No specific toxicity
Steps to be taken if material is spilled	Wash away with water. Material may be slippery, take care

2.7.2 Assay data for 75% GFS™ fucoidan

A typical seaweed sample taken randomly from November 2003 batch assay indicates that galactofucan sulphate (GFS™), fucoidan, is present at a minimum of 75%. This analysis was conducted in accordance with the methods of Stevenson and Furneaux (1991). The material safety data sheet for 75% fucoidan is shown in Appendix-4. Table 2.8 shows the sugar, protein, and sulphate assay data for a typical random sample. Table 2.9 shows the microbiological assay data conducted on 75% GFS™ samples collected randomly from the November 2003 stock.

Table 2.8: Sugar, protein, and sulphate assay data for a purified 75% GFS™ fucoidan extract sample chosen randomly from November 2003 batch.*

Fucose	24.76
Galactose	20.35
Mannose	nil
Sulphate	29.07%
Protein	2.19%
Bound ions	7%
Total GFS™ fucoidan	76.37%

*This analysis was conducted in accordance with the methods of Stevenson and Furneaux. Extraction is undertaken with water and ultra-filtration. No solvent residues are present. Water content less than 5%.

Notes: GFS™ contains small amounts of other sugars (xylose, glucose, uronic acid), and small variations in sulphate levels. Sodium ions remain attached to the sulphate residues during processing. The fucoidan has a small and integral protein component. Polyphenolic materials are also present in this fraction.

Table 2.9: Microbiology assay data for a purified 75% GFS™ fucoidan extract sample chosen randomly from November 2003 batch.*

Test	Specification	Test Method
Aerobic plate count	80cfu/g	Current Version of USP
Yeasts	<1cfu/g	Current Version of USP
Moulds	<1cfu/g	Current Version of USP
Coliforms count	Negative	Current Version of USP
Salmonella	Negative	Current Version of USP
Staphylococcus aureus	Negative	Current Version of USP
Pseudomonas aeruginosa	Negative	Current Version of USP

* All tests were conducted by the Australian Government Analytical Laboratories (AGAL), Melbourne, VIC Australia, a NATA accredited facility.

Table 2.10 shows the metal assay for a typical randomly chosen 75% fucoidan sample and Table 2.11 shows the different physical properties.

Table 2.10: Metal assay data for a purified 75% GFS™ fucoidan extract sample chosen randomly from November 2003 batch tested at AGAL.

Test	Specification
Lead	1.15ppm
Arsenic	Total 5ppm
Mercury	<0.01ppm
Cadmium	0.091ppm
Sodium	3.06g/100g
Potassium	2.53g/100g
Calcium	1.29g/100g
Magnesium	0.85g/100g
Iodine	28.6ppm

Table 2.11: Physical assay data for a purified 75% GFS™ fucoidan extract sample chosen randomly from November 2003 batch tested at AGAL.

Test	Specification	Test Method
Appearance	Off white powder	Visual
Moisture	Less than 10% (w/w)	USP LOD
Identification	Conforms to reference standard IR spectrum	IR analysis
Particle size	100% pass through 50 mesh (300 μm)	USP

2.7.2.1 Determination of the degree of acetylation of fucoidan

This technique allows the observation of all of the protons present in the sample. It measures the relative amounts of fucose and bulk acetyl content in the fucoidan. The resonances for these two moieties are well separated in the ^{1}H Nuclear magnetic resonance (NMR) spectrum. The fucose methyl resonance is at ~1.6 ppm and the acetyl resonance at ~2.5 ppm. The remainder of the spectrum between 3 and 7 ppm contains the HDO peak from the solvent and the sugar backbone protons. The degree of acetylation is the ratio of the acetyl peak(s) to the fucose methyl expressed as a percentage. Since the acetyl groups may be attached to different sugars and the sugars are in different environments, all peaks tend to be broad and may show multiple

peaks. Integration ranges for the two have been set at 2.1-2.9 ppm for the acetyl and 1.0-2.1 ppm for the fucose methyl. No chemical alteration of the sample is required. The method was qualified and limits of quantitation were established.

Procedure:

A 400 MHz wide bore Varian-Inova NMR spectrometer is used with a 10 mm probe. The sample (10 mg ± 1 mg) is dissolved in 99.9% D20, CAS [7789-20-0] (1.0 mL, Cambridge Isotope Laboratories) in a high precision 5mm NMR tube (Wilmad 535-PP). No internal standard is added and the HDO peak is set at 4.75 ppm. The spectrum is acquired without spinning with the following parameters:

Temperature: 60°C
Pulse sequence: standard pi pulse
Frequency: 399.683 MHz
Relaxation delay: 10 seconds
Data points: 128K
Acquisition time: 10.7 seconds
Transients: 128 to 256

Time domain data Fourier transform with a 1Hz exponential window function (line broadening), manually phased, baseline corrected by the polynomial method and drift corrected. To provide a quantitative determination of the acetyl groups, the peaks at 1.6 and 2.5 ppm are integrated (from 2.9 to 2.1 ppm and 2.1 to 1.0 ppm) and the ratio reported as the degree of acetylation. This work was conducted at Marinova Pty Ltd., Hobart, Tasmania.

2.7.2.2 Analysis of sulphur in fucoidan using Magnetic Sector ICP-MS

This work was conducted by Marinova Pty Ltd., Hobart Tasmania. Small amounts (1-10 mg) of provided sample were weighed into tin cups on a highly accurate micro-balance. Samples were dissolved and diluted in ultra-pure water to 100 g (nominally ~100 mL) in 120 mL polycarbonate sealed containers. A 1 g sub-sample was further diluted 10 fold to 10 g, with Indium added as an internal standard (at a concentration level of 100 ppb) and acid added (0.1 ml high purity SEASTAR nitric acid) in 12 mL polycarbonate sample tubes. Dried sodium sulphate was used as an “in-house” standard reference material (S = 22.6 wt %).

Analysis was performed using an ELEMENT magnetic sector ICP-MS (Finnigan, Bremen, Germany). This instrument allows high resolution measurements to be performed, which in this instance means that the major isotope of S (^{32}S) can be spectrally resolved from the interfering $^{16}O_2$ (also of nominal mass 32amu). The instrument was allowed to warm for ~1 hr prior to any analysis. Sulphur standards were prepared from an externally sourced mixed multi-elemental calibration solution (100 ppm stock solution in 10% nitric, QCD Analysts, USA).All calibration blanks and standards were prepared in 1% nitric acid, with Indium present at 100 ppb [*i.e.* calibrants and samples were matched in terms of internal standard and acidity].

A typical calibration consisted of a blank and 1 standard (100 ppb). Calibration accuracy was verified by the analysis of another independent single element S solution, prepared as above to a final concentration of ~1000 ppb. The instrument provides raw data in the form of detector counts of the ^{32}S and ^{115}In isotopes, over 40 scans, and then internally calculates the sulphur concentration in ppb. A 120 second sample uptake time was employed, along with a 150 second rinse with 5% nitric acid between each sample. The method was qualified. This work was conducted at Marinova Pty Ltd., Hobart, Tasmania.

The percentage sulphur values were calculated as follows:

$$((reading\ ppb - blank\ ppb) * dilution\ factor) \div 10^7$$

2.8 Human cells

2.8.1 KG1a cells

The KG-1a cell line was used to establish the methodology for the *in vitro* work. KG1a is a variant sub-line of the human acute myelogenous leukaemia (AML) cell line (KG-i). The KGla cells are morphologically and histochemically undifferentiated blast cells. This cell line is a suitable model for this study as it is an immortal haemopoietic progenitor cell, expressing the CD34 antigen and having similar metabolic requirements to primary cells (Koeffler *et al.*, 1980). The KG1a has a doubling time of 30-35 hours. The cell line was kept at the Graduate School of Biomedical Engineering, UNSW. Fresh cultures were used during the study.

2.8.2 Human peripheral blood $CD34^+$ cells

Isolex-selected $CD34^+$ cells were harvested from consenting multiple myeloma patients or donors provided by Peter MacCallum Cancer Centre (Melbourne, VIC Australia). These cells were stored in liquid nitrogen and thawed immediately at 37°C on the day of the experiment. Then they were washed thrice with 10% FCS in IMDM. $CD34^+$ cells were cultured with fucoidan extracts as described later.

2.8.3 Cord blood $CD34^+$ cells

2.8.3.1 Human umbilical cord blood (HUCB) collection

Cord blood samples, 1–2 days old and stored at 4–10°C, were obtained from the umbilical cord vein following vaginal delivery of babies at full-term from the cord blood bank, Randwick NSW Sydney, Australia. An average volume of 95 mL of HUCB was collected in 250 mL pediatric collection bags containing citrate phosphate dextrose (Baxter Health Care, NSW Australia) and 800 Units of preservative free heparin (Delta West, Bently, WA Australia). The blood was stored at 4°C until processing. Informed consent was obtained from all mothers, and all procedures were approved by both the UNSW Committee on Experimental Procedures Involving Human Subjects and the Research Ethics Committee of the Eastern Sydney Area Health Services.

2.8.3.2 Cord blood $CD34^+$ cells isolation

CB was obtained as described previously in section 2.6.3.1. Briefly, the CB $CD34^+$ cells were MACS (Miltenyi Biotech GmbH, Germany) enriched as follows.

To isolate a mononuclear cell (MNC) concentrate on a Ficoll Histopaque density gradient (Sigma-Aldrich, MO USA), approximately 30 mL of CB was aliquotted into 45 mL Falcon tubes using a 60 mL syringe. Twelve mL of Histopaque was introduced under each CB layer and the tubes were then centrifuged at 400 *xg* for 30 min. The upper layer was then aspirated and discarded and the opaque interface containing the MNC was transferred to a new centrifuge tube and washed with 40 mL PBS-D plus 2 mM EDTA, pH 7.2 (MACS buffer). Residual RBC where then lysed using 40 mL cold ammonium chloride (140 mM) on ice for 15 min before centrifugation at 250 *xg* for 10 min. The cells were then washed twice with MACS buffer, the cells counted and re-suspended in MACS buffer. Cells were magnetically labelled with MACS reagents by adding 100 µL of each FCR blocking reagent and CD34 Multisort MicroBeads per 10^8 cells, incubated for 30 min at 4°C, then washed twice with 12 mL washing buffer by centrifugation for 10 min at 250 *xg*, and re-suspended in MACS buffer at a concentration of $2x10^8$ cells/mL.

The magnetic separation was then performed by applying a positive LS column to the separator. The column was washed first with 3mL of MACS buffer then the labelled cell suspension was applied to the column and the negative cells collected. The column was washed thrice with 3 mL of MACS buffer. After that, the enrichment tube was put in place and the LS column removed from the separator. Five mL of MACS buffer was applied to the reservoir of the column and the cells were flushed out using the plunge supplied with the enrichment tube. The collected $CD34^+$ cells in the enrichment tube were centrifuged for 10 min at 250 *xg* and re-suspended in MACS buffer. The MS column was replaced in the separator and washed with 0.5 mL of MACS buffer and the waste was collected. The labelled cell suspension was reapplied to the column and the negative cells were again collected. The column was washed thrice with 0.5 mL MACS buffer, the enrichment tube was then put in place and the MS column removed from the separator. One mL of MACS buffer was applied to the reservoir of the column and the cells were flushed out using the plunge supplied with the enrichment tube. The enrichment tube containing positive cells was centrifuged for 10 min at 250 *xg*. The cells were resuspended in MACS buffer and counted.

2.8.3.3 MACS of HUCB

HUCB cells expressing the CD34 antigen were immunomagnetically selected using the MACS system (Miltenyi BD, North Ryde, NSW Australia). Briefly, HUCB MNC ($1.6x10^7$-$2.3x10^8$) were incubated at 4°C with 100 µL of blocking reagent (human IgG) and 100 µL CD34 Ab (QBEND/10 mouse IgG_1), per 10^8 cells for 15 min. The cells were washed once in buffer [PBS; 5 mM EDTA (Ajax Chemicals, Auburn, NSW Australia); 0.5% Bovine Serum Albumin (Sigma, USA)], and incubated for 15 min at 4°C with colloidal MACS micro-beads (100 µL/10^8 cells) directed against the haptenised QBEND/10, mouse IgG_1. The cells were washed in buffer, re-suspended and passed through a 70 µm nylon mesh cell strainer (Falcon, USA) to remove clumps. The labelled cells were added to a sterile separation column placed in the magnetic field. The $CD34^-$ fraction passed through the column and was collected into sterile tube. The separation column was then eluted by the addition of 1-2 mL of buffer. The separation column was removed from the magnetic field and the $CD34^+$ cells eluted from the column with buffer. Initial $CD34^+$ cell percentage, total number of $CD34^+$ cells, purity, enrichment, yield and co-expression of CD38 by $CD34^+$ cells were evaluated.

2.8.4 Mononuclear cells preparation

HUCB (15-20) mL was diluted 1:1 with 15 mL of Dulbecco's phosphate buffered saline (PBS-D) (Gibco, Melbourne, Australia) and under-layered with 15 mL of Ficoll (d=1.077 g/mL) (Sigma, Castle Hill, NSW Australia) in a 50 mL tube. The gradients were centrifuged at 400 *xg* for 30 min and the resultant MNC layer was collected. The MNC were washed twice with PBS-D and cell count and viability studies were performed using the trypan blue exclusion method.

2.8.5 Staining $CD34^+$ cells with CFSE

Purified $CD34^+$ cells were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE). The optimum concentration that would give enough staining intensity to enable resolution by flow cytometry for at least six cellular divisions was 2.5 µM. The cells were incubated with the stain for 10 min at 37°C and cold foetal bovine serum (FBS) was added at 3 times the volume of the cell suspension to stop the staining reaction. The cells were then washed twice with IMDM + 10% FBS and re-suspended in 4mL of the same medium. The cells were incubated overnight at 37°C + 5% CO_2.

Next day, the cells were washed once with PBS-D +10% BSA and cultured as needed. Cells were cultured with either DP-GFS-001 or 75% GFS^{TM} at 500 μg/mL in the modified StemlineTM II media containing 100 ng/mL of each of the cytokines (G-CSF, SCF, Flt-3, and TPO). Cells were harvested and analysed every day using flow cytometry for a period of 5 days.

2.8.6 Immunophenotyping

The immunophenotyping of cells before and after cytokine treatment was determined by three-color flow cytometry. On the day of harvesting, 1 μg of human IgG (CSL Ltd., Australia) was added to each well and incubated for 15 min at ambient temperature. A panel of MoAbs was used to distinguish between undifferentiated and differentiated progenitor cells. Cells were harvested and transferred to FACS tubes then incubated with MoAbs for 30 min at 4°C followed by two washes with 3 mL PBS-D + 10% FBS. The cells were re-suspended in 300 μL of FACS-Fix and stored in the dark at 4°C for later analysis. Fluorescence compensation was performed on all channels to minimize spectral overlap and flow cytometric data acquisition was performed with CELLQuest software on a Becton-Dickenson FACSort. Gates were set to include the viable cells and at least $1x10^4$ events were collected per sample. Analysis was performed using Cytomation Summit V3.1, CO USA.

2.8.7 Cell count using beads

Uniform micro-spheres (beads) with a mean diameter of 9.62 μm (Bangs Lab, IN USA) were added to the wells before harvest to provide an internal standard for calculating absolute cell count. Beads were washed thrice with PBS-D + 2% BSA and re-suspended in PBS-D + 10% FBS. Generally, $1x10^5$ beads/mL were added to each culture. The cell count was calculated using the cell to bead ratio (R=cell/bead) as determined by flow cytometry. This ratio was then multiplied by the number of beads added to each sample to calculate the number of cells in that sample.

2.8.8 Cryopreservation of mammalian cells

Most mammalian cells can be stored at temperatures below –130°C for many years. The viability of the cells after cryopreservation depends on their ability to cope with the variety of stresses imposed on them during the freezing and thawing procedures.

Viable cells were frozen in the exponential growth phase. Cells were harvested by centrifugation at 400 *xg* and resuspended in growth medium containing BSA at room temperature to a concentration of $2x10^6$–$2x10^7$ cells per mL. Viable cells were counted. Dimethyl sulphoxide (DMSO) was diluted to 20% in medium, allowed to cool to below 37°C and subsequently added to the cells to a final concentration of 10%.

The cells and cryoprotectant (20% in medium) were mixed at room temperature to final concentration of viable cells in the range between 10^6 and 10^7 cells per mL in 1:1 ratio. The cells were incubated with DMSO for 20 min to allow the DMSO to enter the cells and the cooling procedure was started. One mL of cells was then aliquoted into CryoTube™ vials after mixing.

The cells were then cooled down at a rate of 1°C per minute in the range room temperature to below –70°C, then submerged directly in liquid nitrogen (–196°C). A special cooling container containing ethanol was used.

2.8.9 Thawing of cells

To obtain the best possible survival of the cells, thawing of the cells was performed as quickly as possible once the CryoTube™ vial was removed from the freezer/liquid nitrogen tank. This was achieved by transporting the CryoTube™ in a covered, insulated container before incubation in a 37°C water bath. Once completely thawed the CryoTube™ vials were immersed in 70% ethanol before being taken to the laminar flow cabinet. The cells were washed once in growth medium before being transferred to a TC-flask in fresh growth medium. The DMSO was diluted in a stepwise manner to minimize the osmotic stress imposed upon the cells. A sample of the thawed cell suspension was taken out for a viability test before the cells were cultured.

2.9 Factorial experimental design

In this study we have used a two-level full factorial experimental design where the number of experimental runs is 2^n (n is the number of factors to be examined). For larger numbers of factors, the number of experimental runs can be reduced by grouping together (confounding) higher order interactions (*i.e.* two or more interacting

factors). A fractional factorial design (resolution V, 2^{5-1}, half replicate of 5 factors in 16 runs) was employed to determine single-factor effects and to screen for two-factor interaction.

Two experimental blocks were used to determine the effect of cytokines and fucoidan on HSC expansion and proliferation. In block-A, we examined the effect of fucoidan at low concentrations; the low and high level of fucoidan was 0 and 10 μg/mL, respectively. Block-B determined the effect of higher levels of fucoidan; 100 versus 500 μg/mL.

The 2^{5-1} fractional factorial design was used to investigate the relative effects of five factors on the proliferation and expansion of mobilised peripheral $CD34^+$ cells incubated in serum-free media containing100 ng/mL G-CSF. The experiments were duplicated using two different patient donors. The combinations of factors tested are shown in Table 2. $CD34^+$ cells $2x10^4$ in 1 mL of culture media were grown in 24 well plates at 37°C with 5% CO_2 over a 10 day period. Manual and automated cell counts were determined along with phenotypic analysis, as described later, for each culture condition.

A full factorial design was used to investigate the effect of high levels of growth factors (SCF, Flt-3, TPO at 100 versus 1000 ng/mL) in combination with fucoidan. Four $x10^3$ $CD34^+$ cells in 200 μL of culture media were grown in 96 well plates at 37°C with 5% CO_2 over a 10 day period.

CHAPTER THREE

CLINICAL PATHOLOGY TESTS

Chapter objectives:

- To establish the safety of fucoidan administration at 3g per day in volunteers
- To study and report the clinical, biological and pathological changes after oral doses of fucoidan by examining volunteers' general health, physical and routine laboratory studies including LFT, U+E and haematology tests
- To study the effect of ingesting fucoidan on the lipid profile
- To study the effect of oral fucoidan on NO and insulin levels

Chapter contents:

3.1 Summary

This chapter reports the laboratory results from volunteers screened before the study and judged eligible for entry into the clinical trials. They were monitored during the study for health and organ function by sequential tests for liver and kidney function plus haematology tests. Blood and urine samples were collected at different time points before and after ingesting the treatment capsules. All tests were performed according to the standard methods available at the time at the core pathology laboratory, Royal Hobart Hospital. Volunteers were excluded from the clinical trial if they showed any abnormal values at the start of the survey. All volunteers considered suitable for the study showed good tolerance towards the ingestion of the different fucoidan extracts or placebo.

3.2 Introduction

Fucoidans have a wide spectrum of activity in biological systems. Besides their well-attested anticoagulant and antithrombotic activity, they act on the inflammatory and immune systems, they have anti-proliferative and anti-adhesion effect on cells, they protect cells from viral infection, and can interfere with mechanisms involved in fertilization. All of these properties have been reviewed by Boisson-Vidal and his co-workers (1995).

Most studies have used a commercial preparation of *Fucus vesiculosus* fucoidan, which has been shown to contain heteropolysaccharides of various kinds besides those consisting predominantly of sulphate and fucose (Nishino *et al.*, 1994). Some studies have been published, however, on partly characterized fucoidan fractions, and most of these concern the effects of fucoidan on blood coagulation. Relatively few studies have interpreted the biological activity of fucoidans in terms of molecular structure. Almost all biological studies use a commercially available, crude preparation of sulphated polysaccharides from *Fucus vesiculosus* rather than a purified fucoidan (Mulloy *et al.*, 1994). Recent insights into the structures of fucans from different plant and animal species may help explain their mode of activity, whether as research reagents or as potential therapeutics.

There have been no studies reporting the effect of ingesting fucoidans on general liver and kidney function and on blood parameters, and in particular, the fucoidan extracted from *Undaria pinnatifida*. This emphasised the importance of a study of the well-being of the volunteers and any possible metabolic changes arising from ingestion of this fucoidan.

The liver and kidneys are the main regulator of all body fluids and are primarily responsible for maintaining homeostasis since they receive one fourth of the cardiac output. Study of the function of each of these organs is vital for clinical trials with potential new drugs.

Lipids constitute a wide variety of organic compounds that differ greatly in their chemical and physical properties and their physiological roles. They include a variety of substances such as fatty acids, sterols, triglycerides, phospholipids, fat-soluble vitamins, bile acids, waxes, and other complex fats.

In this chapter the effects of nitric oxide (NO) is also reported. NO is a recently identified biological signal molecule that plays an important role in vascular regulation, immune responses, and neural signal transduction, it maintains blood pressure by dilating blood vessels, and is involved in control of some neoplasms, inhibiting growth and inducing differentiation and apoptosis in acute myeloid leukaemia cells (Hussain *et al.*, 2003). NO is a chain-breaking antioxidant which means it has the ability to react with reactive oxygen species to protect molecules such as proteins and lipids. It reduces oxygen consumption during free radical mediated lipid peroxidation. Nitric oxide synthase (NOS-2) (iNOS) is not expressed constitutively but is rapidly induced by IFN-γ, TNF-α, IL-12 and LPS (Chesler *et al.*, 2002).

NO is produced from the amino acid arginine in many different cell types through four separate nitric oxide synthases (NOS), neuronal (nNOS), endothelial (eNOS), inducible (iNOS) and insulin-activated (iaNOS) (Guha *et al.*, 2002). The iNOS is involved in the dysregulation of haemopoiesis in the BM of patients with myelodysplastic syndromes (MDS) (Kitagawa *et al.*, 1999) and the bioavailability of NO in plasma is affected by the RBC availability and membrane status (Liao *et al.*, 1999; Huang *et al.*, 2001). Therefore the study of the level of NO in the plasma before and after fucoidan treatment is important.

Liver and kidney function tests, blood parameters, lipid profile and other tests such as ESR and NO levels will therefore provide information about how the body organs are responding to the treatment and how they perform under medical stress that might be caused by the ingestion of the fucoidan.

3.3 Materials and methods

Liver function tests (LFT), kidney tests (U+E), haematology analysis and urine analysis were performed on volunteer's blood samples to assess the state of their health and organ function before and during the study to follow any possible effect caused by the treatment capsules. In this clinical trial a total of 42 volunteer's where recruited in three groups as follows:

- Placebo group, 6 volunteers,
- 10% fucoidan group, 6 volunteers,
- 75% fucoidan group, 30 volunteers

The chemistry and haematology tests including (LFT, U+E, electrolytes, and CBC) were performed on 6 volunteers who ingested placebo or 10% fucoidan capsules and on 25 volunteers who ingested the 75% fucoidan capsules. ESR was only tested in the first 11 volunteers who received 75% fucoidan capsules, nitric oxide and insulin were tested in 10 volunteers who ingested the 75% fucoidan capsules and in 3 placebo volunteers. The proceeding volunteers were not tested for ESR, NO or insulin because of the limited funding.

3.3.1 Chemistry tests

All chemistry tests in sections 3.3.1.1 to 3.3.1.16 were measured in samples on the Vitros Chemistry System V950 (Ortho Clinical Diagnostic, Inc. NY USA). Vitros slides are multilayered, analytical elements coated on polyester support.

3.3.1.1 Total protein (TP)

The plasma TP, most of which are made by the liver, was measured in samples (10 µL) using the Vitros TP slides and chemistry products calibrator kit-4. The analysis is based on the biuret reaction, which produces a violet complex when protein reacts with cupric ion (Cu^{+2}) in an alkaline medium. The TBIL was measured colorimetrically at 540 nm and 37°C. The reference range that was used in this study was (60-80) g/L.

$$\text{protein} + \text{copper tartrate} \xrightarrow{\text{LiOH}} \text{coloured complex}$$

3.3.1.2 Albumin (Alb)

Albumin was measured in samples (10 µL) colorimetrically at 630 nm and 37°C using Vitros Alb slides and a chemistry products calibrator kit-4. The colour complex that formed was measured by reflectance spectrophotometry. The reference range that was used in this study was 35-50 g/L.

$$\text{albumin} + \text{bromcresol green (BCG)} \rightarrow \text{BCG - albumin complex}$$

3.3.1.3 Alkaline phosphatase (ALP)

Serum ALP (ortho-phosphoric monoester phosphohydrolase) was measured in samples (11 µL) using the Vitros ALP slides and chemistry products calibrator kit-3. The ALP in the sample catalysed the hydrolysis of the *p*-nitrophenyl phosphate to *p*-nitrophenol at alkaline pH. The *p*-nitrophenol diffused into the underlying layer, and it was monitored by reflectance spectrophotometry by multiple-point rate at 400 nm and 37°C. The reference range that was used in the study was 40-115 IU/L.

$$p\text{ - nitrophenyl phosphate} \xrightarrow{\text{ALP, } Mg^{+2}\text{, AMP}} p\text{ - nitrophenyl} + H_3PO_4$$

3.3.1.4 Alanine transaminase (ALT)

ALT also called Serum Glutamic Pyruvic Transaminase (SGPT) or alanine aminotransferrase was measured in samples (11 µL) using the Vitros ALT slides and chemistry products calibrator kit-3. The rate oxidation of NADH is monitored by reflectance spectrophotometry by multiple-point rate at 340 nm and 37°C. The reference range that was used in this study was <65 IU/L.

$$\text{alanine} + \alpha\text{ - ketoglutarate} \xrightarrow{\text{ALT, pyridoxal-5-phosphate}} \text{pyruvate} + \text{glutamate}$$
$$\text{pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{LDH}} \text{lactate} + NAD^+$$

3.3.1.5 Gamma-glutamyl transferase (GGT)

GGT which is also called Gamma-glutamyl transpeptidase was measured in samples (11 µL) using the Vitros GGT slides and chemistry products calibrator kit-3. The GGT was measured by multiple-point rate at 400 nm and 37°C. The reference range used in this study was < 90 IU/L.

$$\text{L-}\gamma\text{-glutamyl-}p\text{-nitroanilide} + \text{glycylglycine} \xrightarrow{\text{GGT}} p\text{-nitroaniline} + \gamma\text{-glutamylglycylglycine}$$

3.3.1.6 Total bilirubin (TBIL)

The total bilirubin was measured using the Vitros TBIL slides and chemistry products calibrator kit-4. The analysis is based on a modification of the classic diazo reaction. The TBIL was measured in samples (10 µL) colorimetrically at 540 nm and 640 nm at 37°C. The reference range used in this study was < 25 µmol/L.

$$\text{total bilirubin} \xrightarrow{\text{dyphylline,[4-(N-carboxymethylsulfonyl)-benzenediazonium hexafluorophosphate]}} \text{azobilirubin chromophores}$$

3.3.1.7 Glucose

Glucose was measured using the Vitros glucose slides and chemistry products calibrator kit-1. The glucose concentration was measured in 10 µL of the sample colorimetrically at 540 nm and 37°C. The dye system used is closely related to that first reported by Trinder (Trinder, 1969). The chemistry of the glucose slides has been described by Crume and his colleagues (Crume *et al.*, 1978). The reference range used in this study was (3.5-7.9) mmol/L.

$$\beta\text{-D-glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} \text{D-gluconic acid} + H_2O_2$$
$$2H_2O_2 + \text{4-aminoantipyrine} + \text{1,7-dihydroxynaphthalene} \xrightarrow{\text{peroxidase}} \text{red dye}$$

3.3.1.8 Sodium (Na^+)

The Na^+ was measured using the Vitros Na^+ slides and chemistry products calibrator kit-2. The Vitros Na^+ slide uses direct potentiometry for measurement of ionic sodium. The slide consists of two ion-selective electrodes, each containing methyl monensin (an ionophore for Na^+), a reference layer, and a silver and silver chloride layer coated on a polyester support. The Na^+ was measured potentiometrically in 10 µL of the sample at 37°C. The reference range used in this study was (135-145) mmol/L.

3.3.1.9 Potassium (K^+)

The K^+ was measured using the Vitros K^+ slides and chemistry products calibrator kit-2. The Vitros K^+ slide uses direct potentiometry for measurement of ionic potassium. The slide consists of two ion-selective electrodes, each containing valinomycin (an ionophore for potassium), a reference layer, and a silver and silver chloride layer coated on a polyester support. The K^+ was measured potentiometrically in 10 µL of the sample at 37°C. The reference range used in this study was (3.5-5.0) mmol/L.

3.3.1.10 Chloride (Cl^-)

The Cl^- was measured using the Vitros Cl^- slides and chemistry products calibrator kit-2. The Vitros Cl^- slide uses direct potentiometry for measurement of chloride ions. The slide consists of two ion-selective electrodes, each containing a protective layer, a silver layer and silver chloride layer coated on a polyester support. The protective layer inhibits interference from normal levels of bromide and uric acid. The Cl^- was measured potentiometrically in 10 µL of the sample at 37°C. The reference range used in this study was (95-110) mmol/L.

3.3.1.11 Bicarbonate (HCO_3)

Also known as ECO_2 was measured using the Vitros ECO_2 slides and chemistry products calibrator kit-2. The ECO_2 was measured by enzymatic end-point using 10 µL of the sample at 340 nm and 37°C. The reference range used in this study was (22-33) mmol/L.

$$HCO_3^- + \text{phosphoenolpyruvate} \xrightarrow{\text{phosphoenolpyruvate carboxylase}} \text{oxalacetate} + PO_4^{-3}$$
$$\text{oxalacetate} + NADH + H^+ \xrightarrow{\text{malate dehydrogenase}} \text{malate} + NAD^+$$

3.3.1.12 Blood urea nitrogen (BUN/Urea)

The urea was measured using the Vitros BUN/Urea slides and chemistry products calibrator kit-1. The BUN/Urea was measured colorimetrically in 10 µL of the sample at 670 nm and 37°C. The reference range used in this study was (2.7-7.8) mmol/L.

$$H_2NCONH_2 + H_2O \xrightarrow{\text{urease}} 2NH_3 + CO_2$$
$$NH_3 + \text{ammonia indicator} \rightarrow \text{dye}$$

3.3.1.13 Creatinine (Creat)

The Creat was measured using the Vitros Creat slides and chemistry products calibrator kit-1. The Creat was measured by two-point rate at 670 nm and 37°C in 6 µL of the sample. The reference range used in this study was (60-115) µmol/L.

$$\text{creatinine} + H_2O \xrightarrow{\text{creatinine, amidohydrolase}} \text{creatine}$$
$$\text{creatine} + H_2O \xrightarrow{\text{creatine, amidinohydrolase}} \text{sarcosine} + \text{urea}$$
$$\text{sarcosine} + O_2 + H_2O \xrightarrow{\text{sarcosine, oxidase}} \text{glycine} + \text{formaldehyde} + H_2O_2$$
$$H_2O_2 + \text{leuco dye} \xrightarrow{\text{peroxidase}} \text{dye} + 2H_2O$$

3.3.1.14 Cholesterol (Chol)

The Chol was measured using the Vitros Chol slides and chemistry products calibrator kit-2. The method is based on an enzymatic method similar to that proposed by Allain and his colleagues (Allain *et al.*, 1974). The Chol was measured colorimetrically at 540 nm and 37°C in 5.5 µL of the sample. The reference range used in this study was <5.5 mmol/L.

$$\text{lipoprotein} \xrightarrow{\text{TX100}} \text{cholesterol} + \text{cholesterol esters} + \text{proteins}$$
$$\text{cholesterol esters} + H_2O \xrightarrow{\text{cholesterol ester hydrolase}} \text{cholesterol} + \text{fatty acids}$$
$$\text{cholesterol} + O_2 \xrightarrow{\text{cholesterol oxidase}} \text{cholest - 4 - en - 3 - one} + H_2O$$
$$H_2O_2 + \text{leuco dye} \xrightarrow{\text{peroxidase}} \text{dye} + 2H_2O$$

3.3.1.15 Triglyceride (Trig)

The Trig was measured using the Vitros Trig slides and chemistry products calibrator kit-2. The Trig was measured colorimetrically at 540 nm at 37°C in 5.5 µL of the sample. The reference range used in this study was <2.0 mmol/L.

$$\text{lipoproteins} \xrightarrow{\text{surfactant}} \text{triglycerides} + \text{proteins}$$
$$\text{triglycerides} + H_2O \xrightarrow{\text{lipase}} \text{glycerol} + \text{fatty acids}$$
$$\text{glycerol} + \text{ATP} \xrightarrow{\text{glycerol kinase, } MgCl_2} \text{L - } \alpha \text{ - glycerophosphate} + \text{ADP}$$
$$\text{L - } \alpha \text{ - glycerophosphate} + O_2 \xrightarrow{\text{L-}\alpha\text{-glycerol-phosphate-oxidase}} \text{dihydroxyacetone phosphate} + H_2O_2$$
$$H_2O_2 + \text{leuco dye} \xrightarrow{\text{peroxidase}} \text{dye} + 2H_2O$$

3.3.1.16 High density lipoprotein cholesterol (HDL)

The HDL was measured using the Vitros HDL slides and chemistry products calibrator kit-25. The method is based on a non HDL precipitation method similar to one used by Burstein and co-workers (Burstein *at al.*, 1970) followed by an enzymatic detection similar to that proposed by Allain and his colleagues (Allain *et al.*, 1974). The HDL was measured colorimetrically at 670 nm and 37°C in 10 µL of the sample. The reference range used in this study was (0.90-1.81) mmol/L.

$$\text{HDL} + \text{non - HDL} \xrightarrow{\text{PTA/MgCl}_2} \text{HDL} + \text{non - HDL}\ (\downarrow)$$

$$\text{HDL} \xrightarrow{\text{Emulgen B-66}} \text{cholesterol} + \text{HDL cholesterol esters} + \text{proteins}$$

$$\text{HDL cholesterol esters} + \text{H}_2\text{O} \xrightarrow{\text{cholesterol ester hydrolase}} \text{cholesterol} + \text{fatty acid}$$

$$\text{cholesterol} + \text{O}_2 \xrightarrow{\text{cholesterol oxidase}} \text{cholest - 4 - en - 3 - one} + \text{H}_2\text{O}_2$$

$$\text{H}_2\text{O}_2 + \text{leuco dye} \xrightarrow{\text{peroxidase}} \text{dye} + 2\text{H}_2\text{O}$$

3.3.1.17 Low density lipoprotein (LDL)

The LDL was calculated according to Friedwald's formula (Kaplan *et al.*, 2003).

$$\text{LDL (mmol/L)} = \text{TC - (HDL} + (\text{Trig} \div 2.22))$$

3.3.1.18 Osmolality

Osmolality was determined by measuring the colligative properties (determined by the number of particles of solute in a given volume of solvent) using freezing point depression on the advanced micro osmometer (model 3MO plus or 3300, Advanced Instruments, Inc. Norwood, MA USA). The referenced range that was used in this study was (275-295) mmol/Kg. Then the osmolality was calculated using the following formula and both results were compared.

$$\text{Osmolality} = 1.86\,(\text{Na} + \text{K}) + \text{Urea} + \text{Glucose}$$

3.3.1.19 Anion gap

A measurement of the interval between the sum of "routinely measured" cations minus the sum of the "routinely measured" anions in the blood. The anion gap is

representative of the unmeasured anions in the plasma, and these anions are affected differently based on the type of metabolic acidosis.

The anion gap was determined by the following equation

$$\text{Anion gap (mmol/L)} = (\text{Na} + \text{K}) - (\text{Cl} + [\text{HCO}_3^-])$$

3.3.1.20 Nitric oxide (NO)

The NO concentration in plasma was determined by using a colorimetric, non-enzymatic assay for the determination of the total nitrite using a kit and protocol from Bioxytech (OXIS International Inc., USA). The assay procedure was performed as described below.

Half gram cadmium beads (approximately 6-7 beads) was added to a microcentrifuge tube for each sample and for each standard. The cadmium beads were washed twice with 1mL of each of the following in order:

a) H_2O

b) 0.1 M HCl

c) 0.1 M NH_4OH (pH 9.6)

The standards were prepared as detailed below. After that, 50 µL of plasma was added to 140 µL water and then 10 µL of 30% (w/v) $ZnSO_4$ solution was added and vortexed. The mixture was incubated at room temperature for 15 min then centrifuged (2,300 *xg*) for 5 min. The resulting supernatants were transferred to microcentrifuge tubes containing the granulated cadmium and incubated at room temperature overnight with agitation. The microcentrifuge tubes were re-centrifuged (2,300 *xg*) for 5 min and the supernatants were used for the assay. A 100 µL of each sample was added to the assigned wells in duplicate, and then 50 µL of colour reagent number-1 (Sulphanilamide in 3 N HCl) was added. Then, 50 µL of colour reagent number-2 (N-(1-Naphthyl) ethylenediamine dihydrochloride in deionized water) was added and mixed for 5 min at room temperature. After that, the absorbance values were read at A_{540} nm in a microtiter plate reader (MR 5000 Micro-plate Reader, Dynatech Laboratories Inc., VA USA). A standard curve was constructed and the concentration of the samples was estimated from the curve. A series of standards ranging from (0.5-500) µM were prepared and the final working concentration in the wells ranged from (0-50) µM.

To calculate the final NO concentration, the average absorbance value of the blank wells was subtracted from all other pairs of wells. The absorbance values for each pair of duplicate wells were averaged. A standard curve was plotted using the average absorbance value for each standard value versus the concentration and the concentration of each unknown was determined by interpolation from the standard curve. A sample standard curve is shown in [Figure 3.1]. The concentration of the unknowns in the assay was multiplied by the dilution factors to obtain the concentration in the original sample.

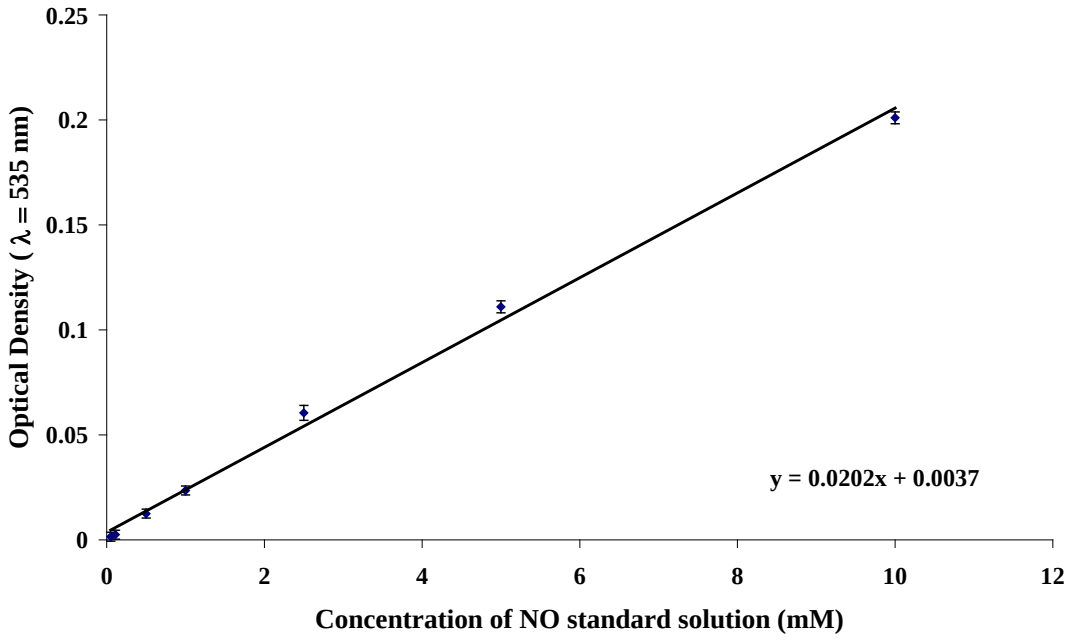


Figure 3.1: NO standard linear regression curve. Each point represents average of duplicate readings ± SD from one sample experiment.

3.3.1.21 Insulin

Insulin level in volunteer blood samples was quantitatively measured using Immulite 2500 Insulin kits on an Immulite 2500 analyser (Diagnostic Products Corporation A Siemens Company, CA USA). The Immulite 2500 Insulin is a solid-phase, two-site chemiluminescent immunometric assay. The reference normal fasting range used in this study is 6-27 µIU/L.

3.3.2 Haematology tests

3.3.2.1 Full blood count (FBC)

The full blood count (FBC), sometimes referred to as a full blood examination or complete blood count (CBC), is one of the most commonly performed blood tests. It is important for diagnosing conditions in which the number of blood cells is abnormally high or abnormally low, or the cells themselves are abnormal.

A complete blood count measures the status of a number of different features of the blood. The complete blood count that was performed included the tests shown in Table 3.1 and was obtained using an automated cell counter (CELL-DYN 4000 System, Abbott Laboratories, Abbot Park, IL USA).

Table 3.1: Tests included in the CBC performed on all volunteers.

Test	Male reference range	Female reference range
Haemoglobin (Hb) g/L	130-175	115-160
Red cell count (RCC) /pL	4.3-6	3.8-5.3
Haematocrit (Hct) %	38-50	33-46
Mean cell volume (MCV) fL	82-96	81-96
Mean cell haemoglobin (MCH) pg	27.5-34	
Red cell distribution width (RDW) %	10-13	
White cell count (WCC) /nL	3.5-11	
Neutrophil count (Neut) /nL	1.5-7.5	
Lymphocyte count (Lymph) /nL	0.8-3.5	
Monocyte count (Mono) /nL	0.0-0.8	
Eosinophil count (Eos) /nL	0.0-0.4	
Basophil count (Baso) /nL	0.0-0.1	
Platelet count (Plat) /nL	160-420	
Mean platelet volume (MPV) fL	7.5-10.4	

3.3.2.2 Erythrocyte sedimentation rate (ESR)

ESR was determined by measuring the change in the opacity of the blood column as the sedimentation progresses under infrared in a Diesse Ves-Matic-20 instrument (Diesse Diagnostica Senese SPA, Monteriggioni Italy). The blood samples were collected in ESR tubes. The test was performed on all tubes within 4h of collection. The 18° slant of the Vacu-tec tubes with respect to the vertical axis causes an acceleration of the sedimentation, allowing results to be obtained in 24 min. The reference range that was used in this study was < 13 mm/h for males and <15 mm/h for females.

3.3.3 Statistical analysis

The standard curve readings were calculated using mean and standard deviation or mean standard error. Two tailed-distribution, paired Student's t-test was used in this chapter to indicate significancy when appropriate. Statistical parameters were calculated using Microsoft Office Excel 2003.

3.4 Results

3.4.1 Chemistry tests

3.4.1.1 Total protein

The TP was measured in all plasma samples collected from all groups of volunteers before and after taking the study capsules. The mean TP concentration ± MSE at baseline was 74.08 g/L ± 0.86 (n = 25). There were no changes in the mean TP concentration during or at the end of the study at all three measurement points.

3.4.1.2 Albumin

The Alb was measured in all plasma samples collected from all groups of volunteers before and after taking the study capsules. The mean Alb concentration ± MSE at baseline was 42.67 g/L ± 0.51 (n = 25). There were no changes in the mean Alb concentration during or at the end of the study at all three measurement points.

3.4.1.3 Alkaline phosphatase

The ALP was measured in all plasma samples collected from all groups of volunteers before and after taking the study capsules. The mean ALP concentration ± MSE at baseline was 70.54 IU/L ± 4.2 (n = 25). There were no changes in the mean ALP concentration during or at the end of the study at all three measurement points.

3.4.1.4 Alanine transaminase

The ALT was measured in all plasma samples collected from all groups of volunteers before and after taking the study capsules. The mean ALT concentration ± MSE at baseline was 28.46 IU/L ± 1.99 (n = 25). There were no changes in the mean ALT concentration during or at the end of the study at all three measurement points.

3.4.1.5 Gamma-glutamyl transferase

The GGT was measured in all plasma samples collected from all groups of volunteers before and after taking the study capsules. The mean GGT concentration ± MSE at baseline was 22.21 IU/L ± 1.8 (n = 25). There were no changes in the mean GGT concentration during or at the end of the study at all three measurement points.

3.4.1.6 Total bilirubin

The TBIL was measured in all plasma samples collected from all groups of volunteers before and after taking the study capsules. The mean TBIL concentration ± MSE at baseline was 12.17 µmol/L ± 1.76 (n = 25). There were no changes in the mean TBIL concentration during or at the end of the study at all three measurement points.

3.4.1.7 Glucose

The glucose was measured in all plasma samples collected from all groups of volunteers before and after taking the study capsules. The mean glucose concentration ± MSE at baseline was 4.48 mmol/L ± 0.12 (n = 25). There were no changes in the mean glucose concentration during or at the end of the study at all three measurement points.

3.4.1.8 Sodium

The Na^+ was measured in all plasma samples collected from all groups of volunteers before and after taking the study capsules. The mean Na^+ concentration ± MSE at baseline was 141.13 mmol/L ± 0.37 (n = 25). There were no changes in the mean Na^+ concentration during or at the end of the study at all three measurement points.

3.4.1.9 Potassium

The K^+ was measured in all plasma samples collected from all groups of volunteers before and after taking the study capsules. The mean K^+ concentration ± MSE at baseline was 4.21 mmol/L ± 0.04 (n = 25). There were no changes in the mean K^+ concentration during or at the end of the study at all three measurement points.

3.4.1.10 Chloride

The Cl^- was measured in all plasma samples collected from all groups of volunteers before and after taking the study capsules. The mean Cl^- concentration ± MSE at baseline was 105 mmol/L ± 0.44 (n = 25). There were no changes in the mean Cl^- concentration during or at the end of the study at all three measurement points.

3.4.1.11 Bicarbonate

The HCO_3 was measured in all plasma samples collected from all groups of volunteers before and after taking the study capsules. The mean HCO_3 concentration ±

MSE at baseline was 27.74 mmol/L ± 0.38 (n = 25). There were no changes in the mean HCO_3 concentration during or at the end of the study at all three measurement points.

3.4.1.12 Blood urea nitrogen

The urea was measured in all plasma samples collected from all groups of volunteers before and after taking the study capsules. The mean urea concentration ± MSE at baseline was 4.94 mmol/L ± 0.25 (n = 25). There were no changes in the mean urea concentration during or at the end of the study at all three measurement points.

3.4.1.13 Creatinine

The Creat was measured in all plasma samples collected from all groups of volunteers before and after taking the study capsules. The mean Creat concentration ± MSE at baseline was 79.33 µmol/L ± 2.66 (n = 25). There were no changes in the mean Creat concentration during or at the end of the study at all three measurement points.

3.4.1.14 Cholesterol

The Chol was measured in all plasma samples collected from all groups of volunteers before and after taking the study capsules. The Chol mean concentration decreased non-significantly when compared to the baseline readings after taking the 75% fucoidan w/w capsules. The results at the four different measurement points (± MSE) are represented in [Figure 3.2].

3.4.1.15 Triglyceride

The Trig was measured in all plasma samples collected from all groups of volunteers before and after taking the study capsules. The Trig mean concentration decreased non-significantly when compared to the baseline readings after taking the 75% fucoidan capsules. The results at the four different measurement points (± MSE) are represented in [Figure 3.3].

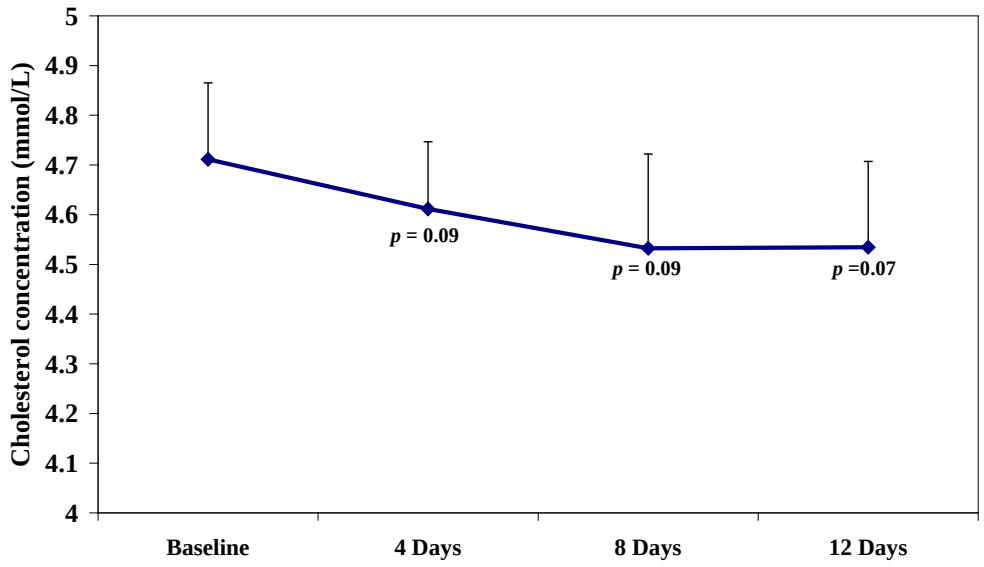


Figure 3.2: Cholesterol mean concentration before and after the active 75% fucoidan treatment. Each point represents the mean of 30 different individual samples ± MSE. The results were considered significant if the *p*-value was <0.05 using Student's t-test when compared to the baseline values.

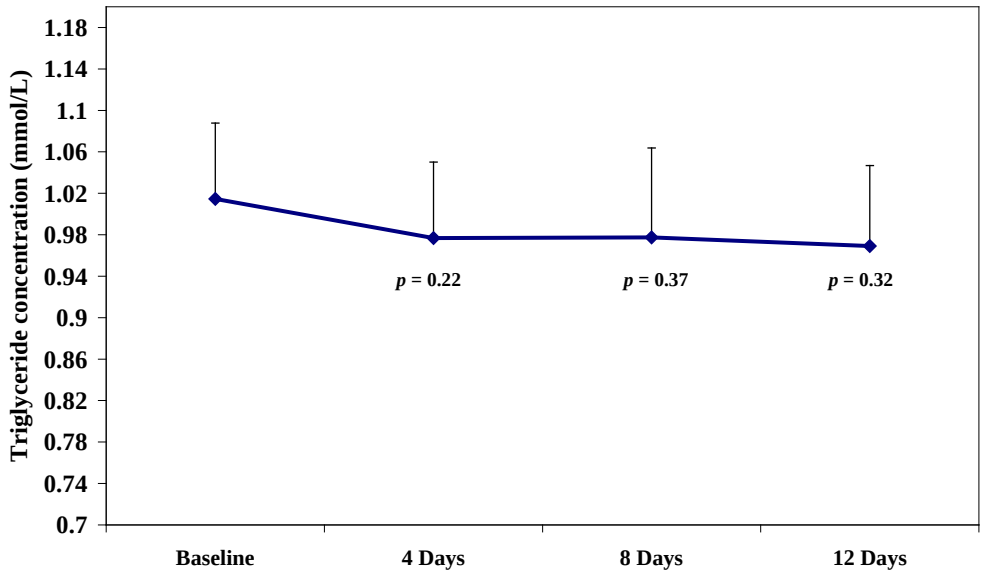


Figure 3.3: Triglyceride mean concentration before and after the active 75% fucoidan treatment. Each point represents a mean of 30 individual samples ± MSE. Results were considered significant if the *p*-value was <0.05 using the Student's t-test when compared to the baseline values.

3.4.1.16 High density lipoprotein cholesterol

The HDL was measured in all plasma samples collected from all groups of volunteers before and after taking the study capsules. The HDL mean concentration increased non-significantly when compared to the baseline readings after taking the 75% fucoidan capsules. The results at the four different measurement points (± MSE) are represented in [Figure 3.4].

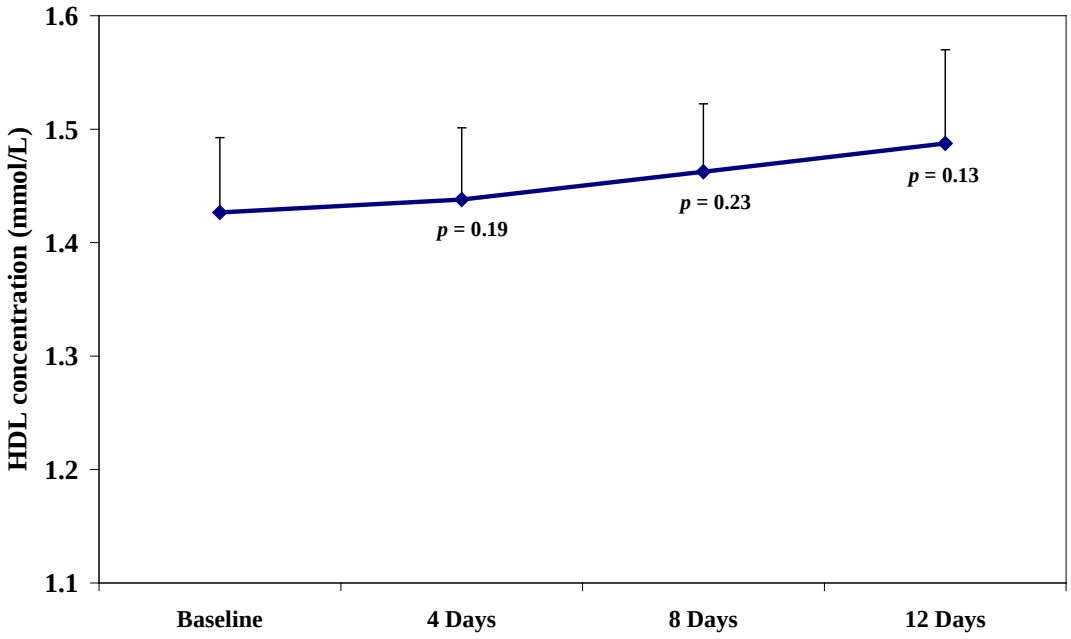


Figure 3.4: HDL mean concentration before and after the active 75% fucoidan treatment. Each point represents a mean of 30 different individual samples ± MSE. Results were considered significant if the *p*-value was <0.05 using the Student's t-test when compared to the baseline values.

3.4.1.17 Low density lipoprotein

The LDL was measured in all plasma samples collected from all groups of volunteers before and after taking the study capsules. The LDL mean concentration decreased non-significantly when compared to the baseline readings after taking the 75% fucoidan capsules. The results at the four different measurement points (± MSE) are represented in [Figure 3.5].

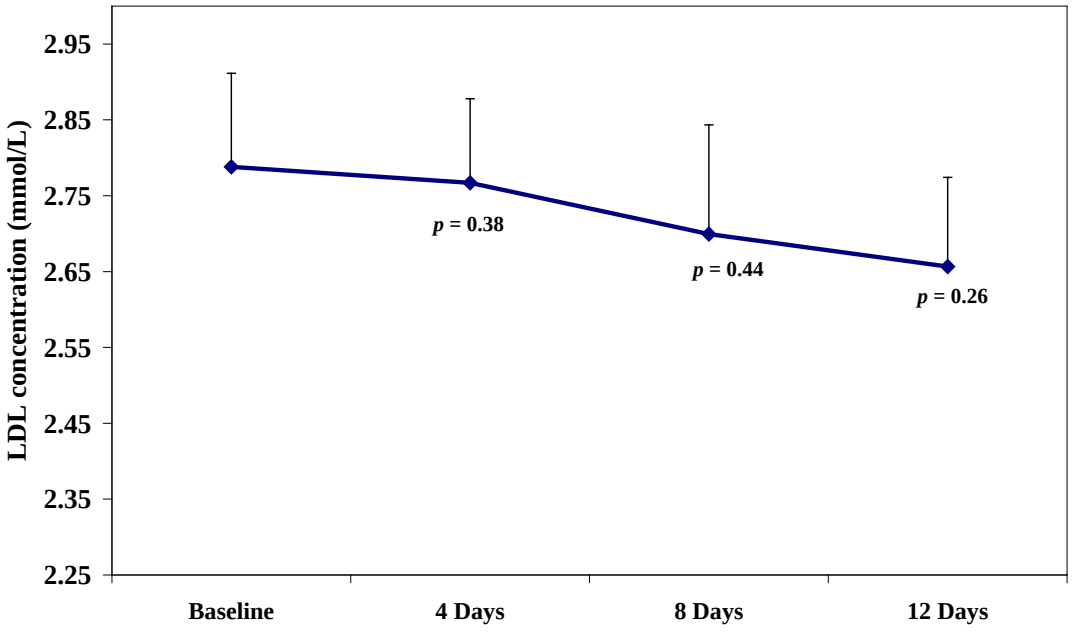


Figure 3.5: LDL mean concentration before and after the active 75% fucoidan treatment. Each point represents a mean of 30 different individual samples ± MSE. The results were considered significant if the *p*-value was <0.05 using the Student's t-test when compared to the baseline values.

3.4.1.18 Osmolality

The osmolality was measured in all plasma samples collected from all groups of volunteers before and after taking the study capsules. The mean osmolality concentration ± MSE at baseline was 290 mmol/Kg ± 0.76 (n = 25). There were no changes in the mean osmolality concentration during or at the end of the study at all three measurement points.

3.4.1.19 Anion gap

The anion gap was measured in all plasma samples collected from all groups of volunteers before and after taking the study capsules. The mean anion gap concentration ± MSE at baseline was 8.43 mmol/L ± 0.52 (n = 25). There were no changes in the mean anion gap concentration during or at the end of the study at all three measurement points.

3.4.1.20 Nitric oxide (NO)

In this study NO was measured in a group of volunteers (n = 10) according to the methodology described earlier in this chapter. The volunteers received the active treatment (75% fucoidan w/w) 3 g tid for a total period of 12 days. Another group of volunteers (n = 3) who received the placebo-control treatment was also tested for their NO level at the same test points. The average NO level decreased in a time dependant manner from 31.2 mM at baseline to (29.3, 27.2 and 26.4) mM at 4, 8 and 12 days, respectively [Figure 3.6]. However, this decrease was not statistically significant when the readings were tested against the baseline reading using the Student's t-test. There was no change in the NO level of the placebo-control group.

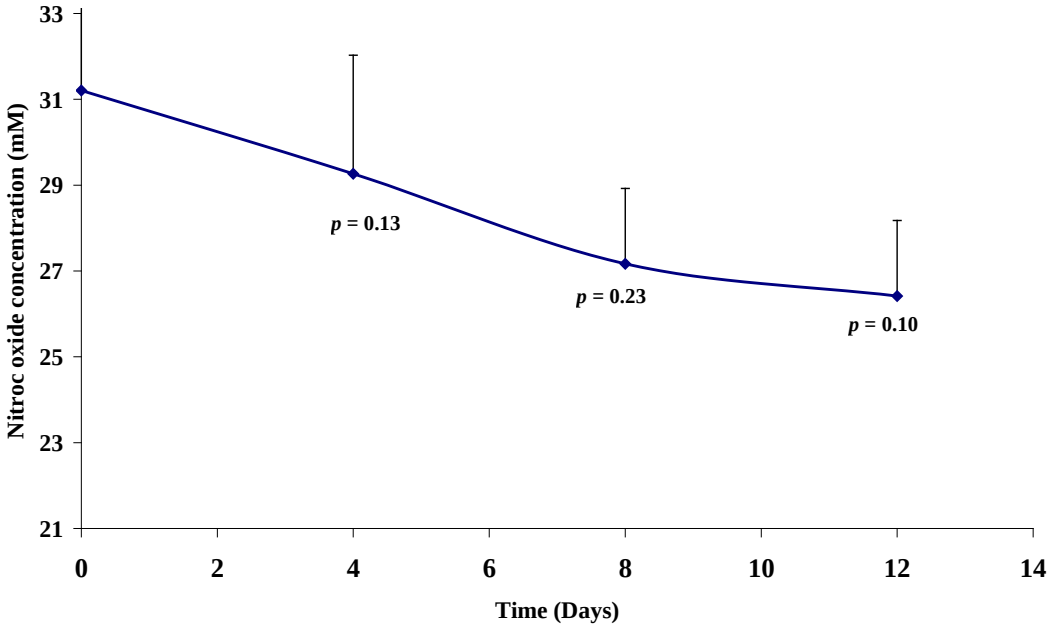


Figure 3.6: Nitric oxide concentration before and after the active 75% fucoidan treatment. Volunteers received 3 g tid of 75% fucoidan over a period of 12 days. Each point represents the average reading for 10 different individuals (5 males and 5 females) ± MSE.

3.4.1.21 Insulin

The insulin level was tested in the same group of volunteers (n = 10) who were tested for their NO level after receiving the active treatment (75% fucoidan w/w) 3 g tid for 12 days. The insulin was tested only at two points, baseline and 12 days from receiving the active treatment. Another group of volunteers (n = 3) who received the placebo-control treatment was also tested for their insulin level at the same test points. There was no change in the average insulin level of the placebo group and a non-significant decrease in the average insulin level of the active treatment group [Figure 3.7].

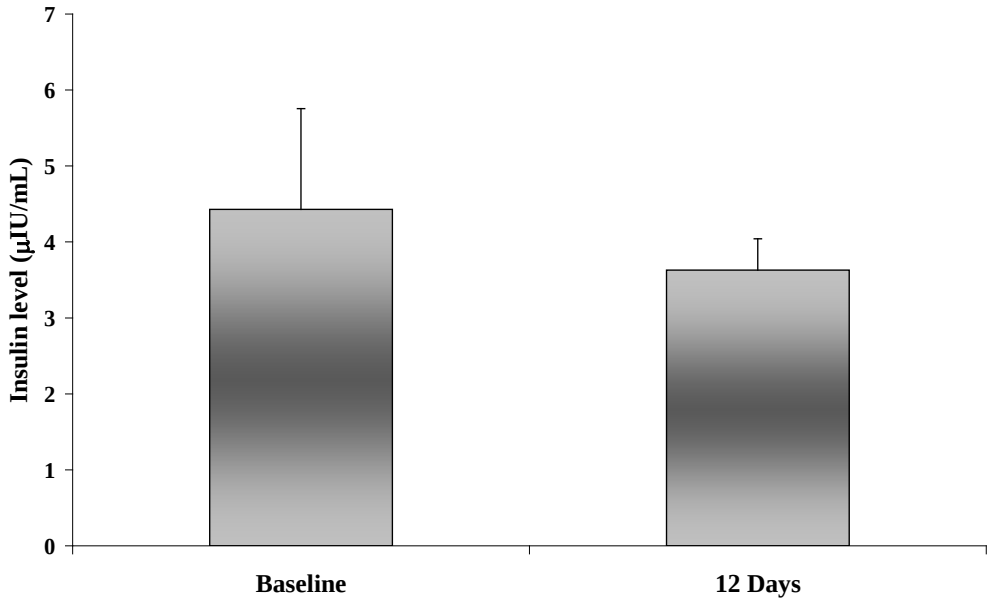


Figure 3.7: Insulin concentration before and after the active 75% fucoidan treatment. Volunteers received 3 g tid of the 75% fucoidan over a period of 12 days. Each point represents the average of 10 different individuals (5 males and 5 females) ± MSE.

3.4.2 Haematology tests

3.4.2.1 Full blood count

Volunteers who received the placebo-control treatment, the 10% fucoidan w/w treatment and the 75% fucoidan w/w treatment were checked for their CBC. The platelet count and platelet parameters are discussed in chapter five while the differential white blood cell count is discussed in chapter six. Generally, no changes were observed in the haematology tests for all volunteers in the three treatment groups. Results are shown in the following tables:

Table 3.2: Haematology tests for the male placebo-control group. Each value represents average reading for the tested independent individuals ± MSE (*p*-value).

Test	Baseline‡	4 Days‡	8 Days‡	12 Days‡
RCC ± MSE (*p*-value)	5.47 ± 0.06	5.48 ± 0.19 (0.91)	5.41 ± 0.25 (0.64)	5.46 ± 0.34 (0.96)
Hct ± MSE (*p*-value)	46.33 ± 1.53	46 ± 2 (0.42)	47 ± 2 (0.18)	46.67 ± 2.31 (0.74)
Hb ± MSE (*p*-value)	151.33 ± 7.23	151.33 ± 5.50 (1)	149 ± 7.21 (0.25)	151.67 ± 9.02 (0.93)
MCV ± MSE (*p*-value)	88.33 ± 1.53	88.67 ± 0.58 (0.67)	88.67 ± 1.15 (0.42)	89 ± 0 (0.53)
MCH ± MSE (*p*-value)	29.33 ± 1.15	29.67 ± 0.58 (0.74)	29 ± 1 (0.42)	29.33 ± 0.58 (1)
RDW± MSE (*p*-value)	12.17 ± 0.21	12.03 ± 0.42 (0.38)	12.1 ± 0.44 (0.74)	12.27 ± 0.51 (0.67)

‡ Number of volunteers (n = 3)

Table 3.3: Haematology tests for the female placebo-control group. Each value represents average reading for the tested independent individuals ± MSE (*p*-value).

Test	Baseline‡	4 Days‡	8 Days‡	12 Days‡
RCC ± MSE (*p*-value)	4.9 ± 0.40	4.91 ± 0.50 (0.89)	4.87 ± 0.32 (0.84)	4.99 ± 0.36 (0.52)
Hct ± MSE (*p*-value)	40.33 ± 3.21	40.67 ± 4.62 (0.74)	40.67 ± 2.08(0.67)	41.33 ± 1.53 (0.42)
Hb ± MSE (*p*-value)	141.33 ± 7.23	138 ± 11.27 (0.29)	139 ± 7.21 (0.25)	138.33 ± 4.62 (0.19)
MCV ± MSE (*p*-value)	87 ± 1	87.67 ± 0.58 (0.42)	87.33 ± 1.15 (0.74)	87.67 ± 0.58 (0.18)
MCH ± MSE (*p*-value)	28.33 ± 0.58	28 ± 1 (0.42)	28.33 ± 0.58 (1)	28.33 ± 0.58 (1)
RDW± MSE (*p*-value)	12.07 ± 0.38	12.23 ± 0.4 (0.04)	12.47 ± 0.4 (0.07)	12.37 ± 0.21 (0.1)

‡ Number of volunteers (n = 3)

Table 3.4: Haematology tests for the male 10% fucoidan group. Each value represents average reading for the tested independent individuals ± MSE (*p*-value).

Test	Baseline‡	4 Days‡	8 Days‡	12 Days‡
RCC ± MSE (*p*-value)	5.70 ± 0.28	5.56 ± 0.29 (0.09)	5.47 ± 0.15 (0.11)	5.54 ± 0.19 (0.18)
Hct ± MSE (*p*-value)	50.33 ± 1.53	50.67 ± 2.89 (0.74)	49.33 ± 2.52 (0.42)	50 ± 3.46 (0.81)
Hb ± MSE (*p*-value)	153.33 ± 12.34	152.33 ± 10.69 (0.85)	149 ± 7.94 (0.40)	152.33 ± 10.26 (0.79)
MCV ± MSE (*p*-value)	85 ± 3.61	86 ± 4.36 (0.23)	86 ± 5.29 (0.42)	85 ± 3.46 (1)
MCH ± MSE (*p*-value)	29 ± 1.73	28.67 ± 2.08 (0.42)	28.67 ± 2.08 (0.42)	29.33 ± 1.53 (0.42)
RDW± MSE (*p*-value)	11.77 ± 0.25	11.7 ± 0.26 (0.42)	11.73 ± 0.15 (0.81)	11.63 ± 0.31 (0.06)

‡ Number of volunteers (n = 3)

Table 3.5: Haematology tests for the female 10% fucoidan group. Each value represents average reading for the tested independent individuals ± MSE (*p*-value).

Test	Baseline‡	4 Days‡	8 Days‡	12 Days‡
RCC ± MSE (*p*-value)	4.67 ± 0.40	4.72 ± 0.50 (0.80)	4.57 ± 0.41 (0.53)	4.68 ± 0.44 (0.96)
Hct ± MSE (*p*-value)	40.33 ± 1.53	40.67 ± 2.89 (0.74)	39.33 ± 2.52 (0.43)	40 ± 3.46 (0.81)
Hb ± MSE (*p*-value)	140 ± 7	139 ± 9 (0.85)	139 ± 7.9 (0.58)	139 ± 7.2 (0.79)
MCV ± MSE (*p*-value)	88 ± 5.29	88 ± 4.36 (1)	88 ± 5.20 (1)	87 ± 3.61 (0.42)
MCH ± MSE (*p*-value)	29 ± 1.73	28.67 ± 2.08 (0.42)	28.67 ± 2.08 (0.42)	29.33 ± 1.53 (0.42)
RDW± MSE (*p*-value)	11.77 ± 0.25	11.7 ± 0.26 (0.42)	11.73 ± 0.15 (0.81)	11.63 ± 0.31 (0.06)

‡ Number of volunteers (n = 3)

Table 3.6: Haematology tests for the male 75% fucoidan group. Each value represents average reading for the tested independent individuals ± MSE (*p*-value).

Test	Baseline‡	4 Days‡	8 Days‡	12 Days‡
RCC ± MSE	5.25 ± 0.20	5.18 ± 0.20	5.11 ± 0.14	5.10 ± 0.14
(*p*-value)		(0.51)	(0.16)	(0.23)
Hct ± MSE	45.56 ± 1.12	44.89 ± 1.30	44.22 ± 1.02	44.44 ± 0.99
(*p*-value)		(0.46)	(0.04)*	(0.23)
Hb ± MSE	150.22 ± 4.35	149.56 ± 4.80	149.33 ± 5.60	147.56 ± 5.35
(*p*-value)		(0.76)	(0.71)	(0.35)
MCV ± MSE	87.67 ± 2.17	86.55 ± 2.10	87.11 ± 2.19	88.88 ± 0.92
(*p*-value)		(0.04)*	(0.37)	(0.17)
MCH ± MSE	28.44 ± 1.00	28.78 ± 1.08	28.78 ± 1.09	29.50 ± 0.42
(*p*-value)		(0.40)	(0.40)	(0.83)
RDW± MSE	12.15 ± 0.28	12.07 ± 0.27	12.18 ± 0.22	11.99 ± 0.25
(*p*-value)		(1)	(0.29)	(0.87)

* *p*-value < 0.05 using Student's t-test
‡ Number of volunteers (n = 9)

Table 3.7: Haematology tests for the female 75% fucoidan group. Each value represents average reading for the tested independent individuals ± MSE (*p*-value).

Test	Baseline‡	4 Days‡	8 Days‡	12 Days‡
RCC ± MSE	4.50 ± 0.08	4.48 ± 0.08	4.45 ± 0.09	4.42 ± 0.08
(*p*-value)		(0.69)	(0.57)	(0.20)
Hct ± MSE	39.69 ± 0.70	39.44 ± 0.72	39.27 ± 0.69	38.93 ± 0.66
(*p*-value)		(0.60)	(0.72)	(0.27)
Hb ± MSE	134 ± 2.09	132.75 ± 1.95	132.53 ± 2.54	131.53 ± 2.35
(*p*-value)		(0.28)	(0.59)	(0.11)
MCV ± MSE	88.44 ± 0.67	88.06 ± 0.71	88.27 ± 0.67	88.21 ± 0.84
(*p*-value)		(0.14)	(1)	(0.66)
MCH ± MSE	29.31 ± 0.27	29.19 ± 0.36	29.47 ± 0.45	29.14 ± 0.38
(*p*-value)		(0.68)	(0.58)	(1)
RDW±MSE	11.77 ± 0.14	11.71 ± 0.14	11.77 ± 0.13	11.76 ± 0.14
(*p*-value)		(0.44)	(0.61)	(0.03)*

* *p*-value < 0.05 using Student's t-test
‡ Number of volunteers (n = 16)

3.4.2.2 Erythrocyte sedimentation rate

In this study the ESR was tested in volunteers who received the active treatment (75% fucoidan w/w) 3 g tid for 12 days. There were no noted changes in the levels of ESR at any time point. The results are shown in Table 3.8.

Table 3.8: The average ESR readings for volunteers treated with 75% fucoidan w/w.

	ESR mm/h	**Baseline**	**4 Days**	**8 Days**	**12 Days**
Males ‡	**Average ± MSE (*p*-value)**	4.67 ± 1.50	2.67 ± 1.48 (0.20)	3.83 ± 1.64 (0.19)	4.8 ± 2.22 (0.59)
Females §	**Average ± MSE (*p*-value)**	12.5 ± 1.50	13 ± 1.91 (0.64)	12.5 ± 1.66 (1)	12.5 ± 1.32 (1)

‡ Number of volunteers in this group (n = 6).
§ Number of volunteers in this group (n = 5).

3.5 Discussion

The general wellbeing of all volunteers is a critical issue during different study treatments involving the ingestion of experimental substances, and changes in any pathological parameters will be important and may give information about how the treatments may act within the body.

The term LFT implies standard tests for measurement of liver function (transaminases), excretory function (bilirubin) and inflammatory activity of hepatocytes (serum aminotransferases). Abnormal LFT are commonly the first indicator of liver disease, but normal or slightly abnormal LFT do not rule out the presence of liver disease nor changes in body metabolism. Likewise, many of the usual laboratory tests other than the level of alanine aminotransferases and bile acids are not specific for hepatic function, and may reflect other extrahepatic pathological processes.

In parallel to the LFT, to help monitor kidney function, blood levels of BUN and creatinine normally are measured. The amount of calcium and phosphate in the blood and the balance of serum and urine electrolytes are measured, as these are often affected by kidney disease. Haemoglobin, which is measured as part of the CBC can also provide information about the kidney function.

The volunteers were checked at the start of the treatment and during the study for their liver and kidney functions. The volunteers were chosen as apparently healthy and disease-free and were specifically informed of possible side effects of the treatment. The seaweed has been part of the Japanese and Korean diets for centuries. These peoples consume large quantities of it daily when compared with others and it is generally considered safe to ingest seaweed. However, there may be some unlikely possible side effects of natural preparations such as seaweed that involve nausea, vomiting, diarrhoea, tiredness and lethargy and muscle pain. These were used as an indication to cease treatment and seek medical advice.

In this work none of the volunteers who ingested any of the treatments experienced any unusual clinical or pathological changes except one female volunteer who reported that she had some odour bloating after taking the 75% fucoidan treatment.

The fucoidan is a highly sulphated compound and the sulphur content may have occasioned the unpleasant odour she experienced.

In a closer look at the results, the proteins which could help in transport of any absorbed or metabolite fucoidan around the body were assessed. Proteins are important constituents of all cells and tissues. TP (albumin and globulins) can reflect nutritional state, kidney disease, liver disease, and many other conditions. Ingesting fucoidan did not actually have an effect on TP serum level. At the same time, there were no changes in Alb level when baseline level was compared with post-treatment level. All values remained within the accepted reference ranges.

Other LFT which have been looked at are the enzymes. No detectable changes in any of the tested enzymes (ALP, ALT and GGT) were observed when the placebo group mean values were compared to the active treatment groups. Therefore, fucoidan ingestion is considered safe in terms of its effect on liver function.

To study the metabolism of haemoglobin, the plasma bilirubin concentration (TBIL) was tested. No changes in the TBIL were detected when levels were measured at baseline and after ingesting the fucoidan as directed.

Ingesting fucoidan seems to have an effect on the lipid profile. It has been shown by this study that volunteers who ingested 3 g tid of 75% fucoidan w/w had their lipid profile changed, although non-significantly. Their Chol, Trig, and LDL levels decreased while the HDL level increased in a period of 12 days. In previous work, aminated fucoidan effectively prevented endotoxin-induced hepatic vein thrombosis in hyperlipemic rats and decreased the elevated levels of serum cholesterol and triglyceride (Soeda *et al.*, 1994).

There were no changes in the measured glucose level before and after taking the fucoidan capsules. Levels of urea, creatinine and plasma electrolytes do not change after the ingestion of fucoidan.

An increase in serum insulin concentration in the rat causes a decrease in blood pressure by stimulating the synthesis of NOS followed by a subsequent increase the

NO concentration. Incubation of various tissues from mice and human erythrocytes or their membrane fractions, with physiological concentrations of insulin, resulted in the activation of a membrane-bound nitric oxide synthase (NOS). Activation of NOS and synthesis of NO were stimulated by the binding of insulin to specific receptors on the cell surface membrane (Khan *et al.*, 2000).

The NO level in the circulation was affected by fucoidan ingestion. It decreased when 3 g of fucoidan was ingested tid for 12 days, but it is not clear if this is a direct or indirect effect. At the same time there was a decrease in the insulin level after 12 days of ingesting the fucoidan. The insulin was reported previously to activate the iaNOS to produce NO. Thus, the decrease in the NO could be due to the decrease in the insulin level that activates iaNOS, or due to an interruption in the activation process caused by fucoidan.

3.6 Conclusions

In conclusion, ingesting up to 3 g fucoidan daily for up to 12 days seems to have no effect on liver and kidney function and does not affect the metabolism. It appears to be safe to use the whole seaweed (which contains 10% fucoidan w/w) or the purified 75% fucoidan (w/w). This extract has a lipid lowering effect and decreases the NO level also.

CHAPTER FOUR

DETECTION OF FUCOIDAN IN PLASMA AND URINE

Chapter objectives:

Develop a method suitable for routine laboratory settings that can measure the level of *Undaria pinattifida* fucoidan (GFS^{TM}) in human plasma and urine samples after oral administration.

Chapter contents:

4.1 Summary

In this chapter the development of an antibody-based method to assess plasma uptake of *Undaria*-derived fucoidan after ingestion by human volunteers is described and discussed. A competitive ELISA, using an Ab raised to sulphated polysaccharides, was adapted to quantitate fucoidan in plasma samples from healthy volunteers who had ingested 3 g/day of whole *Undaria* containing 10% fucoidan w/w, purified 75% fucoidan w/w, or 3 g of a non-sulphated placebo-control polysaccharide over 12 days.

Increasing reactivity to the Ab (1B1) was detected throughout the experiment and assuming the measured material was indeed intact fucoidan, the concentration detected (median) was 4.002 mg/L and 12.989 mg/L when 3 g of 10% or 75% fucoidan respectively was ingested over the period of 12 days. Fucoidan started to appear in the urine 4 h after the first dose and peaked at 8 h after ingestion of the active material. In conclusion, high molecular weight fucoidan can be detected in plasma and urine samples using an ELISA competitive assay based on the 1B1 Ab directed against sulphated polysaccharide.

4.2 Introduction

Sulphated fucans constitute a class of polysaccharides first isolated in 1913 from marine brown algae. The sulphated fucans are often called fucoidans since they are fucose-rich sulphated carbohydrate polymers found in seaweeds such as *Undaria sp.* and *Laminaria sp.*, common in the Japanese diet as wakame and kombu (Shibata *et al.*, 2000; Berteau & Mulloy, 2003). Fucoidan extracts may have therapeutic benefits and are relatively apparently non-toxic, non-allergenic, have no harmful effects on any bodily function or organ and are considered completely safe (Shibata *et al.*, 2000).

It has recently been shown that i.v fucoidan administration produces rapid mobilisation of murine haemopoietic progenitor cells with a long-term BM repopulating potential. Fucoidan administration similarly increased circulating mature WBC and HPC in mice and in nonhuman primates (Sweeney *et al.*, 2000; Frenette & Weiss, 2000; Sweeney *et al.*, 2002) and other research has shown that the orally administered fucoidans have many different biological effects (Nagaoka *et al.*, 2000; Zhang *et al.*, 2003).

The search for new drugs recently has raised interest in the fucoidans. In the past few years the structure of several fucoidans has been resolved, and many aspects of their biological activity have been elucidated. New methods have been developed capable of detecting different polysulphated polysaccharides in urine and blood plasma samples after oral and i.v routes of administration, including the compounds chondroitin sulphate, (Ronca *et al.*, 1998; Conte *et al.*, 1991), dermatan sulphate, and hyaluronan (Sakai *et al.*, 2002). The development of a rapid, accurate and sensitive method for the determination of fucoidan in biological samples such as plasma and urine was necessary to study the effects of these sulphated sugars with a view to possible new drug therapies.

The uptake of fucoidan after oral administration has not been evaluated to date. High molecular weight materials are generally not considered to be easily absorbed, but Structum®, a high molecular weight chondroitin sulphate preparation (17 kD), is accepted as a treatment for arthritis. The uptake of un-degraded chondroitin sulphate occurs across the small intestine, probably via endocytosis (Barthe *et al.*, 2004).

Similarly, heparin can be delivered orally, and has a low but detectable uptake (Hiebert *et al.*, 2005).

The therapeutic use of fucoidan requires knowledge of its absorption, distribution, catabolism, and excretion. Because of their large molecular weight, these polysaccharides may cross capillary walls with difficulty. New methods have been developed to detect different polysaccharides in urine and blood plasma samples after oral and i.v routes of administration (Conte *et al.*, 1991; Ronca *et al.*, 1998; Sakai *et al.*, 2002). Dimethylmethylene blue (DMB) was used by Taylor and Jeffree (1969) (a strongly metachromatic dye) for the histochemical detection of sulphated glycosaminoglycans, and was subsequently used by Humbel (1974) in analytical procedures. Farndale has described a more convenient and reproducible form of the analytical method that has been found useful in the analysis of urine samples (Farndale *et al.*, 1982) and he modified the procedure to eliminate interference by other polyanions (Farndale *et al.*, 1986).

In an effort to understand the clinically observed effects of fucoidan in humans, it is important to determine the uptake of *Undaria* fucoidan after oral administration. A new MoAb (1B1) that possesses reactivity against polysulphated polysaccharides has been developed. The Ab reacts with heparin and semi-synthetic heparin-like substances including dextran sulphate, pentosan polysulphate, and glucoseaminoglycan polysulphate (Kongtawelert & Kulapongs, 2000). This Ab was tested on urine samples to detect the excretion of the fucoidan, which was also detected using a colorimetric method based on the DMB dye. The aim of the current study was to develop a simple technique to determine the concentration of fucoidan in plasma or urine microsamples using the 1B1 Ab.

4.3 Materials and methods

4.3.1 Volunteers

Informed consent and human ethics approval were obtained as described in section 2.1. Healthy human volunteers of either sex aged between 20-46 years (average 29) were chosen as described in sections 2.2 and 2.3.

The study capsules were prepared as described in section 2.5. Volunteers were divided into three groups. The first group of volunteers (n = 6) took 3g of guar gum as a placebo. The guar gum structure is shown previously in [Figure 2.2]. The second group (n = 6) took 3g of whole *Undaria* containing 10% fucoidan w/w. The third group (n = 40) took 3g of 75% fucoidan w/w daily for 12 days. The fucoidan structure is shown previously in [Figure 1.12]. All volunteers took three capsules tid at the same time each day. During the study time, volunteers were asked not to eat any sort of seafood or food containing seaweed derived products and they did not take any drugs or food supplements. Blood samples from the three groups were collected as described later. Not all of the volunteers involved in this study collected their urine samples.
The urine samples were collected only from volunteers who were willing to donate a urine sample.

4.3.2 Blood samples collection

Citrated venous blood was collected from the three groups of volunteers and centrifuged at 1500 *xg* for 5 min. Plasma fractions were collected and stored in aliquots at −80°C within 30 min of collection for later analysis. Some of these samples were pre-treated using pronase enzyme (Sigma-Aldrich Co., Louis, MO USA) or purified using affinity chromatography as described later, in an attempt to increase the 1B1 Ab sensitivity in the fucoidan assay. Other samples were tested directly for fucoidan. The complete blood count was obtained as described in section 3.3.2.1 and the glucose level was measured as described in section 3.3.1.7.

4.3.3 Pronase treated plasma samples

Citrated plasma samples were mixed with an equal volume of pronase solution (1 g/L of actinase E in 0.05 M Tris-acetate buffer (pH 8.0)) and incubated at 37°C overnight. The digested samples were boiled for 3 min in a boiling water bath and cooled in an

ice bath, and then centrifuged at 2500 *xg* for 10 min. The resulting supernatants were collected as pre-treated samples for further assay.

4.3.4 Urine sample collection

Volunteers were asked to complete the label on the urine container by writing their name, date and time of collection before collecting the urine. The volunteers were divided into two groups. The first group was asked to collect their urine every two hours (baseline, 2, 4, 6, 8, 24 and 96) h while they took the capsules tid for only one day, while the second group was asked to collect their urine at three different time points:

- 0 Day: early in the morning (first morning urine) before taking any capsules
- 1 day (24 h): after 1 day of taking the capsules (next morning day)
- 4 days: after 4 days of taking the capsules (morning of the fifth day)

Volunteers were asked to collect their midstream urine according to the given instructions (Appendix-5).

4.3.5 Urine glucose measurement

In most people, blood glucose levels above 10 mmol/L will cause glucosuria, this level being called the 'renal threshold' for glucose. The glucose was measured by a glucose slide method using the Vitros glucose slides and chemistry products calibrator kit-1 on Vitros Chemistry System V950 (Ortho Clinical Diagnostic, Inc. NY USA). The glucose was measured in 10 µL of the sample colorimetrically at 540 nm and 37°C. The dye system used is closely related to that first reported by (Trinder, 1969). The chemistry on the glucose slides has been described by Crume and his co-workers (1978). The reference range used in this study was <1.7 mmol/L.

$$\beta\text{-D-glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} \text{D-gluconic acid} + H_2O_2$$

$$2H_2O_2 + \text{4-aminoantipyrine} + \text{1,7-dihydroxynaphthalene} \xrightarrow{\text{peroxidase}} \text{red dye}$$

4.3.6 Urine pH and specific gravity measurement

The urine pH and specific gravity were measured using a dipstick method (Combur test, Roche Diagnostics, Australia). The reference range for the pH number that was used in the study was (4.7-7.8).

4.3.7 Affinity chromatography

A bi-functional affinity/ion exchange chromatography matrix (CM Affi-Gel Blue; Bio-Rad Laboratories, Hercules, CA USA), which binds both albumin and serum proteases, was used to remove these proteins and purify the plasma samples. The gel was prepared and used according to Bio-Rad instruction manual with slight modification.

Briefly, the gel was washed in a column with 5 bed volume of pre-wash buffer (0.1 mol/L acetic acid, pH 3, 1.4 mol/L NaCl, 40% isopropanol) followed by 7 bed volumes of deionized water, then the gel was washed with 2 bed volumes of 1.4 mol/L NaCl and then with running buffer (10 mmol/L K_2HPO_4, pH 7.25, 0.15 mol/L NaCl). The gel was then packed into a poly-prep chromatography column (Bio-Rad Laboratories, Hercules, CA USA). The plasma sample (1 mL) was applied and the column was washed with two bed volumes of running buffer. The effluent from this step was then collected in fractions in Eppendorf tubes for further analysis.

To use the gel again, the albumin was eluted from the column with 2 bed volumes 1.4 mol/L NaCl in running buffer and the column was regenerated with 2 bed volumes of regeneration buffer (2 mol/L guanidine HCl) followed by 2 bed volumes of running buffer.

4.3.8 Preparation of 1B1 antibody

The biotinylated MoAb isotype IgM was prepared as described previously (Kongtawelert & Kulapongs, 2000). Briefly, this Ab was developed against protamine sulphate (Salmon, from Aventis Pharmaceuticals Inc., NJ USA), heparin (Leo Pharmaceuticals, Ballerup, Denmark or Sigma-Aldrich Co., Louis, MO USA) and heparin-like substances by immunizing Balb/c mice with protamine sulphate and polyolpolysulphate. The MoAb was purified by thiophilic adsorption (T-gel a gift from Dr Jan Carlsson, Pharmacia Diagnostics AB, Uppsala, Sweden) column

chromatography then biotinylated, aliquoted, lyophilized and stored at −20°C. On the day of the experiment, the Ab was reconstituted with 100 µL of distilled water, and diluted with PBS (Oxoid Ltd., Hampshire, England) to the optimum concentration 20 µg/mL.

4.3.9 Microtitre plate preparation

A competitive ELISA technique was used to detect and measure the concentration of fucoidan in plasma samples. Briefly, a 96-well microtitre plate maxisorp surface (Nunc, NalgeNunc International, Denmark) was coated with 100 µL of 200 µg/mL protamine sulphate solution in PBS (pH 7.4) per well overnight at 4°C. Some wells of the plate were treated with 100 µL of distilled water for use as blank wells. All wells in the plate were then blocked with 200 µL of PBS Tween-20 0.1% (v/v) (BDH, Poole, England) (PBS-T) supplemented with 6% BSA (Sigma-Aldrich Co., Louis, MO USA) and incubated at 37°C for 1 h. The plate was washed 3 times with PBS-T and air-dried. These protamine sulphate coated plates were prepared freshly before each assay [Figure 4.1].

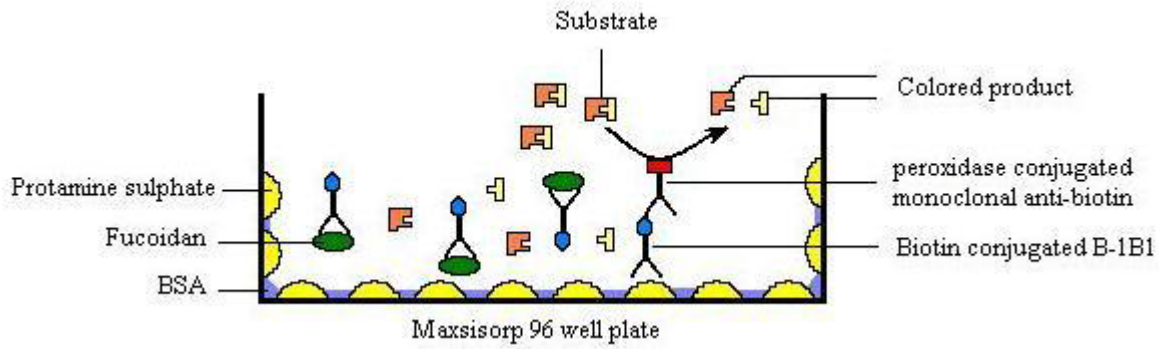


Figure 4.1: Schematic diagram showing the competitive ELISA reactions inside one well.

4.3.10 Construction of standard curves using 1B1

A stock solution of heparin (4 kIU/L) and a stock solution of fucoidan (160 mg/L) were used to each produce a twofold dilution series. Concentrations (0.008-2) kIU/L of heparin and (0.078-80) mg/L of fucoidan are the final working concentrations after diluting heparin or fucoidan with the Ab to prepare “inhibition mixtures”. All

dilutions were made using pooled plasma or pooled urine prepared from baseline plasma or urine as an intra-assay control. Each plate contained a set of serial standard concentrations to construct the standard curve. This was created using algorithm four-parameter logistic fitting, using the computer software, Genesis Lite, version 3.03, Life Sciences, UK. The inhibition percentages for both heparin and fucoidan were calculated using the following formula:

$$\%Inhibition = 100 - ((Unknown \div PositiveControl) \times 100)$$

Blank readings were subtracted from all other readings. The positive control was prepared by mixing equal volumes of 1B1 Ab and plasma or urine (inhibition mixture). A 100 µL of inhibition mixture was then added to the protamine sulphate coated wells in triplicate. The 50% inhibition concentration (IC_{50}) for both heparin and fucoidan standard curves was calculated.

4.3.11 Construction of standard curves using DMB

The fucoidan level in urine was measured after constructing a standard curve. A stock standard solution of fucoidan (10 mg/mL) was prepared by dissolving 10 mg of the 75% fucoidan w/w in 1 mL deionised distilled water. Then a twofold dilution series (0-160) µg/mL was prepared in deionised distilled water and a standard curve was established as shown in [Figure 4.2]. After that, 10 µL of the standard solution was mixed with 2.5 mL of DMB dye solution in a polystyrene tube then transferred to a spectrophotometer cuvette. The absorption was determined at A_{525} nm after a fixed time (20 sec). The assay was calibrated using a reagent blank and standards containing up to 5 µg whale chondroitin sulphate and fucoidan.

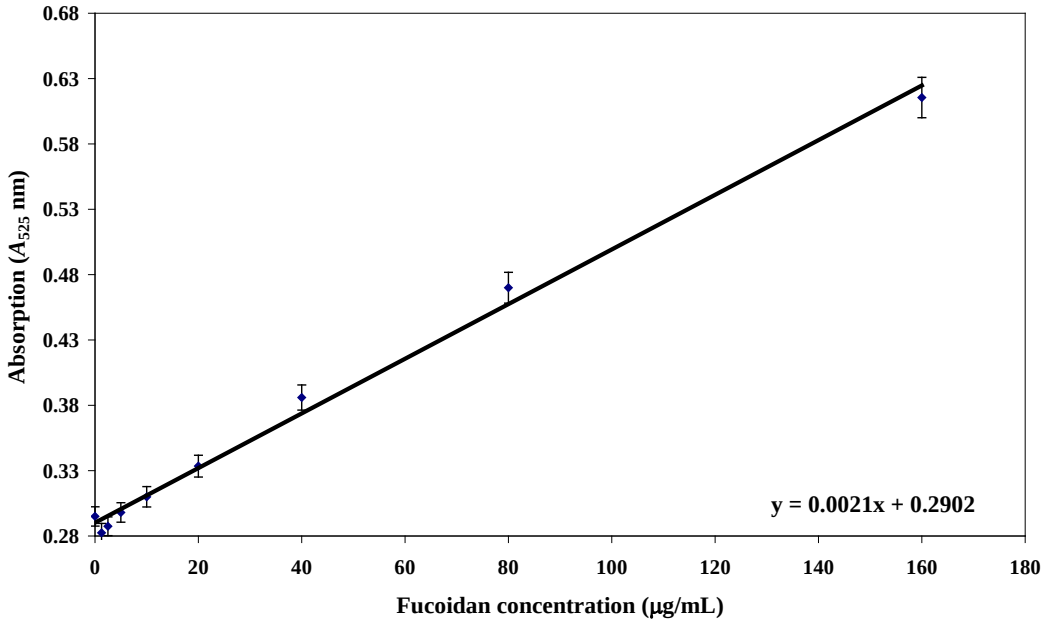


Figure 4.2: Fucoidan standard linear regression curve prepared using DMB dye. Each point represents the mean for duplicate readings ± SD of a sample experiment.

4.3.12 ELISA detection of fucoidan in plasma and urine

A set of serial standard concentrations to generate a standard curve was included in each mircotitre plate. Each standard, blank, control, or sample was mixed in a 1:1 ratio with the pre-prepared MoAb 1B1 and incubated for 1 h at 37°C to prepare "inhibition mixtures". 100 µL of each "inhibition mixture" was added to the designated wells in triplicate and the plate was incubated for 1 h at 37°C. The plate was washed 3 times with PBS-T and air dried, followed by the addition of adding 100 µL of 1:1000 of peroxidase conjugated monoclonal anti-biotin antibody (Zymed Laboratories Inc., South San Francisco, CA USA) to each well (prepared in PBS-T supplemented with 0.5% BSA) and left for 1 h at 37°C. The plate was washed three times with PBS-T and air-dried. Next, 100 µL/well of o-phenylenediamine dihydrochloride substrate (Sigma-Aldrich Co., Louis, MO USA) (0.4 mg/mL) was added. The plate was incubated in the dark for 10 min and the reaction stopped by adding 50 µL of 4M H_2SO_4 to each well. The absorbance was measured at $A_{490/630}$ nm using a microtitre plate reader (MR 5000 Micro-plate Reader, Dynatech Laboratories Inc., VA USA). The amount of fucoidan in plasma or urine samples was calculated from the standard curve.

4.3.13 Spectrophotometric detection of fucoidan in urine

The fucoidan concentration in urine was detected by using a colorimetric method based on the dye 1,9-dimethylmethylene blue. The colour reagent was prepared as described previously by Barthe and co-workers (2004). Briefly, 16 mg of DMB (Sigma Chemical Co., USA) , 3.04 g glycine (aminoacetic acid, Sigma Chemical Co. USA), 2.37 g NaCl (Ajacs Chemicals, Auburn, NSW Australia), and 95 mL 0.1 M HCl (Merck Pty. Ltd., Kilsyth, VIC Australia) were dissolved in 1 L of Milli-Q water. The pH was adjusted to 3 using HCl/NaOH. The reagent was kept in a brown bottle in the dark at ambient temperature. Ten µL of the unknown urine sample was mixed with 2.5 µL of DMB dye solution in a polystyrene tube, mixed and transferred to a spectrophotometer cuvette. The absorption was determined at A_{525} nm after 20 sec. The assay was calibrated by using reagent blank and standards containing fucoidan. All readings were converted from absorbance to concentration (ng/mL) using the standard curve after subtraction of values from the blank reading.

4.3.14 Statistical analysis

The standard curve readings were calculated using mean and standard deviation or mean standard error. All other statistical values are based on non-parametric statistics using the median and 95% confidence interval. Triplicate readings for each standard, control, and sample were averaged and subtracted from the average blank readings. Statistical parameters were calculated using Microsoft Office Excel 2003, and SPSS Version 12.1.

4.4 Results

4.4.1 1B1 affinity for heparin and fucoidan

The reactivity of the 1B1 Ab towards fucoidan was assessed prior to its use in the clinical assay by comparing its affinity for heparin and fucoidan. Clinical samples from the study were assessed using the developed competitive ELISA assay. The reactivity of the Ab towards *Undaria* fucoidan was similar to that of heparin [Figure 4.3].

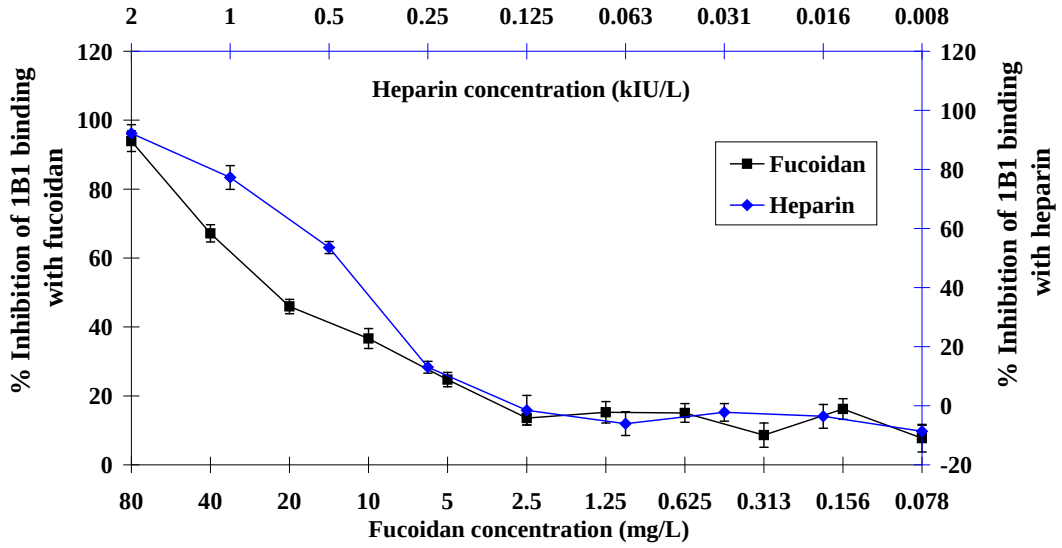


Figure 4.3: Inhibition curves for heparin and fucoidan using 1B1 Ab. It shows a comparison between the affinity of the MoAb 1B1 with both heparin-sulphate and fucoidan at equivalent concentrations. Each point represents the mean of triplicate readings from three different experiments ± SD.

The calculated IC_{50} for each fucoidan standard curve ranged from 18.29-25.61 mg/L with an average of 21.95 mg/L, where the IC_{50} for each heparin standard curve ranged from 0.47-0.57 kIU/L with an average of 0.52 kIU/L (Table 4.1).

Table 4.1: Average inhibition concentration 50% (IC_{50}) for heparin and fucoidan standard curves using 1B1 Ab and the competitive ELISA.

	Heparin (kIU/L)	**Fucoidan (mg/L)**
IC_{50}	0.52 ± 0.05^{a}	21.95 ± 3.66^{b}
Range	0.47-0.57	18.29-25.61

[a] Mean ± SD; n = 3
[b] Mean ± SD; n = 6

4.4.2 Plasma fucoidan level

Plasma samples collected from volunteers were quantified for the presence of fucoidan using the described competitive ELISA method. The median concentration of fucoidan for the three different groups of volunteers after taking the capsules orally is summarized in Table 4.2.

Table 4.2: Concentration of fucoidan (median) in human plasma samples of the three groups of volunteers, measured by the competitive ELISA using the 1B1 Ab.

Volunteers group	**Guar gum** [a]	**GFS™ 10% fucoidan w/w** [a]	**GFS™ 75% fucoidan w/w** [b]
Days	**Fucoidan concentration (median) (mg/L) ± 95% confidence intervals; (lower), (upper)**		
4	−0.852 (2.334), (2.177)	4.002 (0.689), (2.722)	11.906 (1.573), (2.35)
8	−0.281 (2.873), (1.96)	2.087 (1.045), (1.485)	13.015 (1.433,) (1.757)
12	2.394 (3.327), (0.368)	5.082 (2.961), (1.288)	15.746 (4.008), (1.703)
Median for all point readings at 4, 8 and 12 Days	**-0.281 (2.905), (2.675)**	**4.002 (1.915), (2.721)**	**12.989 (1.165), (1.267)**

[a] n = 6, median representing the mean of triplicate readings for each point ± 95% confidence intervals
[b] n = 40, median representing the mean of triplicate readings for each point ± 95% confidence intervals

The apparent median plasma fucoidan concentration in samples from volunteers who received guar gum as placebo was -0.28 mg/L. The plasma fucoidan concentration (median) in samples from volunteers who received 10% or 75% fucoidan was 4.00 mg/L and 12.99 mg/L, respectively [Figure 4.4 & 4.5].

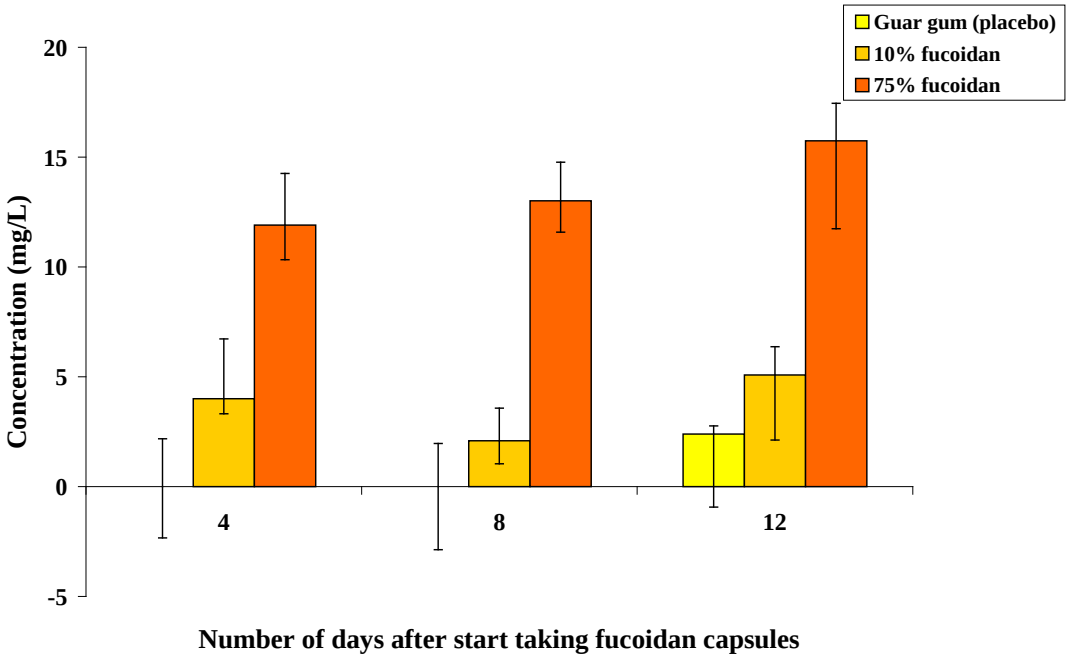


Figure 4.4: Concentration of free fucoidan in plasma measured by 1B1. This histogram shows three different groups at 4, 8, and 12 days after ingestion of the designated capsules. The first group of volunteers (n = 6) ingested capsules containing guar gum (placebo). The second group (n = 6) and third group (n = 40) ingested capsules containing GFS^{TM} fucoidan at two different concentrations (10% and 75%) w/w, respectively. Each point represents the median of readings ± 95% confidence intervals.

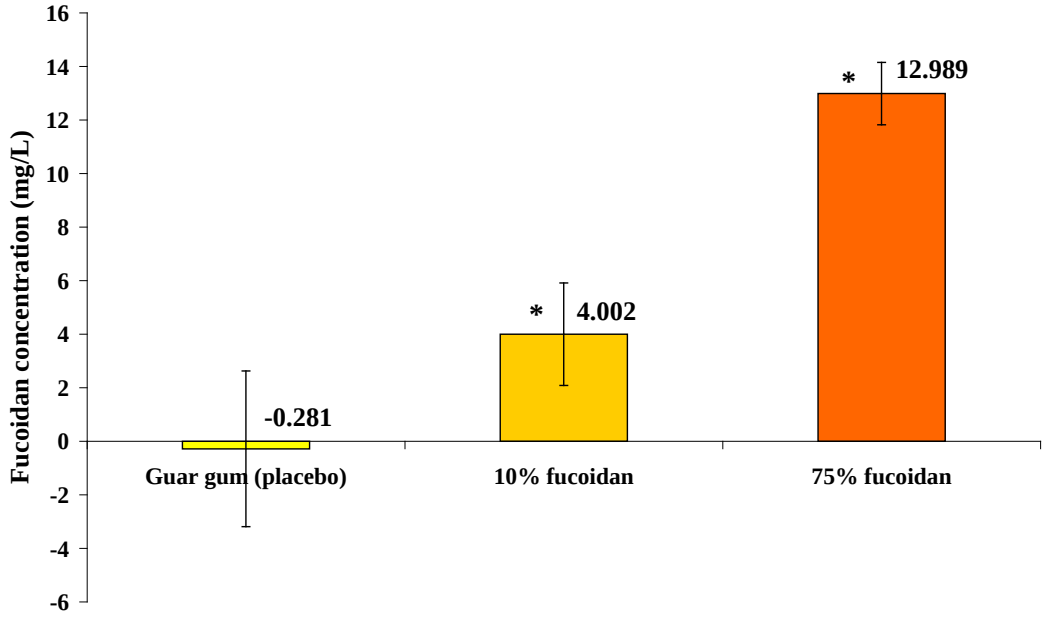


Figure 4.5: Median concentration of free fucoidan in plasma. The concentration at 4, 8, and 12 days for each group was pooled together for each sample group (placebo, 10% fucoidan w/w, and 75% fucoidan w/w) and plotted ± 95% confidence intervals. * *p*-value < 0.05 (ANOVA).

4.4.3 Fucoidan level in protein-purified plasma samples

Another set of clinical plasma samples, together with the standard curve plasma, was protein-purified using the affinity/ion exchange chromatography matrix (CM Affi-Gel Blue Gel). The resulting fractions were tested for the presence of fucoidan using the same ELISA technique. The fucoidan level was low in most of the samples (n = 10) and lower than the level of untreated samples.

Another ten plasma samples from the same volunteers, and a standard curve, were treated with the pronase enzyme and tested for the presence of fucoidan. Fucoidan levels were also too low for detection using this method.

4.4.4 Urine fucoidan level

The urine fucoidan concentration was measured by two different methods as mentioned earlier. The first method was conducted using the 1B1 Ab in an ELISA assay. The urine samples were collected at two different times, baseline and 4 days after ingestion of the fucoidan or placebo capsules. A standard curve was constructed as mentioned in section 4.3.10 and the urine samples collected from the volunteers were tested for fucoidan using the competitive ELISA. The median concentration of fucoidan for the placebo group (n = 3) was 0.334 mg/L and was 1.031 mg/L for the 75% fucoidan group (n = 6).

The second method to detect fucoidan in urine was a colorimetric method and was conducted using the DMB dye. A standard curve was constructed and the urine samples collected from volunteers were quantified for the presence of fucoidan using the described assay. The median concentration of fucoidan for the two different groups of volunteers after taking the capsules orally is shown in [Figure 4.6].

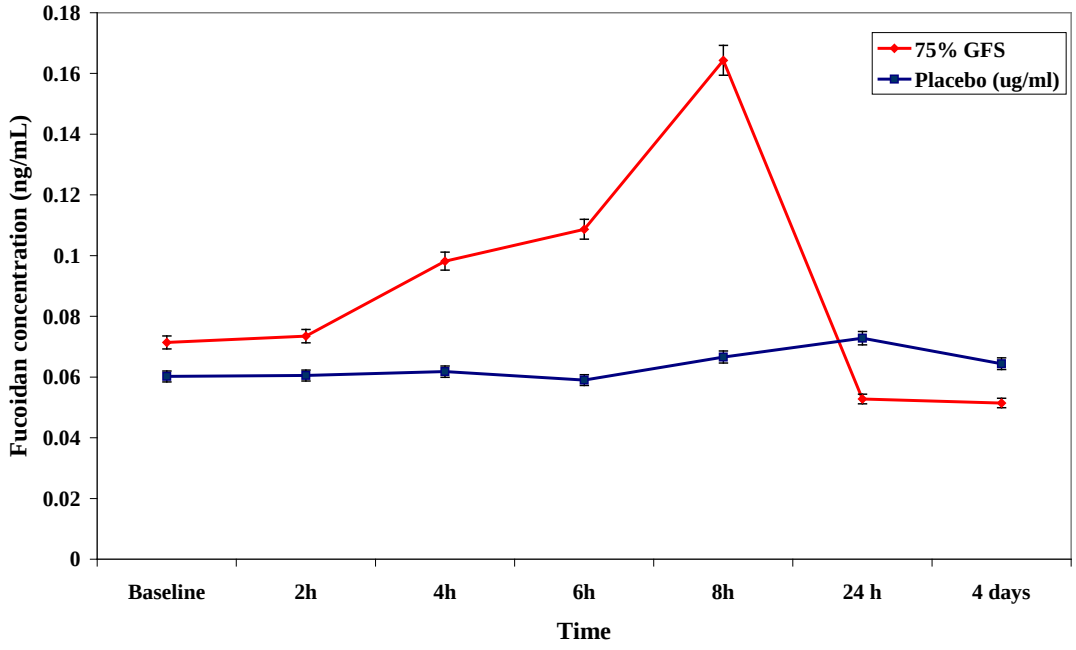


Figure 4.6: Median concentration of fucoidan in urine measure by DMB method. The figures shows two graphs for both 75% fucoidan group (n = 8) and guar gum placebo group (n = 3) at different time points after ingesting the study capsules tid for one day. Each value represents duplicate readings for each urine sample ± MSE.

4.4.5 Urine glucose level

The concentration of glucose in urine samples collected from volunteers has been measured as described previously in section 4.3.5. The mean glucose level in the placebo group at the baseline was <1mmol/L and after 4 days of ingesting the placebo capsules it remained at <1 mmol/L (n = 3). In the 75% fucoidan group of volunteers the mean glucose level at baseline was also <1 mmol/L and after 4 days of ingesting the fucoidan capsules it was still <1.1 mmol/L (n = 6).

4.4.6 Urine specific gravity

The urine specific gravity was measured in both placebo and fucoidan treated volunteers. In the placebo group the average measurement at the baseline was 1.018 and after 4 days it was 1.018 (n = 3). In the 75% fucoidan group the average measurement at baseline was 1.015 and after 4 days it was 1.014 (n = 6).

4.4.7 Urine pH

The urine pH was measured in urine samples collected from both placebo and fucoidan treated volunteers. The average pH value in the placebo group at baseline was 5.3 and after 4 days was 5.0 (n = 3), whereas in the 75% fucoidan group the pH value at baseline was 5.9 and after 4 days it was 6.0 (n = 6).

4.5 Discussion

The data in this chapter demonstrate that fucoidan can be measured in plasma samples using an Ab method and in urine using a colorimetric method. Despite the fact that *Undaria* fucoidan, a large molecular weight material, was given orally, it was possible to detect small amounts of it in the plasma and urine. Whilst acidic conditions in the stomach may cause a limited hydrolysis of the fucoidan, humans do not produce enzymes capable of breaking down fucoidans, and the polysaccharide also appears to be unaffected by human faecal flora (Michel *et al.*, 1996). This suggests that small quantities of orally-administered fucoidan may cross the intestinal wall as whole molecules, probably by the process of endocytosis. This theory mirrors the results reported by Barthe and co-workers for chondroitin sulphate (Barthe *et al.*, 2004). They suggested that chondroitin sulphate (a slow-acting drug taken orally in humans to treat osteoarthritis) cross the upper intestine intact probably via endocytosis. Unlike

fucoidan, chondroitin sulphate is effectively degraded in the distal gastro-intestinal tract presumably by the enzymes of the intestinal flora.

In this study a MoAb was used for fucoidan detection using competitive ELISA. The MoAb 1B1 was raised to react with heparin and heparin-like substances. It also reacts with pentosan polysulphate, dextran sulphate and chondroitin sulphate E. It reacts weakly with heparan sulphates, chondroitin sulphate and dermatan sulphate. It has been found that the 1B1 Ab lower detection limit for dextran sulphate was about 156 mg/L (Kongtawelert & Kulapongs, 2000). The 1B1 was found to have good reactivity against fucoidan in this ELISA assay.

The amount of fucoidan detected in serum was small, correlating with about 0.6 % of the oral dose. It was assumed that all the material detected by this Ab was any sulphated polysaccharide with an affinity for the Ab.

The graph in [Figure 4.3] shows that both heparin and fucoidan have similar binding affinities with 1B1 Ab using plasma as a diluent, which is a clear indication that this Ab can be used to detect fucoidan in biological systems. However, at high concentrations of both heparin (0.25-2 kIU/L) and fucoidan (5-80 mg/L), the avidity of the Ab for heparin was higher than the avidity for fucoidan. This may be because the Ab was originally produced against heparin, which has a different sulphation pattern to that of fucoidan. At lower concentrations of both heparin (0.0078 - 0.25 kIU/L) and fucoidan (0.078 - 5 mg/L), the reactivity of the Ab for both substances was low under the same experimental conditions. This implies a limit on the use of this Ab at very low concentrations of antigen. The data about the inhibition concentration of heparin and fucoidan by 1B1 Ab, as shown in Table 4.1, confirm that heparin and fucoidan have comparable IC_{50} values at the concentrations used in the study.

Owing to the fact that the 1B1 Ab used in this assay is not specific to fucoidan and was raised to sulphated polysaccharides, it is possible that the increased readings in this assay were not due to fucoidan, but due to elevation of another sulphated polysaccharide, such as heparin. The measurement of heparin using this Ab has been reported previously (Kongtawelert & Kulapongs, 2000). An important part of the method was the digestion of plasma samples with broad-spectrum proteolytic enzymes

like pronase. In the previous work, pre-treatment with pronase enhanced the sensitivity of the assay towards heparin. In the work presented here, pre-treatment of the clinical samples with pronase decreased the sensitivity, indicating that the detected molecule was not heparin. The same samples were pre-purified using affinity/ion exchange prior to the assay. This pre-treatment reduced the amount of reacting material in the samples, perhaps due to an association of the fucoidan with proteins removed by the affinity matrix. Fucoidans are charged molecules and may react in ion-exchange procedures.

Guimaraes and Mourao (1997) showed in animal models that the branched polysaccharides, such as the sulphated fucan from brown algae, is cleared only in minor amounts in the urine and that degradation of the anionic molecules occurs during glomerular filtration. In this study the fucoidan concentration in urine was measured using two methods, the antibody-based method and the DMB dye based method. The fucoidan was not detected in high quantities in urine when 1B1 Ab was used, being only 0.697 mg/L higher than the concentration in urine samples collected from the placebo group. This was not significant, especially since the samples were collected 4 days after ingestion. However, when the DMB dye method was used for urine samples, it was possible to study the kinetics at 2 h time intervals. Fucoidan started to appear in the urine samples after 4 h and peaked at 8 h, but after 1 and 4 days it was not detected in the urine samples, meaning that the body possibly had cleared most of the ingested fucoidan. It is possible that some fucoidan is still available within the body, but bound to cells or proteins or in another form.

This implies that the glomerular permeability for fucoidan is limited because of the structure and the size of the molecule. However, a reduction in size for excreted fucoidan is possible and may occur in the kidney or other organs by the non-specific action of free radicals. It is likely that the urinary excretion of fucoidan is therefore underestimated because the degraded compound possibly is less metachromatic. The finding that fucoidan was excreted between 4 and 8 h agrees with previous work showing than different sulphated polysaccharides are excreted in the first 12 h (Guimaraes *et al.*, 1997).

4.6 Conclusion

In conclusion, this work has shown that the Ab 1B1 can be used to detect the bio-available free fucoidan, whole or as fragments, in human plasma samples using a quantitative competitive ELISA assay. The level of fucoidan in plasma was low after the ingestion of 3 g of the two different fucoidans probably due to the large molecular weight and lack of hydrolytic enzymes in the human intestine. Competitive ELISA using the 1B1 Ab was not sensitive enough to detect urinary fucoidan concentration. However, fucoidan excreted in urine between 4 and 8 h after ingestion was detected by the DMS dye. The ELISA assay may be useful for monitoring fucoidans when used for therapeutic purposes. Further development of the assay may be needed to measure the total fucoidan concentration and to increase the Ab specificity and sensitivity.

CHAPTER FIVE

ANTICOAGULANT ACTIVITY OF FUCOIDAN

Chapter objectives:

- Determine the *in vitro* effects of fucoidan on coagulation pathways.
- Determine the *in vivo* effects of fucoidan on haemostasis by studying the different coagulation tests in single blinded placebo controlled clinical trial.

Chapter contents:

5.1 Summary

Seaweed derived heparin-like substances such as fucoidan have been extensively studied *in vitro* as potential blood anticoagulants. However, there have been no studies to date investigating the anticoagulant activity of fucoidan when administered *orally* to humans. The aim of this chapter was to study the *in vitro* effect of fucoidan on the coagulation pathways and to assess in a clinical trial the *in vivo* effect of oral intake of fucoidan on haemostasis by testing the safety of ingesting fucoidan and determine if there was any clinical consequence after ingestion.

Thirty one healthy human volunteers were randomly allocated to placebo or active-treatment groups in a single blinded clinical trial. The placebo group (n = 6) ingested 3 g of guar gum capsules and the active-treatment group (n = 25) ingested 3g of 75% fucoidan in capsules for a maximum of 12 days. Activated partial thromboplastin time (aPTT), antithrombin-III (AT-III), thrombin time (TT), anti-factor-Xa, prothrombin time (PT), and Prothrombin Ratio/International Normalised Ratio PR (INR) were analysed according to the standard methods for both *in vitro* and *in vivo* tests plus platelet count for the latter.

The *in vitro* anticoagulant activity of fucoidan was prominent. It increased aPTT, TT, and PT at low fucoidan levels (0-0.1) mg/mL. No clot formation was detected at higher concentrations (5-50) mg/mL but anti-Xa results were increased. In the active-treatment group, significant changes were noted in aPTT, TT and AT-III, but all changes remained within normal clinical parameters. Non-significant changes were noted in PT and PR (INR). There were no effects on anti factor-Xa or platelet count. Although there were significant changes in some of the coagulation factors, all were still within the accepted reference ranges.

Thus, oral intake of fucoidan in quantities up to 3 g is not clinically effective in inducing anticoagulation. The minor changes however suggest that further investigation to assess the potential for a therapeutic use especially developed fractions with greater uptake, may be worthwhile.

5.2 Introduction

Over the last 60 years, seaweed derived heparin-like substances have been extensively studied as possible blood anticoagulants. The first report on marine algal extracts possessing anticoagulant properties was made in 1936 by Chargaff and his colleagues (Chargaff *et al.*, 1936). An extract of a red alga *Iridae laminarioides*, which contains 'galactan sulphuric acid ester', demonstrated anticoagulant activity with 30 U/mg of heparin equivalence. Subsequent studies described similar anticoagulant properties in agar and carrageenan (Elsner *et al.*, 1937; Elsner & Hoppe-Seyler's Z, 1938; Hokkaio, 1941; Houck *et al.*, 1957).

About 150 species representing three major divisions of marine algae, Rhodophyta (red algae), Phaeophyta (brown algae) and Chlorophyta (green algae), have been reported to have blood anticoagulant activities (Shanmugam & Mody, 2000). However, there is a greater incidence of anticoagulant activity in extracts from the brown algae compared to red and green algae (McLellan & Jurd, 1992).

The first isolation of sulphated fucan from marine brown algae was reported almost ninety years ago as fucoidin (Killing, 1913). Sulphated fucans are widely present among all the brown algae (Phaeophyceae) so far investigated. On the other hand sulphated fucans seem to be absent from green algae (Chlorophyceae), red algae (Rhodophyceae) and golden algae (Xanthophyceae), and from terrestrial plants. The composition of sulphated fucan polymers varies according to the algal species (Percival & Ross, 1950; Percival & McDowell, 1967), the extraction procedures (Mabeau *et al.*, 1990), the season of harvest and local climatic conditions (Black, 1954; Von Holdt *et al.*, 1955; Wort, 1955; Honya *et al.*, 1999).

The general sulphated polysaccharides of Phaeophyta, brown marine algae, are called fucans. These include the compounds fucoidin, fucoidan, ascophyllan, sargassan and glucuronoxylofucan. They comprise families of polydisperse heteromolecules based on L-fucose, D-xylose, D-glucuronic acid, D-mannose and D-galactose (McLellan & Jurd, 1992).

The anticoagulant component of brown marine algal extracts resides with the fucans, and of these, fucoidan has been the most extensively studied. The most active

fucoidan fractions consist predominantly of sulphated fucose residues. Thrombin inhibition activity of these fractions exceeded that of heparin (Springer *et al.*, 1957).

Early studies used whole blood clotting times and the United States/British pharmacopoeial assays to determine the anticoagulant activity, whereas more recent studies have used clot end points such as PT, aPTT and TT (McLellan & Jurd, 1992). It has been reported that fucoidans exhibit a potent anticoagulant activity related to their capacity to catalyse the inhibition of thrombin by its natural inhibitors antithrombin and heparin cofactor II (Colliec *et al*., 1991; Mauray *et al.*, 1995). The anticoagulant activity of high molecular weight fucans extracted from *Ecklonia kurome* was reported to be dependent on their sulphate content and molecular weight like numerous other sulphated polysaccharides (Nishino *et al.*, 1991). Fucoidan produces a dose-dependent anticoagulant effect, which is similar to that of heparin.

There is a market need for new, non-animal derived and preferably oral anticoagulants. Heparin has several side effects, such as the development of thrombocytopenia (Warkentin, 1999; Visentin, 1999); it has a haemorrhagic effect (Kelton & Hirsh, 1980; Kakkar *et al.*, 1986); it is ineffective in correcting congenital or acquired antithrombin deficiencies and it cannot inhibit thrombin bound to fibrin (Weitz, 1992; Liaw *et al.*, 2001). Heparin is generally extracted from pig intestine or bovine lung, where it occurs in low concentrations, so there is a potential risk of transmission of prion-related diseases. The increasing requirement of antithrombotic therapy suggests the need to look for alternative sources of anticoagulant and antithrombotic compounds.

The aim of this chapter is to investigate the effect of fucoidan derived from *Undaria pinnatifida* on the *in vitro* coagulation pathways and the *in vivo* coagulation process in human volunteers after oral administered.

5.3 Materials and methods

5.3.1 Volunteers

Informed consent and human ethics approval were obtained as described in section 2.1. Thirty one human volunteers aged between 23-58 years (average 40 years) were included in this clinical study after meeting the study criteria as described in sections 2.2 and 2.3.

In the plasma level of fucoidan assay all 25 volunteers took the active treatment (75% fucoidan) and 6 other volunteers took the placebo treatment. Only 17 volunteers were tested for aPTT and 10 volunteers for each of (TT, AT-III, PT, and INR) tests. The ELISA test was performed on 10 volunteers of the active treatment group and 6 other volunteers of the placebo group.

5.3.2 Blood sample collection and plasma preparation

Venous blood from the antecubital vein was collected using citrated tubes for the anticoagulant experiments. EDTA blood was used for all other tests. The plasma was prepared within 30 min of blood collection by centrifuging the tubes at 300 *xg* for 5 min at room temperature. The plasma was then aspirated into a transfer tube to be used later in the different tests.

5.3.3 Plasma level of fucoidan

The 75% fucoidan concentration in plasma was measured using the 1B1 antibody according to the method described previously in section 4.3.12 using competitive ELISA assay (Irhimeh *et al.*, 2005a). The amount of fucoidan in plasma samples was calculated after creating the standard curve.

5.3.4 Full blood count

FBC including platelet count, MPV and PDW were obtained for all volunteers before and after taking the 75% GFS^{TM} using an automated cell counter (CELL-DYN 4000 System, Abbott Laboratories, Abbot Park, IL USA). PDW was calculated as (σ x 100) (fL)/MPV (fL) after log transformation of the MPV.

5.3.5 Preparation of fucoidan serial dilutions for *in vitro* experiments

For the *in vitro* study of the 75% fucoidan effect on the coagulation assays 1 g of fucoidan extract was dissolved in 10 mL pooled normal plasma (PNP) to prepare a stock solution at 100 mg/mL. The fucoidan stock solution was diluted with PNP to give serial dilutions ranging from zero to 50 mg/mL.

5.3.6 Coagulation study

The aPTT, AT-III, TT, anti factor Xa and PT tests were all determined using an automated coagulation analyser (Sysmex CA1500, Kobe Japan) and STA anticoagulation assay machine (Diagnostica Stago, France). The two machines were standardised and calibrated to match each other. The results from both machines were averaged for each test.

5.3.6.1 Activated partial thromboplastin time (aPTT)

The aPTT was determined using Dade Actin FSL Activated PTT Reagent according to the manufacturer specifications (Dade Behring Marburg GmbH, Germany). The citrated test plasma was incubated with the optimal amount of phospholipids and a surface activator. This activated factors of the intrinsic coagulation system. The addition of calcium ions triggers the coagulation process allowing measurement of the clotting time.

5.3.6.2 Antithrombin-III (AT-III)

The AT-III was determined using a chromogenic assay kit Berichrom Antithrombin III (A) according to the manufacturer specifications (Dade Behring Marburg GmbH, Germany). The AT-III in the citrated test plasma sample was converted into an immediate inhibitor by heparin inactivates thrombin present. The residual thrombin content is determined in a kinetic test by measuring the increase in absorbance at 405 nm. AT-III reference range used in this study is (90-140) %.

5.3.6.3 Thrombin time (TT)

The TT was determined using a Thromboclotin assay kit according to the manufacturer specifications (Dade Behring Marburg GmbH, Germany). The time to clot when thrombin is added in the citrated test plasma sample was assayed

automatically according to the instructions. TT reference range used in this study is (14-17) sec.

5.3.6.4 Anti factor Xa

Anti factor Xa was determined using a chromogenic assay kit Spectrolyse heparin (Xa), according to the manufacturer specifications (Trinity Biotech plc., Ireland). The citrated plasma was added to a tube containing both AT-III and heparin in excess. Then, factor Xa and factor Xa-specific chromogenic substrate are added and the absorbance was determined.

5.3.6.5 Prothrombin time (PT)

PT was quantitatively determined using RecombiPlasTin, a thromboplastin reagent based on recombinant human tissue factor, according to the manufacturer specifications (Instrumentation Laboratory Company, USA). Basically, the addition of the tissue thromboplastin to the human plasma in the presence of calcium ions initiates the activation of the extrinsic pathway. This results ultimately in the conversion of fibrinogen to fibrin, with formation of a solid gel. PT time reference range used in this study is (11-13) sec.

5.3.6.6 PR (INR)

Because of differences between the source and batch of tissue factor, the International Normalized Ratio (INR) system was devised to achieve standardisation. Each manufacturer gives an ISI (International Sensitivity Index) for any instrument and tissue factor they make. The ISI value indicates how the particular batch of tissue factor compares to an internationally standardized sample.

The ISI is normally between 1.0 and 1.4. The INR is the ratio of a patient's prothrombin time to a normal (control) sample, raised to the power of the ISI value for the thromboplastin used. The PR (INR) reference range used in this study is <1.2.

$$INR = \left(\frac{PT_{test}}{PT_{normal}} \right)^{ISI}$$

5.3.7 Statistical analysis

The fucoidan standard curve readings were calculated using mean and standard deviation. All other statistical values were based on non-parametric statistics (chi-square test) using the median and 95% confidence interval (CIM). To calculate the fucoidan concentration in the plasma, triplicate readings for each standard, control, and sample were averaged and subtracted from the average blank readings. Statistical parameters were calculated using Microsoft Office, Excel and SPSS, Version 12.1.

5.4 Results

5.4.1 *In vitro* anticoagulant activity of fucoidan

The *in vitro* effect of the 75% fucoidan extract was tested on different coagulation assays including aPTT, TT, AT-III, PT, PR (INR) and anti-Xa. The fucoidan prolonged the aPTT time from 25.8 sec for the PNP to 135.8 at 0.063 mg/mL 75% fucoidan. There was no detectable clot formation for concentrations higher than 0.1 mg/mL 75% fucoidan (Table 5.1). The TT was also prolonged but at greater rate, where it was 15.6 sec at 0.0078 mg/mL 75% fucoidan and 80.4 sec at 0.0156 mg/mL 75% fucoidan. No clot was detected at higher concentrations (Table 5.1).

The AT-III decreased from 107.5% at zero fucoidan concentration (PNP) to 50% at 0.5 mg/mL (Table 5.1). Low concentrations of the 75% fucoidan, ranging from 0.0078 to 0.063 mg/mL, had no effect on PT, but at 0.125 mg/mL it began to increase (Table 5.1).

The PR (INR) calculation was also affected, and it increased although not consistently, reflecting the changes in the PT. Interestingly, the 75% fucoidan at high levels (1 to 50) mg/mL had a very strong effect on the anti-Xa assay whereas at low levels the effect was not obvious (Table 5.1).

At high fucoidan concentrations (*i.e.* 25 or 50 mg/mL) the solution viscosity increases which could affect the coagulation tests especially if the test is based on the movement of the magnetic bead in the cuvette. The other tests which are based on spectrophotometry would also be affected as the highly viscose fucoidan solution will interfere with the light beam. These possible effects of the highly concentrated fucoidan may have caused the increase in anti-Xa results observed. It also may have

caused prolongation in the coagulation time in some other tests. At such high fucoidan concentrations assays should not be performed.

Table 5.1: The *in vitro* effect of the 75% fucoidan on the different coagulation assays.*

75% fucoidan (mg/mL)	aPTT (sec)	TT (sec)	AT-III (%)	PT (sec)	PR (INR)	Anti-Xa
0 (PNP)	25.8	15.6	107.5	11.3	0.98	0.14
0.0078	32.3	80.4	97	9.4	0.84	0
0.0156	39.7	>120	94	9.6	0.88	0.04
0.031	63.8	>120	93	9.2	0.87	0.08
0.063	135.8	>120	87	9.5	1.01	0.01
0.125	>180	>120	85	12.1	1.49	0
0.25	>180	>120	73	18.6	2.76	0.03
0.5	>180	79.6	50	39.1	6.9	0.13
1	>180	>120	113	no clot	-----	0
5	>180	>120	96	8.8	0.77	0.09
10	>180	>120	86	14.2	1.25	0.25
25	>180	>120	63	no clot	-----	0.52
50	>180	>120	40	no clot	-----	0.87

* Each reading represent the average of 3 samples each read on both Sysmex and Diagnostica machines and then averaged.

5.4.2 Clinical studies for the anticoagulant activity of fucoidan

There were significant changes in aPTT, TT and AT-III tests for volunteers after ingesting the orally administered fucoidan. The aPTT increased significantly as shown in [Figure 5.1] from 28.4 sec at baseline to 30.06 sec after 12 days (n = 17, $p = 0.007$) (Table 5.2). The individual changes in aPTT for each volunteer are shown in [Figure 5.2]. TT decreased significantly from 18.62 sec at baseline to 17.55 sec after 4 days (n = 10, $p = 0.04$), and in contrast to the *in vitro* data AT-III increased significantly from 113.5% at baseline to 117% after 4 days (n = 10, $p = 0.02$) (Table 5.3). The TT and AT-III levels were not tested after 8 and 12 days of ingesting the treatment.

Table 5.2: aPTT readings for both placebo control and 75% fucoidan groups. The reference range (22-32) sec.

	Placebo group ± MSE [§]	**Fucoidan group ± MSE** [‡]
Baseline	27.7 ± 0.76	28.41 ± 0.65
4 Days (*p*-value)	27.5 ± 0.72 (0.61)	29.29 ± 0.7 (0.07)
8 Days (*p*-value)	27.2 ± 0.6 (0.36)	29.5 ± 0.53 (0.03)[*]
12 Days (*p*-value)	27.2 ± 0.79 (1)	30.06 ± 0.46 (0.007)[*]

[§] n = 6
[‡] n = 17
[*] $p < 0.05$ using Students' t-test

For the other parameters PT and PR (INR), no significant changes were noted. The PT level was 11.54 sec at baseline and 11.85 sec after 4 days (n = 10, $p = 0.11$). The PR (INR) was 0.95 at baseline and 0.96 after 4 days (n = 10, $p = 0.59$). Anti factor Xa was not affected under the treatment conditions (Table 5.3).

Table 5.3: Average readings and reference range for the coagulation tests for the healthy volunteers who ingested 3 g tid of 75% fucoidan.

Test	**Baseline**	**4 Days**
TT ± MSE sec (*p*-value)	18.62 ± 0.49	17.55 ± 0.39 (0.04)
AT-III ± MSE % (*p*-value)	113.5 ± 2.81	117 ± 2.07 (0.02)
PT time ± MSE sec (*p*-value)	11.54 ± 0.17	11.85 ± 0.17 (0.11)
PR (INR) ± MSE (*p*-value)	0.95 ± 0.02	0.96 ± 0.02 (0.59)
anti-Xa (LMWH)	0	0

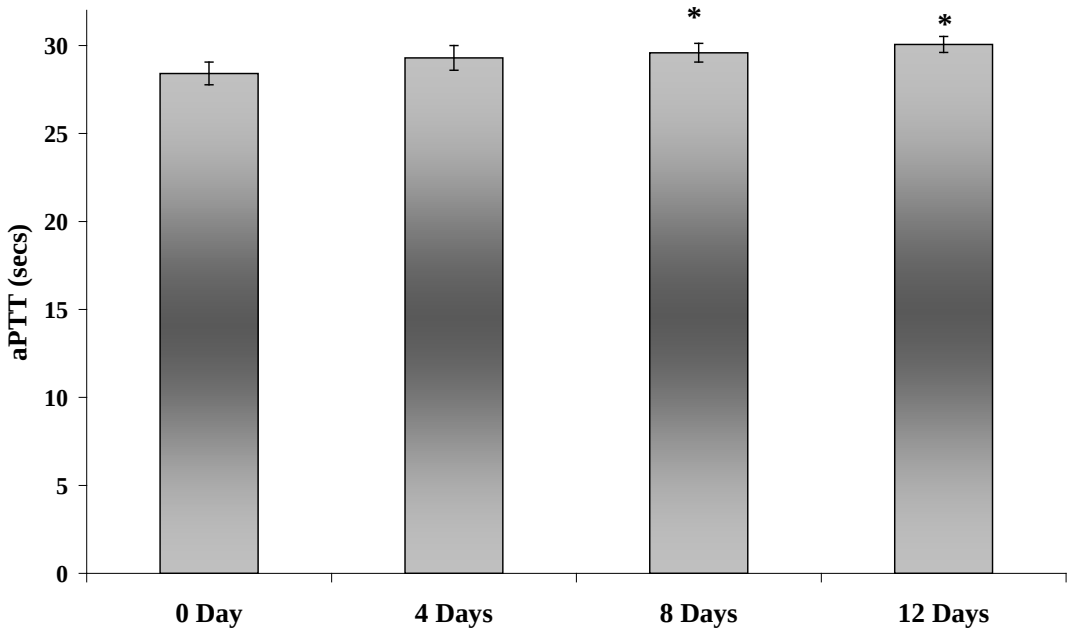


Figure 5.1: aPTT measurements in volunteers treated with 3 g of 75% fucoidan tid for 12 days. Each point represents the average measurements for 17 different volunteers ± MSE. * $p < 0.05$ when compared with baseline measurements.

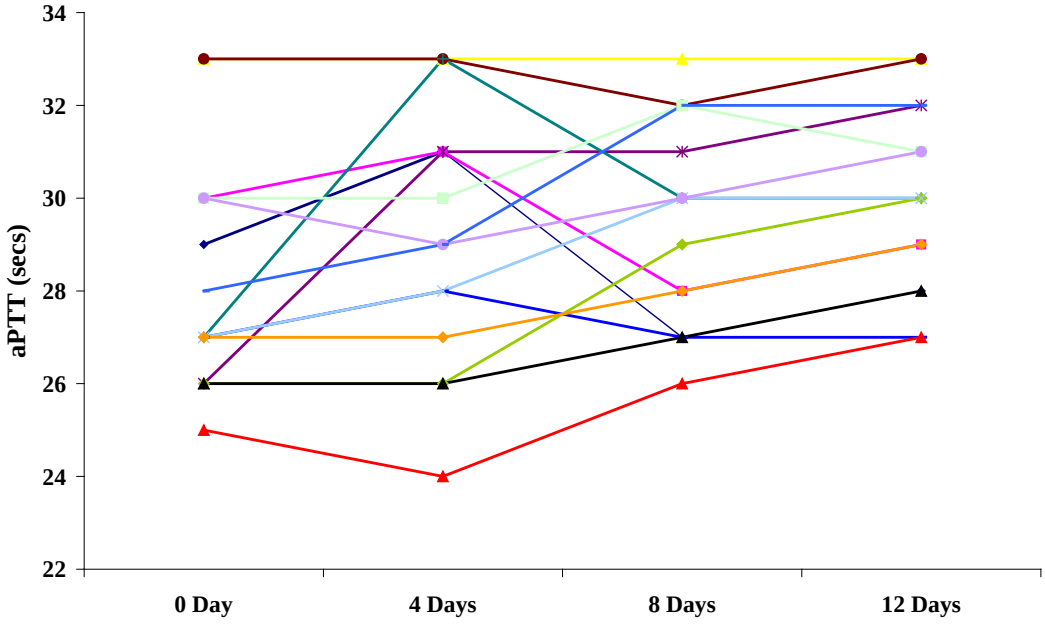


Figure 5.2: Individual aPTT in volunteers treated with 75% fucoidan. Each line represents the aPTT levels in one individual treated with 3 g tid of 75% fucoidan w/w for 12 days at 4 time points.

In comparison with the placebo group, there were no significant changes in platelet count, MPV, and PDW when volunteers ingested the 75% fucoidan daily for up to 12 days. Both the placebo group and the 75% fucoidan group mean platelet parameters did not change after ingesting the treatment capsules when compared to the baseline reading. The platelet parameters for the placebo group are shown in Table 5.4 where the platelet parameters for the 75% fucoidan group are shown in Table 5.5.

Table 5.4: Placebo control group average readings for platelet count, mean platelet volume (MPV) and platelet distribution width (PDW). Volunteers (n = 6) ingested 3 g tid of guar gum placebo capsules for 12 days.

	Platelet count /nL ± MSE	MPV (fL) ± MSE	PDW (fL) ± MSE
Baseline	327 ± 66	8.96 ± 1.19	16.23 ± 0.15
4 Days	333 ± 69	8.77 ± 0.93	15.87 ± 0.12
8 Days	313 ± 55	8.79 ± 0.89	16 ± 0.51
12 Days	306 ± 44	8.52 ± 0.80	16.1 ± 0.49

Table 5.5: Active treatment group average readings for platelet count, mean platelet volume (MPV) and platelet distribution width (PDW). Volunteers (n = 25) ingested 3 g tid of 75% fucoidan capsules for 12 days.

	Platelet count /nL ± MSE	MPV (fL) ± MSE	PDW (fL) ± MSE
Baseline	280 ± 12	8.29 ± 0.1	16.07 ± 0.18
4 Days	279 ± 14	8.29 ± 0.19	15.9 ± 0.15
8 Days	285 ± 13	8.09 ± 0.19	15.81 ± 0.1
12 Days	285 ± 13	8.00 ± 0.19	16.03 ± 0.12

Plasma samples from sixteen volunteers were quantified for fucoidan using the described competitive ELISA method. The median concentration of material measured as fucoidan for the placebo group was 0.166 mg/L (CIM 0.018; n = 6) and 13.058 mg/L (CIM 0.031; n = 10) for the 75% fucoidan group after 12 days of oral intake.

5.5 Discussion

In this chapter fucoidan derived from brown seaweed (*Undaria pinnatifida*) was tested *in vitro* and in clinical study as a possible anticoagulant compound. This type of fucoidan differs from the common fucoidan that previously has been extensively investigated. It is derived from a different seaweed which makes the specific sulphation pattern in particular different from that of the common commercially available fucoidans (*e.g.* Sigma fucoidan).

Fucoidans are the most abundant sulphated polysaccharides of non-mammalian origin. Percival and Ross (1950) described the fucoidan from the common brown algae *Fucus vesiculosus* as a polysaccharide based on L-fucose with mainly α (1→2) glycosidic linkages and sulphate groups at position 4. They found branches of sulphated fucose every five units. In 1993 Patankar and co-workers (Patankar *et al.*, 1993) re-investigated the structure of the sulphated fucan from *Fucus vesiculosus*. Their model differs in the nature of the main glycosidic linkage; α (1→3) instead of α (1→2). The position of the sulphate, in accordance with the previous model, was found to be mainly in position 4. Recently, the fucoidan was described as being composed of α (1→3) and α (1→4) fucosyl units mostly sulphated at positions 2 and 3 (with branching sulphate or additional monosaccharides at free positions) (Haroun-Bouhedja *et al.*, 2000). Fucoidans are considered to have similarities to the much smaller mammalian molecule heparin. They have a complex and heterogeneous structure and it is not always possible to define a repetitive unit.

The fucoidan which was used in this study is about 75% pure with high sulphate composition of 23% and it contains about 35% acetyl group. The molecular weight of the used fucoidan is very large and larger than heparin. Therefore, it is probably expected to see a greater anticoagulant activity for the fucoidan than heparin.

Heparin, which is usually administered intravenously or subcutaneously to treat venous thrombosis or for the prevention of thromboembolism, belongs to the glucosaminoglycans family. These compounds are heterogeneous and highly negatively charged with variant molecular weights and sulphation patterns. Heparin can bind to endothelial cells and plasma proteins which affect its availability, distribution and activity (Hiebert, 2002). As mentioned earlier, there are several side

effects associated with heparin administration and the search for alternative therapies, especially orally available anticoagulants, is important.

Scientists have started investigating substances similar in structure to heparin, including fucoidan. Many reports suggest that fucoidan has anticoagulant activity. *Fucus vesiculosus* fucoidan was found to have a specific "anticoagulant activity" by the aPTT assay of 9-13 U/mg, as compared with 167 U/mg for heparin (Nishino *et al.*, 1994). Fucoidan from *Laminaria brasiliensis* has a higher specific activity (30 U/mg); though its sulphate content is lower (Mourao & Pereira, 1999). In this study the *in vitro* effect of the *Undaria pinnatifida* 75% fucoidan w/w was evaluated. This fucoidan was found to have a specific anticoagulant activity by the aPTT assay at low concentration, in the range of 0-0.125 mg/mL. At concentrations higher than 0.125 mg/mL the fucoidan prevented clot formation, corresponding to the previously reported results with fucoidans from other seaweed species.

The aPTT is used to evaluate coagulation alterations in the intrinsic pathway and will also detect severe functional changes in factors II, V, X, or fibrinogen. The aPTT has been used to monitor the effectiveness of heparin therapy. Engelberg has shown that there is a small but significant effect on aPTT (averaged at 2.3 sec increase) after the administration to 45 patients of 20,000 units of oral heparin (Engelberg, 1995). Hiebert and co-workers have also observed an increase in the aPTT levels in an HIV^+ trial after long term oral administration of dextran sulphate (8 kD, 1 g, 4 times daily) (Hiebert *et al.*, 1999).

In the present study it was found that orally administered fucoidan may prolonged the aPTT from 28.4 sec at baseline to 30.06 sec after 12 days ($n = 17$, $p = 0.007$) (1.65 sec increase), which indicates that fucoidan may alter the intrinsic coagulation pathway. The mechanism by which the fucoidan affects the aPTT is hard to predict but it could be through the circulating levels of factors II, VII, IX, and X. The level of these factors has been shown to be depressed in patients receiving oral anticoagulants when the aPTT could be expected to be prolonged (Berteau & Mulloy, 2003). This is supported by the demonstration that orally administered heparin (intact and LMWH) had antithrombotic activity ranging between 50% and 100% in rat models (Hiebert, 2002). This was associated with the presence in plasma of about 1% of the orally

administered heparin dose. Similarly, our findings indicate that the average level of fucoidan in plasma after 4 days was 0.6% of the oral dose (Irhimeh *et al.*, 2005a).

It is possible that fucoidan has heparin like action (*i.e.* prolong aPTT via anti-Xa and anti-IIa activity). The mechanism of action of heparin is achieved when it binds to the enzyme inhibitor AT-III causing a conformational change which results in its active site being exposed. The activated AT-III then inactivates thrombin and other proteases involved in blood clotting, most notably factor Xa. The rate of inactivation of these proteases by AT-III increases 1000-fold due to the binding of heparin.

The observed changes in the aPTT and TT were minimal when 75% fucoidan was ingested at 3 g daily. These changes have no obvious clinical significance other than the fact that administration of 3 g of 75% fucoidan daily has no clinical implications on volunteers and is safe. However, a dose response relationship is predicted and the increase in the bio-available fucoidan in plasma may alter the aPTT and TT readings significantly. This is supported by the in vitro trial were large shift the aPTT and TT readings was observed when plasma sample were treated with the 75% fucoidan. It is possible that a change in the fucoidan administration route will increase its bio-availability in plasma, thus a clinically significant changes in aPTT and TT may occur.

It has been found that purified fucoidan fractions from *Ecklonia kurome* (Nishino *et al.*, 1999), *Ascophyllum nodosum* (Millet *et al.*, 1999), and *Pelvetia caniculata* (Colliec *et al.*, 1994) have activity mediated both by antithrombin and by another plasma serine protease inhibitor (serpin) and heparin cofactor II (HCII) (Colwell *et al.*, 1999). Heparin interacts with HCII by means of the regular repeating units, not the antithrombin-binding sequence. These serpins act against several of the coagulation system proteases, including thrombin, factor Xa, and factor IXa (Mauray *et al.*, 1998). All of these factors may be involved in the ability of fucoidan to prevent venous thrombosis (Millet *et al.*, 1999). The release of the tissue factor pathway inhibitor from endothelium, which is stimulated by fucoidan more potently than by heparin, may also have an antithrombotic effect (Giraux *et al.*, 1998). The *in vitro* assays in this study showed that *Undaria* fucoidan has the ability to alter the AT-III assay. It decreased the readings in a concentration dependent manner.

The anticoagulant and antithrombotic activities of fucoidan fractions from *Ascophyllum nodosum* increase with increasing molecular weight and sulphate content. However, fractions in which the native pattern of sulphation was intact were more potent than fractions of equivalent molecular weight and overall degree of sulphation but where the pattern had been disrupted by partial de-sulphation (Boisson-Vidal *et al.*, 2000). In this study the fucoidan used was in its native structure.

Fucoidans may also promote fibrinolysis by potentiating plasminogen activators (Nishino *et al.*, 2000). The predominant pattern of sulphation in *Ascophyllum nodosum* fucoidan is the trisulphated disaccharide motif, similar to that found in heparin. A heavily sulphated substituted disaccharide is also the repeat unit of an anticoagulant galactan isolated from red algae (Farias *et al.*, 2000).

PT measures the presence and activity of five different blood clotting factors (factors I, II, V, VII, and X) which characterizes the extrinsic mechanism of coagulation. Prothrombin (factor II) as well as factors II, VII, XI, and X are produced by the liver in the presence of adequate amounts of vitamin K. Circulating levels of factors II, VII, IX, and X are depressed in patients receiving oral anticoagulants (Berteau & Mulloy, 2003). In this study, it was found that orally administered fucoidan has no significant effect on the PT level, and suggests that this dose of fucoidan does not affect the activity of any of these clotting factors.

The *in vitro* TT assay showed that the *Undaria* fucoidan alters the coagulation pathway in a concentration dependent manner. Prolonging the TT is also seen with heparin and in patients with dysfibrinogenaemia or fibrinogenaemia. The clinical trial showed that TT decreased significantly from 18.62 sec at baseline to 17.55 sec after 4 days ($n = 10$, $p = 0.04$) after ingesting fucoidan. The TT is considered to characterize the final stage of coagulation and so implies that fucoidan interferes with the final stage of the coagulation process. Fibrinogen levels were not tested in this study however; the results from the *in vitro* study may indicate that fucoidan interferes with fibrin production therefore preventing the formation of the clot or it inhibits thrombin mediated conversion of fibrinogen to fibrin. The increase in the TT *in vitro* may reflect the inhibitory effect on fibrinogenolysis which is not seen *in vivo*.

Correlation of the sulphation patterns of less highly sulphated fucans and galactans with their anticoagulant activity (Mourao & Pereira, 1999; Pereira *et al.*, 2002) has used various invertebrate fucans. It was found, for example, that a 3-linked, regularly 2-O-sulphated galactan has anticoagulant activity lacking in the corresponding 3-linked, 2-O-sulphated fucan or a 4-linked, regularly 3-O-sulphated galactan. The regular, linear sulphated fucans express anticoagulant activity, which is not simply a function of charge density but depends critically on the exact structure of the polysaccharide.

Although platelet parameters, including the mean platelet volume (MPV) and the platelet distribution width (PDW) have been routinely available to clinicians for some time, their role in the diagnosis and management of patients remains unclear. No effect of fucoidan on the number of platelets in the blood stream has been observed but it may possess an effect on platelet aggregation, which has not yet been studied. It has been shown that fucoidans of different molecular weights and degree of sulphation can enhance platelet aggregation *in vitro*. However, fucoidan-induced platelet aggregation was found to depend on the molecular weight of the fucoidan (Durig *et al.*, 1997). In contrast, a study using baboons found fucoidan to be a potent inhibitor of platelet aggregation *in vivo* (Alwayn *et al.*, 2000). Despite that, it is now known that fucoidan can act as a ligand for either L- or P-selectins, both of which interact with sulphated oligosaccharides. Most likely, fucoidan is acting like heparin or heparan sulphate, presenting a spatial pattern of sulphated saccharide structures that imitates the clustering of sulphated, sialylated, and fucosylated oligosaccharides on the cell surface. Platelet function and fibrinolysis were not assessed in this study.

This clinical trial showed no effect of the fucoidan on the anti-Xa assay although the *in vitro* assay showed that high levels of fucoidan had an effect, similar to that of heparin. The fucoidan absorption and plasma level in volunteers may have been too low to have an impact on the anti-Xa assay. It is still difficult to explain why fucoidan had no effect at low concentrations *in vitro*. Low molecular weight heparins and fondaparinux (pharmaceutical anticoagulants whose chemical structure is almost identical to the structure of the AT-III binding pentasaccharide sequence) target anti-factor Xa activity rather than anti-thrombin (IIa) activity, with the aim of facilitating a more subtle regulation of coagulation and an improved therapeutic index. Fucoidan in

this study did not have aan effect on anti-Xa in the *in vitro* or *in vivo* settings meaning that it probably works as an anticoagulant through inhibition of a different pathway.

A fucoidan from *Laminaria brasiliensis* has a higher specific activity than heparin (30 U/mg); though its sulphate content is lower (Mourao & Pereira, 1999). The high potency of heparin depends on a specific pentasaccharide sequence with high affinity for the serine protease inhibitor antithrombin, a sequence that is absent from this fucoidan.

The specific step of the coagulation pathway at which the sulphated polysaccharide acts can be determined using assays based on the amidolytic activity of the purified target protease (thrombin or factor Xa) and purified coagulation inhibitor (antithrombin or heparin cofactor II). They can determine the IC_{50} for each sulphated polysaccharide.

5.6 Conclusion

In conclusion, the results reported here demonstrate that *Undaria* fucoidan has a strong anticoagulant activity *in vitro* similar to that of heparin. The small quantity of bio-available fucoidan after oral administration to volunteers had a significant effect on some of the coagulation factors whilst they still remained within the reference ranges. Oral fucoidan thus could be a possible coagulation regulator, and these effects require further investigation to assess the potential for therapeutic use.

CHAPTER SIX

HAEMOPOIETIC STEM CELLS AND FUCOIDAN

Chapter objectives:

- Examine the direct effect of oral fucoidan on the level of pro-inflammatory cytokines SDF-1, IFN-γ and IL-12.
- Examine the effect of oral fucoidan on CD4, CD8, CD16 and CD19 cell subsets.
- Study the effect of ingested fucoidan on PB MNC using CFU-GM assay.
- Define the possible effects of fucoidan on HPC and HSC in BM and PB by studying the PB $CD34^+$ count and trafficking receptors.

Chapter contents:

6.1 Summary

Transplantation of HPC is an important treatment for a variety of neoplastic diseases. HPC collection for transplantation with G-CSF may be unsuccessful in patients who have received prior chemotherapy or for other reasons, and methods to improve the mobilisation of HPC are required. Disruption of the interaction between the cell surface receptor CXCR4 and its ligand SDF-1 is thought to be one of the mechanisms for HPC release from the BM into the PB.

In this chapter a clinical trial was carried out to evaluate the effects of ingestion of fucoidan (a putative HPC mobilising agent) on circulating $CD34^+$ cells, CXCR4, CD3, CD4, CD8, CD16, CD19 expression, and plasma levels of SDF-1, IFN-γ and IL-12. Following ingestion of fucoidan, $CD34^+$ cells increased significantly in the PB from 1.64 to 1.84 cells/μL after 4 days. The proportion of $CD34^+$ cells that expressed CXCR4 increased from 45% to 90% after 12 days of treatment, while the plasma level of SDF-1 increased from 1978 to 2010 pg/mL and the IFN-γ level increased from 9.04 to 9.89 pg/mL.

Oral fucoidan significantly increased the $CXCR4^+$ HPC population. The ability to mobilise HPC using sulphated polysaccharides and mobilise more cells with high levels of CXCR4 expression could be clinically valuable.

6.2 Introduction

Autologous transplants of HPC are used to treat a variety of neoplastic and other diseases. HPC can be mobilised from the BM niche into the PB via the administration of G-CSF or other agents such as AMD3100 or fucoidan. The mechanisms for the mobilisation may include modulation of serine proteases, metalloproteases, and of SDF-1/CXCR4 interactions (Katayama *et al.*, 2006).

The success of subsequent engraftment may be associated with the expression of the receptor CXCR4 on $CD34^+$ HPC. This effect can be observed both clinically (Spencer *et al.*, 2001) and experimentally (Brenner *et al.*, 2004; Lapidot *et al.*, 2005). Mobilisation of adequate numbers of HPC, including $CD34^+$ $CXCR4^+$ is not always successful. Failure to achieve sufficient mobilisation can occur for example in patients who have received multiple cycles of chemotherapy.

Previous research has shown that i.v fucoidan has a pronounced and extended mobilising effect on HPC in mice and on nonhuman primates (Sweeney *et al.*, 2000; Sweeney & Papayannopoulou, 2001; Frenette & Weiss, 2000; Sweeney *et al.*, 2005). This effect is postulated to be the result of dissociation of the chemokine SDF-1 from the BM stroma, creating an attractive gradient into the peripheral circulation. Disruption of the interaction between the CXCR4 and its ligand SDF-1 is one of the mobilising mechanisms in common with that of G-CSF (Petit *et al.*, 2002), and newer agents such as AMD3100 (Devine *et al.*, 2004; Broxmeyer *et al.*, 2005).

Fucoidan is a generic term for the sulphated, fucose rich polysaccharides derived from brown marine macro-algae (Berteau & Mulloy, 2003). In animal models, ingestion of marine macro-algae or fucoidan fractions has inhibitory effects on tumours, which appears to be associated with a rise in IFN-γ, IL-12, and stimulation of innate immunity (Funahashi *et al.*, 2001; Maruyama *et al.*, 2003; Mavier *et al.*, 2004) and in human (Irhimeh *et al.*, 2005b). *In vitro* treatment of bone marrow mononuclear cells with IFN-γ can up-regulate the expression of CXCR4 on granulocyte precursors and monocytes (Funahashi *et al.*, 2001).

Despite the evidence in the available literature that i.v fucoidan has an effect in tumour animal-models and in the mobilisation of HPC, there are no reports to date about the clinical use of oral or i.v fucoidan to modulate or mobilise human HPC. Although the fucoidans synergize with G-CSF to increase mobilisation 11 times over G-CSF alone in non-human primates, to date no clinical trials in patients have been reported. In this chapter, the effects of orally ingested *Undaria pinnatifida* fucoidan on the PB stem cells, the expression of CXCR4 and other receptors, and plasma levels of SDF-1, IL-12, and IFN-γ will be examined.

6.3 Volunteers, materials, and methods

6.3.1 Human volunteers

Informed consent and human ethics approval were obtained as described in section 2.1. Healthy human volunteers of either sex aged between 20-46 years (average 29) were chosen as described in sections 2.2 and 2.3. In a single blind, randomized, placebo-controlled clinical trial, thirty seven volunteers were divided into three groups.

The first group of volunteers (n = 6) took 3 g of guar gum as placebo. The second group (n = 6) took 3 g of whole *Undaria* containing 10% fucoidan w/w. The third group (n = 25) took 3 g of 75% fucoidan w/w daily for 12 days. The fucoidan capsules used in this study were prepared as described in section 2.5. Blood samples from the three groups were collected as described later.

6.3.2 Blood samples collection

Venous blood from the antecubital vein from the three groups of volunteers was collected using EDTA tubes as described in section 2.4. Blood samples collected for the IFN-γ and IL-12 studies were centrifuged at 1000 *xg* for 10 min at 4°C, and plasma fractions were collected and stored in aliquots at −80°C within 30 min of collection for later analysis. Blood samples collected for the SDF-1 study were centrifuged for 15 min at 1000 *xg* at 4°C, the separated plasma samples were centrifuged at 10,000 *xg* for 10 minutes at 4°C for complete platelet removal and stored in aliquots at −80°C within 30 min of collection for later analysis.

6.3.3 Blood parameters

Full blood counts were obtained as described in section 3.3.2.1 Venous blood samples were collected after overnight fasting for monitoring of blood glucose level during the study, using the glucose oxidase colorimetric method as described in section 3.3.1.7.

6.3.4 Flow cytometry analysis

The expression of different surface membrane markers on normal human PB HPC was evaluated by FACS direct immunofluorecence. MoAb raised against CD 3, 4, 8, 16, 19, 34, 45 and CXCR4 were used in staining.

Briefly, freshly collected, less than 2 h, EDTA whole blood was washed once with PBS-D (Oxoid Ltd., Hampshire, England) supplemented with 0.5% BSA and 0.1% NaN_3 (Sigma-Aldrich Co., Louis, MO USA) and centrifuged at 115 *xg* for 15 min for platelet depletion. The cells were washed twice using the same buffer by centrifugation at 300 *xg* for 5 min to remove the contaminating serum components.

Before staining with antibodies the cells were Fc-blocked with 1 µg of human IgG/10^5 cells (Zymed Laboratories Inc., South San Francisco, CA USA) for 15 min at room temperature, and then the cells were stained with the designated MoAb, using 10 µL of each antibody with 100 µL of the Fc-blocked cell suspension.

FITC-conjugated mouse monoclonal anti-human CD4, CD8, CD16, CD19 and CD34; Cy5-conjugated mouse monoclonal anti-human CD45 (Becton Dickinson, San Jose, CA USA); PerCP-conjugated mouse monoclonal anti-human CD3 (Becton Dickinson, San Joes, CA USA); and PE-conjugated mouse MoAb anti-human CXCR-4 (R&D Systems Inc., MN USA) were used in the study. The cells were then incubated for 40 min at 4°C with the antibodies.

A negative control tube was prepared identically but contained isotype controls IgG_1, or IgG_{2a} antibodies (Becton Dickinson, San Jose, CA USA) Table 6.1. The stained cells were washed with the buffer at 300 *xg* for 5 min, the erythrocytes were lysed in FACS lysing solution (Becton Dickinson, San Jose, CA USA) and the remaining cells were washed once in the buffer. All cells were fixed using FACS-Fix for later analyses using a FACScan flow cytometer and the Cell Quest software package

(Becton Dickinson, San Jose, CA USA) or Cytomation Summit V3.1 (Cytomation Inc. CO USA).

Table 6.1: Monoclonal antibody panels.

Tube number	Description	FL1	FL2	FL3
1	Early BM progenitor, mobilisation and homing subset	CD34- FITC	CXCR4 IgG_{2a} PE	CD45 Cy5
1c	Control for tube 1	CD34-FITC	IgG_1-PE	CD45-Cy5
2	T-cell, myeloid	CD16- FITC	CXCR4 IgG_{2a}-PE	CD3-PerCP
2c	Control for tube 2	CD16-FITC	Mouse IgG_{2a}-PE	CD3-PerCP
3	T-cell, T-suppressor	CD8-FITC	CXCR4 IgG_{2a}-PE	CD3-PerCP
3c	Control for tube 3	CD8-FITC	Mouse IgG_{2a}-PE	CD3-PerCP
4	T-cell, T-helper	CD4-FITC	CXCR4 IgG_{2a}-PE	CD3-PerCP
4c	Control for tube 4	CD4-FITC	Mouse IgG_{2a}-PE	CD3-PerCP
5	B-cell	CD19-FITC	CXCR4 IgG_{2a}-PE	----
5c	Control for tube 5	CD19-FITC	Mouse IgG_{2a}-PE	----

6.3.5 Preparation of mononuclear cells and CFU-GM assays

Human PB mononuclear cells were isolated from healthy subjects using a Histopaque-1077 kit and protocol from Sigma-Aldrich Co. St Louis, MO USA. Briefly, 3 mL of EDTA blood was layered onto the Histopaque-1077 and centrifuged at 400 *xg* for 30 min at ambient temperature. The upper layer was aspirated and the opaque interface transferred to a new centrifuge tube, mixed with 10 mL isotonic PBS and centrifuged at 250 *xg* for 10 min. The cell pellet was washed with 5 mL PBS twice and resuspended in 0.3 mL PBS. PBMC were plated at densities of $1x10^5$ viable nucleated cells per 35 mm plate in growth medium MethoCult GFH4534, (StemCell Technologies, Vancouver, BC Canada) in preparation for the colony forming units-granulocyte macrophage assay (CFU-GM).

Petri dishes were incubated for fourteen days at 37°C in a humidified atmosphere containing 5% CO_2. CFU-GM colonies defined as clusters of≥ 30 cells were counted using an inverted microscope and recorded as the mean of quadruplicate counts. Plates were discounted if contaminated or if over 50 colonies were present, since such high levels have an inhibitory effect on CFU-GM. Calculations were made to determine the CFU-GM /µL whole blood.

6.3.6 Cell viability

Viable nucleated cells were enumerated using a haemocytometer and the exclusion dye trypan blue. Equal volumes of both cell suspension and trypan blue dye (0.4%) were mixed in a dilution tray and allowed to stand for 5 min at room temperature. The viable and non-viable cells were counted in a haemocytometer using a light microscope (Olympus BX50F, Olympus Optical Co. Ltd., Japan).

6.3.7 Plasma cytokines and cytokine assays

PB was collected from volunteers according to the designated schedule in tubes containing EDTA. Platelet poor plasma (PPP) was prepared within 30 min of blood collection and stored at−80°C for later analysis. Three diffe rent cytokines, SDF-1, IFN-γ and IL-12 levels were analysed directly after thawing plasma samples on the day of testing by ELISA, using kits and protocols from R&D Systems Inc., MN USA.

6.3.7.1 Human SDF-1α immunoassay protocol

I. Principle of the assay

This assay employs a quantitative direct ELISA. A MoAb specific for SDF-1α has been pre-coated onto a micro-plate. Standards and samples are pipetted into the wells and any SDF-1α present is bound by the immobilised Ab. After washing away any unbound substances, an enzyme-linked PoAb specific for SDF-1α is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and colour develops in proportion to the amount of SDF-1α bound in the initial step. The colour development is stopped and the intensity of the colour measured.

II. Plasma samples collection and storage

EDTA plasma samples were collected on ice and centrifuged for 15 min at 1000 *xg* within 30 min of collection. An additional centrifugation step at 10,000 *xg* for 10 min at 2-8°C was introduced to remove any platelets from the plasma. Samples were then aliquotted and stored at −80°C for later analysis.

III. Reagent preparation

All reagents were brought to room temperature before use. The wash buffer concentrate was mixed to dissolve any formed crystal and 20 mL diluted with MilliQ water to prepare 500 mL of wash buffer. The substrate solution from the colour reagents A and B were mixed together in equal volumes within 15 min of use and stored away from light.

IV. SDF-1α standard

The SDF-1α standard was reconstituted with 1 mL of MilliQ water to produce a stock solution of 100,000 pg/mL. It was then mixed and allowed to sit for a minimum of 30 min with gentle agitation prior to making dilutions. Serial dilution tubes were prepared and labelled. A 900 µL of calibrator diluent (RD6Q) was added into the 10,000 pg/mL tube and 500 µL of calibrator diluent was added into the remaining tubes. Serial doubling dilutions allowed preparations of standards from 0 to 10,000 pg/mL of SDF-1α.

V. Assay procedure

All reagents and samples were brought to room temperature before use and samples and standards assayed in duplicate. A 100 µL assay diluent (RD1-55) was added to each well of the microplate. A 100 µL of standard or sample was added per well and then covered with an adhesive strip, followed by an incubation for 2 h at room temperature on a microplate shaker (0.12” orbit) set at 500 ± 50 rpm. Each well was aspirated and washed four times with 400 µL of wash buffer using a multi-channel pipette. After the last wash, any remaining wash buffer was removed by decanting and blotting against paper towels, then air-drying. Two hundred µL of SDF-1α conjugate was added to each well; covered with a new adhesive strip and incubated for 2 h at room temperature on the shaker followed by washing as above. Two hundred µL of substrate solution was added to each well and incubated for 30 min at room

temperature in the dark. The reaction was stopped by adding 50 µL of stop solution to each well and mixed to ensure colour uniformity through out the plate. The optical density was determined within 30 min, using a microplate reader set at 450/570 nm (MR 5000 Micro-plate Reader, Dynatech Laboratories Inc., VA USA).

VI. Calculation of results

The duplicate readings for each standard, control, and sample were averaged and subtracted from the average zero standard optical density. A standard curve [Figure 6.1] was plotted using the optical density for the standards versus the concentration of the standards and the SDF-1α concentration of each sample was found.

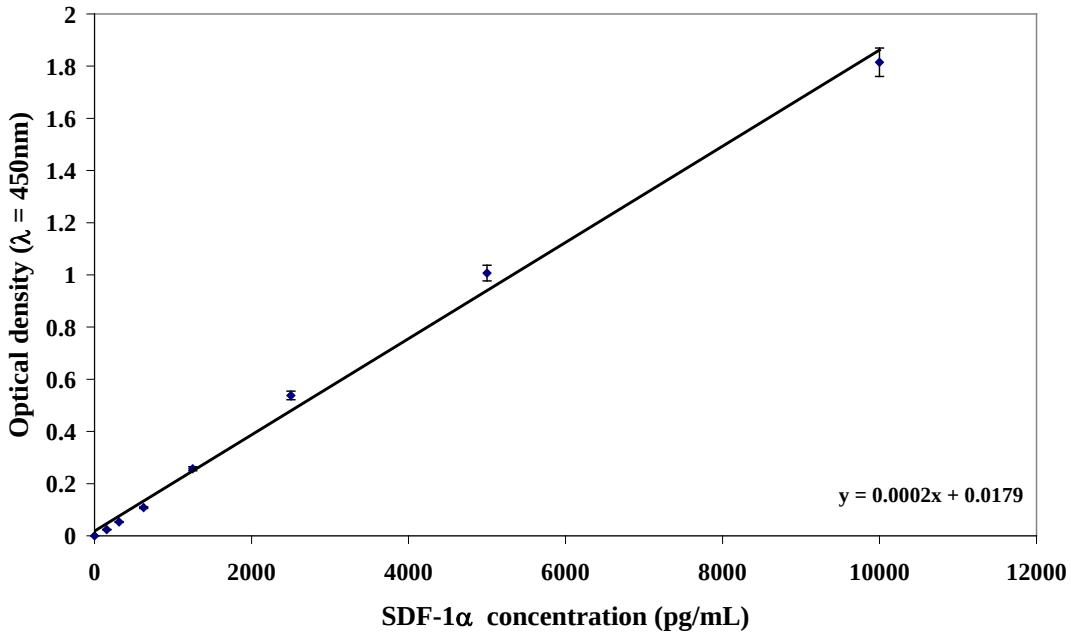


Figure 6.1: SDF-1 standard linear regression curve. Each point represents the mean for two different duplicate experiments ± SD.

6.3.7.2 Human IFN-γ immunoassay protocol

I. Principle of the assay

This assay employs a quantitative direct ELISA. A PoAb specific for IFN-γ has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any IFN-γ present is bound by the immobilised Ab. After washing away any unbound substances, an enzyme-linked PoAb specific for IFN-γ is added to the wells.

Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and the colour develops in proportion to the amount of IFN-γ bound in the initial step. The colour development is stopped and the intensity of the colour measured.

II. Plasma samples collection and storage

EDTA plasma samples were centrifuged at 1000 *xg* within 30 min of collection. The samples were aliquotted and stored at −80°C for later analysis.

III. Reagent preparation

Reagents were brought to room temperature, and the wash buffer concentrate mixed and diluted with MilliQ water to prepare 500 mL of wash buffer. The substrate solution was prepared by mixing colour reagents A and B in equal volumes within 15 min of use and away from light.

IV. IFN-γ standard

The IFN-γ standard was reconstituted with the calibrator diluent (RD6-21) producing a stock solution of 1000 pg/mL. It was mixed and allowed to stand for a minimum of 30 min with gentle agitation prior to making dilutions. Serial doubling dilution tubes were prepared to give a range of values between 0 and 1,000 pg/mL of IFN-γ.

V. Assay procedure

All reagents and samples were brought to room temperature before use and samples and standards were assayed in duplicate. One hundred µL of assay diluent (RD1-51) was added to each well followed by 100 µL of standard or sample per well. The wells were covered with an adhesive strip and incubated for 2 h at room temperature. Each well was aspirated and washed with four washes of 400 µL wash buffer using a multi-channel pipette. After the last wash the plate was inverted and blotted against paper towelling and air-dried.

Two hundred µL of IFN-γ conjugate was added to each well and covered with a new adhesive strip before incubation for 2 h at room temperature. The aspiration/washes were repeated. Two hundred µL of substrate solution was added to each well and

incubated for 30 min at room temperature in the dark. The reaction was stopped by adding 50 μL of stop solution to each well followed by mixing. The optical density was determined within 30 min, using a microplate reader set at 450/570 nm (MR 5000 Micro-plate Reader, Dynatech Laboratories Inc., VA USA).

VI. Calculation of results

The duplicate readings for each standard, control, and sample were averaged and subtracted from the average zero standard optical density. A standard curve (four parameter logistic curve-fit) [Figure 6.2] was plotted using the optical density for the standards versus the concentration of the standards and the IFN-γ concentration for each sample calculated.

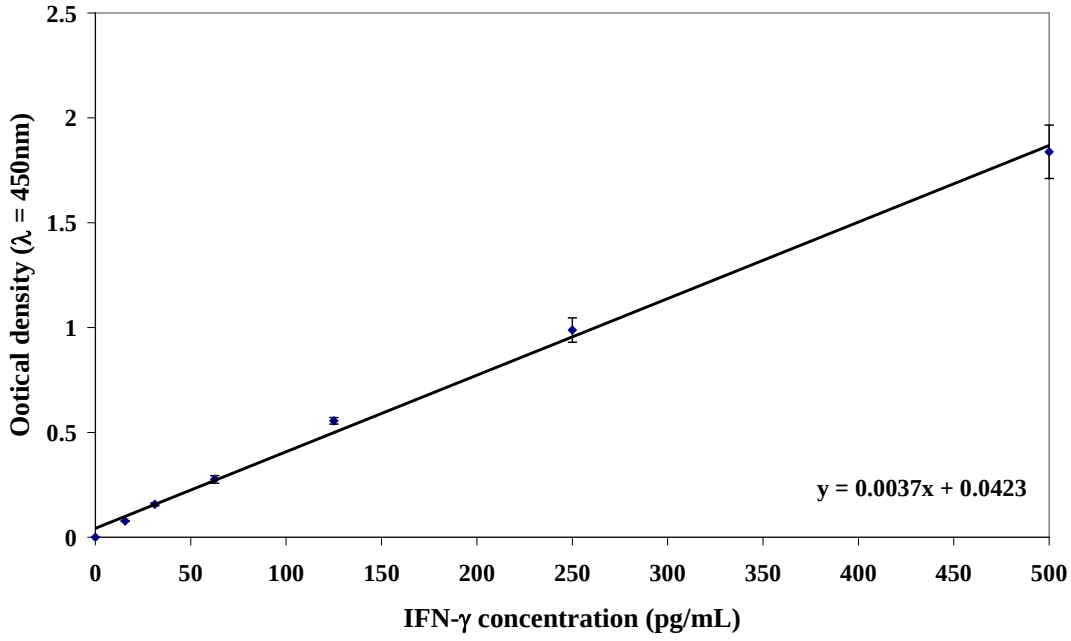


Figure 6.2: IFN-γ standard linear regression curve. Each point represents the mean for two different duplicate experiments ± SD.

6.3.7.3 Human IL-12 immunoassay protocol

I. Principle of the assay

This assay employs a quantitative direct ELISA. A MoAb specific for IL-12 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any IL-12 present is bound by the immobilised Ab. After washing away any unbound

substances, an enzyme-linked polyclonal Ab specific for IL-12 is added to the wells. Following a wash to remove any unbound Ab-enzyme reagent, a substrate solution is added to the wells and colour develops in proportion to the amount of IL-12 bound in the initial step. The colour development is stopped and the intensity of the colour is measured.

II. Plasma samples collection and storage

EDTA plasma samples were collected and centrifuged at 1000 *xg* within 30 min of collection, then aliquotted and stored at −80°C for later analysis.

III. Reagent preparation

All reagents were brought to room temperature before use. The wash buffer concentrate was mixed with MilliQ water to prepare 500 mL of wash buffer. The calibrator diluent concentrate (5X) (RD5C) was diluted with MilliQ water to yield 100 mL. The substrate solution was prepared by mixing colour reagents A and B together in equal volumes within 15 min of use and kept in the dark.

IV. IL-12 standard

The IL-12 standard was reconstituted with 5 mL of calibrator diluent (1X) to produce a stock solution of 500 pg/mL, which was allowed to sit for a minimum of 15 min with gentle agitation prior to making dilutions. Serial doubling dilutions were performed to give standards ranging from 0 pg/mL to 500 pg/mL IL-12.

V. Assay procedure

All reagents and samples were brought to room temperature before use and all samples and standards were assayed in duplicate. Fifty µL of assay diluent (RD1F) was added to each well. Two hundred µL of standard or sample were added per well. The plate was covered by an adhesive strip and incubated for 2 h at room temperature. Each well was aspirated and washed three times with 400 µL wash buffer using a multi-channel pipette. After the last wash the plate was inverted and blotted against paper towelling and air-dried. Two hundred µL of IL-12 conjugate was added to each well and covered with a new adhesive strip and incubated for 2 h at room temperature. The aspiration/wash was repeated as described above.

Two hundred µL of substrate solution was added to each well and incubated for 20 min at room temperature in the dark. Fifty µL of stop solution was added to each well and mixed. The optical density was determined within 30 min, using a microplate reader set at 450/570 nm (MR 5000 Micro-plate Reader, Dynatech Laboratories Inc., VA USA).

VI. Calculation of results

The duplicate readings for each standard, control, and sample were averaged and subtracted from the average zero standard optical density. A standard curve (four parameter logistic curve-fit) [Figure 6.3] was plotted using the optical density for the standards versus the concentration of the standards and the IFN-γ concentration of each sample was calculated.

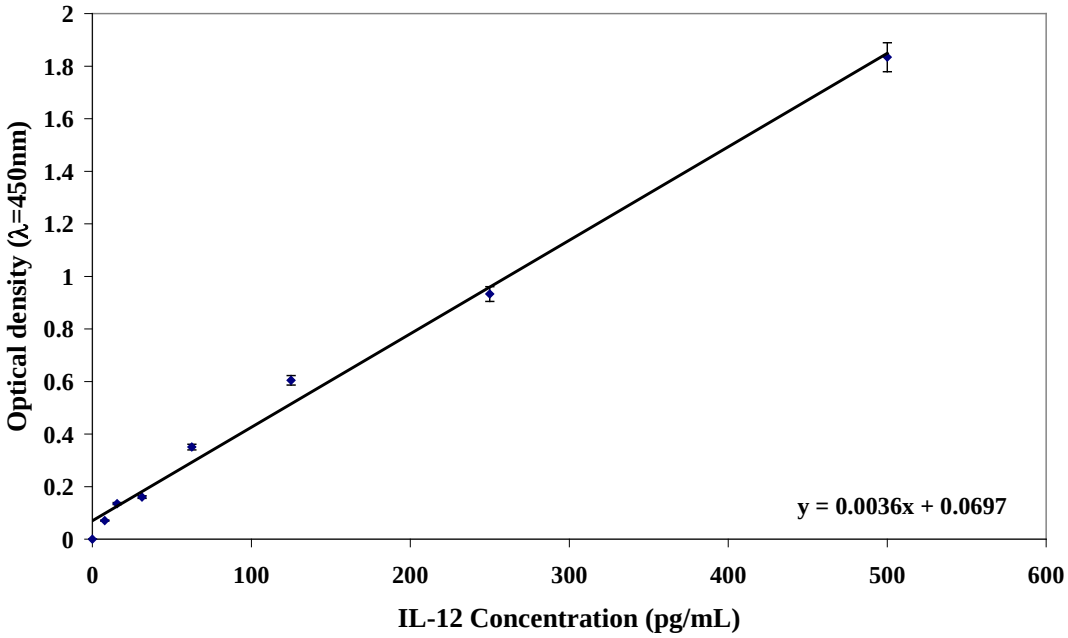


Figure 6.3: IL-12 standard linear regression curve. Each point represents the mean for two different duplicate experiments ± SD.

6.3.8 Statistical analysis

The Student's t-test and ANOVA were used to analyse data. A *p*-value of 0.05 was chosen as the limit of statistical significance. Duplicate readings for each sample were averaged. All statistical parameters were calculated using Microsoft Office, Excel and SPSS, v.12. Parametric data was represented by histograms while non-parametric data was represented by box plots.

6.4 Results

No side effects were reported and none of the volunteers exhibited evidence of toxicity when 3 grams of guar gum, whole *Undaria* containing 10% fucoidan w/w, or 75% fucoidan w/w extracts were taken orally every day for a period of 12 consecutive days.

6.4.1 Fucoidan ingestion caused mild leucopenia and lymphopenia but has no effect on neutrophils

A slight non-significant decrease in the total number of leucocytes in the PB was observed when whole *Undaria* containing 10% fucoidan w/w was ingested but when 75% fucoidan w/w was ingested the decrease was significant after 12 days. Results are summarized in Tables 6.2, 6.3 and 6.4. There was a decrease in the total number of leucocytes from 5.74 cells/nL at baseline to 5.37 after 12 days (p = 0.05). Of the leucocyte fractions, lymphocytes were most affected. Ingestion of either 10% fucoidan w/w or 75% fucoidan w/w decreased the lymphocyte count but the decrease was only significant when 75% fucoidan was ingested. The absolute number of lymphocytes decreased from 2.18 cells/nL at baseline to 1.98 after 12 days (p = 0.03) (Table 6.4). Neutrophil count was not affected after ingesting guar gum, 10% fucoidan, or 75% fucoidan.

Table 6.2: The average-readings of all of the tests at 4 time points; baseline and on the 4th, 8th and 12th day after ingesting 3 g tid of guar gum as a placebo-control. Zero (0) day represents the baseline readings.

	0 day ‡	**4 days**	**8 days**	**12 days**	
Test		**Average ± MSE (*p*-value)**			**n**
Leucocyte (cells/nL)	7.37 ± 0.23	7.38 ± 0.61 (0.87)	7.03 ± 0.87 (0.11)	7.22 ± 0.71 (0.38)	6
Lymphocyte (cells/nL)	2.80 ± 0.13	2.82 ± 0.16 (0.9)	2.65 ± 0.15 (0.14)	2.83 ± 0.18 (0.76)	6
Neutrophil (cells/nL)	3.63 ± 0.44	3.52 ± 0.38 (0.41)	3.45 ± 0.32 (0.45)	3.43 ± 0.26 (0.47)	6
SDF-1 (pg/mL)	1977 ±11	1978 ± 8 (0.95)	1981 ± 2 (0.71)	1974 ± 16 (0.91)	3
IFN-γ (pg/mL)	9.03 ± 0.18	9.14 ± 0.15 (0.97)	9.2 ± 0.15 (0.32)	9.17 ± 0.08 (0.9)	3

n = number of volunteers, different volunteers have been used for each treatment

‡ The mean value at 0 day (baseline) has been used in the t-test as the first set of data to which other groups are compared

Table 6.3: The average-readings of all of the tests at 4 time points; baseline and on the 4th, 8th and 12th day after ingesting 3 g tid of 10% fucoidan w/w. Zero (0) day represents the baseline readings.

	0 day ‡	4 days	8 days	12 days	
Test		**Average ± MSE (*p*-value)**			**n**
Leucocyte (cells/nL)	7.48 ± 0.35	6.98 ± 0.29 (0.05)*	7.00 ± 0.51 (0.18)	6.95 ± 0.59 (0.26)	6
Lymphocyte (cells/nL)	2.87 ± 0.12	2.73 ± 0.16 (0.06)	2.6 ± 0.30 (0.35)	2.62 ± 0.26 (0.22)	6
Neutrophil (cells/nL)	3.73 ± 0.28	3.42 ± 0.18 (0.10)	3.42 ± 0.24 (0.07)	3.35 ± 0.31 (0.12)	6
SDF-1 (pg/mL)	1973 ± 8	1977 ± 5 (0.77)	1980 ± 25 (0.80)	1979 ± 15 (0.65)	3
IFN-γ (pg/mL)	9.01 ± 0.13	9.01 ± 0.38 (0.85)	9.05 ± 0.19 (0.63)	8.97 ± 0.27 (0.77)	3

n = number of volunteers, different volunteers have been used for each treatment

* $p < 0.05$ using paired Students' t-test

‡ The mean value at 0 day (baseline) has been used in the t-test as the first set of data to which other groups are compared

Table 6.4: The average readings of all of the tests at 4 time points; baseline and on the 4th, 8th and 12th day after ingesting 3 g tid of 75% fucoidan w/w. Zero (0) day represents the baseline readings.

	0 day ‡	4 days	8 days	12 days	
Test		**Average ± MSE (*p*-value)**			**n**
Leucocyte (cells/nL)	5.74 ± 0.28	5.62 ± 0.33 (0.41)	5.48 ± 0.30 (0.15)	5.37 ± 0.37 (0.05)*	25
Lymphocyte (cells/nL)	2.18 ± 0.14	2.06 ± 0.13 (0.06)	1.95 ± 0.14 (<0.01)†	1.98 ± 0.14 (0.03)*	25
Neutrophil (cells/nL)	2.94 ± 0.19	2.97 ± 0.22 (0.80)	2.90 ± 0.19 (0.81)	2.82 ± 0.22 (0.33)	25
SDF-1 (pg/mL)	1978 ± 26	1996 ± 31 (0.27)	2101 ± 33 (0.00005)†	2059 ± 47 (0.079)	23
IFN-γ (pg/mL)	9.04 ± 0.42	9.41 ± 0.49 (0.13)	9.89 ± 0.39 (<0.01)†	9.82 ± 0.57 (0.04)*	20

n = number of volunteers, different volunteers have been used for each treatment

* $p < 0.05$ using paired Students' t-test

† $p < 0.01$ using paired Students' t-test

‡ The mean value at 0 day (baseline) has been used in the t-test as first set of data to which other groups are compared

6.4.2 Increase in $CD34^+$ cell count in PB after fucoidan ingestion

A slight increase in the number of circulating HPC was observed after ingesting fucoidan. When moderate quantities (3 g/day) of 10% fucoidan w/w were ingested, a small non-significant increase in the total number of HPC ($CD34^+$) in PB was observed. This increase was from 1.07 to 1.29 cells/µL ($p = 0.06$, n = 6) after 12 days. However, when 75% fucoidan capsules were ingested daily, the number of $CD34^+$ cells increased significantly from 1.64 cells/µL to 1.84 cells/µL ($p = 0.05$) after 4 days of the treatment [Figure 6.4]. Some volunteers presented a large increase in the total number of $CD34^+$ cells on days 8 and 12. These are represented in [Figure 6.4] as outliers. They were not considered by the box plot graph, generated by the SPSS to calculate the median.

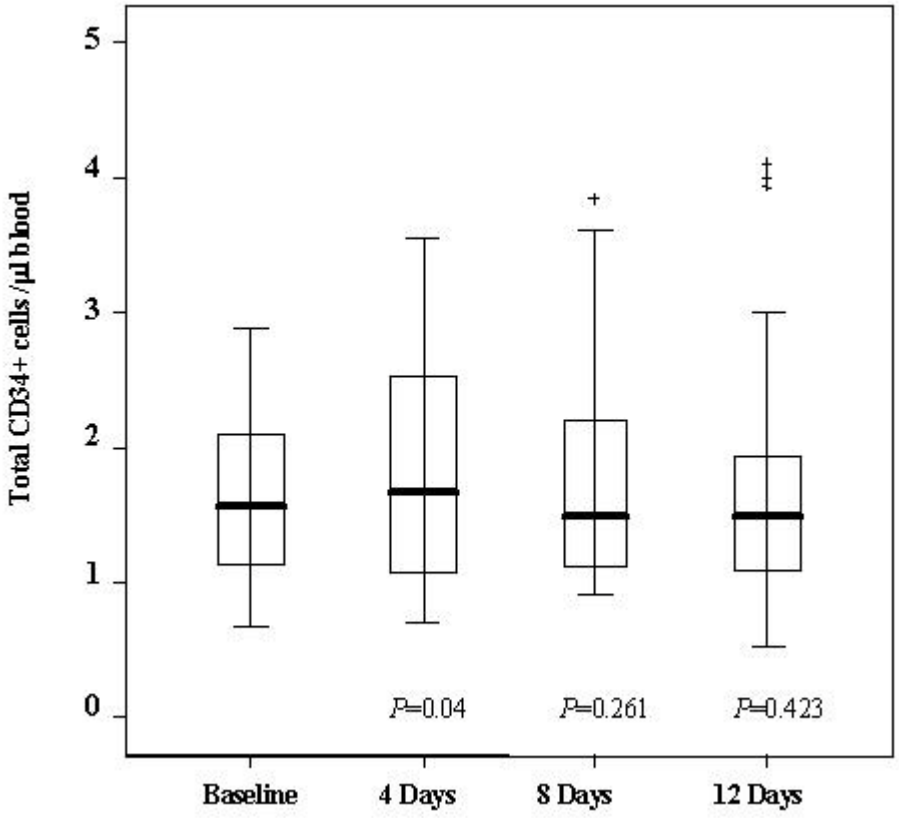


Figure 6.4: Total number of PB circulating CD34$^+$ cells at baseline and after 4, 8 and 12 days of taking 75% fucoidan. Black horizontal lines inside the boxes represent the medians for duplicates from 23 volunteers.

$^+$ Outliers or high responders.

6.4.3 Increase in the expression of CXCR4 on CD34$^+$ cells after fucoidan ingestion

When 3 g/day of 10% fucoidan was ingested, a small but non-significant increase in the total number of HPC that expressed CXCR4 surface receptor was observed. However, when 3 g/day of the 75% fucoidan was ingested, the number of CD34$^+$/CXCR4$^+$ cells increased significantly (p< 0.0002) from 0.75 cells/µL at baseline to 1.65 cells/µL after 12 days [Figure 6.5]. The flow cytometry study of HPC in one subject treated with 75% fucoidan is illustrated in [Figure 6.6]. The proportion of CD34$^+$ stem cells that expressed CXCR4 increased from 45% to 90% after 12 days of treatment (Table 6.5) [Figure 6.7]. A few volunteers showed a large increase in the

total number of $CD34^+$ cells. These are represented in [Figure 6.5] as outliers. They were not considered by the box plot graph, generated by the SPSS to calculate the median.

Table 6.5: The average-percentage of cells that are $CXCR4^+$ out of the total $CD34^+$ cells at 4 time points; baseline and on the 4^{th}, 8^{th} and 12^{th} day after ingesting 3 g tid of each treatment. Zero (0) day represents the baseline readings. Guar gum is used as a placebo-control.

	Average % of $CD34^+/CXCR4^+$ ± MSE (*p*-value)				
Treatment [§]	**0 day** [‡]	**4 days**	**8 days**	**12 days**	**n**
Guar gum	62.33 ± 4.64	64.15 ± 2.81 (0.685)	66.43 ± 3.18 (0.285)	63.66 ± 3.09 (0.805)	6
10% fucoidan	67.27 ± 8.33	65.49 ± 9.13 (0.63)	71.78 ± 9.01 (0.59)	71.96 ± 13.09 (0.4)	6
75% fucoidan	49.75 ± 7.19	84.57 ± 4.74 (<0.001)[†]	84.4 ± 5.57 (<0.001)[†]	91.15 ± 3.63 (<0.001)[†]	23

n = number of volunteers, different volunteers have been used for each treatment
[†] $p < 0.01$ using paired Students' t-test
[‡]The mean value at 0 day (baseline) has been used in the t-test as first set of data.
[§] Volunteers ingested 3g tid of each treatment daily

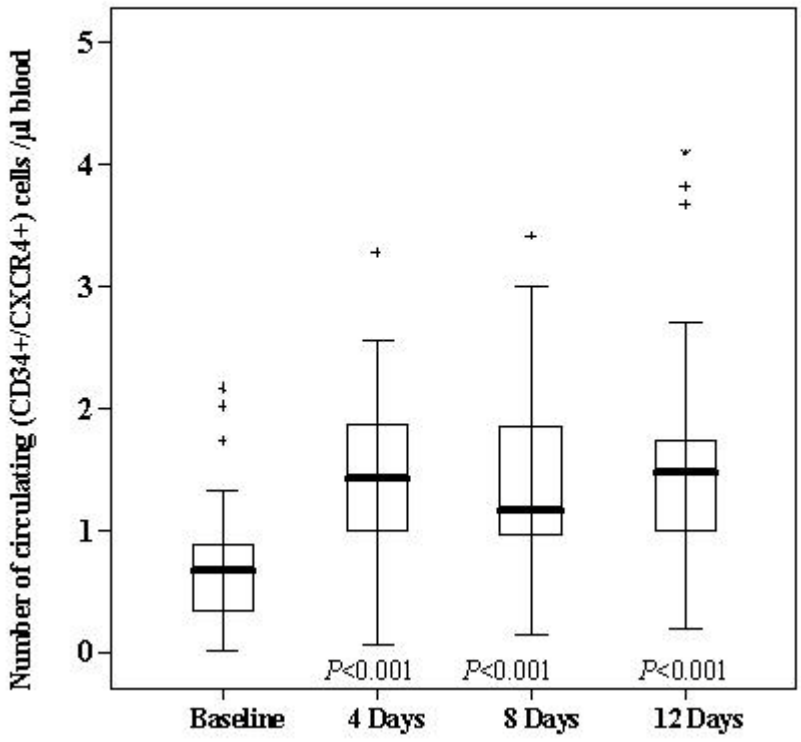


Figure 6.5: Total number of PB $CD34^+/CXCR4^+$ cells at baseline and after 4, 8 and 12 days of taking 75% fucoidan. Black horizontal lines inside the boxes represent the medians for duplicate tests of 23 volunteers.

$^+$ Outliers or high responders.

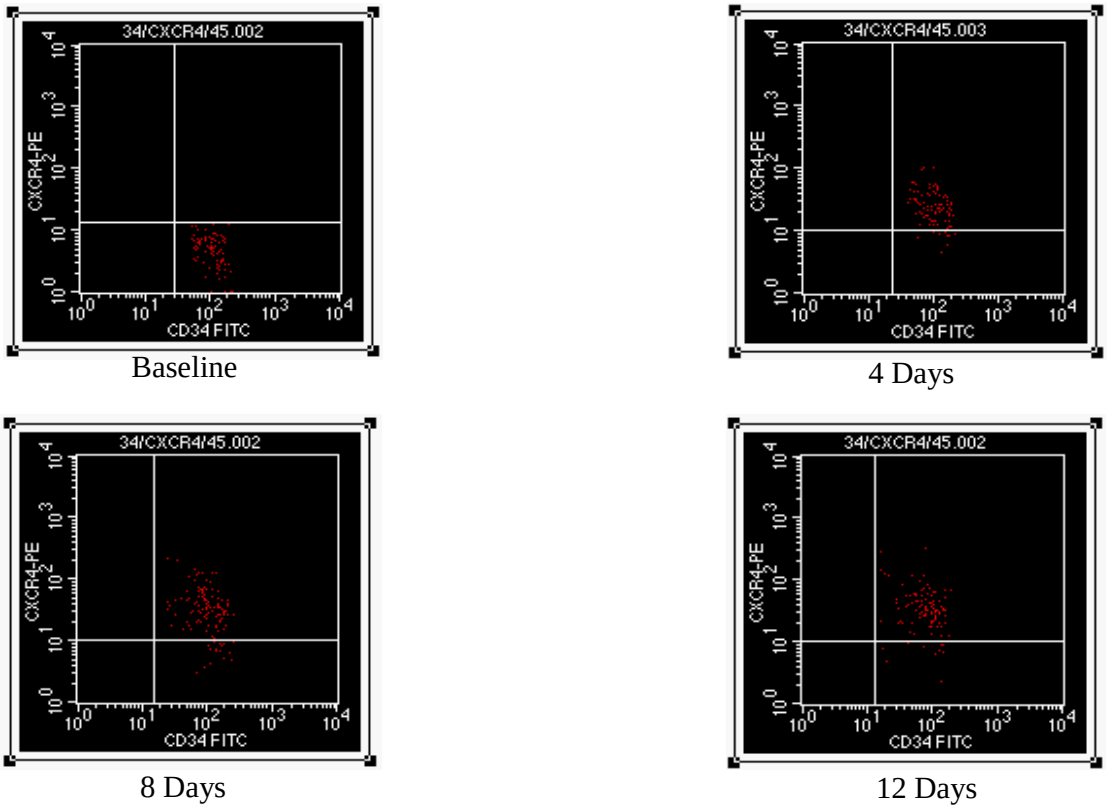


Figure 6.6: Flow cytometric histograms for one subject after ingesting 75% fucoidan, showing $CD34^+$ cells stained with CD34-FITC and CXCR4-PE. Baseline histogram, 100% of cells are $CD34^+/CXCR4^-$. Four Days histogram, 89% of cells are $CD34^+/CXCR4^+$. Eight Days histogram, 85% of cells are $CD34^+/CXCR4^+$. Twelve Days histogram, 91% of cells are $CD34^+/CXCR4^+$.

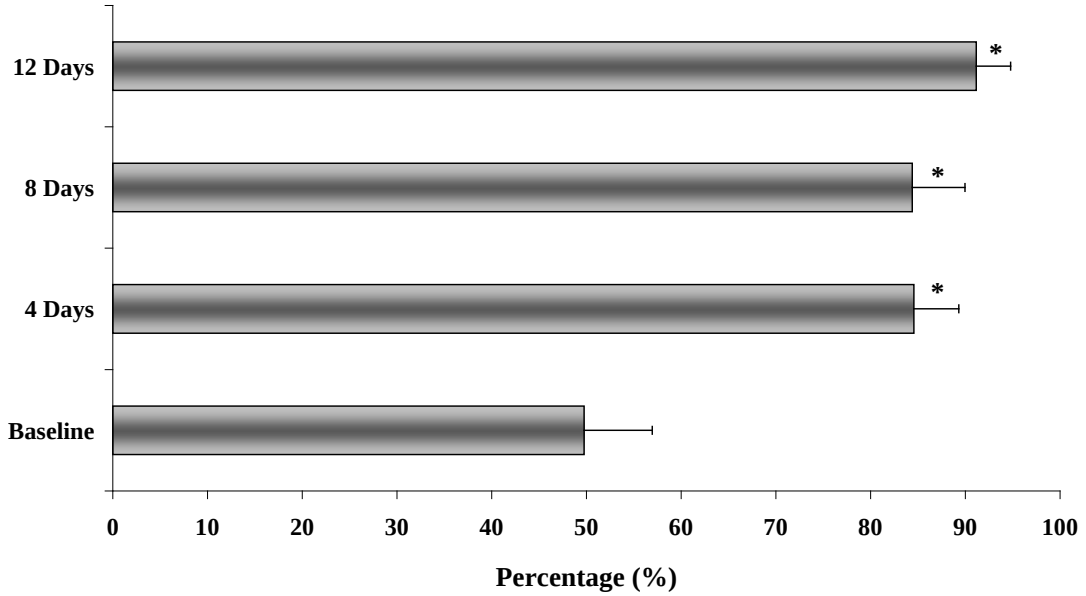


Figure 6.7: Percentage of total number of PB $CD34^+CXCR4^+$. Means are for duplicate tests + MSE on 23 volunteers who took 3 g tid of 75% fucoidan for 12 days. * *p*-value < 0.001 compared to baseline.

6.4.4 Fucoidan ingestion has no effect on PB mononuclear cells in CFU-GM

Generally, there was a non-significant decrease in the number of colony forming unit-granulocyte-macrophage (CFU-GM) per µL blood after ingesting the 75% fucoidan. The number of CFU-GM at baseline was 1.87/µL and decreased to 1.71, 1.40, and 1.31 after 4, 8 and 12 days (p = 0.50, 0.19 and 0.14) respectively [Figure 6.8A&B].

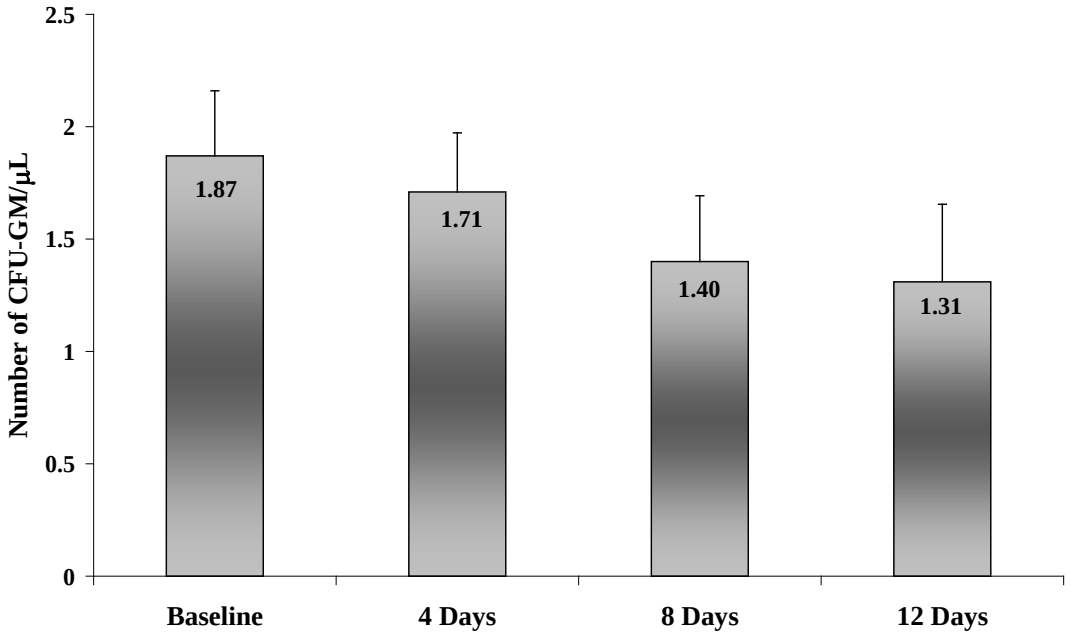


Figure 6.8A: Total number of CFU-GM per µL blood. Each point represents the mean for 13 different readings from 13 different volunteers + MSE.

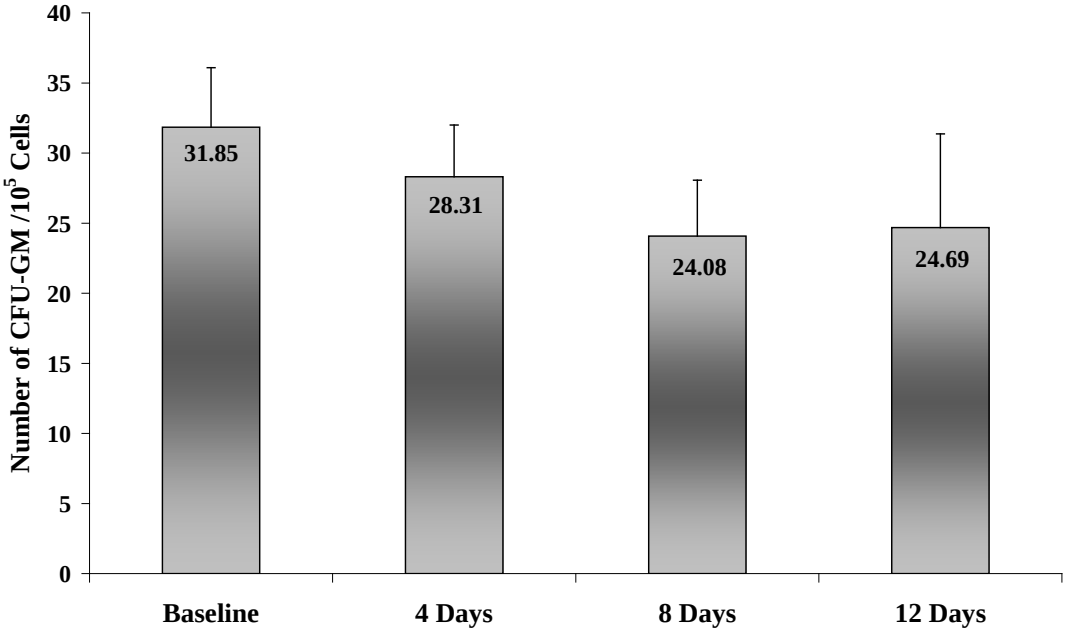


Figure 6.8B: Total number of CFU-GM per 10^5 blood cells. Each point represents the mean for 13 different readings from 13 different volunteers + MSE.

6.4.5 Fucoidan treatment and CD4 cell population

A significant decrease was observed in the total number of $CD4^+$ cells after 4, 8 and 12 days compared to the baseline. This decrease was from 0.978 to 0.886, 0.763, and 0.831 cells /μL, respectively [Figure 6.9A]. On the other hand, the percentage of CD4 cells that express CXCR4 decreased significantly from 0.615 at baseline to 0.53, 0.439, and 0.488 cells /μL at 4, 8, and 12 days respectively, whereas the percentage of $CD4^+CXCR4^-$ stayed relatively the same [Figure 6.9B&C].

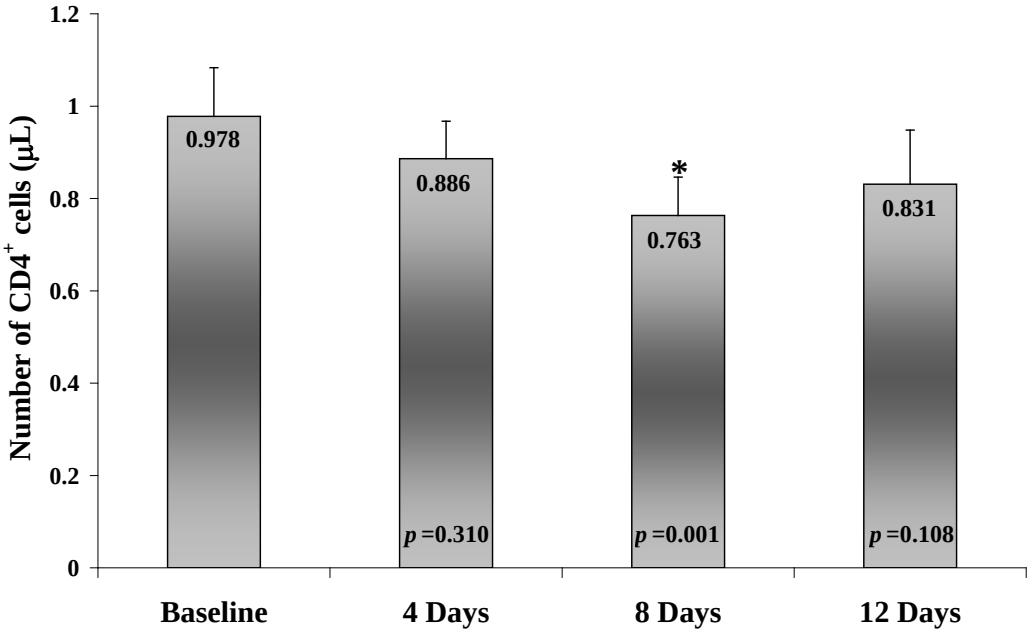


Figure 6.9A: $CD4^+$ lymphocyte counts before and after active 75% fucoidan treatment. Means are for duplicate tests on 23 volunteers + MSE.

* *p*-value <0.05 compared to baseline readings.

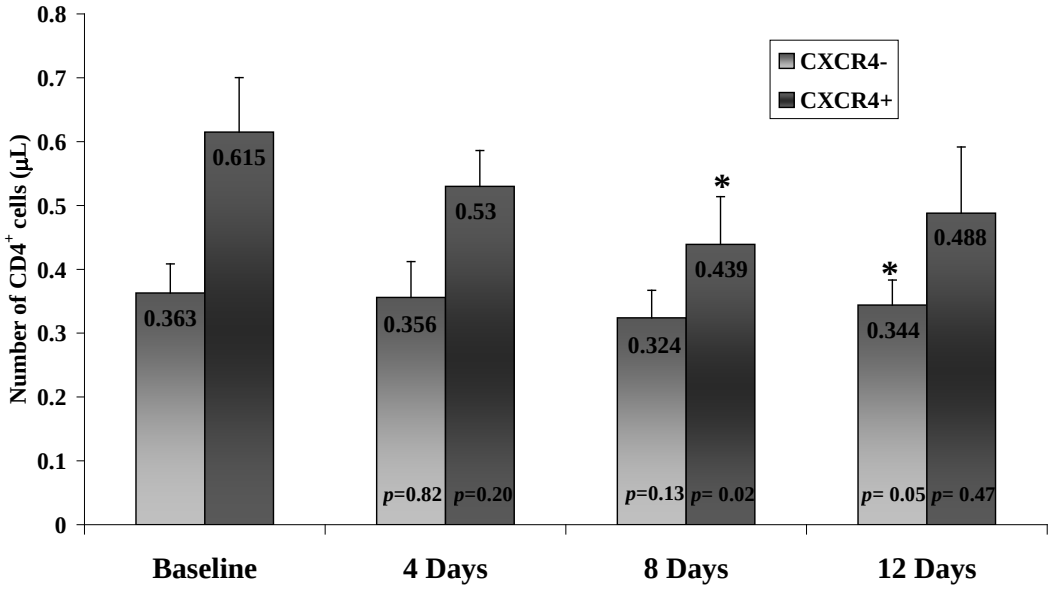


Figure 6.9B: $CD4^+CXCR4^-$ and $CD4^+CXCR4^+$ lymphocyte counts before and after active 75% fucoidan treatment. Means are for duplicate tests on 23 volunteers + MSE. * *p*-value <0.05 compared to baseline readings.

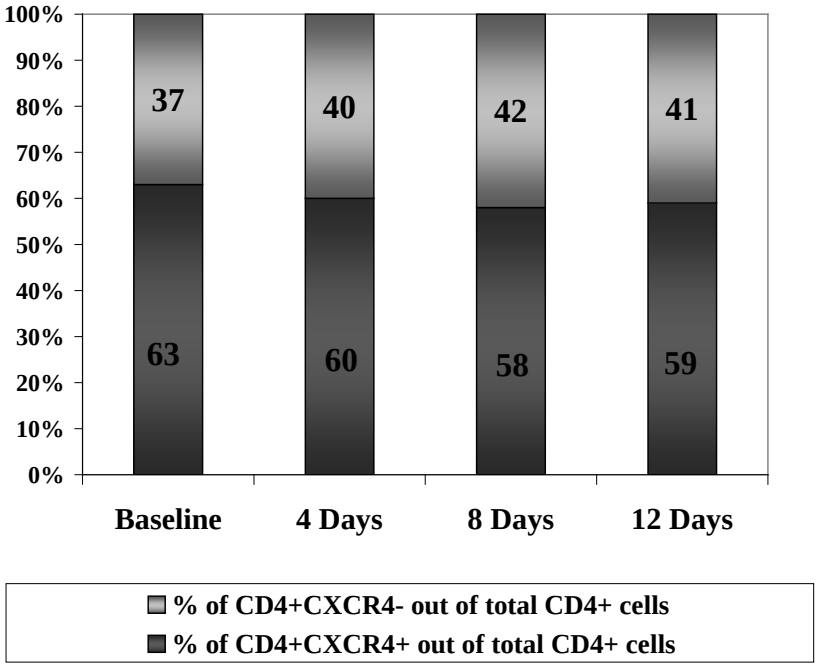


Figure 6.9C: Percentage of $CD4^+CXCR4^-$ and $CD4^+CXCR4^+$ before and after active 75% fucoidan treatment. Means are for duplicate tests on 23 volunteers + MSE. * *p*-value <0.05 compared to baseline readings.

6.4.6 Fucoidan treatment and the CD8 cell population

A small decrease was observed in the total number of CD8 cells after 4, 8 and 12 days of active 75% fucoidan treatment compared to baseline readings. This decrease was from 0.468 to 0.465, 0.414, and 0.419 cells /μL, respectively (Figure 6.10A). On the another hand, the percentage of CD8 cells that express CXCR4 decreased significantly from 0.24 at baseline to 0.207, 0.182, and 0.181 cells /μL at 4, 8, and 12 days respectively, when the percentage of $CD8^{+}CXCR4^{-}$ stayed relatively stable [Figure 6.10B&C].

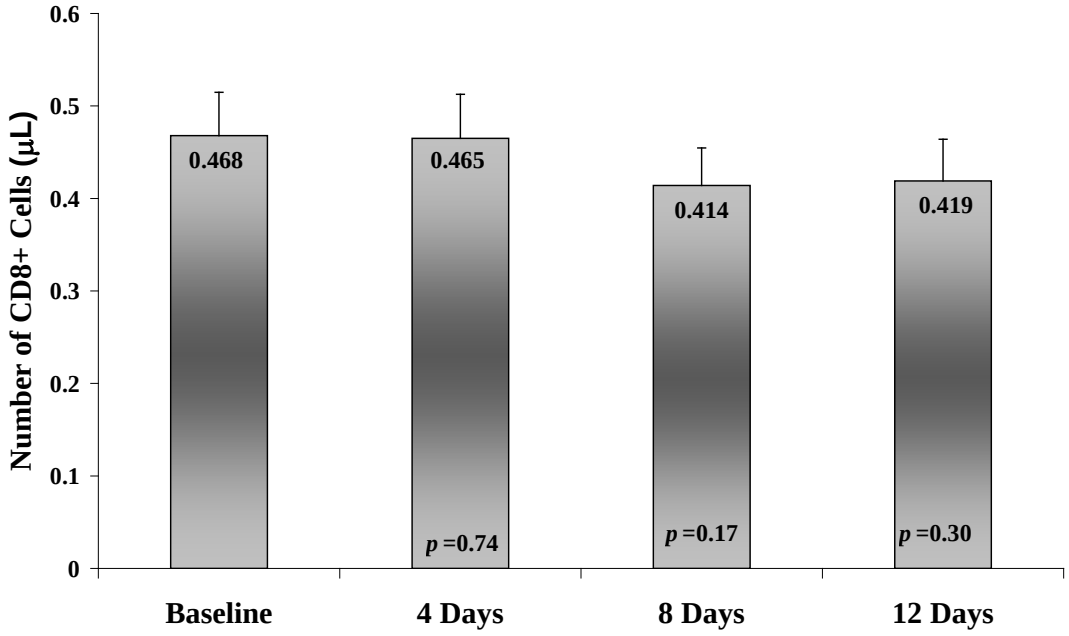


Figure 6.10A: $CD8^{+}$ lymphocyte counts before and after active 75% fucoidan treatment. Means are for duplicate tests on 23 volunteers + MSE.

* *p*-value <0.05 compared to baseline readings.

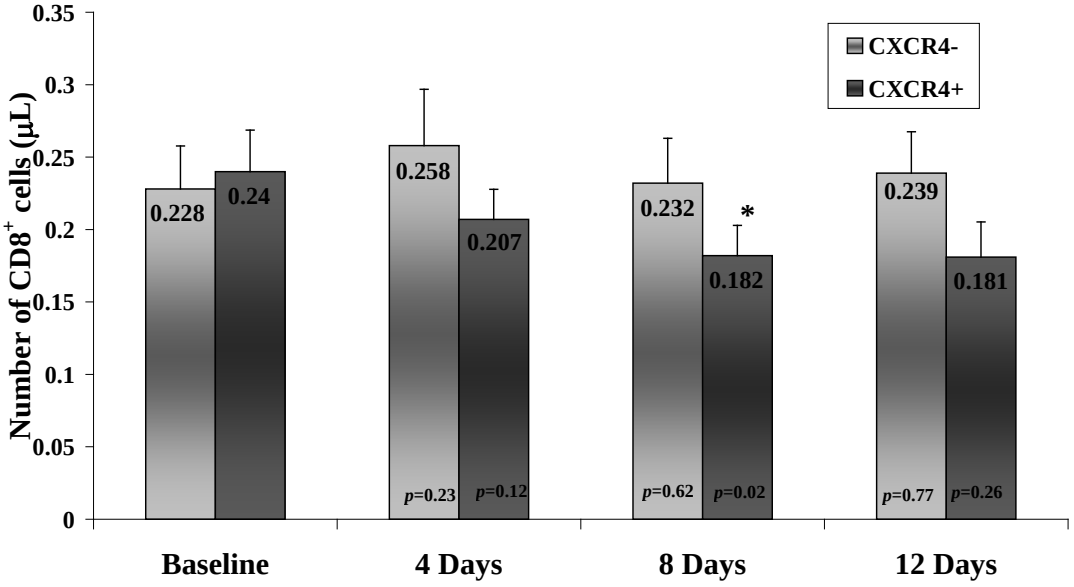


Figure 6.10B: $CD8^+CXCR4^-$ and $CD8^+CXCR4^+$ lymphocyte counts before and after active 75% fucoidan treatment. Means are for duplicate tests on 23 volunteers + MSE. * *p*-value <0.05 compared to baseline readings.

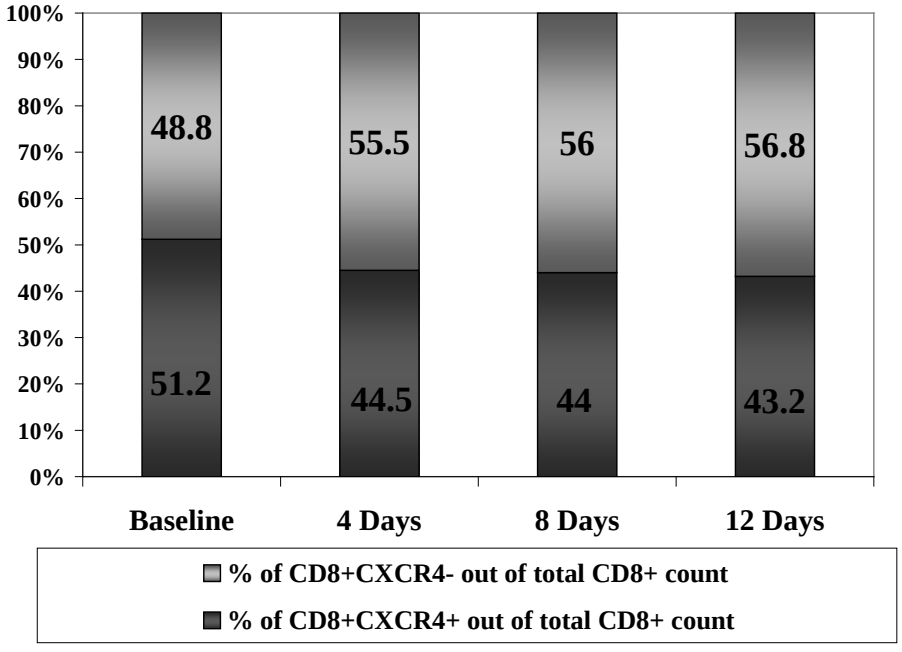


Figure 6.10C: Percentage of $CD8^+CXCR4^-$ and $CD8^+CXCR4^+$ before and after active 75% fucoidan treatment. Means are for duplicate tests on 23 volunteers. * *p*-value <0.05 compared to baseline readings.

6.4.7 Fucoidan treatment and CD16 and CD19 cell populations

A small decrease was observed in the total number of CD16 cells after 4 days of ingestion of the 75% fucoidan compared to baseline reading. This decrease was from 0.25 to 0.20 cells /µL. The $CD16^+$ population increased back up to the baseline reading (0.25 cells /µL) during further ingestion of the fucoidan capsules [Figure 6.11]. The B-cell population encountered a significant decrease (p = 0.04) in the $CD19^+$ cell population (0.18 cells/µL) when compared to the baseline readings (0.21 cells /µL) after 4 days of ingestion of the 75% fucoidan. Again, as with the $CD16^+$ population, the $CD19^+$ cells started to increase (0.19 cells /µL after 8 days) and returned to the baseline readings [Figure 6.12].

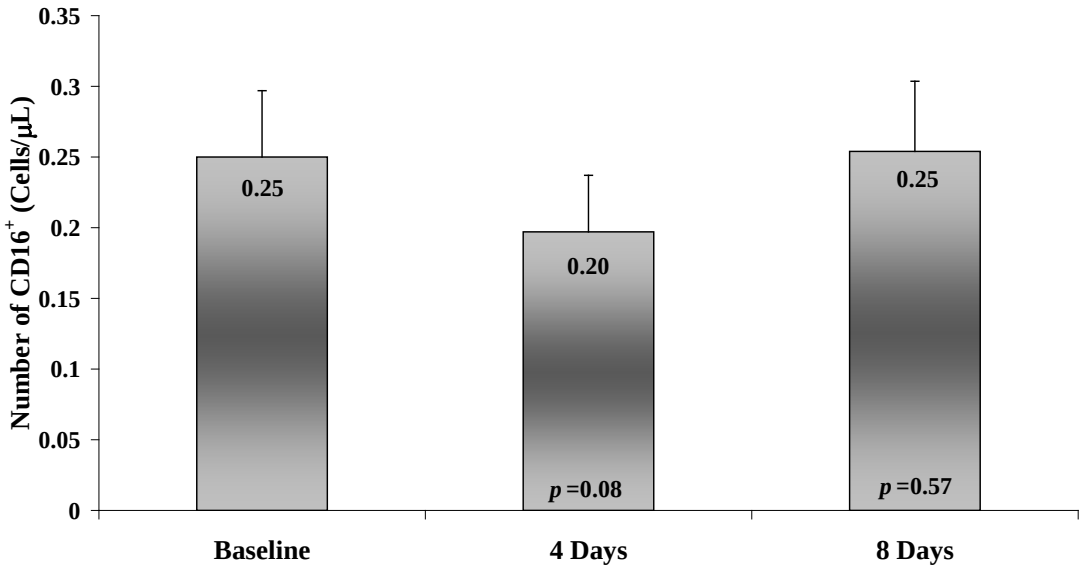


Figure 6.11: $CD16^+$ myeloid cell count before and after active 75% fucoidan treatment. Each point represents the mean for duplicate tests on 10 volunteers + MSE. * p-value <0.05 compared to baseline readings.

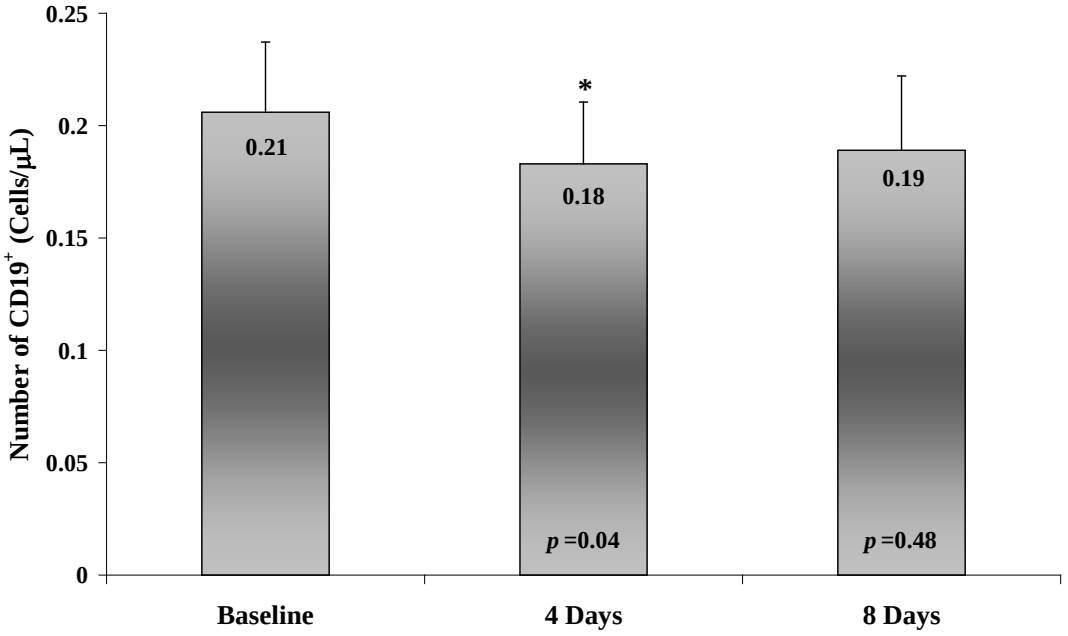


Figure 6.12: CD19$^+$ B-cell count before and after active 75% fucoidan treatment. Each point represents the mean for duplicate tests on 10 volunteers + MSE.* *p*-value <0.05 compared to baseline readings.

6.4.8 Increase in plasma levels of SDF-1 and IFN-γ with fucoidan ingestion

Ingestion of guar gum and 10% fucoidan did not affect the levels of SDF-1 and IFN- γ in plasma (Tables 6.2 and 6.3). Volunteers who ingested 3 g of the 75% fucoidan had an elevation in the serum level of SDF-1 after 8 and 12 days from 1978.5 pg/mL to 2101 pg/mL (p = 0.045) and 2059 (p = 0.05), respectively [Figure 6.13]. The plasma level of IFN-γ was assayed because of its association with the up-regulation of CXCR4. Volunteers who ingested 3 g of the 75% fucoidan showed a significant elevation (p = 0.04) in the serum IFN-γ level from 9.04 pg/mL to 9.82 pg/mL after 12 days (Table 6.4) [Figure 6.14]. However, there was no detectable change in the IL-12 plasma level, when all readings remained around 0 pg/mL, baseline level (n = 20).

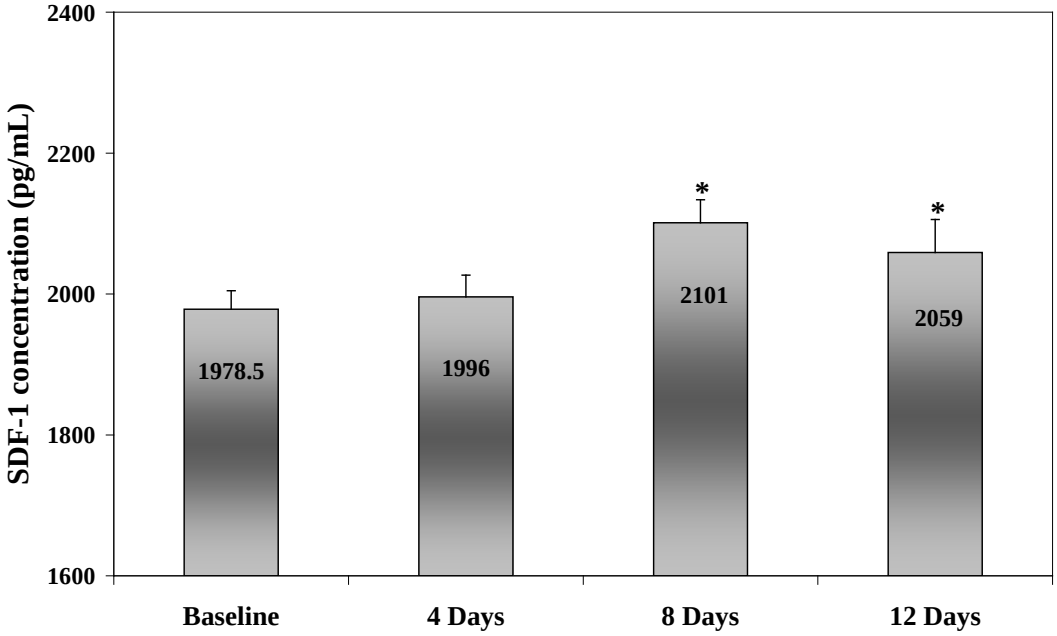


Figure 6.13: SDF-1 plasma level before and after ingesting 3 g of 75% fucoidan for 12 days. Each point represents the mean for 20 different volunteers + MSE. * *p*-value <0.05 compared to baseline readings.

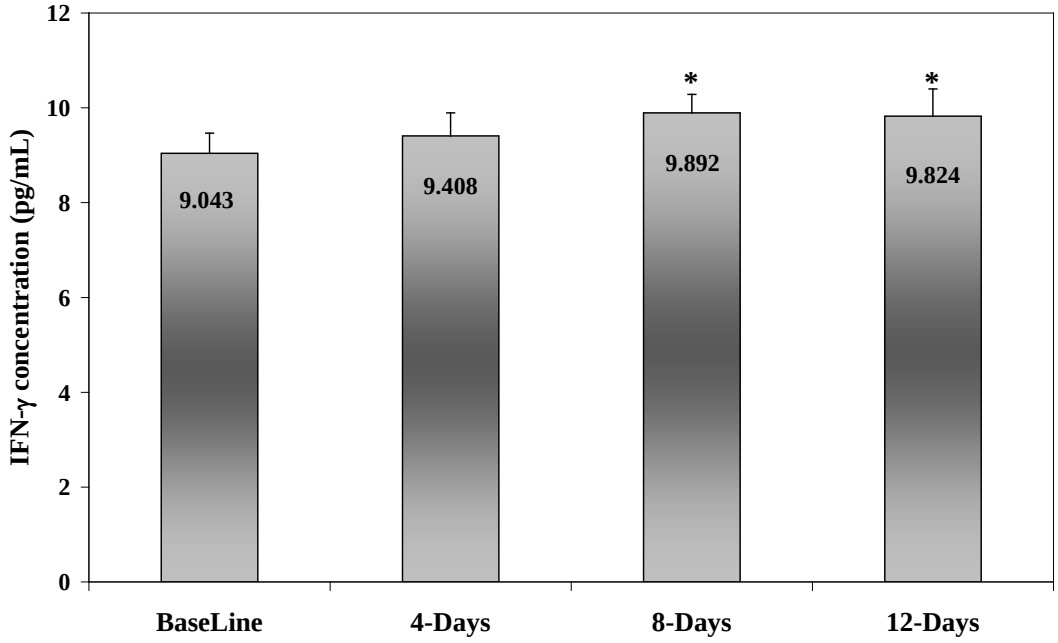


Figure 6.14: IFN-γ plasma level before and after ingesting 3 g of 75% fucoidan for 12 days. Each point represents the mean for 20 different volunteers + MSE. * *p*-value < 0.05 compared to baseline readings.

6.5 Discussion

This clinical study was carried out to determine the effects of ingested *Undaria* derived fucoidan on PB. In this study there was a small increase in total numbers of $CD34^+$ cells, and a profound increase, from 40% to 90% in the proportion of $CD34^+$ cells that expressed CXCR4 when 75% *Undaria* fucoidan was ingested. A smaller increase was noted when *Undaria* containing 10% w/w fucoidan was ingested. At the same time a small but significant increase was observed in IFN-γ and SDF-1 in the 75% fucoidan group, but not in the control or 10% groups. No increase was observed but rather a non-significant decrease in CFU-GM despite the increase in the number of $CD34^+$ cells.

Previous studies have shown that i.v fucoidan produces rapid mobilisation of murine HPC with long-term BM repopulating potential in mice and nonhuman primates (Sweeney *et al.*, 2000; Frenette & Weiss, 2000; Sweeney & Papayannopoulou, 2001; Petit *et al.*, 2002). CXCR4 was not assayed in those studies. Fucoidan has also been used as a tool to examine the effects of binding SDF-1. Mavier and co-workers (Mavier *et al.*, 2004) demonstrated that after experimental hepatic destruction, intravenous fucoidan blocked the SDF-1 expression of liver stem cells and markedly decreased their accumulation. Studies have shown that CXCR4 is present on platelets and has a high affinity for SDF-1 (Kowalska *et al.*, 1999; Abi-Younes *et al.*, 2000). Therefore, to measure circulating levels of SDF-1, PPP should be collected. It should be noted that many protocols for plasma preparation, including procedures recommended by the National Committee for Clinical Laboratory Standards (NCCLS), result in incomplete removal of platelets from blood, which will cause variable and irreproducible results for assays of factors contained in platelets and released by platelet activation. In this study we have used a cold blood collection to minimise platelet activation and a protocol to prepare PPP.

Ingestion of fucoidan or seaweed preparations has been shown to inhibit tumours of various kinds (Funahashi *et al.*, 2001), an effect that may be attributable to a stimulation of the non-specific immune system (Maruyama *et al.*, 2003; Shimizu *et al.*, 2005). Fucoidans are well known experimental selectin blockers. *In vitro*, the binding of L-selectin on lymphocytes by fucoidan enhanced the expression of surface CXCR4 in lymphocytes (Ding *et al.*, 2003). Clinical use of a fucoidan preparation

dates from the 1960s and Claudio and Stendardo (1966) reported favourable results from patients with leucopenia and leucocytosis with general improvement in their condition.

'Fucoidan' is a generic term for a class of fucose-rich sulphated carbohydrate polymers found in brown macroalgae (Berteau & Mulloy, 2003) and echinoderms (Mulloy *et al.*, 1994). In the prior work using intravenous fucoidans to mobilise HPC two preparations have been used; a sulphated linear fucan isolated from the sea urchin *Lytechinus variegatus* (FucS) given at 100 mg/kg i.v (Sweeney *et al.*, 2000; Sweeney & Papayannopoulou, 2001) and a branched fucoidan fraction derived from *Ascophyllum nodosum* (obtained from Fluka) given at 25 mg/kg i.p (Sweeney *et al.*, 2002). Despite the differences in the type of fucoidan in those studies, similar mobilisations were observed and attributed to the creation of an SDF-1 gradient into the PB.

The fucoidan used in this study was derived from the edible brown alga *Undaria pinnatifida*. The antibody based detection method described in chapter four indicated that when 3 g of 75% *Undaria* fucoidan was ingested daily, plasma concentration was elevated up to 4 mg/L after 4 days, and 13 mg/L after 12 days despite the fact that fucoidan has a large molecular weight (Irhimeh *et al.*, 2005a). Acidic conditions in the stomach may cause a limited hydrolysis of the fucoidan. Humans do not produce enzymes capable of breaking down fucoidans, and the latter also appear to be unaffected by human faecal flora (Michel *et al.*, 1996). Hypothetically, small quantities of orally administered fucoidan may cross the intestinal wall as whole molecules probably by the process of endocytosis. These relatively low concentrations of fucoidan may have caused the observed changes in this study. The slight but significant changes in HPC noted in this study indicate potential for the development of fucoidans as therapeutic agents.

AMD3100, which is a reversible inhibitor of the binding of SDF-1α to its cognate receptor CXCR4, is currently in clinical trials as a mobilising agent. It significantly improves the mobilisation capacity of G-CSF when used in combination in mice (Cottler-Fox *et al.*, 2003). Fucoidan may have a similar action on the SDF-1/CXCR4

interaction. This could play a role in the mobilisation of $CD34^+$ cells from BM to PB especially if fucoidan were to be used intravenously.

The increase in CXCR4 expression on total CD34 cells was marked in this study, whilst the increase in the total number of $CD34^+$ cells was small. Previously, CXCR4 expression on HPC has been shown to increase after administration of G-CSF in both human and murine BM, reaching peak levels at the time of mobilisation (Petit *et al.*, 2002), although SDF-1 levels do not rise. Interestingly, in this present study, a non-significant decrease was observed in CFU-GM over 12 days in the 75% fucoidan group. This effect may perhaps be attributed to the rise in IFN-γ. Constitutive expression of low levels of IFN-γ by stromal cells has been noted to have a profound inhibitory effect on haemopoiesis (Selleri *et al.*, 1996).

CXCR4 plays an important and unique role in regulating the trafficking of normal HPC and their homing to and retention in the BM. The same axis also modulates several biological processes in more differentiated cells from the granulocyte-monocytic, erythroid, and megakaryocytic lineages (Majka & Ratajczak, 2006).

In this study, a small but significant decrease in the total number of leucocytes and lymphocytes was observed after 12 days of ingestion of 75% fucoidan, although this decrease was within the normal clinical range. This modest leucopenia may result from the decrease in committed HPC available to proliferate into mature leucocytes. In previous studies, there was a fall in circulating leucocytes immediately after G-CSF was given (Wright *et al.*, 1936; Bradford *et al.*, 1997; Abkowitz *et al.*, 2003).

The presence of a normal number of functional neutrophils is important for mobilisation of HPC. In this study, it was demonstrated that ingestion of 3 g of fucoidan has no effect on neutrophil count and does not cause neutropenia.

It was observed that there was an increase in the plasma level of IFN-γ, consistent with a previous study which showed an increase in HPC that was accompanied by an increase in the level of SDF-1, IFN-γ, and IL-12 (Sweeney & Papayannopoulou, 2001). IFN- γ is a multifunctional protein produced by T-lymphocytes and NK cells and is now known to be both an inhibitor of viral replication and a regulator of

numerous immunological functions. IL-12 is produced by macrophages and B lymphocytes, and has multiple effects on T-cells and NK cells. These include inducing the production of IFN- γ and TNF by resting and activated T- and NK cells. In its role as an initiator of cell-mediated immunity, it has been suggested that IL-12 has therapeutic potential as a mediator of cell-mediated immune responses to microbial pathogens, metastatic cancer and viral infections. However, the level of IL-12 in this study did not change. It was shown previously that in vitro treatment of BM mononuclear cells with IFN-γ can up-regulate the expression of CXCR4 on granulocyte precursors and monocytes (Lee *et al.*, 1999). This may, in part, reflect our observation of increased expression of CXCR4 on $CD34^+$ cells.

6.6 Conclusion

Findings from this chapter conclude that ingestion of fucoidan (75%) up-regulates the plasma level of IFN- γ and SDF-1 after 8-12 days with no apparent effect on IL-12. Fucoidan ingestion has an effect on CD4 and CD8 populations. Both subsets decreased significantly after 8 days of oral intake. At the same time $CD4^+CXCR4^+$ and $CD8^+CXCR4^+$ subpopulations decreased significantly. However, fucoidan ingestion appears to have no effect on CD16 and CD19 cells.

Oral administration of fucoidan increased the PB $CD34^+$ cells and significantly amplified the $CXCR4^+$ HPC population. The ability to mobilise HPC with high levels of CXCR4 expression could be clinically valuable, especially with an orally delivered, non-toxic substance. However, the engraftment success of fucoidan elicited CXCR4 expression has not yet been assessed. Future work in this area would focus on animal models of engraftment.

CHAPTER SEVEN

IN VITRO HAEMOPOIETIC CELL EXPANSION

Chapter objectives:

- Establish an accurate technique using beads to calculate the number of cells in culture using FACS.
- Study the toxicity of beads on haemopoietic cell cultures.
- Study the toxicity of different fucoidan preparations on haemopoietic cell cultures using KG1a cells.
- Examine the effects of different fucoidan preparations on *in vitro* human $CD34^+$ cells proliferation and differentiation.
- Determine the interaction between fucoidan and growth cytokines in the expansion system.

Chapter contents:

7.1 Summary

Sulphated glycans such as fucoidan have been shown to induce rapid HPC mobilisation in mice and primates. The results from the previous clinical study in chapter six has demonstrated up-regulation of CXCR4 on peripheral $CD34^+$ cells in volunteers who had received oral doses of 75% fucoidan w/w. The aims reported in this chapter were to establish the cytotoxicity level of fucoidan in human haemopoietic cell cultures, to investigate the *in vitro* effects of fucoidan on a cytokine-mediated $CD34^+$ cell expansion system and to characterize the fucoidan effects on $CD34^+$ proliferation and differentiation.

The effect of the different fucoidan extracts on cell viability was tested on the KG1a cell line and then on PB HSC and CB HSC. Fractional and full factorial design experiments were used to investigate the interaction between fucoidan fractions and cytokines. The output of the expansion system was analysed by flow cytometry.

At 100 ng/mL and at saturating levels of Flt3, SCF, TPO and G-CSF (1000 ng/mL), depyrogenated fucoidan (DP-GFS-001) at 10 µg/mL was observed to reduce the number of $CD34^+$ cells produced per input $CD34^+$ cell (1.09 ± 0.07 versus 0.84 ± 0.05, n = 16, $p = 0.002$) and have a negative effect on the percentage of $CD34^+$ cells that express CXCR4 (87.2 ± 1.8 versus 77.4 ± 2.3, n = 16, $p = 0.001$). There was neither significant effect of DP-GFS-001 on the total cellular output nor other cell subsets examined ($CD41a^+CD14^-$, $CD15^+$, $CD14^+$). PB and CB $CD34^+$ cells stained with CFSE and treated with fucoidan showed a significant decrease in the cell cycle time and a greater number of undifferentiated cells were found in the early divisions when compared to the control.

In conclusion, fucoidan down-regulates the expression of CXCR4 on $CD34^+$ cells *in vitro* and slows the cell cycle significantly. It may have value as an artificial BM-matrix. This may provide opportunities for the future improvement of *ex vivo* manipulated grafts. Hypothetically, fucoidan acts *in vitro* and *in vivo* via different mechanisms and the loss of acetyl groups changes the fucoidan mode of action.

7.2 Introduction

In the previous chapter orally ingested fucoidan was found to have an effect on the human haemopoietic system although its bio-availability was very low probably due to its large size. To increase the plasma levels of fucoidan, smaller molecular weight molecules have to be used, which could negatively affect the fucoidan bio-activities owing to the change in chemical structure. Another way for increasing systemic levels could be by using an i.v form, but this has yet to be established.

The study of the direct effect of the fucoidan on the haemopoietic system *in vitro* is therefore of great importance. In this chapter the *in vitro* effects of different fucoidan preparations which vary in chemical structure were tested on an *ex vivo* haemopoietic expansion system.

Ex vivo haemopoietic cell expansion using soluble cytokines has been proposed as a technology for production of various haemopoietic lineages in cell-based therapies. Neutropenia and thrombocytopenia are significant problems that limit the efficacy of cancer therapies. Large-scale production and administration of expanded mobilised PBSCs has been shown to abrogate post-myeloablative cytopenia (Prince *et al.*, 2004). In the future it may be possible to manufacture myeloid progenitors using HPC from alternate sources such as CB.

The use of *ex vivo* expanded haemopoietic stem cells promises to play a major role in marrow transplantation. It has a great potential to generate clinically relevant grafts from a limited number of stem cells. The expansion of certain cell populations using cytokines depends on many factors including cell maturity. The cells undergo extensive proliferation and differentiation in response to stimulation with different combinations of cytokines (Moore, 1991) and other compounds (*e.g.,* fucoidan). The choice of cytokines along with the cell type and media used influences the outcome of expansion strategies for the *ex vivo* culture of pluripotent stem cells and progenitors in stroma-free suspension cultures.

An understanding of the mechanisms regulating HPC engraftment is of great importance in the clinical use of cultured transplants. Functional HPC should have the ability to home to the BM microenvironment and rapidly repopulate the recipient with

both myeloid and lymphoid cells. This has been shown when human $CD34^+CD38^-$ or $CD34^+CD38^{-/low}$ cells engrafted the BM of sub-lethally irradiated immune-deficient mice with high levels of myeloid and lymphoid cells (Larochelle *et al.*, 1996; Kollet *et al.*, 2000) and where $CD34^+CD38^+$ or $CD34^-CD38^-$ cells were also found to have a limited repopulation potential (Conneally *et al.*, 1997; Bhatia *et al.*, 1998).

Intravenous injection of human BM or HUCB into NOD-SCID mice resulted in the engraftment of a small number of human cells capable of proliferating and differentiating in the murine BM. These cells were capable of producing long term culture-initiating cell (LTC-IC), colony forming cells (CFCs), immature $CD34^+Thy1^+$ and $CD34^+CD38^-$ cells, along with mature myeloid, erythroid and lymphoid cells (Larochelle *et al.*, 1996).

To potentially improve the efficacy of CB transplantation, attempts have been made to increase the available cell dose. One such approach is the transplantation of multiple cord blood units; another is the use of *ex vivo* expansion. Evidence for a functional and phenotypic heterogeneity exists within the HSC population and one concern associated with *ex vivo* expansion is that expansion of “lower quality” HPC occurs at the expense of “higher quality” HPC, thereby impacting upon the quality of the graft.

The current *ex vivo* expansion processes include the use of a system where $CD34^+$ or $CD133^+$ cells are selected and cultured in medium containing factors which target the proliferation and self-renewal of primitive haemopoietic progenitors. A second approach involves a co-culture expansion system where un-manipulated CB cells are cultured with stromal components of the haemopoietic microenvironment, specifically mesenchymal stem cells (MSC), in a medium containing GFs. A third approach uses a continuous perfusion where CB HPC are cultured with GFs in 'bioreactors' rather than in static cultures. Ultimately, the goal of *ex vivo* expansion is to increase the available dose of the CB cells responsible for successful engraftment, thereby reducing the time to engraftment and reducing the risk of graft failure (Robinson *et al.*, 2005).Therefore, the role of fucoidan as a large interface molecule interacting and binding to GFs in an expansion medium is of great interest and importance.

To home into the BM microenvironment from the blood circulation, HPC must first connect with the E and P selectins, which are expressed on the BM vascular endothelial cells (Page *et al.*, 1992; Schweitzer *et al.*, 1996; Frenette *et al.*, 1998). Human $CD34^+$ cells require surface-bound SDF-1 on human endothelial cells for the development of integrin-mediated adhesion to the vascular endothelium under physiologic shear flow (Peled *et al.*, 1999). In addition, SDF-1 regulates interactions between immature human $CD34^+$ cells and the BM microenvironment, *i.e.* the stromal cells and extracellular matrix, by activating the major integrins LFA-1, VLA-4, and VLA-5 that are crucial for engraftment of severe combined immunodeficient repopulating cells (Peled *et al.*, 2000). Homing of human $CD34^+$ cells was shown to be dependent on VLA-4, using a foetal sheep model (Zanjani *et al.*, 1999).

Homing of blood stem cells to the BM and the peripheral circulation requires CXCR4. Stimulation of $CD34^+$ cells with cytokines increased the expression of CXCR4 (Yong *et al.*, 2002), while pre-treatment of enriched human $CD34^+$ cells with antibodies for CXCR4 or major integrins VLA-4, VLA-5, and LFA-1 inhibited the homing (Kollet *et al.*, 2001). Moreover, primitive $CD34^+CD38^{-/low}CXCR4^+$ cells also homed in response to a gradient of human SDF-1 directly injected into the BM or spleen of non-irradiated NOD/SCID mice (Kollet *et al.*, 2001).

Other studies have shown that orally administered fucoidans have many biological effects (Nagaoka *et al.*, 2000; Zhang *et al.*, 2003). In previous chapters, fucoidan ingestion was shown to mobilise HSC from BM to PB and at the same time it amplified the expression of CXCR4 on $CD34^+$ cells and up-regulated the SDF-1 plasma level (Irhimeh *et al.*, 2005b). Sulphated glycans (polyanions) such as fucoidan have been shown to induce rapid HPC mobilisation in mice at 25 mg/kg i.v. They were believed to act as inhibitors of selectin-mediated interactions between HPC and BM endothelial cells (Frenette & Weiss, 2000). However, the effects of fucoidan on the haemopoietic expansion system *ex vivo* are still unknown and need to be investigated.

It has been shown that mice myeloid cells bind to fucoidan via SDF-1/CXCR4, L-selectin, and the integrin αMβ2. Fucoidan binding has been shown to degrade CXCR4 through the release of serine proteases (Hidalgo *et al.*, 2004). However, the primary

mechanism of action may involve the release into the blood of large quantities of chemokines and cytokines, particularly SDF-1 and kit ligand, sequestered in heparin sulphates and glycosaminoglycans that coat the surfaces of BM endothelial cells, stromal cells and extracellular matrix (Sweeney *et al.*, 2000). Although fucoidans synergize with G-CSF to increase mobilisation 11 times over that of G-CSF alone in non-human primates (Winkler & Levesque, 2006), to date no clinical trials in patients have been reported.

The establishment of stromal cell layers, which is the current method for long-term maintenance or expansion of blood stem cells, are unsuitable for clinical trial. There would be considerable clinical interest in a synthetic matrix that was suitable for blood stem cell maintenance or expansion. Fucoidan preparations may provide an easily manufactured proteoglycan component of an artificial BM matrix, since it displays sulphate sugars that interact with heparin-binding proteins, integrins, and selectins.

The present studies were designed to directly examine the characterization of fucoidan effects on *in vitro* human $CD34^+$ cells proliferation and differentiation and to determine the interaction between fucoidan and cytokines in the expansion system. Factorial design experiment and flow cytometry were used to identify these interactions. Human mobilised PB and CB isolated $CD34^+$ cells were cultured under conditions that stimulated short-term expansion and proliferation.

7.3 Materials and methods

7.3.1 Cells and serum free media

Institutional human ethics committees (UNSW, Sydney Eastern Area Health Service and the Peter MacCallum Research Centre) approved the use of human PB or CB $CD34^+$ cells for the study of expansion processes as outlined in section 2.1. Cells were obtained from consenting patients or from donors as explained previously in section 2.4. The medium base used to culture human $CD34^+$ cells was StemlineTM II Haemopoietic Stem Cell Expansion media (Sigma-Aldrich Co., MO USA). This serum-free media was modified by the addition of antibiotics (penicillin and streptomycin, $1x10^4$ U/L, JRH, USA) and human cytokines (see section 7.3.11).

7.3.2 KG1a cells

KG1a cells were cultured with different fucoidan extracts in Iscove's Modified Dulbecco's Medium (Gibco, VIC Australia) supplemented with 10% foetal calf serum (IMDM + 10% FCS) to determine whether these extracts had direct cytotoxicity. The IMDM was prepared by mixing 17.7 g of the powder and 2 g/L of sodium bicarbonate (Sigma, USA) in 1 L of sterile water for irrigation (Baxter Healthcare Pty. Ltd., NSW Australia). The pH was then adjusted to 7.2 using HCl/NaOH and filter-sterilised (0.22 µm). 10 mL of sterile FCS was added to each 200 mL if needed and it was kept at 4°C.

Filter-sterilized fucoidan extracts were prepared at 10 mg/mL in IMDM + 10% FCS and serial dilutions made. Cells were then seeded at $2x10^4$ cells /mL and incubated at 37°C, 5% CO_2 for 4–10 days. Beads coated with BSA were added to the cultures at $2x10^5$ bead/mL. Cultures were analysed using the FACS-Scan as described later in the section 7.3.10. Sub-cultures of the cell line were made by passaging the cells in IMDM + 10% FCS before the cell count reached 2 x 10^6/mL.

7.3.3 Peripheral blood $CD34^+$ cells

Isolex-selected $CD34^+$ cells were harvested from the blood of multiple myeloma patients attending the Peter MacCallum Cancer Centre (Melbourne, VIC Australia) as described in section 2.8.2. These cells were stored in liquid nitrogen and thawed immediately before use at 37°C on the day of the experiment. The thawed cells were diluted on ice with 10 mL of IMDM + 20% BSA, adding the media to the cells drop-

wise with mixing to avoid osmotic shock. The cells were centrifuged at 328 *xg* for 5 min and washed twice with 10 mL of IMDM + 20% BSA and finally re-suspended in StemlineTM II medium. The cells were counted using a haemocytometer. $CD34^+$ cells were cultured with fucoidan extracts as described later.

7.3.4 Cord blood $CD34^+$ cells

CB was obtained as described in section 2.8.3.1. The CB $CD34^+$ cells were isolated as described in section 2.8.3.2 and MACS enriched as described in section (2.8.3.3). $CD34^+$ cells were cultured with fucoidan extracts as described later.

7.3.5 Fucoidan preparation

Stock solutions of the four different fucoidan preparations (75% GFS^{TM}, DP-GFS-001, NOV-1 and NOV-6) were prepared by dissolving 100 mg of the fucoidan preparation in 10 mL IMDM supplemented with 10% FCS. The solutions were filter-sterilised through 0.22 µm filters. Serial doubling dilutions were made to prepare different fucoidan concentrations from 5 to 0.2 mg/mL. The fucoidan doses used in the *in vitro* study were chosen as doses that are in the range of the actual levels of the bio-available fucoidan in blood after ingesting 3 g of the 75% GFS^{TM}.

The fucoidan extracts (Marinova Pty. Ltd., TAS Australia) studied are listed in Table 7.1. Depyrogenated fucoidan (DP-GFS-001; N3) was used predominantly in this study. In factorial experiments N3 was studied at 4 levels (0, 10, 100, and 500) µg/mL as described later in this chapter. A stock solution of 50 mg/mL was prepared freshly for each experiment. Fucoidan extracts were dissolved in the modified StemlineTM II medium and filter sterilized. The different concentrations were prepared by serial doubling dilutions using the modified StemlineTM II medium.

Table 7.1: Properties of fucoidan fractions used in the study.

Name	Fucoidan concentration (%)	Sulphation (%)	Acetylation (%)
75% GFS^{TM}	75	23	35
NOV1-GFS (N1)	~100	22.2	44
NOV6-GFS (N2)	~100	4.5	23
DP-GFS-001 (N3)	~100	32	Zero

7.3.6 Bead preparation and counting cells using beads

Uniform micro-spheres (beads) with a mean diameter of 9.62 μm (Bangs Lab, IN USA) were added to the wells before harvest to provide an internal standard for calculating the absolute cell count. Beads were prepared for the cultures by adding a volume of the original stock suspension into a centrifuge tube containing 10 mL of saline (0.9% NaCl for irrigation, sterile, non-pyrogenic, Baxter Healthcare Pty. Ltd., NSW Australia) with 1% BSA (Sigma USA). The bead suspension was centrifuged at 328 *xg* for 5 min. The supernatant was discarded and the beads were washed three times by re-suspending them in 10 mL of saline/BSA. The beads were washed once with culture medium (IMDM, 10% FCS) and re-suspended in 10 mL of culture medium. The beads were counted using a haemocytometer and centrifuged again and re-suspended at an appropriate concentration.

Generally, $1x10^5$ beads/mL were added to each culture. The cell count was calculated using the cell to bead ratio (cell/bead) as determined by flow cytometry. This ratio was then multiplied by the number of beads added to each sample to calculate the number of cells in that sample.

$$Absolute\ number\ of\ cells = \left(\frac{output\ cell\ count\ (\ flow)}{input\ bead\ count\ (\ flow)} \right) \times input\ bead\ count\ (/\ mL)$$

$$Cells\ \ fold\ increase = \frac{Absolute\ number\ of\ \ cells}{Input\ cell\ count\ of\ \ the\ starting\ inoculum}$$

$$Cell\ Expansion = \frac{output\ cell\ count - input\ cell\ count}{input\ cell\ count}$$

$$Doubling\ time = \frac{Ln_2}{Ln_{\ fold\ \ increase}} \times time\ of\ \ culture\ (h)$$

7.3.7 Studying the growth of KG1a cells in the presence of beads

To study the growth of the KG1a cells in the presence of beads in the same culture, the cell-line was cultured with beads in T-flasks with 10 mL of IMDM + 10% FCS at 20,000 cells/mL and 200,000 beads/mL. The control flask contained only cells at 20,000 cells/mL in 10 mL culture medium. The flasks where incubated at 37°C in 5% CO_2 and FACS analysed each day to obtain a growth curve for up to 10 days. The cell cycle time was calculated and compared with the cell cycle time of cells that were cultured without beads as a control.

7.3.8 Culturing KG1a cells with fucoidan and beads

KG1a cells were cultured with the four different fucoidan preparations (75% GFS^{TM}, N1, N2 and N3) in the presence of beads to study the cell growth and any sign of toxicity. Briefly, beads were counted after being prepared for the culture by coating them with BSA as described before in section 7.3.6. The cells were also counted from the original flask using a haemocytometer. The cell suspension was mixed with beads in a ratio of 1:10, respectively. Serial dilutions of the fucoidans were prepared as described before in section 7.3.5. Cell/bead suspensions were added to tubes containing fucoidan at around 20,000:200,000 per mL in duplicate, respectively. The suspension was mixed and 1 mL of each suspension added to wells in a 16 well culture plate in duplicate. As a positive control, 1 mL of IMDM + 10% FCS containing 20,000 cell/mL with no fucoidan was added to the plate in duplicate. As a negative control, 1 mL of PBS containing 20,000 cell/mL with no fucoidan was added to the plate in duplicate. The plates were incubated at 37°C in a 5% CO_2 incubator for 5-10 days. The cells were counted and analysed using the FACS-Scan. To establish the growth curve for the cell-line the cultures were FACS analysed each day.

7.3.9 Effect of cytokines and N3 on PB or CB CD34$^+$ cultures

Beads were prepared as described before in section 7.3.6 and they were re-suspended in StemlineTM II culture medium. Cells were prepared as described in section 7.3.3 and section 7.3.4. Both beads and cells were counted separately using a haemocytometer and the mixtures of cells and beads prepared at a 1:10 ratio.

A StemlineTM II medium was modified by adding the GFs (G-CSF, Flt3, TPO, and SCF) at a final concentration of 100 ng/mL for each GF. Then N3 was dissolved in

part of the modified medium and filter-sterilised. The remainder of the modified medium was filter-sterilised and serial doubling dilutions made. SDF-1 was added to the designated tubes at a final concentration of 100 ng/mL. The cell/bead mixture was added to each well in the culture plate and the plate incubated at 37°C in 5% CO_2 for 10 days. The cells/beads were harvested and analysed on the FACS scan.

7.3.10 Immunophenotyping

The immunophenotyping of cells before and after cytokine treatment was determined by three-colour flow cytometry. On the day of harvest, 1 μg of human IgG (CSL Ltd., Australia) was added to each well and incubated for 15 min at ambient temperature as an Fc-blocking step. A panel of MoAbs was used to distinguish between undifferentiated and differentiated progenitor cells (Table 7.2). Cells were harvested and transferred to FACS tubes and incubated with MoAbs for 30 min at 4°C followed by two washes with 3 mL D-PBS + 10% FBS. The cells were re-suspended in 300 μL of FACS-Fix and stored away from light at 4°C for later analysis. Fluorescence compensation was performed on all channels to minimize spectral overlap and flow cytometric data acquisition was performed with CELLQuest software on a Becton-Dickenson FACSort. Gates were set to include the viable cells and at least $1x10^4$ events were collected per sample. Analysis was performed using Cytomation Summit V3.1, CO USA.

Table 7.2: Monoclonal antibody panels.

Tube number	Specificity	FL1	FL2	FL3
1	Megakaryocytic, monocytic and granulocytic cells	CD15 IgM FITC	CD14 IgG_{2a} PE	CD41 IgG_1 PerCP-Cy5.5
2	CXCR4 and primitive $CD34^+$ cells	CD38 IgG_1 FITC	CXCR4 IgG_{2a} PE	CD34 IgG_1 PerCP-Cy5.5
1a	Isotype control for tube 1	Mouse IgM FITC	Mouse IgG_{2a} PE	Mouse IgG_1 PerCP-Cy5.5
2a	Isotype control for tube 2	Mouse IgG_1 FITC	Mouse IgG_{2a} PE	Mouse IgG_1 PerCP-Cy5.5

7.3.11 Growth factors (cytokines)

Recombinant human haemopoietic cytokines sourced from genetically engineered *Escherichia coli*, lyophilized and filter-sterilised were used throughout the study. These were G-CSF, SCF, TPO, Flt-3, and SDF-1 (Cedarlane Laboratories Ltd., Ontario Canada). The cytokines were used at two different concentrations, 100 ng/mL or 1000 ng/mL. The cytokines were added in various combinations to StemlineTM II medium (Table 7.3 and Table 7.4).

Table 7.3: Fractional factorial growth factors combinations. High level (+), low level (-)

Run		A	B	C	D	E=ABCD	Treatment combination	
	GCSF	SCF	Flt-3	TPO	SDF1	GFS™		
1	+	-	-	-	-	+	e	GFS
2	+	+	-	-	-	-	a	SCF
3	+	-	+	-	-	-	b	Flt-3
4	+	+	+	-	-	+	abe	SCF+Flt-3+GFS
5	+	-	-	+	-	-	c	TPO
6	+	+	-	+	-	+	ace	SCF+TPO+GFS
7	+	-	+	+	-	+	bce	Flt-3+TPO+GFS
8	+	+	+	+	-	-	abc	SCF+Flt-3+TPO
9	+	-	-	-	+	-	d	SDF-1
10	+	+	-	-	+	+	ade	SCF+SDF-1+GFS
11	+	-	+	-	+	+	bde	Flt-3+SDF-1+GFS
12	+	+	+	-	+	-	abd	SCF+Flt-3+SDF-1
13	+	-	-	+	+	+	cde	TPO+SDF-1+GFS
14	+	+	-	+	+	-	acd	SCF+TPO+SDF-1
15	+	-	+	+	+	-	bcd	Flt-3+TPO+SDF-1
16	+	+	+	+	+	+	abcde	SCF+Flt-3+TPO+SDF-1+GFS

Table 7.4: Full factorial design growth factors combination. High level (+), low level (-).

Run		A	B	C	D	Treatment combination	
	G-CSF	SCF	Flt-3	TPO	GFS		
1	+	-	-	-	-	(-1)	nil
2	+	+	-	-	-	a	SCF
3	+	-	+	-	-	b	Flt-3
4	+	+	+	-	-	ab	SCF+Flt-3
5	+	-	-	+	-	c	TPO
6	+	+	-	+	-	ac	SCF+TPO
7	+	-	+	+	-	bc	Flt-3+TPO
8	+	+	+	+	-	abc	SCF+Flt-3+TPO
9	+	-	-	-	+	d	SDF-1
10	+	+	-	-	+	ad	SCF+GFS
11	+	-	+	-	+	bd	Flt-3+GFS
12	+	+	+	-	+	abd	SCF+Flt-3+GFS
13	+	-	-	+	+	cd	TPO+GFS
14	+	+	-	+	+	acd	SCF+TPO+GFS
15	+	-	+	+	+	bcd	Flt-3+TPO+GFS
16	+	+	+	+	+	abcd	SCF+Flt-3+TPO+GFS

7.3.12 Experimental design

In this study a two-level full factorial experimental design has been used where the number of experiments is 2^n (n is the number of factors to be examined). For larger numbers of factors, the number of experiments could be reduced by grouping together (confounding) higher order interactions (*i.e.* 2 or more interacting factors). A fractional factorial design (resolution V, 2^{5-1}, half replicate of 5 factors in 16 runs) was employed to determine single-factor effects and to screen for two-factor interaction.

Two experimental blocks were used to determine the effect of cytokines and fucoidan on cell expansion and proliferation. In block-A, the effect of fucoidan was examined at low concentrations; the low and high level of fucoidan was 0 and 10 μg/mL, respectively. Block-B determined the effect of higher levels of fucoidan; 100 versus 500 μg/mL.

The 2^{5-1} fractional factorial design was used to investigate the relative effects of five factors on the proliferation and expansion of mobilised peripheral $CD34^+$ cells incubated in serum free medium containing 100 ng/mL G-CSF. The experiments were duplicated using two different patient donors. The combinations of factors tested are shown in Table 7.3. $CD34^+$ cells $2x10^4$/mL of culture media were grown in 24 well plates at 37°C with 5% CO_2 over a 10 day period. Manual and automated cell counts were determined along with phenotypic analysis, as described later, for each cultural condition. An example of one experimental fractional factorial design is shown in Table 7.5. The gated regions that were analysed in detail are listed in Table 7.6.

A full factorial design was used to investigate the effect of high levels of growth factors (SCF, Flt-3, TPO at 100 versus 1000 ng/mL) in combination with fucoidan (Table 7.4). $4x10^3$ $CD34^+$ cells in 200 μL of culture medium were grown in 96 well plates at 37°C with 5% CO_2 over a 10 day period.

Table 7.5: Example for one 2^{5-1} fractional factorial design experiment in two blocks

Block	Run	GCSF[1]	SCF[1]	Flt-3[1]	TPO[1]	SDF1[1]	GFS[2]
Block 1	1	100	0	0	0	0	10
	2	100	100	0	0	0	0
	3	100	0	100	0	0	0
	4	100	100	100	0	0	10
	5	100	0	0	100	0	0
	6	100	100	0	100	0	10
	7	100	0	100	100	0	10
	8	100	100	100	100	0	0
	9	100	0	0	0	100	0
	10	100	100	0	0	100	10
	11	100	0	100	0	100	10
	12	100	100	100	0	100	0
	13	100	0	0	100	100	10
	14	100	100	0	100	100	0
	15	100	0	100	100	100	0
	16	100	100	100	100	100	10
Block 2	17	100	0	0	0	0	500
	18	100	100	0	0	0	100
	19	100	0	100	0	0	100
	20	100	100	100	0	0	500
	21	100	0	0	100	0	100
	22	100	100	0	100	0	500
	23	100	0	100	100	0	500
	24	100	100	100	100	0	100
	25	100	0	0	0	100	100
	26	100	100	0	0	100	500
	27	100	0	100	0	100	500
	28	100	100	100	0	100	100
	29	100	0	0	100	100	500
	30	100	100	0	100	100	100
	31	100	0	100	100	100	100
	32	100	100	100	100	100	500

[1] ng/ml

[2] µg/ml

Table 7.6: PB HSC and CB HSC phenotypes analysed.

Phenotype	Description
Total	All cell types
$CD41^+CD14^-$	Megakaryocyte lineage subset
$CD14^+$ $CD15^-$	Monocytes lineage subset
$CD15^+CD14^-$	Granulocyte lineage subset
$CD34^+$	Early progenitors capable of producing all of the haemopoietic lineages
$CD34^+CXCR4^+$	Early progenitors subset capable of mobilisation and homing
$CD34^+(CXCR4^{+/-}$ ratio)	Index of engraftment potential

7.3.13 Staining $CD34^+$ cells with CFSE

Purified $CD34^+$ cells were stained with the carboxyfluorescein diacetate succinimidyl ester (CFSE). The optimal concentration that gave sufficient staining intensity for flow cytometric analysis through to at least six cellular divisions was 2.5 μM. The cell suspension was incubated for 10 min at 37°C before incubation with the stain at a final concentration of 2.5 μM for 10 min at 37°C. Three times the volume of cell suspension of cold FBS was added to stop the reaction. The cells were washed twice in IMDM + 10% FBS and re-suspended in 4 mL of the same medium. The cells were incubated overnight at 37°C + 5% CO_2. The next day, cells were washed once with D-PBS + 10% BSA and cultured with either 75% GFS™ or N3 at 500 μg/mL in the modified StemlineTM II medium containing 100 ng/mL of each of the cytokines (G-CSF, SCF, Flt-3, and TPO). Cells were harvested and analysed using flow cytometry every day for a period of 5 days.

7.3.14 Viability staining

Cell viability was evaluated by flow cytometry on a Becton Dickonson FACSort flow cytometer. Propidium iodide (PI) penetrates the membrane of dying or dead cells where it intercalates with the cellular DNA. PI is excited at 488 nm and fluoresces at 550-670 nm. 0.2-0.3 mL of cell suspension containing 10,000-50,000 cells is placed in a flow cytometry tube and 10 μL of PI stock solution (100 μg/mL in PBS) was added with mixing. The cells were analysed by flow cytometry on the FL2/FL3 channel.

7.3.15 Statistical analysis

Statistical analyses were performed with the Student's t-test or the Wilcoxon Signed Rank test. The Stat Works program was used and p-values ≤ 0.05 were considered to be statistically significant. For experiments where factorial design was used Page: 215 results were expressed as contrasts or effects (see below). ANOVA was used to compare the variances of these effects (see below). A linear regression model was fitted to the data to describe the effect of cytokines and fucoidan. The coefficients for the linear statistical model of the response to cytokines and fucoidan are derived from the experimental data. The regression equation can then predict the response ($\hat{y}$) given a specified combination of cytokines.

$$\hat{y} = \bar{y} + \frac{E_A}{2}\chi_A + \frac{E_B}{2} + \chi_B + \ldots + \frac{E_{AB}}{2}\chi_{AB} + \ldots + \varepsilon$$

where $\hat{y}$ is the average response, E_A, E_B,…, are the "effects" for single factor interactions, $E_{AB,\ldots,}$ are effects of two factor interactions, χ_A, χ_B, $\chi_{AB,\ldots,}$ are the corresponding indicator variables (+1 or -1 if the factor is present or not present, respectively), and ε is a normally distributed random variable (experimental error). The effects for single factors or combinations of factors are calculated from the "contrasts" using the following formula:

$$AB\ldots K = \frac{C_{AB\ldots K}}{n \times 2^{k-1}}$$

where AB…K is the effect of the cytokine combination, *n* is the number of replicates, *k* is the order of the design, and $C_{AB\ldots K}$ is the "contrast" for the cytokine combination. Contrasts are calculated by subtracting the mean response of those combination that include the factor(s) from those where it (they) is (are) absent. Therefore calculation of contrasts involves the addition or subtraction of the various responses. A useful formula to calculate the signs of each term is provided by algebraic expansion of the following expression:

$$C_{AB\ldots K} = (a + \chi_A)(b + \chi_B)\ldots(k + \chi_k)$$

where a, b, ab, c, ac, bc, ,abc,… are the responses to the various factor combinations, (1) corresponding to the response when no factors are present. For example

$$C_{AB} = (a+1)(b+1)(c-1)(d-1)$$
$$= abcd - abc - abd + ab + acd - ac - ad + a + bcd - bc - bd + b + cd - c - d + (1)$$

Analysis of variance is used to determine the statistical significance of these calculated effects. The ratio of the mean square of the effect ($MS_{AB...K}$), to that of the mean square of the experimental error (MS_{error}), provides the F statistic (F_0), and the F-distribution is used to test the hypothesis that the variances are not significantly different (reject null hypothesis if $p < 0.01$). All of these calculations were set up as EXCEL spreadsheets.

$$F_0 = \frac{MS_{AB...K}}{MS_{error}} \quad where \; MS_{AB...K} = \frac{C_{AB...K^2}}{n2^k}$$

7.4 Results

7.4.1 Studying the growth of KG1a cells in the presence of beads

The growth of KG1a cells in culture media containing beads was studied. It has been observed that beads have no effect on the growth of cells. They were not toxic and the cell fold expansion indices for both control (without beads) and test (with beads) remained the same. A bivariate dot-plot for the cultured KG1a cells control and test is shown in Figure 7.1. Growth curves in one experiment when cells were cultured with and without beads are shown in Figures 7.2 and 7.3.

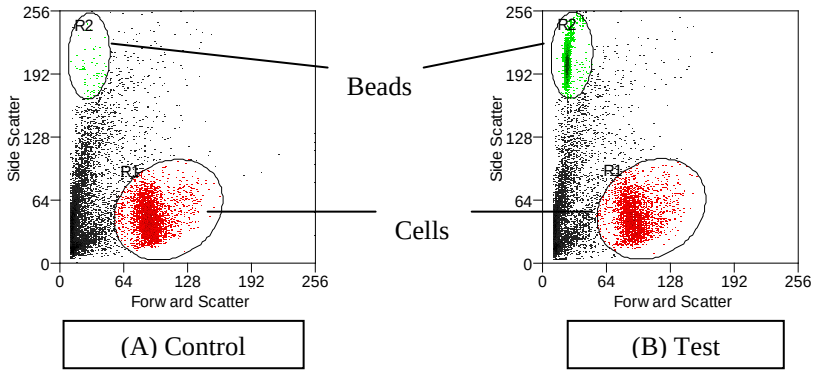


Figure 7.1: Two bivariate dot-plots showing the KG1a cells (R1) with respect to beads (R2). The first dot-plot (A) shows an analysis of cells from the control, cultured without beads while the second dot-plot (B) shows cells from the test flask, cultured with beads.

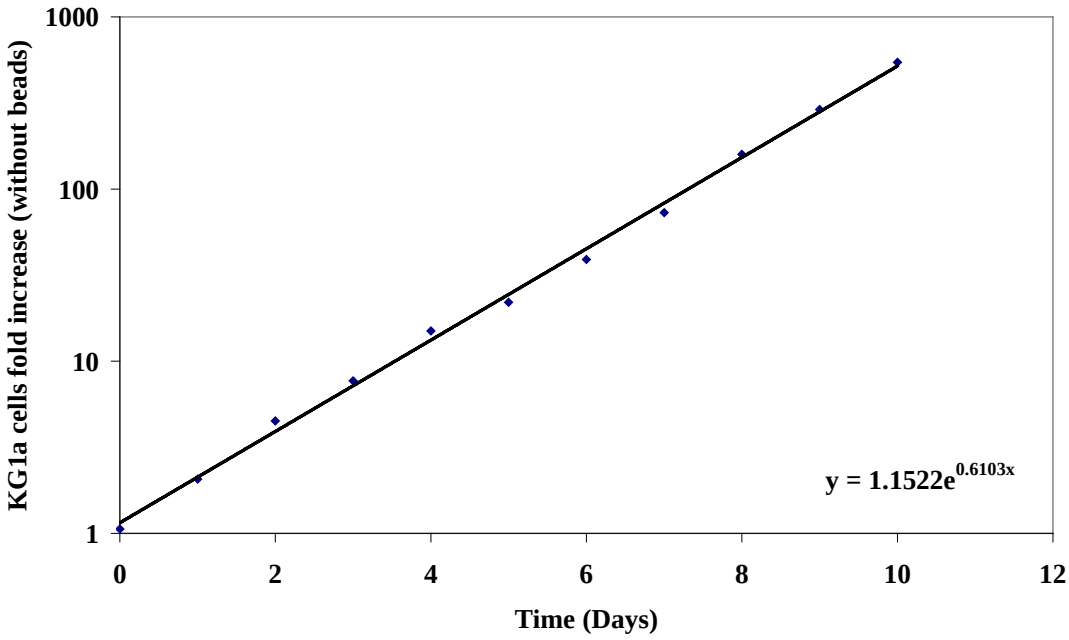


Figure 7.2: Growth curve for KG1a cells cultured without beads. The cells were cultured without beads (control) at a cell to bead ratio of 1:10 at day 0, equivalent to a 1:1 ratio at the end of the experiment.

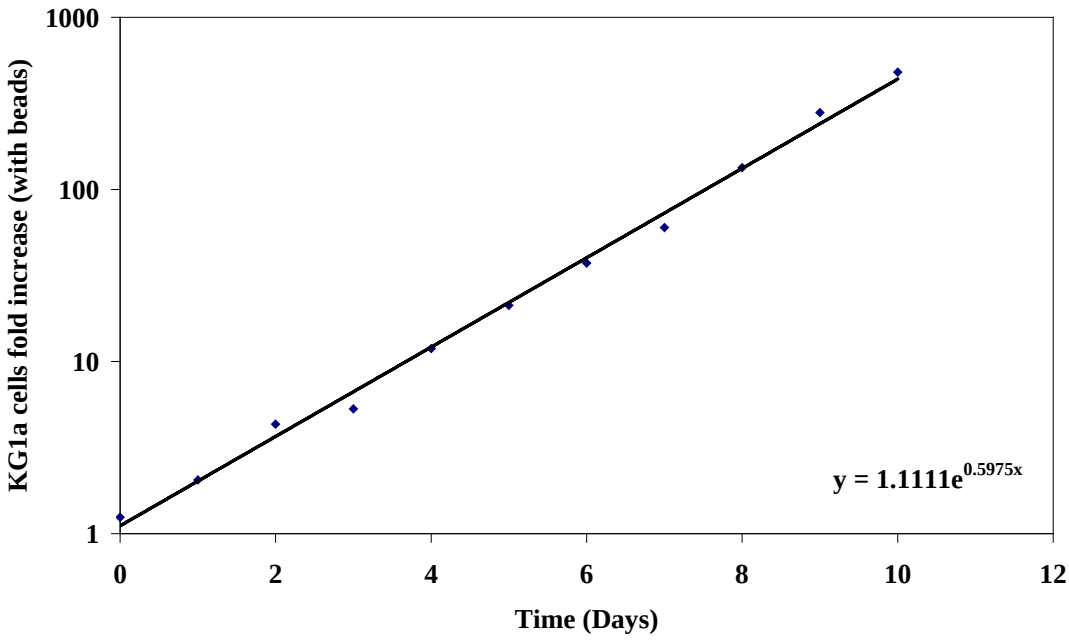


Figure 7.3: Growth curve for KG1a cells cultures with beads. The cells were cultured with beads (test) at a cell to bead ratio of 1:10 at day 0, equivalent to a 1:1 ratio at the end of the experiment.

7.4.2 Cytotoxicity of fucoidan against KG1a cells

Initially a determination was made of the toxicity of the fucoidan preparations on KG1a, a $CD34^+$ cell line. After culturing the cells with a range of fucoidan concentrations in multi-well plates for 5 days they were stained and labelled with Abs as described earlier in this chapter. A representative FACS analysis bivariate dot-plot of one experiment showing the FACS analysis at all concentrations is shown for 75% GFS^{TM} in Figure 7.4 and for N3 fucoidan in Figure 7.5.

Generally, growth of KG1a cells was inhibited in both 75% GFS^{TM} and N3 when the fucoidan concentration was greater than 0.5 mg/mL [Figures 7.6 and 7.7]. Any concentration below 0.5 mg/mL was considered non-toxic for the cells. The other fucoidan extracts (N1 and N2) were not tested in such detail, with growth inhibition occurring somewhere between 0.08 and 2.5 mg/mL, see Figure 7.8.

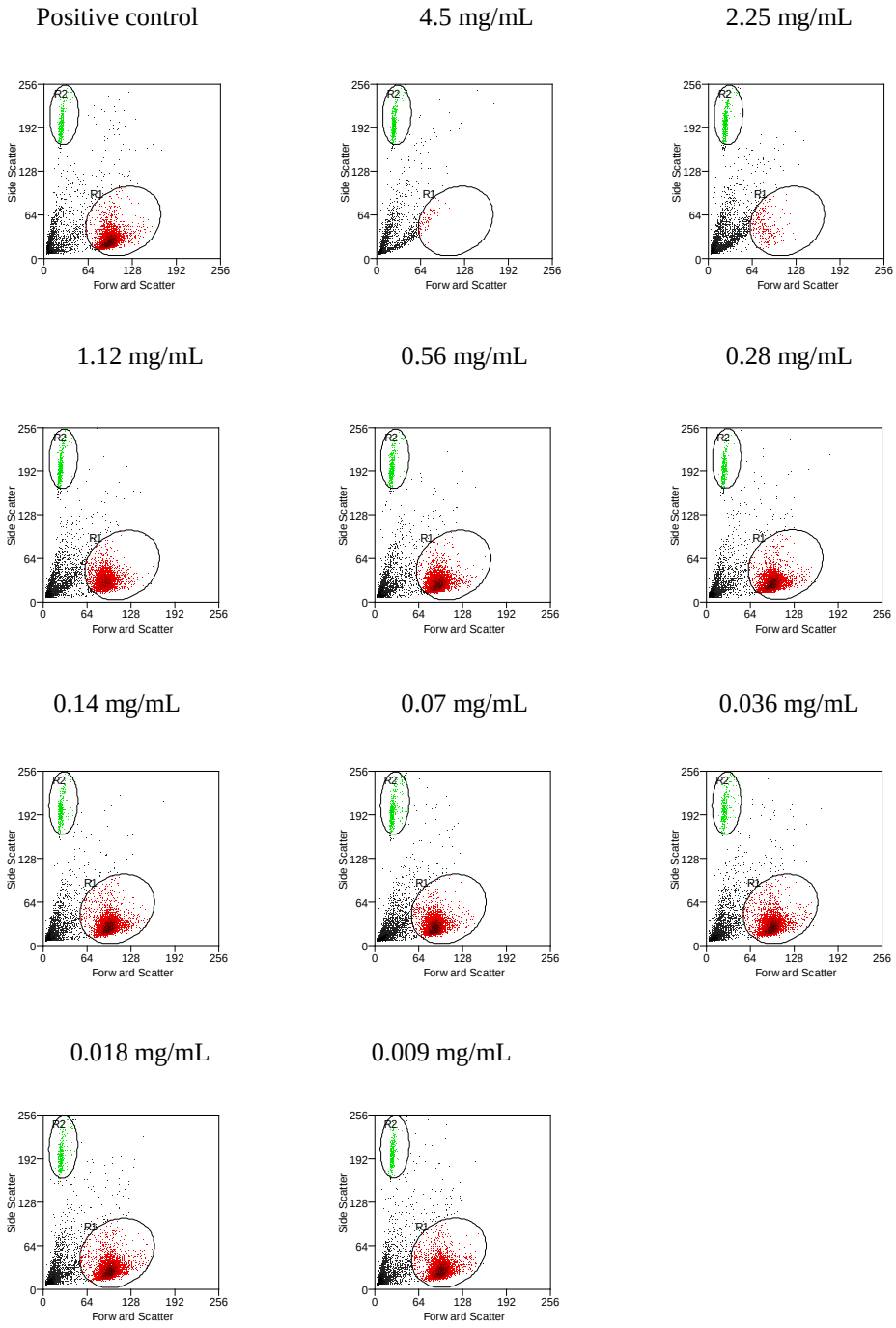


Figure 7.4: Bivariate dot-plots showing the KG1a cells (R1) with respect to beads (R2). The cells were cultured with different concentrations of 75% fucoidan w/w for 5 days. The positive control contained cells cultured without fucoidan.

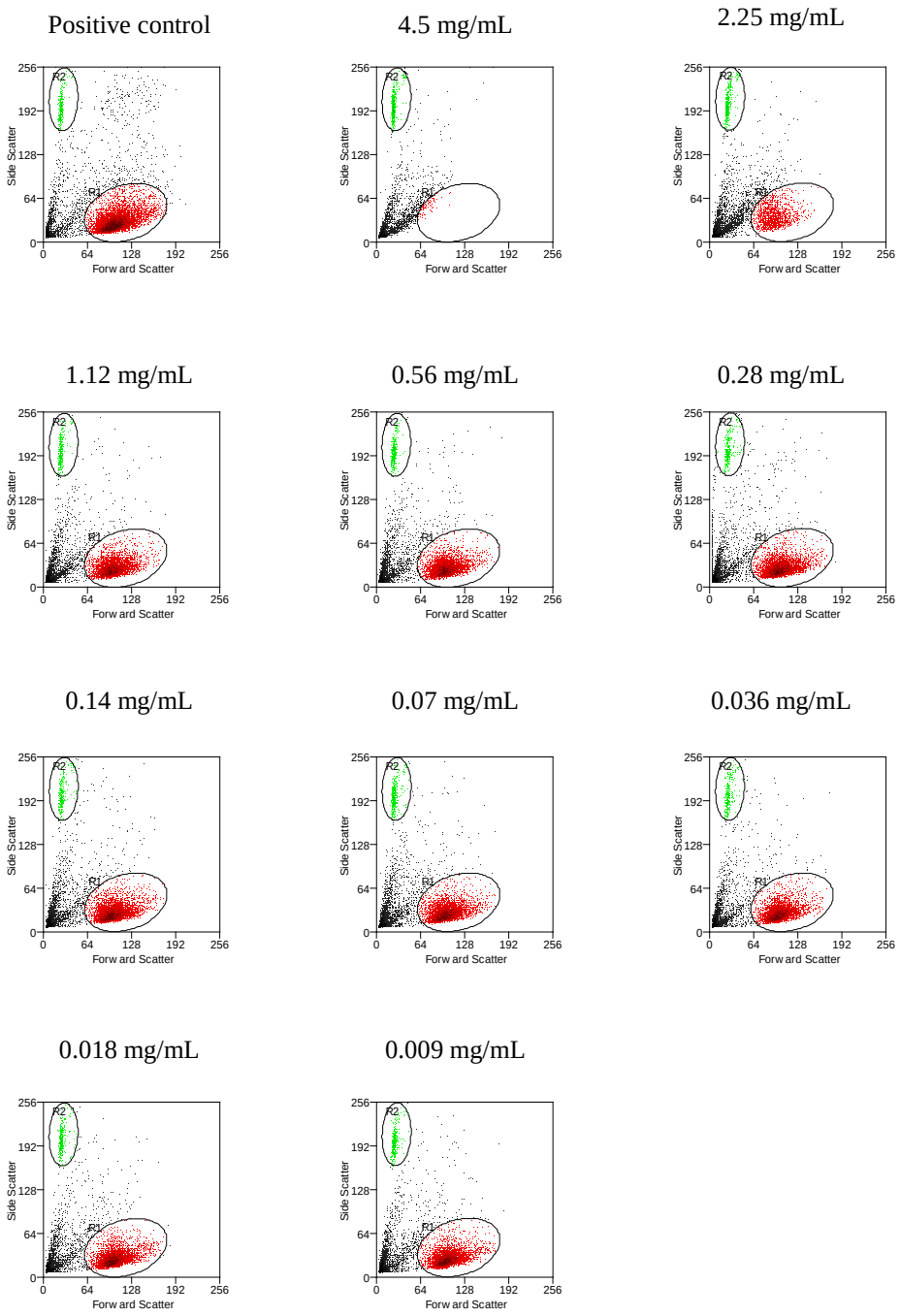


Figure 7.5: Bivariate dot-plots showing the KG1a cells (R1) with respect to beads (R2). The cells were cultured with different concentrations of N3 for 5 days. The positive control contained cells cultured without fucoidan.

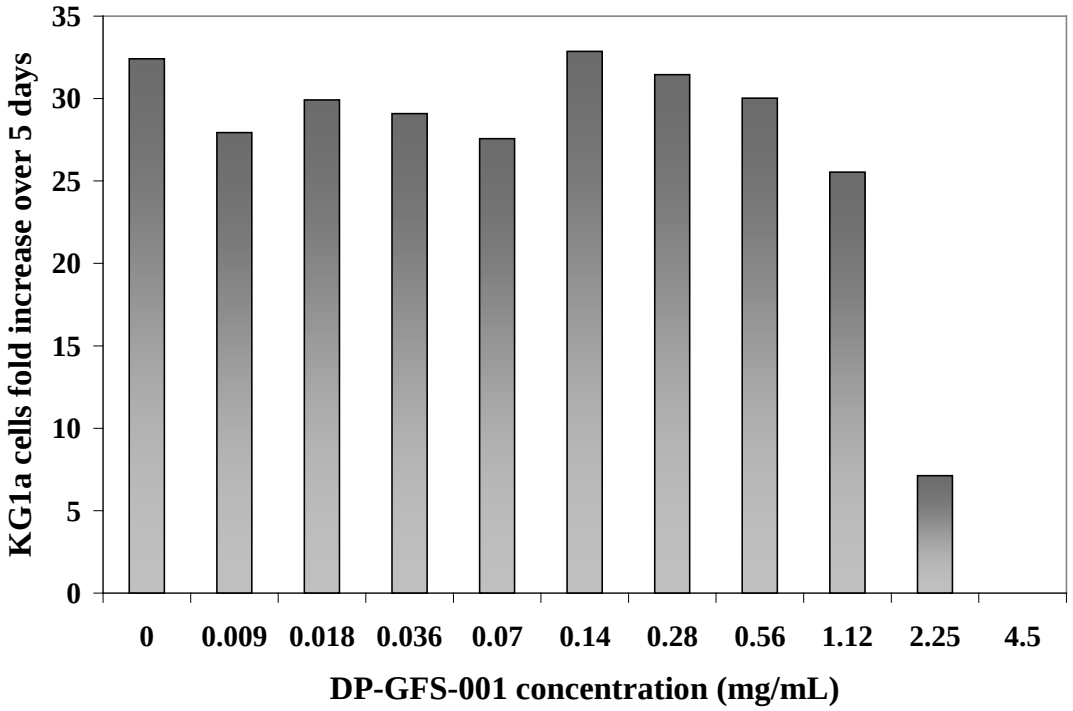


Figure 7.6: Effect of DP-GFS-001 (N3) on the growth of KG1a cells over 5 days. The increase in cells is expressed as the output/input.

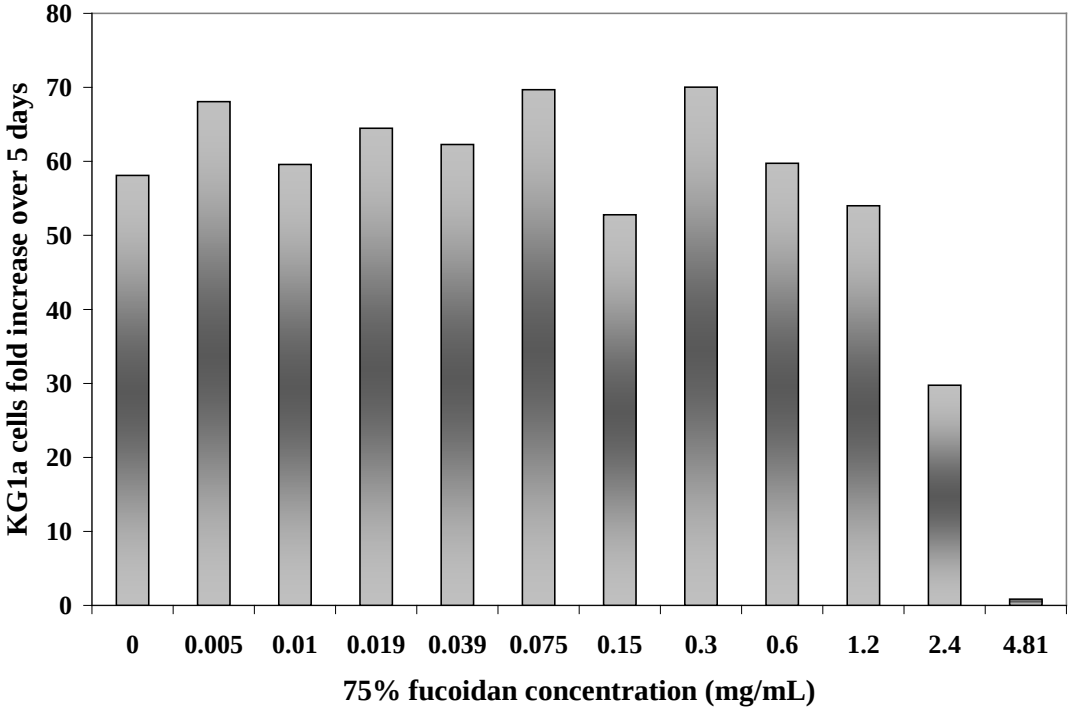


Figure 7.7: Effect of 75% GFS^{TM} on the growth of KG1a cells over 5 days. The increase in cells is expressed as the output/input.

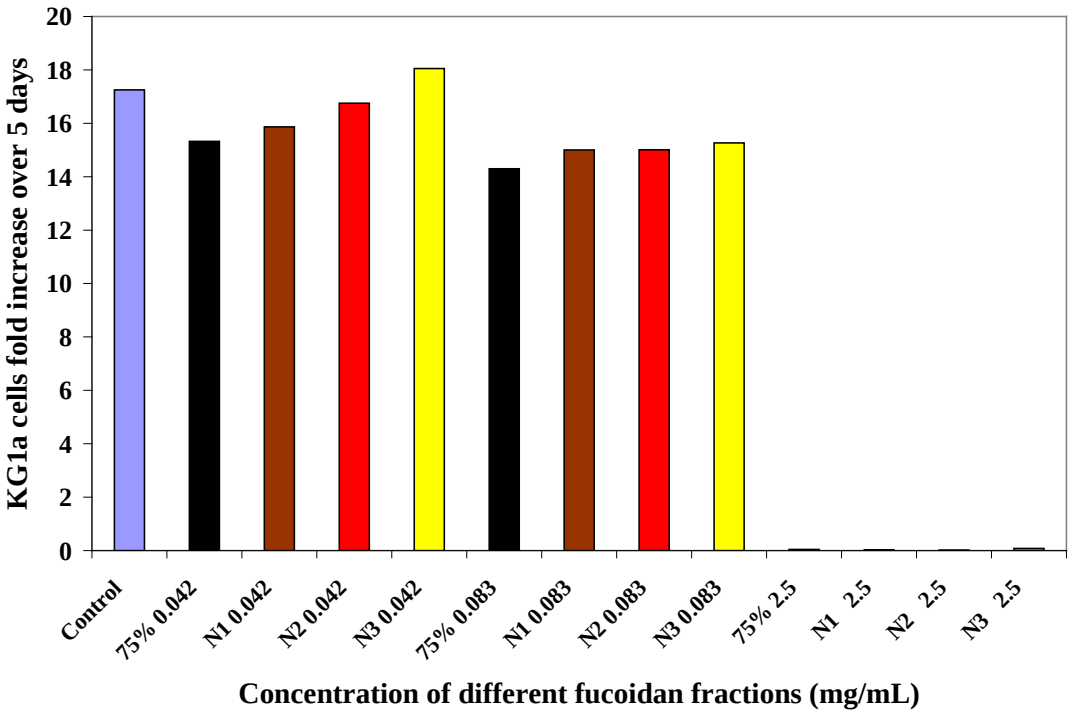


Figure 7.8: Effect of different fucoidan extracts (75% GFS^{TM}, N1, N2 and N3) on the growth of KG1a cells at three different concentrations. The increase in cells is expressed as the output/input. Each point represents an average of duplicate experiments.

It has been observed that the presence of the DP-GFS-001 in the culture media with the other main expansion cytokines (G-CSF, SCF, Flt-3 and TPO) has a positive effect on the expansion of the PB CD34$^+$. The cellular response to varying combinations of cytokines and DP-GFS-001 concentrations is shown in Figure 7.9. The primitive haemopoietic cells (CD34$^+$ subset) did not expand optimally in the presence of DP-GFS-001. The CD34$^+$ subset response to variable combinations of cytokines and DP-GFS-001 concentrations is shown in Figure 7.10.

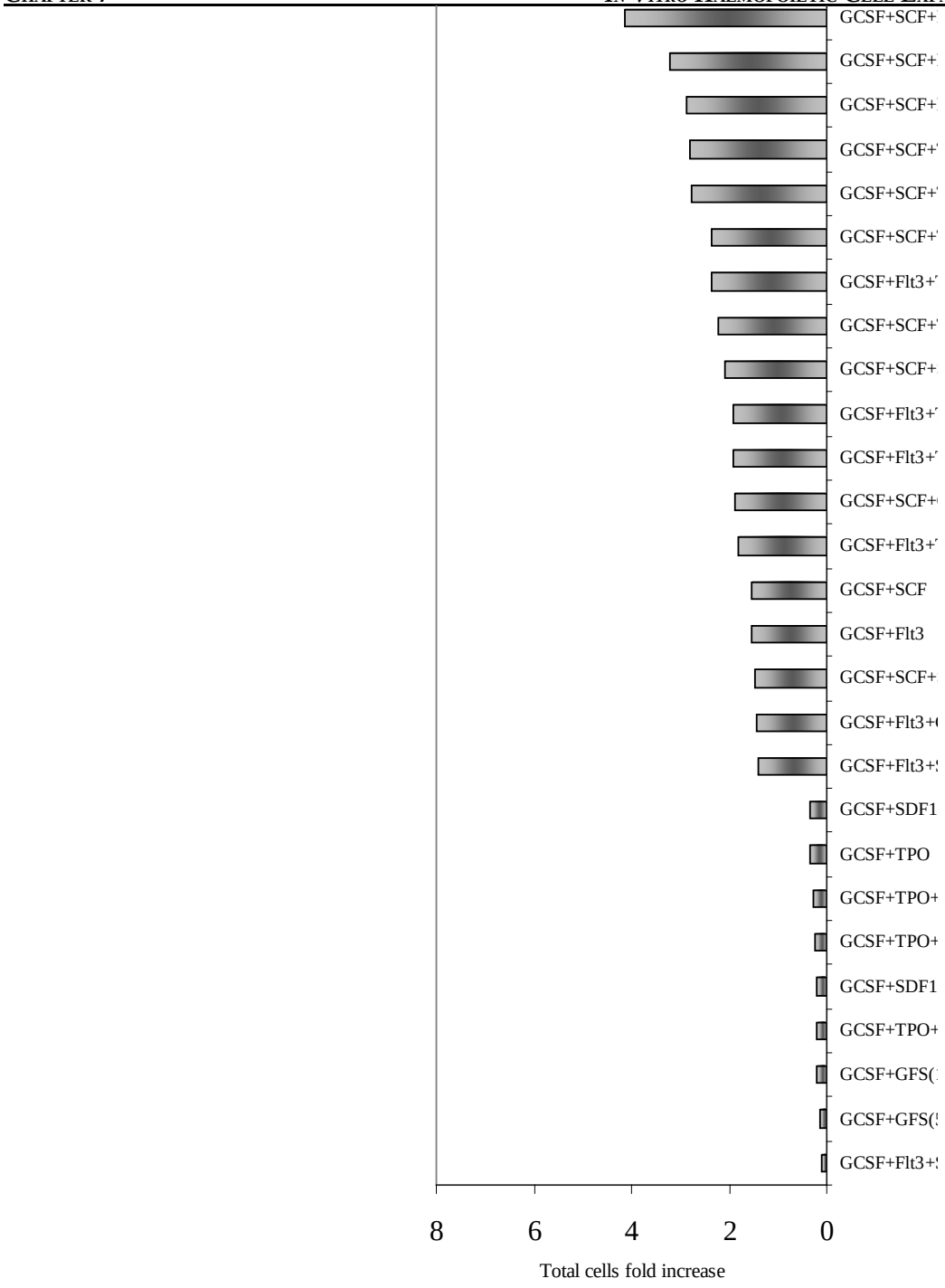


Figure 7.9: Fractional factorial design to investigate the interactions of growth factors with total PB mobilised HSC in the presence of DP-GFS-001. Increase in total cells expressed as the output/input (fold increase) over 10 days.

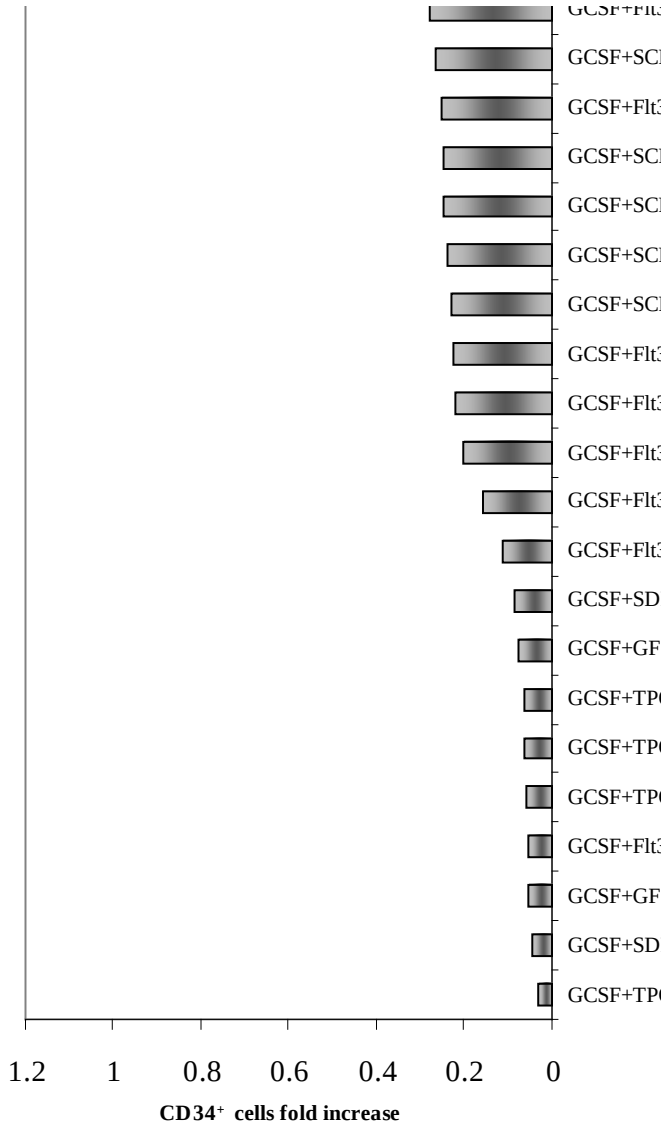


Figure 7.10: Fractional factorial design to investigate the interactions among growth factors, DP-GFS-001 and CD34$^+$ cells. Increase in CD34$^+$ cells subset expressed as the output/input (fold increase) over 10 days.

7.4.3 Factorial design experiment examining the interaction of growth factors and fucoidan on the expansion of mobilised CD34$^+$ cells

The first factorial experiment examined the effect of DP-GFS-001, SCF, Flt-3, TPO and SDF-1 on haemopoietic expansion of mobilised PB CD34$^+$ cells. Analysis of variance was performed on duplicate experiments to determine which effects were statistically significant. CD34$^+$ cell production was significantly enhanced by SCF (p< 0.001), Flt-3 (p< 0.001) and TPO (p = 0.027). The same growth factors increased the production of megakaryocytes and granulocytes (p< 0.001). There was an inhibition of megakaryocyte production by fucoidan at higher levels (100 versus 500 µg/mL, p< 0.001). Monocyte production was increased by Flt-3, SCF, TPO and SDF-1 at low fucoidan levels, however only Flt-3 and SCF were able to increase monocyte production for high levels of fucoidan (100 versus 500 µg/mL) (Appendix-6), see Figures 7.11 and 7.12.

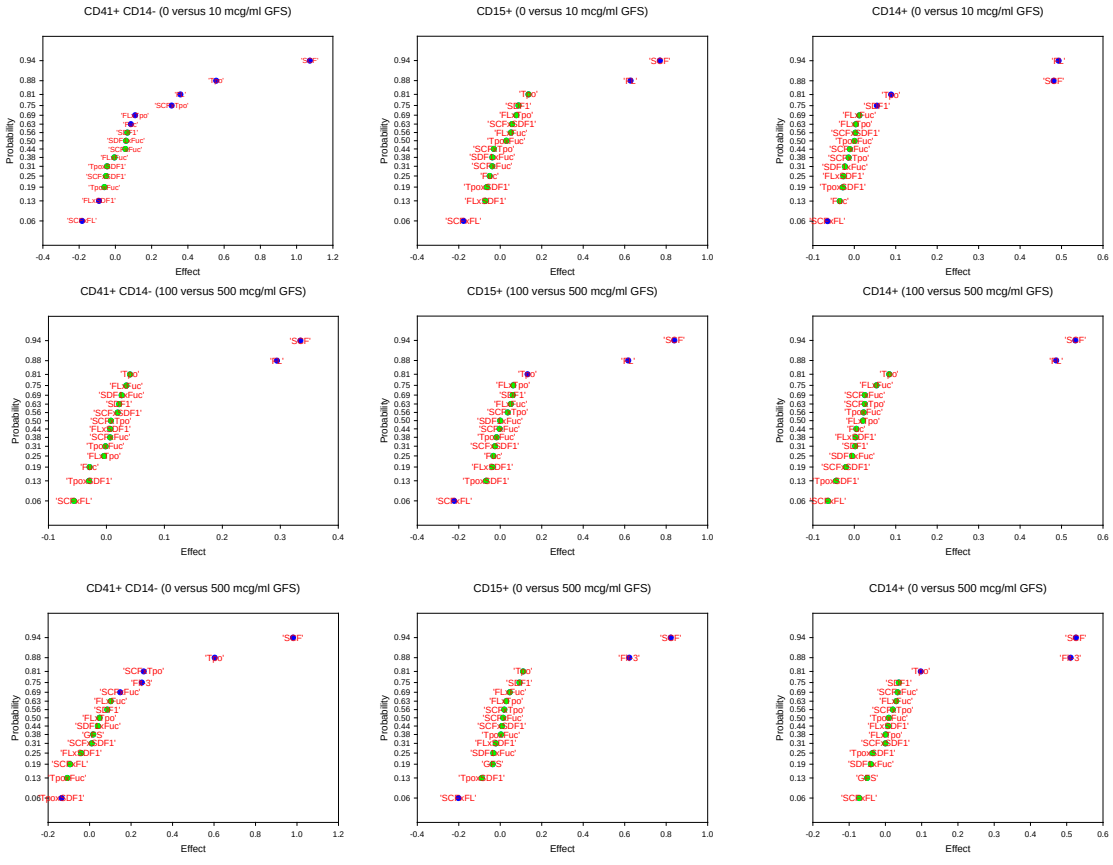


Figure 7.11: Contrast analysis of the effect of various growth factor and GFS (high = 500 µg/mL, low = 100 µg/mL) combinations on the growth of different cell lineages. [(.) p< 0.05].

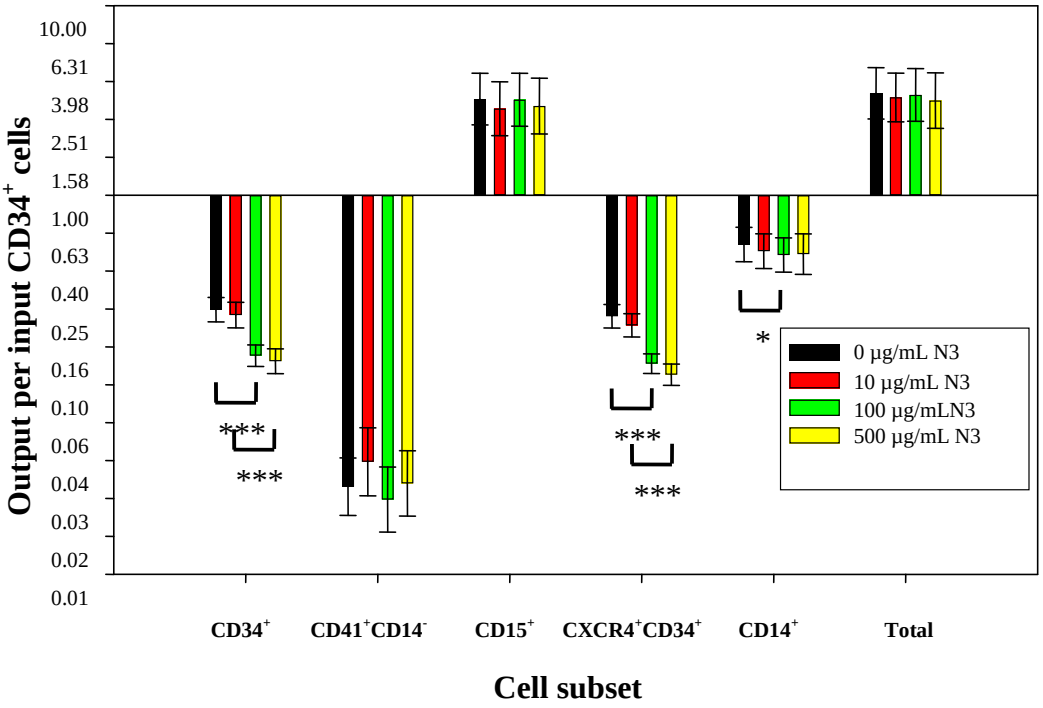


Figure 7.12: Effect of fucoidan (N3) on PB CD34$^+$ cell expansion at 10 days.
*** p< 0.001; * p< 0.05 (paired Students' t-test, n = 16).

At low fucoidan levels (0 versus10 µg/mL), Flt-3 (p = 0.009), SCF (p = 0.016) and fucoidan (p = 0.037) decreased the percentage of CD34$^+$ cells expressing CXCR4. This effect was more marked at high fucoidan levels (100 versus 500 µg/mL, p< 0.001), Figure 7.13.

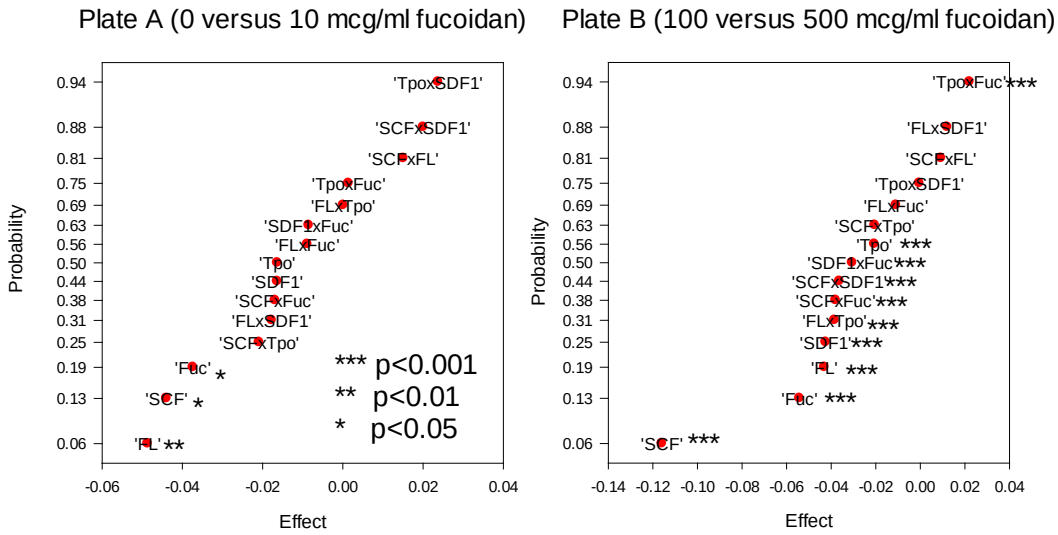


Figure 7.13: Effect of growth factors (0 versus 100 ng/mL) and fucoidan on the percentage of CD34$^+$ cells that are CXCR4$^+$. (Plate A, low fucoidan levels: 0 versus 10 μg/mL; plate B, high fucoidan levels; Fuc = fucoidan, 100 versus 500 μg/mL). Replicate experiments.

At higher fucoidan levels there were a number of second order (two factor) inhibitory effects on CXCR4 expression. A two factor interaction indicates that the combination of factors add to the main single factor effect. For example the inhibitory effect of fucoidan was enhanced when combined with SCF or SDF-1 (p< 0.001), see Figure 7.13.

A full factorial experiment was performed to determine the effect of fucoidan (Plate A: 0 versus 10 μg/mL; Plate B 100 versus 500 μg/mL) on high levels of haemopoietic growth factors (100 versus 1000 ng/mL). The data show that fucoidan down-regulates CXCR4 receptor expression on CD34$^+$ cells and is shown in detail in Appendix-7.

The "low" level of growth factors in this full factorial design was 100 ng/mL. At this concentration the growth factor receptors are saturated, and a maximal growth response occurs, explaining why an increase in any of the growth factors from 100 to 1000 ng/mL did not have a significant effect. The only significant effect was the inhibition of CXCR4 expression on CD34$^+$ cells by fucoidan (0 versus 10 μg/mL) as shown in Figure 7.14.

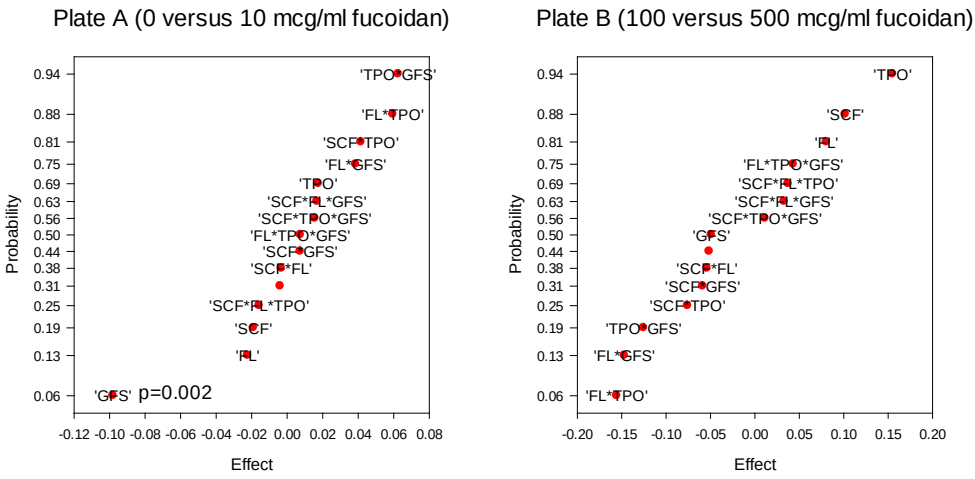


Figure 7.14: Effect of growth factors (100 versus 1000 ng/mL) and fucoidan on the percentage of CD34$^+$ cells that are CXCR4$^+$. (Plate A, low fucoidan levels: 0 versus 10 µg/mL; plate B, high fucoidan levels; 100 versus 500 µg/mL). Replicate experiments.

There is no further inhibition of CXCR4 expression on CD34$^+$ cells at high levels of fucoidan (100 versus 500 µg/mL). Furthermore, the down-regulation of CXCR4 expression appears to be restricted to the CD34$^+$ cell subset, as there was no inhibitory effect on CD34$^-$CXCR4$^+$ cells. There is a modest inhibition of CD34$^+$ cell output by fucoidan (p = 0.03) as shown in Figure 7.15, but no significant effect on the total cell output.

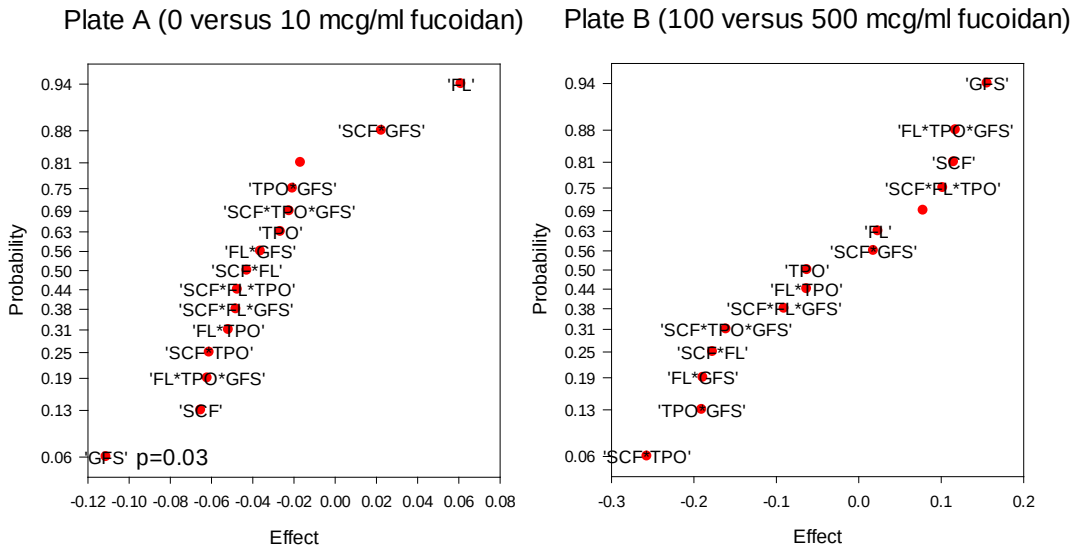


Figure 7.15: Effect of growth factors (100 versus 1000 ng/mL) and fucoidan on the percentage output of CD34$^+$ cells. (Plate A, low fucoidan levels: 0 versus 10 µg/mL; plate B, high fucoidan levels; 100 versus 500 µg/mL). Replicate experiments.

7.4.4 Effect of fucoidan on PB expansion

The growth of PB cells cultured with 0.335 mg/mL of DP-GFS-001 over a period of 5 days with 100 ng/mL of all GFs (G-CSF, SCF, Flt-3 and TPO) was slower compared the growth of the control cells grown without fucoidan using the expansion index. In contrast, the 75% GFS^{TM} effect on the PB expansion was quite the opposite to that of the DP-GFS-001. The growth of the cells was greater that that of the control as seen in Figure 7.16. The cell cultures were analysed after 5 days and the viable cells were gated using the PI exclusion as described before. The bivariate dot-plots are shown in Figure 7.17.

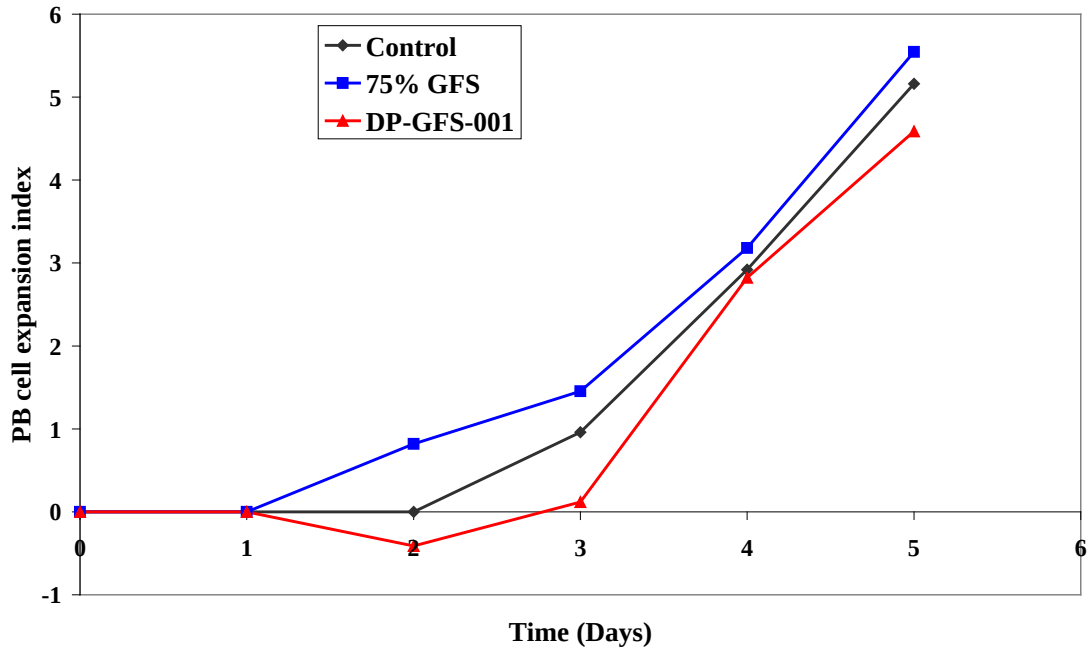


Figure 7.16: PB cell expansion when cells were cultured with either 75% GFS™ or DP-GFS-001 at a concentration of 0.335 mg/mL over a period of 5 days.

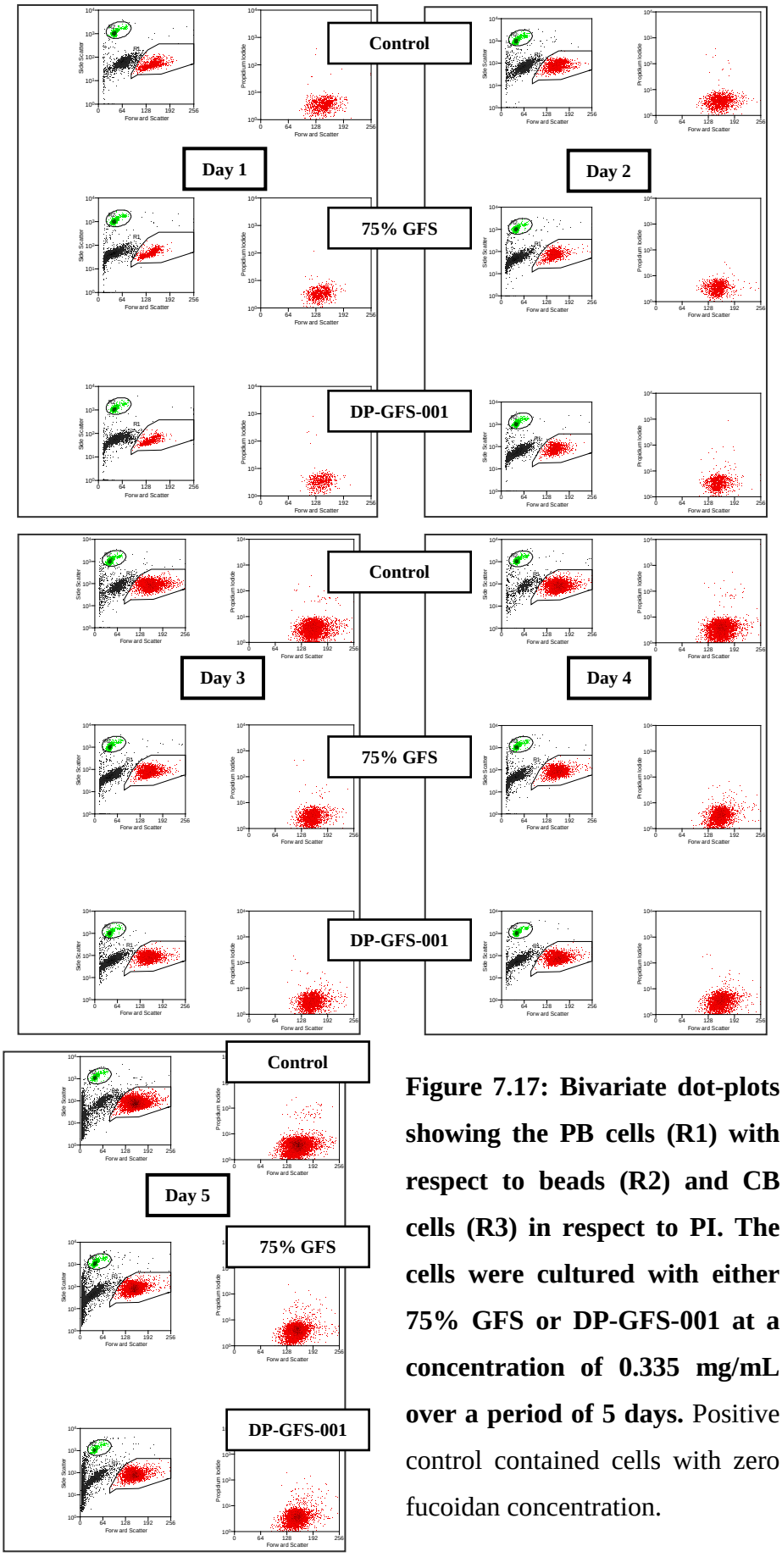


Figure 7.17: Bivariate dot-plots showing the PB cells (R1) with respect to beads (R2) and CB cells (R3) in respect to PI. The cells were cultured with either 75% GFS or DP-GFS-001 at a concentration of 0.335 mg/mL over a period of 5 days. Positive control contained cells with zero fucoidan concentration.

7.4.5 Effect of fucoidan on CB expansion

The growth of CB cells which were cultured with 0.335 mg/mL of either 75% GFS™ or DP-GFS-001 over a period of 5 days with 100 ng/mL of all GFs (G-CSF, SCF, Flt-3 and TPO) was slower when compared to the growth of the control cells (no fucoidan) using the expansion index, shown in Figure 7.18. The cell cultures were analysed after 5 days and the viable cells were gated using the propidium iodide method as described. The bivariate dot-plots are shown in [Figure 7.19].

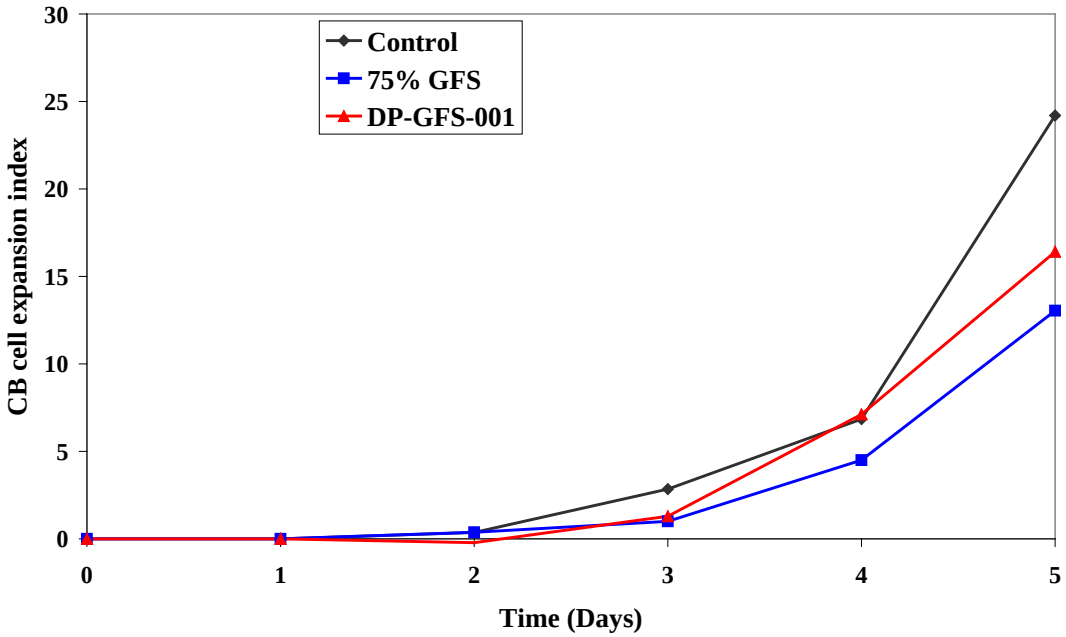


Figure 7.18: CB cell expansion when cells were cultured with either 75% GFS™ or DP-GFS-001 at a concentration of 0.335 mg/mL over a period of 5 days.

The PB cells were cultured with either 75% GFS™ or DP-GFS-001 over a period of 5 days with 100 ng/mL of all GFs (G-CSF, SCF, Flt-3 and TPO) and stained with the early progenitor markers (anti-CD34 and CD38) and CFSE. The cells showed a decrease in the doubling time. The cell growth for the fucoidan-treated cells was slower than the cell growth for the control cultures. The 75% GFS™ slowed the cell cycle more than the DP-GFS-001. Results from one experiment are shown as dot-plot graphs in Figure 7.20.

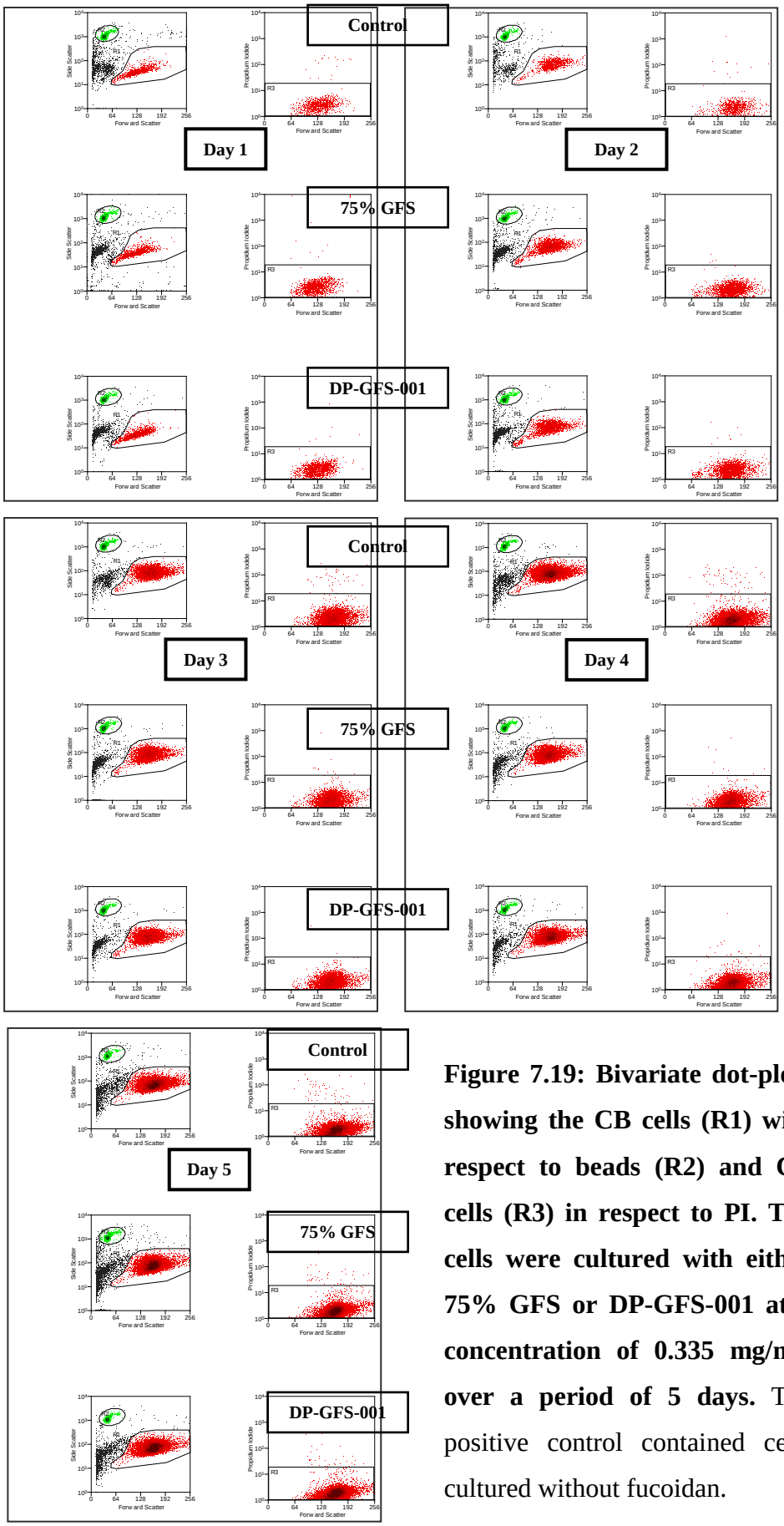
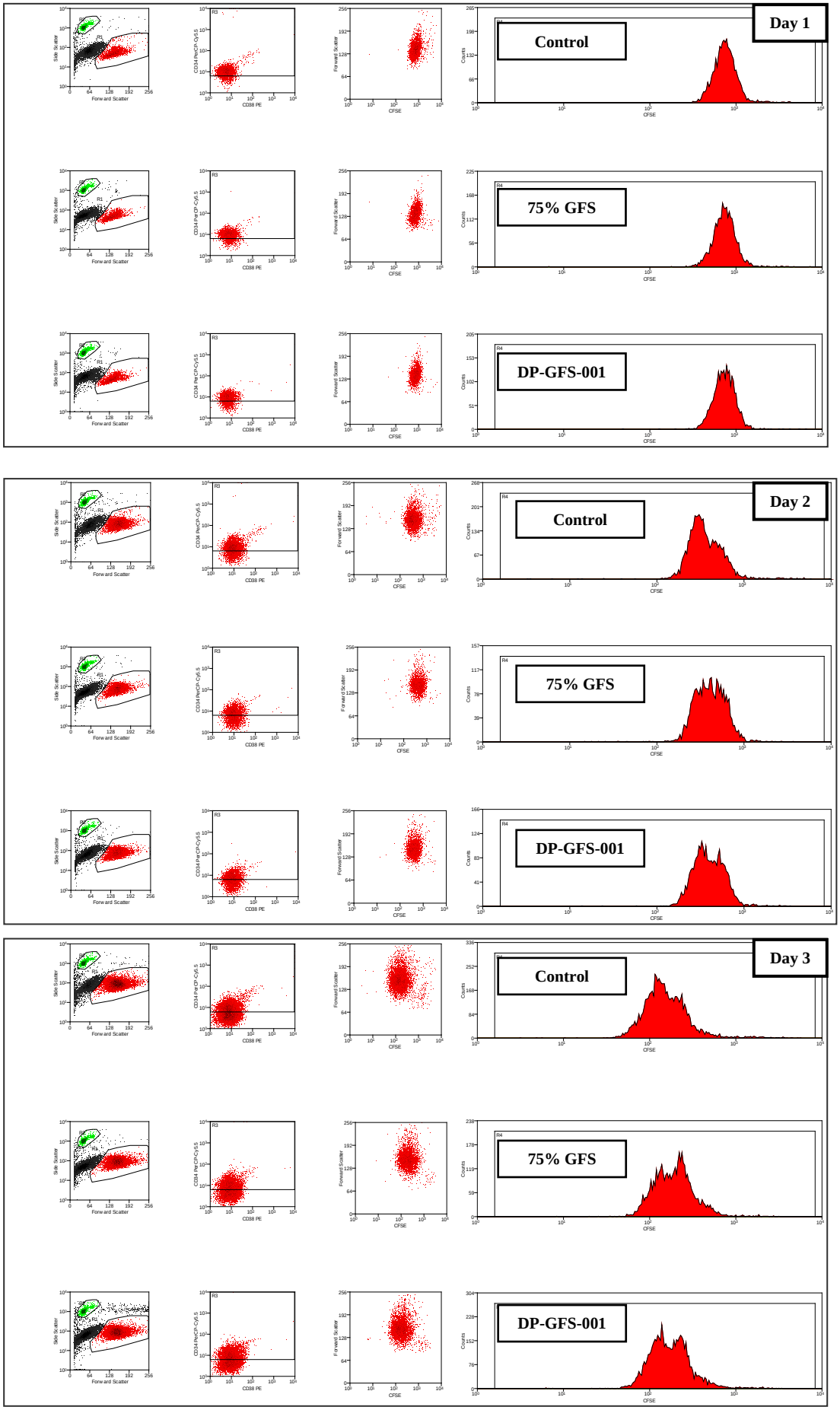


Figure 7.19: Bivariate dot-plots showing the CB cells (R1) with respect to beads (R2) and CB cells (R3) in respect to PI. The cells were cultured with either 75% GFS or DP-GFS-001 at a concentration of 0.335 mg/mL over a period of 5 days. The positive control contained cells cultured without fucoidan.


Day 1
Control
75% GFS
DP-GFS-001
Day 2
Control
75% GFS
DP-GFS-001
Day 3
Control
75% GFS
DP-GFS-001

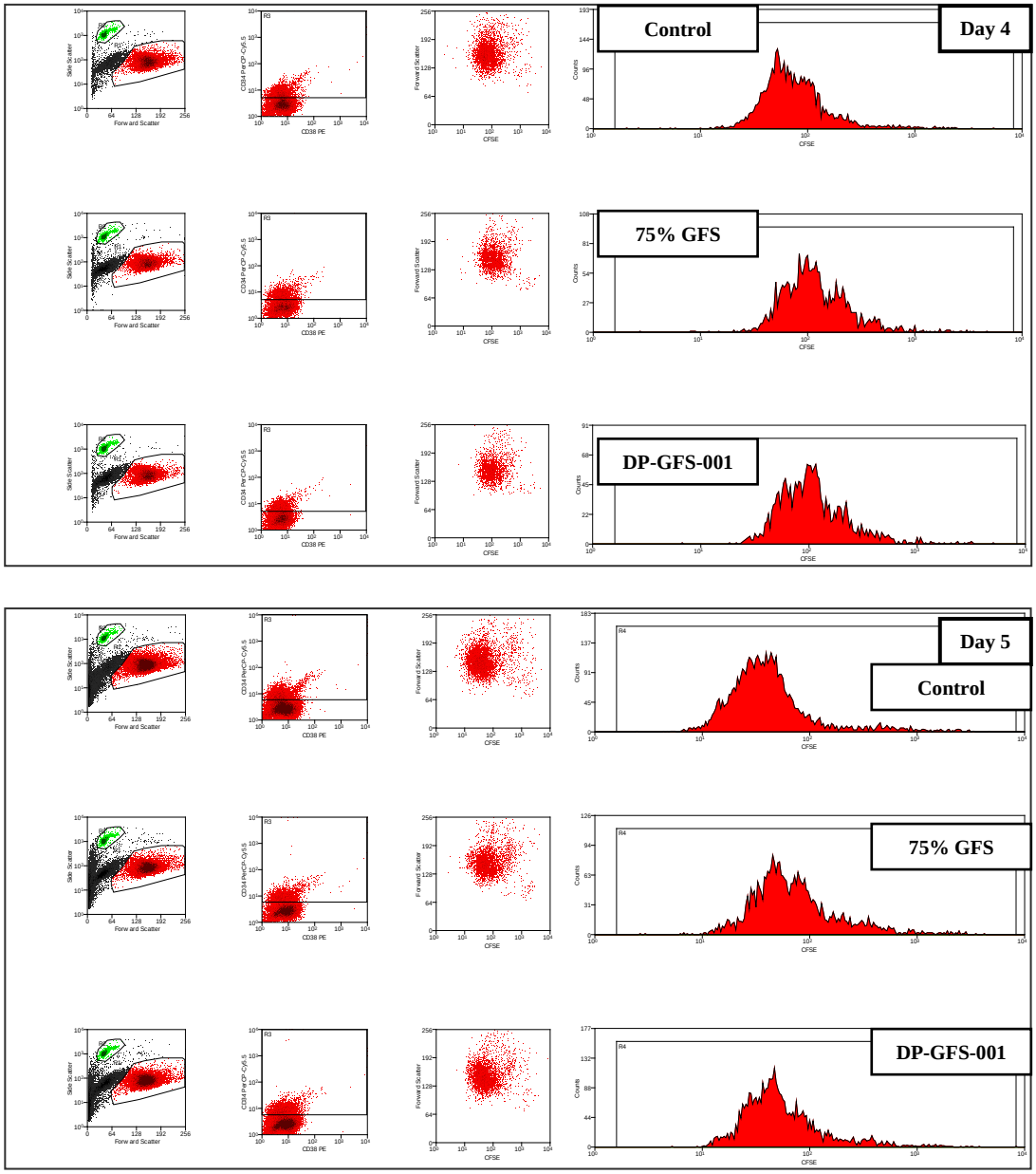


Figure 7.20: Bivariate dot-plots showing the PB cells (R1) with respect to beads (R2). The cells were cultured with either 75% GFS^{TM} or DP-GFS-001 at a concentration of 0.335 mg/mL over a period of 5 days and stained with anti CD34, CD38 and CFSE. Positive control contained cells with zero fucoidan concentration. Number of divisions and slow shifting in the histogram for the cells treated with fucoidans to the left compared to controls can be seen.

Figure 7.21 shows the results for a single experiment where MACS purified $CD34^+$ cells were grown in StemlineTM II medium containing the growth factors SCF, Flt-3, TPO and G-CSF all at 100 ng/mL. The four fucoidan fractions (Table 2.3) were added to separate cultures at 100 μg/mL. The control culture contained no fucoidan.

There was a more marked inhibition of growth of CB $CD34^+$ derived cells by the various fucoidans. The fucoidan fractions in order of increasing inhibition of growth were N3, N2, 75% GFS^{TM}, and N1 (Table 7.7). The inhibition affected total cell count, $CD34^+$ (early progenitors), $CD41^+CD14^-$ (megakaryocytes), $CD15^+$ (granulocyte precursors), $CD14^+$ (monocyte precursors) and $CD34^+CXCR4^+$. The ratio of $CD34^+CXCR4^+/CD34^+$ cells was also reduced by the presence of fucoidan. The relative frequency of the $CD34^+CXCR4^+$ subset with respect to $CD34^+$ cells (the number of $CD34^+CXCR4^+$ divided by the number of $CD34^+$ cells) decreased for all fucoidan preparations when compared to the control.

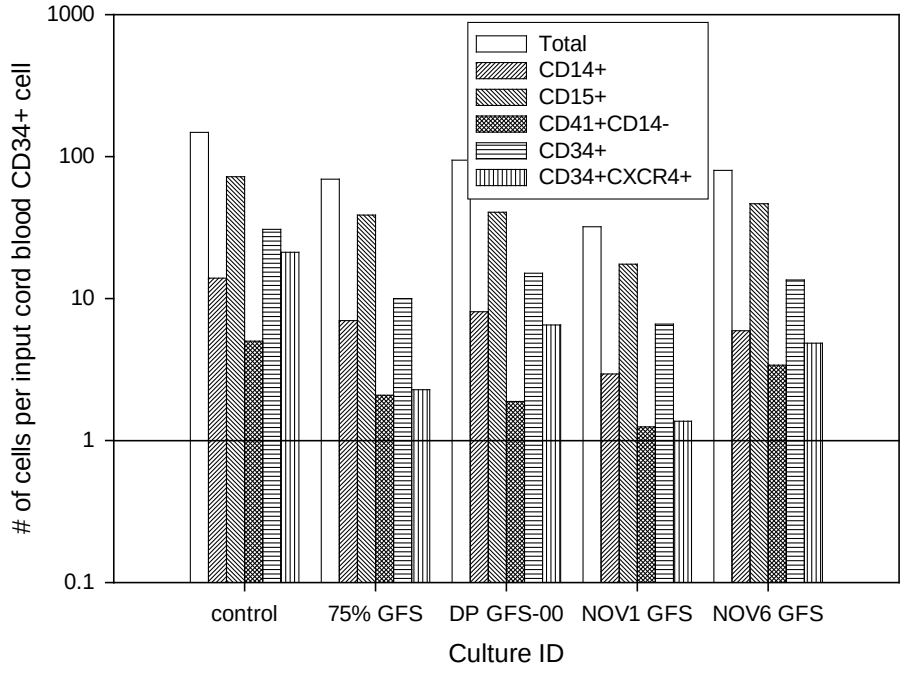
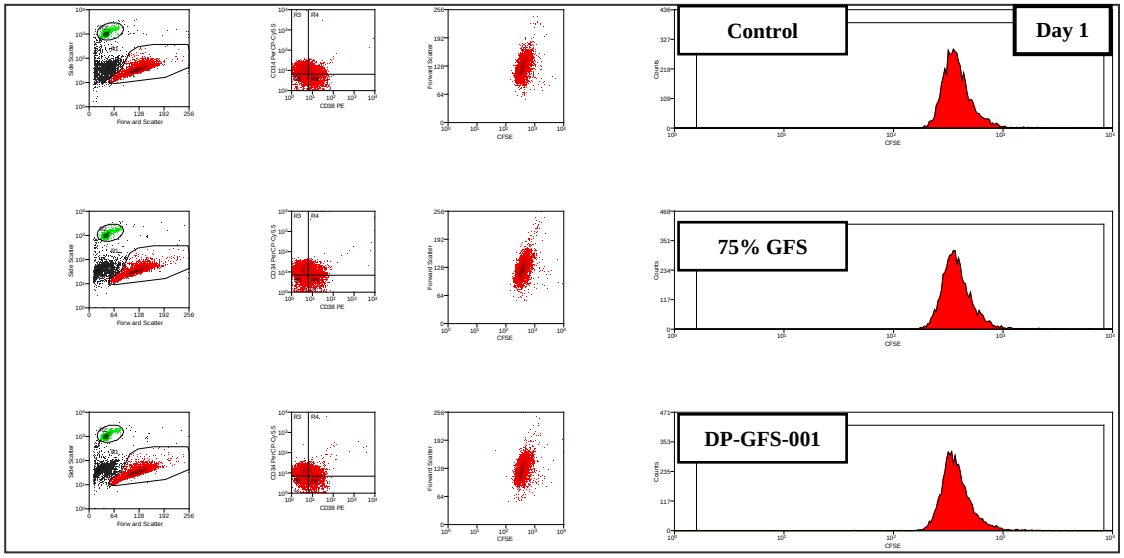


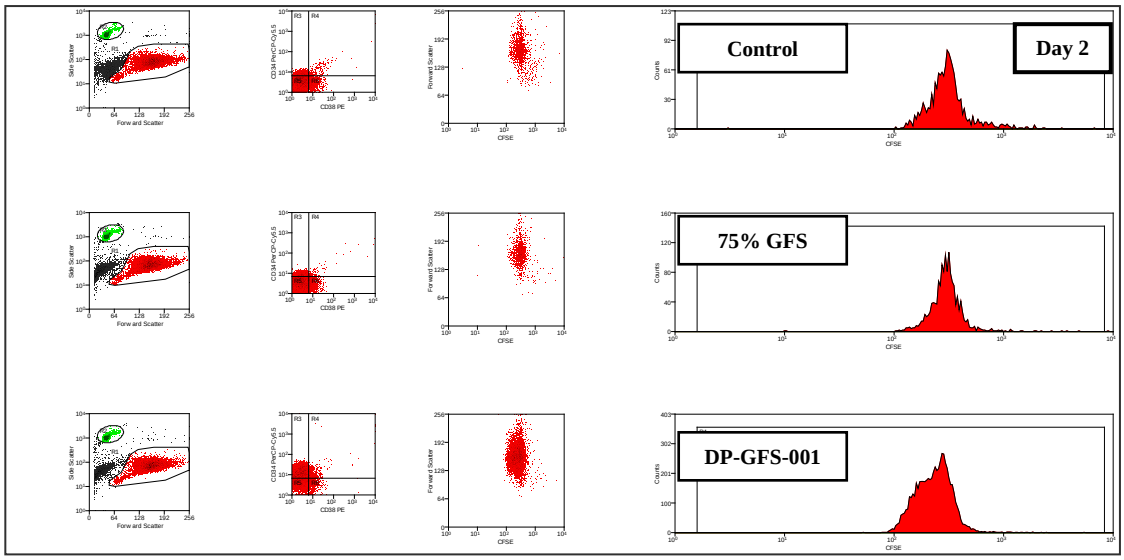
Figure7.21: Effect of the different fucoidan fractions on cord blood $CD34^+$ cell expansion. The test cells were cultured with 0.335 mg/mL of individual fucoidan fractions over a period of 5 days. The positive control cells were cultured without fucoidan.

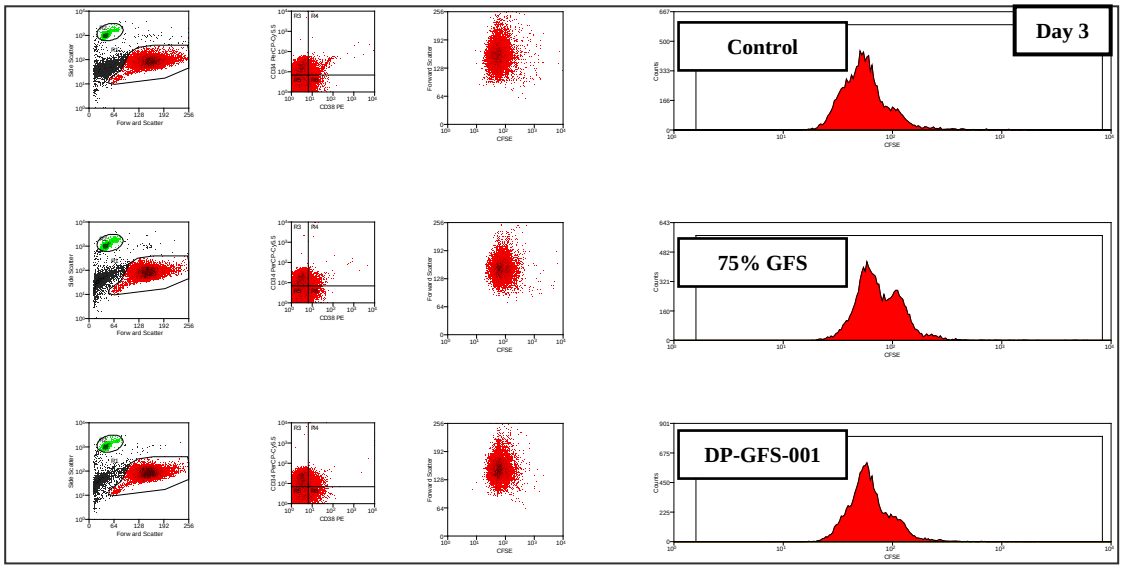
Table 7.7: Effect of fucoidan fractions on the growth of cord blood $CD34^+$ cells

	Control	**75% GFS™**	**N1**	**N2**	**N3**
Total	148.11	69.38	32.03	79.93	94.32
$CD14^+$	13.93	6.99	2.95	5.94	8.09
$CD15^+$	72.16	38.78	17.48	46.60	40.58
$CD41^+CD14^-$	5.02	2.09	1.25	3.40	1.89
$CD34^+$	30.80	10.02	6.63	13.55	15.12
$CD34^+CXCR4^+$	21.23	2.28	1.37	4.86	6.53
$CD34^+CXCR4^+$ /$CD34^+$	0.69	0.23	0.21	0.36	0.43

The CB cells were cultured with either 75% GFS™ or DP-GFS-001 and 100 ng/mL of all GFs (G-CSF, SCF, Flt-3 and TPO). The cells were stained for early progenitor markers (anti CD34 and CD38) and CFSE after a period of 5 days. The fucoidan-treated cells showed a marked decrease in the doubling time compared with the control cultures. The 75% GFS™ slowed the cell cycle more than the DP-GFS-001. Results from one experiment are shown as dot-plot graphs in Figure 7.22.


Control
Day 1
75% GFS
DP-GFS-001


Control
Day 2
75% GFS
DP-GFS-001


Control
Day 3
75% GFS
DP-GFS-001

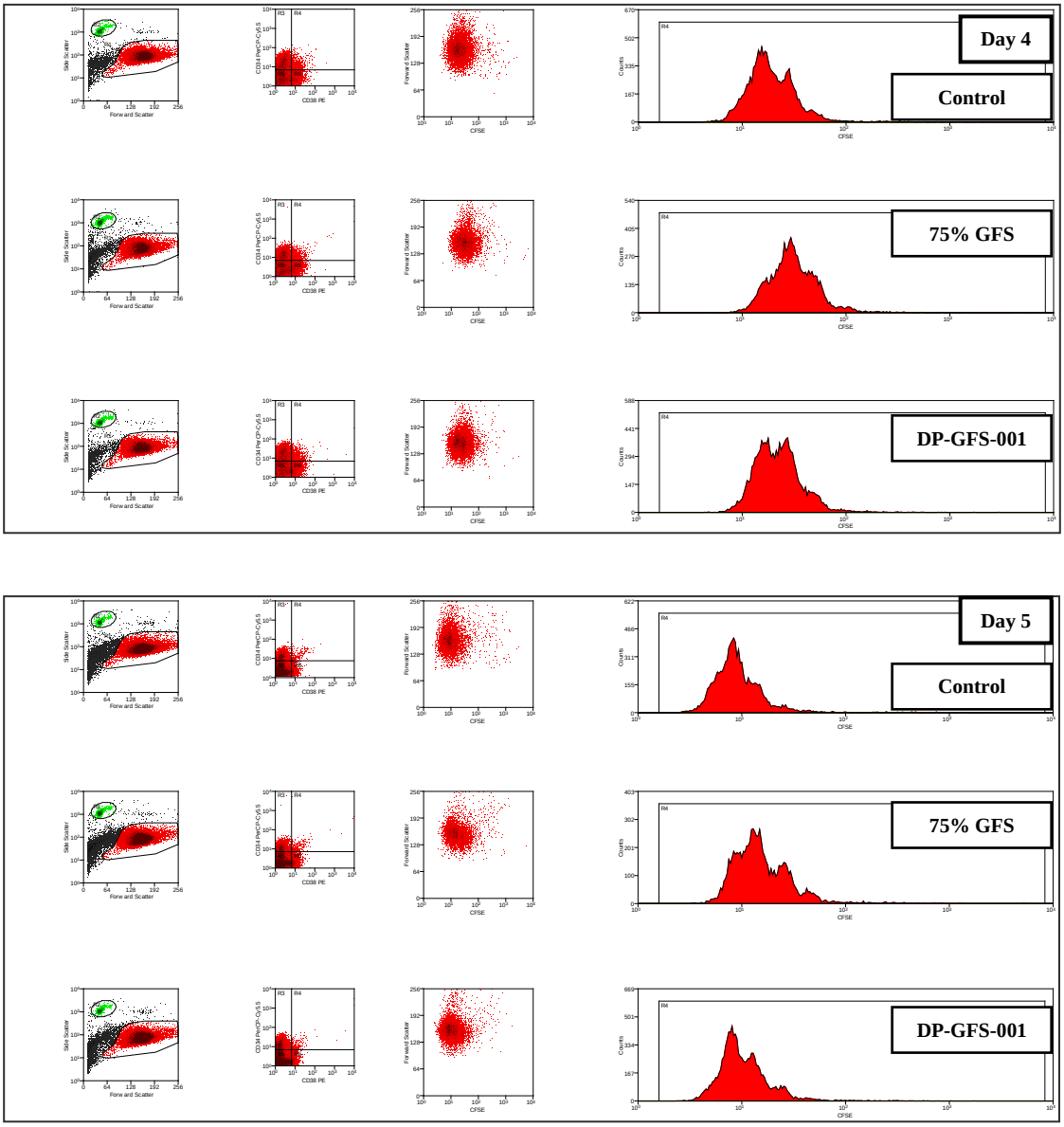


Figure 7.22: Bivariate dot-plots showing the CB cells (R1) with respect to beads (R2). The cells were cultured with either 75% GFS^{TM} or DP-GFS-001 at a concentration of 0.335 mg/mL over a period of 5 days and stained with anti CD34, CD38 and CFSE. Positive control contained cells with zero fucoidan concentration. Number of divisions and slow shifting in the histogram for the cells treated with fucoidans to the left compared to controls can be seen.

7.4.6 Effect of fucoidan on the appearance of cultures

High concentrations of fucoidan have a distinctive effect on the appearance of the cultures; cells aggregate to form a single multi-layered cluster towards the centre of the Petri dish as shown in Figure 7.23. Without fucoidan, cells grow as a single-cell, non-adherent culture with a diffuse distribution across the culture plate. With fucoidan (N3 at 500 μg/mL), there is a formation of dense colonies with occasional spindle shaped cells.

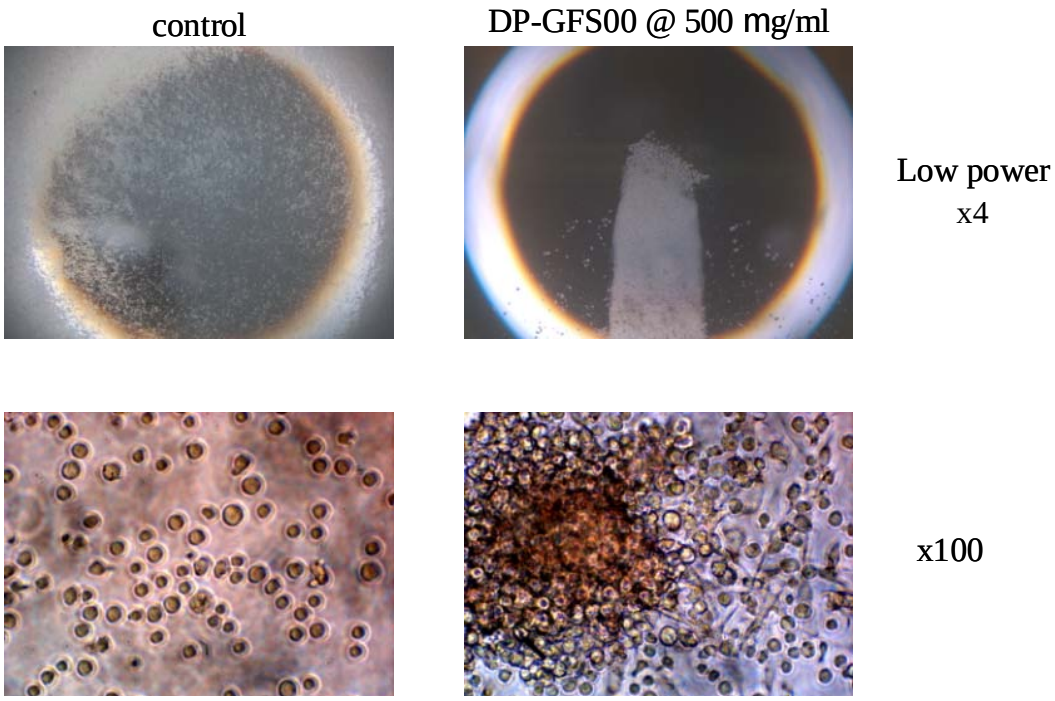


Figure 7.23: Effect of fucoidan on the appearance of cell cultures. All contained G-CSF, SCF, Flt-3 and TPO at 100 ng/mL, with or without N3.

7.4.7 Effect of fucoidan on cell proliferation

Both CB and PB $CD34^+$ cell expansion was inhibited when these cells were incubated with either 75% GFS^{TM} or N3 compared to the control. N3 decreased the expansion slightly, while 75% GFS^{TM} decreased it to almost half after 5 days culture. The doubling time slowed when these cells were incubated with either fucoidan extract. This effect was also studied by evaluating the mean CFSE fluorescent dye in each cell population.

When both CB $CD34^+$ cells and PB $CD34^+$ cells were cultured for 5 days without fucoidan (control) the mean CFSE fluorescence for the cells was 14.91 and 91.13, respectively. The same cells retained more CFSE stain when cultured with the 75% GFS^{TM} where the mean CFSE fluorescence was 22.31 for CD $CD34^+$ cells and 129.75 for PB $CD34^+$ cells. Culturing these cells with DP-GFS-001 did not have a big influence on the mean CFSE fluorescence where it was 13.61 for CB $CD34^+$ cells and 98.38 for PB $CD34^+$ cells.

The percentage increase in the CB $CD34^+$ cells when compared to the control culture (3.1 times) was only 0.7 times when cells were cultured with 75% GFS^{TM} and 2.4 times when cells were cultured with DP-GFS-001 after 5 days culture. The percentage increase in the PB $CD34^+$ cells when compared to the control culture (2.5 times) was only 1.2 times when cells were cultured with 75% GFS^{TM} and 2.7 times when cells were cultured with DP-GFS-001 after 5 days culture.

7.5 Discussion

This study was carried out to investigate the effects of different fucoidan fractions directly applied to stem cells in culture. The work was carried out to examine the potential utility of fucoidan as a stem cell expansion co-factor, given prior research findings. Previous work (Sweeney *et al.*, 2000; Frenette & Weiss, 2000) has demonstrated the pronounced increase in peripheral blood stem cells after i.v administration of fucoidan, and previous work in this study (see chapter 6), demonstrated a marked increase in $CXCR4^{+}CD34^{+}$ cells, and a modest increase in the total number of $CD34^{+}$ cells, in subjects that consumed fucoidan.

There is a clinical need for effective *in vitro* expansion factors, which could be used to maintaining phenotype whilst expanding HSC populations suitable for engraftment. The maintenance or enhancement of CXCR4 levels on $CD34^{+}$ cells is highly desirable, as CXCR4 levels are directly correlated with engraftment success.

Haemopoietic development from blood stem cells requires multiple cytokines that act synergistically. For example, it is now well established that optimal $CD34^{+}$ cell production requires SCF, Flt-3, and TPO. The addition of G-CSF promotes granulocyte development. TPO also promotes megakaryocyte and platelet formation. The chemokine SDF-1 has a variety of actions on blood stem cells, depending on its concentration and the stage of cell development. Fucoidan may have a direct action on haemopoietic cells by binding to selectin receptors, or it may act indirectly by binding to the haemopoietic growth factors or SDF-1.

The experimental approach was to apply a factorial experimental design and analysis to determine the *in vitro* effects of fucoidan and haemopoietic growth factors on human mobilised PB and CB $CD34^{+}$ cell expansion (Case *et al.*, 2001). The response of the culture system was assayed by flow cytometry with enumeration of early blood precursor cells ($CD34^{+}$), megakaryocytes ($CD41^{+}CD14^{-}$), granulocyte precursors ($CD15^{+}$), and monocytes ($CD14^{+}$). The relative expression of the CXCR4 receptor on $CD34^{+}$ cells was also determined. Additional studies examined the effect of fucoidan on human CB $CD34^{+}$ cell expansion, and compared the effect of various fucoidan fractions.

The data suggest that fucoidan has a direct effect on CXCR4 expression, which may modulate the homing of expanded $CD34^+$ cells to BM. Fucoidan appears to have adhesive properties, which are most likely related to selectin-mediated binding of haemopoietic stem cells on to the deposited fucoidan matrix. Selectin signalling may also be responsible for the down regulation of CXCR4 (Hidalgo *et al.,* 2004).

As mentioned earlier, fucoidans have been shown to induce rapid HPC mobilisation in mice (25 mg/kg i.v) (Frenette & Weiss, 2000). The mechanism for mobilisation is quite complex involving SDF-1 gradients, metalloproteases and adhesion molecules. Intravenous infusion of fucoidan in monkeys and mice results in a dramatic increase in serum SDF-1 levels, and perhaps provides the SDF-1 gradient that is required for blood stem cell mobilisation (Sweeney *et al.,* 2002). It has been shown that fucoidan binds SDF-1 via its heparin-binding domain. Thus fucoidan displaces SDF-1 from bone into the periphery, and creates a positive gradient for blood stem cell migration.

A recent publication by Katayama and his colleagues suggested that blood stem cell egress from the BM is regulated by the adrenergic tone innervating the bone and marrow. UDP-galactose ceramide galactosyltransferase-deficient ($Cgt^{-/-}$) mice exhibit aberrant nerve conduction and display virtually no HPC egress from BM following G-CSF or fucoidan administration. Adrenergic tone, osteoblast function, and bone SDF-1 are dysregulated in $Cgt^{-/-}$ mice. Pharmacological or genetic ablation of adrenergic neurotransmission indicates that norepinephrine (NE) signalling controls G-CSF-induced osteoblast suppression, bone SDF-1 down-regulation, and HPC mobilisation. This model suggests that norepinephrine is responsible for osteoblast suppression, leading to lower levels of SDF-1 in bone, and stem cell mobilisation (Katayama *et al.,* 2006). Thus it is possible that both mobilisation mechanisms operate by release of SDF-1 from bone marrow stores. The mechanism for the modulation of sympathetic tone by mobilising agents (G-CSF or fucoidan) remains to be defined.

Previous studies have examined the *in vitro* growth of mouse haemopoietic cells on a fucoidan matrix. Hidalgo and co-workers (Hidalgo *et al.,* 2004) demonstrated that mouse BM myeloid cells bind to fucoidan via L-selectin and the integrin αMβ2 (MAC 1). They were able to show that binding induces secretion of granular serine proteases, thought to mediate the complex process of blood stem cell mobilisation. Serine

proteases degrade VCAM-1, SDF-1 and CXCR4. The negative effect of fucoidan on CXCR4 expression may therefore be related to breakdown of the CXCR4 receptor by serine proteases. Hidalgo and co-workers (2004) also observed the adhesion of human myeloid cells to a fucoidan matrix.

Monomers and polymers of the sugar residue sialyl-Lewis A within fucoidan molecules mediate binding to selectin receptors that are expressed by endothelium, platelets and neutrophils (Ushakova *et al.,* 2005). Selectins have an important role in the initial capture of neutrophils at sites of inflammation.

The recent clinical study using *Undaria* fucoidan (Irhimeh *et al.,* 2005b & Irhimeh *et al.,* 2007 and chapter 6 in this thesis) has demonstrated up-regulation of CXCR4 on peripheral $CD34^+$ cell in patients who had received oral doses of fucoidan. The *in vitro* results were more consistent with a recent clinical study that demonstrates down-regulation of CXCR4 receptors on $CD34^+$ cells in G-CSF or GM-CSF mobilised blood (Dlubek *et al.,* 2006). This inconsistency may be related to the mode of mobilisation (G-CSF versus fucoidan) and the dose of the mobilising agent (oral with low adsorption, versus parenteral). Variation in the fucoidan structure may also affect the expression of CXCR4. In the previous clinical trial (Irhimeh *et al.,* 2005b & Irhimeh *et al.,* 2007) and as described in chapter 6, the 75% GFS^{TM} (impure but with an intact structure) was used, whereas in this study mainly N2 (pure fucoidan lacking acetyl groups) was used.

Different patients normal have different mobilization response when mobilized with G-CSF. It seems that the BM responds to different treatments and in particular mobilization regimen in different levels. The blood samples used in this study as a source for $CD34^+$ cells were from a small number of patients. The number of patients used was minimal in-order to minimize the inter-patient variability in responses to the active treatment. The inter-patient variability due to the small number of patients used was minimal in this work.

It has been shown that circulating mobilised HPC in the blood are quiescent in phase G_0/G_1 of the cell cycle, while a portion of their counterparts in the BM actively divide. The cycling HPC have impaired adhesive and chemotactic properties *in vitro* when

compared to HPC in G_0/G_1 phase, and have impaired homing *in vivo* (Winkler & Levesque, 2006). Finding ways to send HPC into quiescence remain to be identified, but culturing $CD34^+$ cells with fucoidan slowed down the cell cycle significantly. It is possible that fucoidan as an extrinsic factor affects the intrinsic pathways of HPC.

Fucoidan may have value as an artificial BM matrix, particularly if it is combined with other matrix molecules thought to be part of the so-called blood stem cell niche. Its effect on CXCR4 expression may be of significance in the development of *ex vivo* expansion protocols for generation of transplantable haemopoietic progenitors. It may be possible to improve engraftment potential of cytokine-mediated expanded blood stem cells by expression of cell receptors that facilitate stem cell homing. One may speculate that homing and more rapid haemopoietic reconstitution will be facilitated by a phenotype that correlates with the phenotype generated by mobilisation of blood stem cells (*e.g.*, low CXCR4 expression on $CD34^+$ cells (Dlubek *et al.*, 2006)). The molecular players in this complex process are thought to be selectins, integrins (β2, α-4/VCAM-1 α-6/laminin interactions), SDF-1/CXCR4 and hyaluronic acid/CD44 (Scott *et al.*, 2003; Avigdor *et al.*, 2004; Qian *et al.*, 2006).

The demonstration of a substance that selectively reduces that expression of CXCR4 on $CD34^+$ cells and provides a matrix for adherent blood progenitor culture poses opportunities for the future improvement of *ex vivo* manipulated grafts. The *in vitro* action of fucoidan using human blood stem cells parallels that found in the mouse model. Future work should test whether adhesion of human marrow cells to a fucoidan matrix is via L-selectin and the human analogue of the integrin αMβ2. It may be of value to explore the transcription profile of fucoidan-stimulated cells and to learn more about the secondary effects of selectin or integrin receptor engagement. Further studies should aim to recapitulate a BM microenvironment using various combinations of extra-cellular matrix molecules and cell adhesion molecules. Animal blood stem cell transplantation models would determine if the BM matrix preparation could replace a stromal feeder layer. Human studies would follow if it were established that the matrix was suitable for blood stem cell maintenance or expansion.

7.6 Conclusion

In this chapter haemopoietic cells (KG1a, PB $CD34^+$ cells and CB $CD34^+$ cells) were successfully cultured in the presence of beads as an internal standard for calculating the absolute cell count. This technique makes the assay procedure easier and much more accurate. Fucoidans (75% GFS^{TM}, N1, N2 and N3) were all non-toxic for haemopoietic cells when used in concentrations less than 0.08 mg/mL although when concentrations were greater than 2.5 mg/mL the growth was completely inhibited. Fucoidan was found to have a direct effect on CXCR4 expression. This plays an important role in modulating the HSC trafficking. Fucoidan has some adhesive properties which could be related to the selectins expressed on HSC.

Different fucoidans (75% GFS^{TM}, N1, N2 and N3) have different modes of action on CXCR4, SDF-1 and the mobilisation of HSC. The intact fucoidan structure would be more suitable for mobilisation studies, as it has the ability to slow the cell cycle for HSC and may have value as an artificial BM matrix.

CHAPTER EIGHT

GENERAL DISCUSSION AND CONCLUSIONS

This study encompassed investigations of an *Undaria pinnatifida* derived fucoidan in both clinical (oral dose) and *in vitro* situations. A novel assay method was developed and demonstrated serum uptake of fucoidan at low levels. Clinical studies indicated that the ingestion of fucoidan, or of whole seaweed, was non-toxic over the study period. Significant changes occurred in lipid profile, IFN-γ, and in the number of $CD34^+CXCR4^+$ cells. Additional significant changes were noted in the numbers of $CD4^+$ and $CD8^+$ cells. There were also significant increases in clotting time, although this remained within normal clinical parameters.

Fucoidan is a naturally occurring, water soluble component of brown macroalgae, and is regularly ingested as part of the seaweed based dietary items in the Asian diet. Fucoidan derived from *Fucus vesiculosus* has been used for decades as a research tool, as it is a well known selectin inhibitor that blocks cellular adhesion.

The fucoidan used in this study is not the same as those used in most reported literature, being derived from the common edible seaweed “wakame” (*Undaria pinnatifida*) by a neutral cold process and ultrafiltration which leaves the material with its native structure with the major fraction having a high moelcular weight of about one million kD (Marinova Pty. Ltd.). The most commonly used “research fucoidan” is probably *Fucus* fucoidan (Sigma Pty. Ltd.), which is obtained by acid hydrolysis and ethanol precipitation, and has a heterogeneous lower molecular weight profile (Mabeau *et al.*, 1990). Other studies, which have been mentioned in chapter one such as Frenette studies, have used individualised preparations of fucoidan. Comparative studies of fucoidans from different species (prepared by the same methods) indicate that there are differences in activity (Cumashi *et al.*, 2007).

The *Undaria pinnatifida* fucoidan used in these clinical studies has proven to be safe when ingested for 12 consecutive days in capsules containing 3 g as either 10%

fucoidan or 75% fucoidan. There were no side effects associated with ingestion of the fucoidan. The volunteers were generally happy with the taste and few showed concern about the relatively large size of the capsules.

Ingestion of the capsules did not alter the LFT or U+E tests of the volunteers. All liver enzymes and blood electrolytes remained normal and the blood glucose level did not change. The levels of metabolic by-products such as bilirubin were all normal.

On the other hand, the lipid profile in volunteers treated with the 75% fucoidan did change. Blood levels of Chol and Trig decreased significantly after ingestion of the active treatment, while the HDL increased and the LDL decreased, thus reflecting the changes in Chol and Trig. The volunteers were not kept on a controlled diet during the course of the study, but were asked not to consume seafood, seaweed-containing products or large quantities of alcohol. Had the diet been more controlled, then the changes in the lipid profile may have been more pronounced. The mechanism by which the fucoidan regulates the lipid profile is yet to be identified. However, the large size of the fucoidan molecule may interfere with lipid absorption in the intestine and the secretion of bile products. Similar bile binding by seaweed soluble fibres has been demonstrated (Wei *et al.*, 2001).

Nitric oxide level in the plasma decreased non-significantly after ingestion of the active treatment (75% fucoidan). This decrease was associated with a decrease in the insulin level in a time-dependant manner. NO is believed to be partly controlled via an insulin activated NOS. Any changes in the blood insulin level may affect this enzyme and consequently affect the NO level. On the other hand, NOS-2 (also called iNOS) can be induced by IFN-γ and other cytokines such as IL-12 (Chesler *et al.*, 2002).

In this study, ingestion of fucoidan increased the level of IFN-γ in the circulation but there was no change in the levels of IL-12. The increase in IFN-γ may have been expected to trigger or activate the NOS-2, making more NO. However, this was not the case in this study, when the NO level went down. The direct relationship between both NOS-2 and IFN-γ could have been over ridden by the NO scavenging effects of the large, highly charged molecular structure of the fucoidan.

The fucoidan had an effect on haemopoiesis, and may also have affected erythropoiesis, which would then alter the blood profile. However, in this study, the profiles for the volunteers treated with any of the active treatments, or the placebo group, remained unchanged. Parameters including RCC, Hct, Hb, MCV, MCH and RDW did not change over the course of 12 days of ingestion of fucoidan (10% or 75%).

Fucoidan ingestion increased the pro-inflammatory, "anti-cancer" cytokine IFN-γ in this study, and there is other evidence that fucoidans have immuno-regulatory and anti-tumour properties. *Undaria* fucoidan is proven to have anti-viral effects via the inhibition of receptor binding (Thompson & Dragar, 2004). The observed increase in IFN-γ cytokine may have been expected to modulate inflammatory processes, thus affecting ESR readings. In addition, fibrinogen may be affected by fucoidan, which has been reported to be a potent anticoagulant activator. The ESR tests were used as a non-direct way to detect the high levels of fibrinogen which cause rouleaux formation and faster sedimentation of the RBC. However, in this study all the groups' ESR levels remained unchanged. This suggests that the anticoagulant activity of fucoidan is not through fibrinogen itself. This is further evidenced by the *in vitro* and *in vivo* studies reported here.

Fucoidan is well known to be an *in vitro*, and *in vivo* anticoagulant when administered i.p or i.v (Berteau & Mulloy, 2003). Whilst the material used in this study was a high molecular weight material, this property correlated with previously known effects. The *in vitro* tests in this present study showed that fucoidan from *Undaria pinnatifida* is a potent anticoagulant that can prolong aPTT, TT and PT while it can shorten the AT-III. The basic structure of the fucoidan being large, branched and highly sulphated and acetylated may be the factor behind those changes in coagulation tests. According to Cumashi group findings, two structural differences in the fucoidan may contribute to the absence of the anticoagulant activity of *Cladosiphon okamuranus* seaweed that they tested. The low amount of sulphate in its polysaccharide backbone and the presence of vicinal 2,3-branching point formed by 2-O-α-D-glucuronyl substituents (Cumashi *et al.*, 2007).

At the same time, *in vitro* tests on volunteers taking oral doses of fucoidan proved that it has the ability to prolong the aPTT and PT but shortens the TT and AT-III at the same time. The increase in the aPTT and PT was expected and reflects the findings reported in the literature. The measured changes in these anticoagulation tests are significant yet clinically not of value. This does not mean that fucoidan cannot be used as an anticoagulant molecule *in vivo*, because the amount absorbed after oral ingestion is similar to that of oral heparin, *i.e.* equivalent to about 1% of the ingested amount. This means that if the bio-availability of fucoidan can be increased by either increasing its intestinal uptake or by changing the route of delivery (*i.e.* intravenous or intraperitoneal routes) then it may be possible to see greater effects on haemostasis and on other processes in the body (*i.e.* mobilisation of HSC).

The level of the fucoidan in the plasma that was detected in the clinical trials was measured after developing a new competitive ELISA assay. In this assay an antibody 1B1 was used. The 1B1 is not specific for fucoidan but has the ability to bind with it as was shown in this study. The Ab was developed against sulphated polysaccharides such as heparin and chondroitin sulphate. Because fucoidan has sulphated polysaccharides similar to the heparin structure, it was possible to use this Ab to assay for the presence of this seaweed-derived molecule in human plasma. The fucoidan level detected in the plasma was very low compared to the amount taken orally (<1%), which made detection more difficult. In addition, the fucoidan taken up in its intact form in the intestine either by a process of endocytosis or by some other means (*i.e.* active transport or as catabolites) may not be detected by the Ab because all is not free or available.

The fucoidan has the ability to bind to epithelial cells and membranes and is a selectin inhibitor. Part of the ingested fucoidan may adhere to the intestinal walls (from which a fraction will be absorbed and the rest will activate the white blood cells residing in the intestinal wall). Another portion of the absorbed fucoidan may adhere to the walls of blood vessels, while the free fucoidan may bind to many proteins (*i.e.* transport proteins, enzymes, cytokines, etc.). Free fucoidan in the circulation will be therefore a small fraction of the absorbed fucoidan.

Since the level of free fucoidan in the plasma detected in this study was relatively low, it was not expected to see big effects, especially on the rare circulating $CD34^+$ cells, which comprise only a tiny percentage of all white blood cells (< 1%). However, a first indication that fucoidan does have effects on the process of cell formation came from the clinical screening tests performed on the volunteers who ingested the fucoidan. The WCC and the lymphocyte count went down significantly after 12 days of ingestion of the 75% fucoidan. This suggested that the fucoidan directly or indirectly alters the process of cell formation and especially of the haemopoiesis in BM. The second indication arose following FACS analysis of circulating $CD4^+$ and $CD8^+$ cells. Cell counts of both went down after the oral intake of fucoidan (75%) and were accompanied by a decrease in $CD4^+CXCR4^+$ and $CD8^+CXCR4^+$ cells.

The most important evidence came through FACS analysis of the $CD34^+$ cells. This rare population of cells in the circulation represents early progenitor cells which are capable of producing all of the haemopoietic lineages. After ingestion for 12 days of 75% fucoidan, the level of these progenitor cells increased in the circulation. This increase was not sufficient as a source for $CD34^+$ cell collection for transplantation. However, when this population was tested for the mobilisation receptor (CXCR4) it was observed that there was a 50% increase in the $CD34^+CXCR4^+$ cells in the circulation. This indicates that orally delivered fucoidan could play an important role in mobilising stem cells for the purpose of cell transplantation, as the expression of CXCR4 is directly correlated with the success of engraftment (Winkler & Levesque, 2006).

Does an increase in the available plasma fucoidan cause a further increase in the number of $CD34^+$ cells? Does the fucoidan act directly on the CD34 cells to increase the expression of CXCR4? This posed the next set of questions. As discussed previously, the level of fucoidan in the plasma could not be increased without altering its structure (*i.e.* a smaller size) so that it could be more readily absorbed, or by delivering it i.v or i.p. Because of this challenge, the effects of high doses of fucoidan on $CD34^+$ cells were continued in the laboratory. The results are outlined in chapter seven.

Although more control over the cells and the fucoidan concentration and structure was achieved in the laboratory, other factors became of concern. The $CD34^+$ cells are out of their own microenvironmental niches and are cultured in a container lacking many very specific and important influences. Nevertheless, culturing cells *in vitro*, especially $CD34^+$ cells, is a standard and successful practice.

Cells cultured with fucoidan *in vitro* in the presence of different cytokines to enhance the expansion introduced a new challenge. If any change occurred, then what was the effective agent? Was it the fucoidan or one of the cytokines or a combination of factors? This problem instigated a new approach in designing a factorial design for these experiments.

$CD34^+$ cells from two sources, peripheral blood and umbilical cord blood, were cultured in the presence of the different fucoidan extracts and with different cytokines and growth factors at different concentrations. The different fucoidans which were tested were made through different extraction processes, so they varied in their chemical structure. One was highly purified but not sulphated; another was sulphated but was not pure. One was sulphated and acetylated and still another was not acetylated. Would the impurities have any effect on the cells and would the sulphate and acetyl groups have an effect also?

Tests were conducted using a cell line (KG1a) which is similar in its properties to $CD34^+$ cells. The impure fucoidan showed no greater toxicity than the pure fucoidan extracts. When any of the four different fucoidan extracts was tested on the KG1a cells the cells did not grow properly after the concentration exceeded 1 mg/mL. Thus the valuable $CD34^+$ cells obtained from either donors or patients should be cultured with fucoidan concentrations lower than 1 mg/mL.

It was important to make sure that the FACS analysis used for counts of different cell subsets was sufficiently accurate to give reproducible data. Microspheric beads as an internal control were added to the cultures. These were shown not to have any sort of toxicity.

After studying the effects of the different experimental factors on the KG1a cell line, tests using $CD34^+$ cells were begun. A certain fucoidan extract (N3), highly purified, and highly sulphated as it is in the native fucoidan but lacking acetyl groups, was used in these *in vitro* factorial design experiments.

Culturing $CD34^+$ cells sourced from either PB or CB with saturating levels of growth factors (1000 ng/mL) and with N3 did not show an increase in the cell count but rather a decrease. The level of the $CD34^+CXCR4^+$ cells decreased at the same time in contrast to the results found in the clinical trial. This indicated that either the interaction of the cytokines at very high level with the fucoidan inhibited the cell growth or the fucoidan extract itself, without the acetyl groups, contributed to the inhibition, or indeed through both factors. Further factors not yet identified could also be responsible and the mechanism of action of the fucoidan *in vitro* may be different to its mechanism of action *in vivo*.

This study has also indicated that the N3 fucoidan has no effect on the total expansion of the $CD34^+$ cells. All other subsets which were analysed by FACS for an estimate of cell growth in the presence of N3 fucoidan also did not change. These subsets represented the megakaryocyte, granulocyte and monocytes lineages.

An important observation was made when $CD34^+$ cell division was estimated using the CFSE stain. The N3 fucoidan significantly slowed cell division, when more cells were found in the first and second divisions and fewer cells found in the last division (*i.e.* fifth division) compared to the control. The whole cell cycle was found to be slower in the fucoidan-treated cells, which retained more CFSE stain over the cell culture time span. This could mean that fucoidan has the ability to send some of these cells into quiescence and slow down the cell cycle, probably through interacting with cell surface receptors. It may be of value to explore the transcription profile of fucoidan-stimulated cells and to learn more about the secondary effects of selectin or integrin receptor engagement. Extended studies would aim to recapitulate a BM microenvironment using various combinations of extra-cellular matrix molecules and cell adhesion molecules. Animal blood stem cell transplantation models would determine if the BM matrix preparation could replace a stromal feeder layer. Human studies would follow if it were established that the matrix was suitable for blood stem cell maintenance or expansion.

This work concluded that fucoidan is a potential drug to treat different blood disorders. It has the ability to lower blood lipids and the market for such natural therapy is huge. The fucoidan has also anticoagulant activity which is stronger than heparin when tested *in vitro*. The medical field is in need for a heparin substitute which is not animally derived. However, oral intake of fucoidan is not the best choice and the route of administration to increase the fucoidan bio-availability remains to be determined.

Future work should test whether adhesion of human marrow cells to a fucoidan matrix is via L-selectin and the human analogue of the integrin αMβ2. It may be of value to explore the transcription profile of fucoidan-stimulated cells and to learn more about the secondary effects of selectin or integrin receptor engagement. Further studies should aim to recapitulate a BM microenvironment using various combinations of extra-cellular matrix molecules and cell adhesion molecules. Animal blood stem cell transplantation models would determine if the BM matrix preparation could replace a stromal feeder layer. Human studies would follow if it were established that the matrix was suitable for blood stem cell maintenance or expansion.

Further clinical studies are needed to test the synergetic effect of fucoidan with G-CSF and other cytokine therapies. Thus, the development of an i.v form of fucoidan is extremely important for different clinical applications. The development of an i.v form of fucoidan is a challenging project that if performed will help answer many questions and may lead to novel therapies.

This thesis has now established the methodology in answer to the questions set at the beginning of the study. Treating people with fucoidan for the study period of 12 days is safe and no side effects are associated with the ingestion of moderate quantities of 75% fucoidan. There are almost no changes in the pathology tests performed on volunteers who ingested the fucoidan.

A new antibody assay has been developed for the detection of the fucoidan in plasma and another spectrophotometric assay to detect fucoidan in urine was also developed. The fucoidan can be used as a lipid profile modifying agent but more controlled diet studies need to be performed. It is also possible to use the fucoidan as an anticoagulant

but new strategies to deliver a higher concentration of fucoidan need to be developed. Fucoidan increases the percentage of CD34 cells expressing CXCR4 when used *in vivo*, which may be useful in a clinical setting. The non-acetylated fucoidan can be used to slow the stem cell cycle *in vitro*.

Finally, developing a new fucoidan extract which is both acetylated and sulphated and which can be delivered i.v or i.p may be useful in the treatment of defects of coagulation and in the mobilisation of stem cells.

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APPENDICES

Appendix-1: Volunteer information sheet (Sample)

ROYAL HOBART HOSPITAL

UNIVERSITY OF TASMANIA
School of Medicine

Volunteer Information Sheet

Study Title: Effects of natural seaweed extract (GFS^{TM}) on mobilisation of human bone marrow stem cells and immune system activation.

Principal investigators: Mohammad Irhimeh and Ray Lowenthal

Introduction:
You are being invited to take part in a research study to evaluate the effect of a natural supplement on human stem cell mobilisation and activation of the immune system. The supplement is extracted from dried seaweed that has been harvested from the east coast of Tasmania, and up to this point has not been available in Australia. This will be one of the first trials of this supplement on human stem cell mobilisation and immune system activation. Nevertheless, this seaweed is currently used in another study testing its effect in advanced solid tumour patients in Royal Hobart Hospital.
This seaweed has been part of normal diets all around the world for many years. Investigations on the effect of seaweed in women with breast cancer are currently being performed in the United States and elsewhere. Early work in laboratory animals suggests that the supplement can enhance the mobilisation of stem cells and increase the activity of the immune system. All research done on fucoidan (seaweed extract) up to date shows that it is safe, non-allergenic and it has no harmful effects on any bodily function or organ.

Taking part in this study is entirely voluntary and in order to give informed consent, it is important that you read the following information and understand the procedures, benefits and risks involved in participating in this study.

Please take time to read the following information carefully and also listen to the explanation given by the investigator. Feel free to discuss this with your family and friends and ask questions relating to any queries. If you wish to participate you will be asked to sign the attached informed consent form and will be given a copy to keep as a record. You may withdraw from the study at any time.

The purpose of the study:
The purpose of this study is to evaluate the effect of seaweed grown in Tasmania, which has the scientific name *Undaria pinnatifida*, on bone marrow stem cell mobilisation and the activity of human immune system. Information about your bone marrow, blood and immune system response will be collected to help determine the benefits of this seaweed. 200 participants will be enrolled in this study.

Study plan:
You will commence taking three capsules three times daily for a total of 12 days. There will be three groups of volunteers, two active treatment groups and a placebo group. The active treatment groups will receive the seaweed extract capsules (10% or 75%) fucoidan, where the placebo group will receive capsules that contain guar gum dietary fibre (a white powder which constitutes of plant fibres). You will not be informed to which group you are assigned.

Study procedures:
Pre study tests:
After you have given your consent to participate in this study, a blood sample and urine sample may be collected.
It is important that you inform us about any prescription and/or over-the-counter drugs, herbal preparations and nutritional supplements you are taking.

Study capsules:
You will be given enough capsules for the period of the study. These capsules will be dispensed through the RHH pharmacy or the investigator. At the end of the study if you have any unused capsules, these should be returned to the investigators or RHH pharmacy. There are three types of study capsules:

- Guar gum dietary fibres capsules, placebo or no treatment
- 10% fucoidan capsules
- 75% fucoidan capsules

Ongoing study tests:
While you are taking study capsules, you will return to the hospital or clinical school for blood and/or urine collection. You will come to the hospital after 4, 8 and 12 days of full dose capsule intake or according to the schedule given by the investigator. Samples will be collected for the testing of your blood cells and to check the function of your immune system. Please inform us if you have any unusual or unpleasant feelings or symptoms since being on study capsules.

Possible side effects of Capsules:
Seaweed is a part of normal diets in people around the world, and has been classified as safe in most circumstances by Food and Drug Administration in the United States. The side effects listed below are only possible side effects and **it is considered very unlikely you will experience any at all.**

Possible side effects:
Nausea, vomiting, diarrhoea, tiredness and lethargy, muscle pain, increased blood glucose levels, nerve pain (if you have had a history of nerve pain), skin dryness and changes in thyroid gland function.

What are the possible benefits?
You may or may not experience a direct benefit from taking these capsules. If you take part, you will help to provide valuable information that may lead to better understanding of how your immune system responds to this seaweed which will help in treating patients in the future.

What are the costs?
The capsules are being provided free of charge. You will receive no payment for taking part in this study.

What happens if something goes wrong?
In the unlikely event that you become ill or suffer any injury as a direct result of the study extract or trial procedure you should inform us to arrange for the correct treatment.

Will my taking part in this study be kept confidential?
Yes. The study information will be recorded in secure password protected computers and closed cabinets.

What will happen to the results of the research study?
The results will be published, discussed or disclosed to other people, though you will not be identified in any report or publication.

Ethical considerations:
The Southern Tasmanian Health and Medical Human Research Ethics Committee has approved this study. If you have any concerns of an ethical nature or complaints about the manner in which the project is conducted, you may contact the Chairperson or Executive Officer of the Ethics Committee.
Chair: Dr Helen McArdle 6222 8430
Executive Officer: Amanda McAully 6226 2763

What to do if you have problems?
If you develop any of the side-effects mentioned, or if you have any concerns or questions about your condition, please contact:
Discipline of medicine on
(03) 6226 4841; (03) 6226 4846; Mobile: 0405 466 000 between the hours of 9:00am to 5:00pm (Monday to Friday). The switchboard at Royal Hobart Hospital on (03) 6222 8308 will put you in touch with the investigators on call at all other times.

Appendix-2: Informed consent form (Sample)

UNIVERSITY OF TASMANIA

School of Medicine

Informed Consent Form

Study Title: Effects of natural seaweed extract (GFS^{TM}) on mobilisation of human bone marrow stem cells and immune system activation.

Principal investigators: Mohammad Irhimeh and Ray Lowenthal

a. Statement by the subject:

1. I have read and understood the "Information Sheet" for this study.
2. The nature and possible effects of the study have been explained to me.
3. I understand that the study involves the following:
   - samples of up to 30ml blood before, during and after the study
   - urine samples collection before, during and after the study
   - the ingestion of up to 3g of brown seaweed extract in capsule form for 12 days
   - the provision of some personal information
4. I understand that there may be risks and discomfort from the blood sampling, and that there may be possible unknown effects from the extract even though none have been recorded.
5. Any questions that I have been asked have been answered to my satisfaction.
6. I voluntarily agree to participate in this research study and am free to withdraw at any time without giving reasons. I agree that information gained from this study may be published provided that I cannot be identified as a subject. I understand that I will be given a signed copy of this consent form.

By signing this consent form, I have not waived any of the legal rights that I otherwise have as a participant in a research study.

_____

Name of volunteer (BLOCK LETTERS)

_____/_____/_____

Signature, Date

_____

Name of witness (BLOCK LETTERS)

_____/_____/_____

Signature, Date

b. Statement by the researcher:

I have explained the nature and purpose of the study to the named volunteer above and have given the opportunity to ask questions and time to decide whether to participate.

_____

Name of investigator (BLOCK LETTERS)

_____/_____/_____

Signature, Date

Appendix-3: Information sheet and consent form used at UNSW to donate cord blood

Consent Form to Donate Cord Blood

You are invited to consent firstly, to have your cord blood collected, and secondly, to have it banked and stored at the Australian Cord Blood Bank, Sydney Children's Hospital. If suitable, the cord blood will be stored anonymously to be used for the treatment of blood disorders.

What is cord blood and how is it collected?
Cord blood is the blood of the placenta collected via the umbilical cord. Cord blood is rich in bone marrow stem cells that are the building blocks of blood cells. The placenta, umbilical cord and its blood (called cord blood) are normally discarded. In this project, cord blood is collected after your baby is delivered and the umbilical cord cut. The baby is then independent of the umbilical cord and placenta. A needle is inserted into a vein of the umbilical cord/placenta and the blood drained into a collection bag. This blood is not taken from you or your baby. The procedure for normal delivery is not interfered with.

How will the cord blood be used?
The banked cord blood unit will be available anonymously to patients needing treatment with stem cells. If the cord blood cannot be banked, it will either be used for research into its stem cells and the treatment of diseases using protocols approved by hospital or university Ethics Committees, or disposed of appropriately.

Are there additional requirements, if I wish the cord blood to be banked?
Yes. Before the cord blood can be banked for use by a patient, both you and the cord blood have to be tissue typed and tested for possible transmission of blood and genetic disorders and infections such as HIV, Hepatitis, HTLV virus, cytomegalovirus and syphilis. You will be asked to:

- Answer and sign a donor declaration about your risk of infection,
- Provide a personal and family history,
- Give a sample of blood around the time of the baby's birth, and again 6 months later,
- Provide follow up information on yours and your baby's health,
- Give permission for reference samples to be stored for future testing relevant to the cord blood and to review the medical records of yourself and your baby. A piece of the placenta and/or umbilical cord may be kept for infection testing and tissue typing.

HIV testing?
Your decision to donate cord blood will involve an HIV antibody test on both maternal and cord blood. Information and implications of HIV testing are given on the attached infection information sheet. You must have read this before consenting to donate cord blood.

Are there other risks involved?
By donating your cord blood you are agreeing to the use of it by anyone needing treatment. The Australian Cord Blood Bank is part of a national an international resource for anyone in need. This is available to you and your family. Thus, although there are no direct benefits to you or your baby, by participating in this study your donation is further developing an international resource. Since the blood tests will check the infection status of you and your baby, if any are positive, early treatment can be undertaken if appropriate.

Is my privacy and that of my baby protected?
Yes. The cord blood will be made available anonymously to patients needing a transplant. All information identifying you and your baby will be kept confidential. Group results or characteristics on the use of the cord blood may be published or discussed to tother people in a way that will not identify you.

Will I incur expenses?
You will incur no expenses nor will you be paid for giving your cord blood. Participation is voluntary. You will suffer no penalty should you decide not to donate your cord blood, nor will this affect your ability to receive medical care. You are free to withdraw your consent to donation at any time without prejudice.

NB: you should NOT agree to donate if the answer to any of the following categories for you or your partner is 'YES':
If you and your partner:

- are HIV AIDS positive
- carry Hepatitis B or C virus
- have injected drugs in the last 5 years
- have been paid for having sex
- have haemophilia and been treated with blood products before 1980
- and if you have: had sex in the last year with anyone in the above groups or with a bi-sexual man.

If you have any question at any time, please ask your hospital attendant, or the Bank at Sydney Children's Hospital (02 9382 0371). If you wish to participate in the study, please fill in and sign the Consent form. You will be given a copy of the form to keep.

Material Consent

☐ I have read and understood the accompaniment information and I am therefore willing to consent to:

1. The collection and storage of cord blood after delivery of my baby.
2. Give a sample of blood to test for HIV, Hepatitis and other infections around the time of delivery, answer medical questions and complete a Donor Declaration.
3. Give a sample of blood in six months for tests for HIV, Hepatitis and other infections.
4. Let the Bank know if serious or inherited disorders occur in the family.

☐ Use the cord blood for research projects which have been approved by relevant ethics committees or for quality control in the cord blood bank.

Mother's Name _____Mother's Signature _____
(please print)

Witnessed by Staff: Name:_____
(please print)

Signature:_____ Date:_____/_____/_____

What is your expected date of delivery? Date: ____/____/_____Hospital:_____

Certification of Investigator or Associate

I certify that I have discussed the risks that may be involved in terms readly understood by the patient.
Signature:_____ Date:_____/_____/_____

Name of Investigator/Associate/Attending Doctor/Nurse/Interpreter_____
(please print)

Revocation of Patient's Consent

I hereby wish to WITHDRAW my consent to participate in Cord Blood donation and understand that such withdrawal WILL NOT jeopardise any treatment or my relationship with the Hospital or my medical attendants.
Signature:_____ Date:_____/_____/_____

This project is approved by the Ethics Review Committee of the participating hospitals. If you have concerns or complains about the conduct of the project, please contact the Ethics Committee Secretary of the appropriate hospital or the Director, Australian Cord Blood Bank 02 9382 0371

Appendix-4: Material safety data sheet for 75% fucoidan

MARINOVA

MATERIAL SAFETY DATA SHEET

MANUFACTURER
Marinova Pty Ltd
Level 7, 39 Murray Street
Hobart, Tasmania 7000
Australia

EMERGENCY PHONE
+61 3 6231 9988 (International)
03 6231 9988 (Australia)

PRODUCT INFORMATION

MATERIAL
GFS Powder

CHEMICAL NAME AND MOLECULAR FORMULA	CAS NO.(s)
Not applicable	Not available
SYNONYMS Undaria 75% Galactofucan Sulphate Fucoidan/75% GFS™	CHEMICAL FAMILY Not applicable

HAZARDOUS COMPONENTS

Materials or Components	%	Hazard Data (TLV, LD%), LC50, etc.
Dried, powdered extract of *Undaria pinnatifida*.		

PRODUCT IDENTIFICATION

BOILING POINT (DEGREE) F	Not applicable	SPECIFIC GRAVITY (H20 = 1)	Not applicable
VAPOR PRESSURE (mm Hg.)	Not applicable	% VOLATILE BY VOLUME (%)	Not applicable
VAPOR DENSITY (Air = 1)	Not applicable	EVAPORATION RATE water = 1	Not applicable
SOLUBILITY IN WATER	Dissolves readily in water giving a viscous solution	pH	5.0-6.5
APPEARANCE & ODOR	Off white powder		

FIRE & EXPLOSION DATA

FLASH POINT (Method used)	FLAMMABLE	Lel	Uel
Not applicable	Not flammable, will char if burned	Not applicable	Not applicable

EXTINGUISHING MEDIA
Water, carbon dioxide

SPECIAL FIRE FIGHTING PROCEDURES
None

UNUSUAL FIRE AND EXPLOSION HAZARDS
None

REACTIVITY DATA

STABILITY:	CONDITIONS TO AVOID
UNSTABLE ☐ STABLE ☒	None known

INCOMPATIBILITY (Materials to avoid)
None reported

HAZARDOUS DECOMPOSITION PRODUCTS
None known

HAZARDOUS POLYMERIZATION		CONDITIONS TO AVOID
May Occur ☐	Will Not Occur ☒	Not applicable

TOXICITY IDENTIFICATION

ORAL/PARENTERAL
None known

DERMAL (acute)
None known

EYE	INHALATION
None known	None known

CHRONIC
None known. .

CARCINOGENICITY:	NTP? Not listed	ARC Monographs? No	OSHA Regulated? No

HEALTH HAZARD INFORMATION
EFFECTS OF EXPOSURE

ORAL INGESTION
Can be ingested without side effects.

EYE CONTACT
May be a mild irritant.

SKIN CONTACT
Nil, material is non toxic

INHALATION
No specific toxicity.

HEALTH HAZARD INFORMATION
EMERGENCY FIRST AID

ORAL INGESTION
Not applicable.

EYE CONTACT
Wash affected eye with copious amounts of water. Seek medical attention if irritation persists.

SKIN CONTACT
Wash contacted area with warm soapy water.

INHALATION
Not applicable.

SPILL OR LEAK

STEPS TO BE TAKEN IN CASE MATERIAL IS RELEASED OR SPILLED
Wash away with water. Material may be slippery, take care

WASTE DISPOSAL METHOD (Comply with applicable federal, state, and local regulations.)
Comply with applicable federal, state and local regulation. Disposal to sanitary sewer should be considered providing pH conforms to regulations.

SPECIAL PROTECTION INFO.

RESPIRATORY PROTECTION (Specify Type)
As with all powders, wear dust protection if handling.

VENTILATION	LOCAL EXHAUST None	SPECIAL None
	MECHANICAL (general) None	OTHER None
PROTECTIVE GLOVES Not required		EYE PROTECTION Safety glasses may be worn

OTHER PROTECTIVE EQUIPMENT
None required

SPECIAL PRECAUTIONS

PRECAUTIONS TO BE TAKEN IN HANDLING AND STORING
Storage less than 25ºC, dark, sealed packaging.

OTHER PRECAUTIONS
None required

This MSDS is based on a limited review of Marinova's files and standard toxicology handbooks.

The information herein is furnished without warranty of any kind. This information should be used only as a supplement to information already in your possession concerning this product. The determination of whether and under what conditions the product should be used by your employees is yours to make.

Appendix-5: Midstream urine collection instructions

I. Urine collection technique for women

- Wash hands with soap and water
- Spread labia with one hand and hold apart for collection
- clean area and dry it
- Void into toilet for a few seconds and then stop
- Restart urine stream and collect in sterile container
- Cap and avoid touching inside of container
- Put the container in the provided special plastic bag

II. Urine collection technique for men

- Wash hands with soap and water
- Retract foreskin if needed
- Clean tip of penis and dry it
- Void into toilet for a few seconds and then stop
- Restart urine stream and collect in sterile container
- Cap and avoid touching inside of container
- Put the container in the provided special plastic bag

Appendix-6: Fractional factorial design 2^{5-1} to analyse the interaction of fucoidan (0 versus 10 µg/mL, 100 versus 500 µg/mL) and growth factors (0 versus 100 ng/mL).

Plate A (0 versus 10 mcg/ml GFS)																
'CD34+'	Effect	P	'CD41+CD14-'	Effect	P	'CD15+'	Effect	P	CXCR4+CD34+	Effect	P	'CD14+'	Effect	P	'CXCR4/CD34'	Effect	P
'TpoxSDF1'	-0.095	0.009	'TpoxSDF1'	-0.261	0.004	'SCFxFL'	-0.177	0.032	'TpoxSDF1'	-0.083	0.020	'SCFxFL'	-0.065	0.010	'FL'	-0.049	0.009
'SCFxFL'	-0.089	0.013	'SCFxFL'	-0.221	0.011	'FLxSDF1'	-0.072	NS	'SCFxFL'	-0.082	0.022	'Fuc'	-0.035	NS	'SCF'	-0.044	0.016
'SCFxSDF1'	-0.034	NS	'FLxSDF1'	-0.094	NS	'TpoxSDF1'	-0.065	NS	'Fuc'	-0.048	NS	'TpoxSDF1'	-0.028	NS	'Fuc'	-0.037	0.037
'SCFxTpo'	-0.031	NS	'TpoxFuc'	-0.050	NS	'Fuc'	-0.051	NS	'SCFxTpo'	-0.042	NS	'FLxSDF1'	-0.026	NS	'SCFxTpo'	-0.021	NS
'FLxTpo'	-0.029	NS	'SCFxSDF1'	-0.048	NS	'SCFxFuc'	-0.039	NS	'FLxTpo'	-0.028	NS	'SDF1xFuc'	-0.023	NS	'FLxSDF1'	-0.018	NS
'Fuc'	-0.029	NS	'FLxTpo'	-0.010	NS	'SDF1xFuc'	-0.038	NS	'SCFxSDF1'	-0.024	NS	'SCFxTpo'	-0.014	NS	'SCFxFuc'	-0.017	NS
'SDF1xFuc'	-0.018	NS	'SDF1xFuc'	-0.006	NS	'SCFxTpo'	-0.031	NS	'SDF1xFuc'	-0.023	NS	'SCFxFuc'	-0.011	NS	'SDF1'	-0.016	NS
'SCFxFuc'	-0.013	NS	'SCFxFuc'	-0.003	NS	'TpoxFuc'	0.029	NS	'SCFxFuc'	-0.021	NS	'TpoxFuc'	0.000	NS	'Tpo'	-0.016	NS
'TpoxFuc'	0.006	NS	'SDF1'	0.039	NS	'FLxFuc'	0.052	NS	'FLxSDF1'	-0.001	NS	'SCFxSDF1'	0.003	NS	'FLxFuc'	-0.009	NS
'SDF1'	0.008	NS	'Fuc'	0.131	NS	'SCFxSDF1'	0.057	NS	'SDF1'	0.000	NS	'FLxTpo'	0.003	NS	'SDF1xFuc'	-0.008	NS
'FLxSDF1'	0.009	NS	'SCFxTpo'	0.208	0.016	'FLxTpo'	0.077	NS	'TpoxFuc'	0.007	NS	'FLxFuc'	0.012	NS	'FLxTpo'	0.000	NS
'FLxFuc'	0.012	NS	'FLxFuc'	0.276	0.003	'SDF1'	0.088	NS	'FLxFuc'	0.007	NS	'SDF1'	0.054	0.025	'TpoxFuc'	0.001	NS
'Tpo'	0.077	0.027	'FL'	0.426	0.000	'Tpo'	0.136	NS	'Tpo'	0.069	0.047	'Tpo'	0.089	0.001	'SCFxFL'	0.015	NS
'FL'	0.323	0.000	'Tpo'	0.661	0.000	'FL'	0.628	0.000	'FL'	0.300	0.000	'SCF'	0.481	0.000	'SCFxSDF1'	0.020	NS
'SCF'	0.335	0.000	'SCF'	0.831	0.000	'SCF'	0.770	0.000	'SCF'	0.313	0.000	'FL'	0.493	0.000	'TpoxSDF1'	0.024	NS
Plate B (100 versus 500 mcg/ml GFS)																	
'CD34+'	Effect	P	'CD41+CD14-'	Effect	P	'CD15+'	Effect	P	CXCR4+CD34+	Effect	P	'CD14+'	Effect	P	'CXCR4/CD34'	Effect	P
'SCFxFL'	-0.056	NS	'SCFxFL'	-0.184	0.000	'SCFxFL'	-0.222	0.000	'Fuc'	-0.058	NS	'SCFxFL'	-0.064	NS	'SCF'	-0.116	0.000
'TpoxSDF1'	-0.030	NS	'FLxSDF1'	-0.090	0.000	'TpoxSDF1'	-0.069	NS	'SCFxFL'	-0.053	NS	'TpoxSDF1'	-0.044	NS	'Fuc'	-0.054	0.000
'Fuc'	-0.029	NS	'TpoxFuc'	-0.061	NS	'FLxSDF1'	-0.038	NS	'TpoxSDF1'	-0.031	NS	'SCFxSDF1'	-0.020	NS	'FL'	-0.043	0.000
'FLxTpo'	-0.004	NS	'SCFxSDF1'	-0.051	NS	'Fuc'	-0.033	NS	'FLxTpo'	-0.026	NS	'SDF1xFuc'	-0.005	NS	'SDF1'	-0.042	0.000
'TpoxFuc'	-0.002	NS	'TpoxSDF1'	-0.046	NS	'SCFxSDF1'	-0.026	NS	'SCFxFuc'	-0.015	NS	'SDF1'	0.002	NS	'FLxTpo'	-0.038	0.000
'SCFxFuc'	0.006	NS	'FLxFuc'	-0.005	NS	'TpoxFuc'	-0.018	NS	'SCFxTpo'	-0.003	NS	'FLxSDF1'	0.003	NS	'SCFxFuc'	-0.038	0.000
'FLxSDF1'	0.006	NS	'SCFxFuc'	0.055	NS	'SCFxFuc'	-0.003	NS	'SDF1'	-0.001	NS	'Fuc'	0.005	NS	'SCFxSDF1'	-0.036	0.000
'SCFxTpo'	0.008	NS	'SDF1xFuc'	0.059	NS	'SDF1xFuc'	-0.001	NS	'SCFxSDF1'	-0.001	NS	'FLxTpo'	0.021	NS	'SDF1xFuc'	-0.030	0.000
'SCFxSDF1'	0.019	NS	'SDF1'	0.066	NS	'SCFxTpo'	0.036	NS	'SDF1xFuc'	0.008	NS	'TpoxFuc'	0.022	NS	'Tpo'	-0.021	0.000
'SDF1'	0.022	NS	'Fuc'	0.085	0.000	'FLxFuc'	0.051	NS	'TpoxFuc'	0.009	NS	'SCFxTpo'	0.025	NS	'SCFxTpo'	-0.020	NS
'SDF1xFuc'	0.026	NS	'FLxTpo'	0.109	0.000	'SDF1'	0.060	NS	'FLxSDF1'	0.012	NS	'SCFxFuc'	0.025	NS	'FLxFuc'	-0.011	NS
'FLxFuc'	0.034	NS	'SCFxTpo'	0.310	0.000	'FLxTpo'	0.062	NS	'FLxFuc'	0.028	NS	'FLxFuc'	0.053	NS	'TpoxSDF1'	0.000	NS
'Tpo'	0.041	NS	'FL'	0.359	0.000	'Tpo'	0.131	0.000	'Tpo'	0.030	NS	'Tpo'	0.084	NS	'SCFxFL'	0.009	NS
'FL'	0.294	0.000	'Tpo'	0.556	0.000	'FL'	0.617	0.000	'FL'	0.273	0.000	'FL'	0.487	0.000	'FLxSDF1'	0.012	NS
'SCF'	0.335	0.000	'SCF'	1.073	0.000	'SCF'	0.839	0.000	'SCF'	0.276	0.000	'SCF'	0.533	0.000	'TpoxFuc'	0.022	0.000

Appendix-7: Full factorial design to analyse the interaction of fucoidan (0 versus 10 µg/mL, 100 versus 500 µg/mL) and growth factors (100 versus 1000 ng/mL).

Plate A (0 versus 10 mcg/ml GFS)										
total	**Effect**	**P**	**CD34+**	**Effect**	**P**	**CD41+CD14-**	**Effect**	**P**	**CD15+**	**Effect**	**P**
'FL*TPO'	-0.164	0.247	'GFS'	-0.111	0.029	'FL*TPO'	-0.214	0.275	'FL*TPO'	-0.166	0.160
'FL*GFS'	-0.147	0.297	'SCF'	-0.065	0.178	'FL*GFS'	-0.213	0.277	'FL*GFS'	-0.152	0.197
'TPO*GFS'	-0.111	0.427	'FL*TPO*GFS'	-0.062	0.199	'TPO*GFS'	-0.118	0.540	'TPO*GFS'	-0.115	0.325
'SCF*TPO'	-0.078	0.573	'SCF*TPO'	-0.061	0.205	'SCF*FL'	-0.108	0.574	'SCF*FL'	-0.079	0.497
'SCF*FL'	-0.076	0.586	'FL*TPO'	-0.052	0.280	'SCF*TPO'	-0.096	0.620	'SCF*TPO'	-0.068	0.553
'SCF*FL*GFS'	-0.060	0.668	'SCF*FL*GFS'	-0.048	0.314	'SCF*FL*TPO'	-0.068	0.723	'SCF*FL*GFS'	-0.056	0.628
'SCF*TPO*GFS'	-0.014	0.921	'SCF*FL*TPO'	-0.047	0.321	'SCF*FL*GFS'	-0.061	0.751	'SCF*TPO*GFS'	-0.016	0.886
'GFS'	-0.001	0.996	'SCF*FL'	-0.043	0.369	'SCF'	-0.043	0.824	'GFS'	0.006	0.958
'SCF*FL*TPO'	0.017	0.901	'FL*GFS'	-0.036	0.445	'FL*TPO*GFS'	-0.034	0.861	'SCF*FL*TPO'	0.015	0.893
'SCF*GFS'	0.022	0.873	'TPO'	-0.026	0.575	'SCF*TPO*GFS'	-0.027	0.889	'SCF*GFS'	0.020	0.863
'SCF*FL*TPO*GFS'	0.037	0.789	'SCF*TPO*GFS'	-0.022	0.639	'SCF*GFS'	-0.021	0.914	'SCF*FL*TPO*GFS'	0.033	0.772
'FL*TPO*GFS'	0.039	0.777	'TPO*GFS'	-0.021	0.662	'SCF*FL*TPO*GFS'	-0.015	0.939	'FL*TPO*GFS'	0.036	0.754
'SCF'	0.061	0.663	'SCF*FL*TPO*GFS'	-0.017	0.723	'TPO'	0.027	0.888	'FL'	0.065	0.571
'FL'	0.076	0.585	'SCF*GFS'	0.023	0.632	'GFS'	0.089	0.643	'SCF'	0.071	0.537
'TPO'	0.153	0.279	'FL'	0.061	0.204	'FL'	0.174	0.371	'TPO'	0.135	0.251
CXCF4+CD34+	**Effect**	**P**	**CD14+**	**Effect**	**P**	**CD34+CXCR4+/CD34+**	**Effect**	**P**	**CXCR4+CD34-**	**Effect**	**P**
'GFS'	-0.164	0.004	'SCF*FL*GFS'	-0.089	0.459	'GFS'	-0.098	0.002	'FL*TPO'	-2.189	0.023
'SCF'	-0.075	0.141	'FL*TPO'	-0.085	0.480	'FL'	-0.022	0.425	'GFS'	-1.420	0.123
'FL*TPO*GFS'	-0.056	0.263	'FL*GFS'	-0.026	0.824	'SCF'	-0.019	0.495	'TPO*GFS'	-1.334	0.146
'SCF*FL*TPO'	-0.056	0.271	'SCF*FL*TPO'	-0.022	0.850	'SCF*FL*TPO'	-0.016	0.571	'FL*GFS'	-0.928	0.303
'SCF*FL'	-0.045	0.369	'FL*TPO*GFS'	-0.016	0.891	'SCF*FL*TPO*GFS'	-0.004	0.889	'SCF*TPO'	-0.890	0.322
'SCF*FL*GFS'	-0.038	0.442	'SCF'	-0.008	0.949	'SCF*FL'	-0.003	0.908	'SCF*FL*GFS'	-0.691	0.440
'SCF*TPO'	-0.037	0.458	'GFS'	0.003	0.980	'SCF*GFS'	0.007	0.788	'SCF*FL'	-0.537	0.546
'SCF*FL*TPO*GFS'	-0.020	0.682	'FL'	0.004	0.971	'FL*TPO*GFS'	0.008	0.786	'SCF*FL*TPO*GFS'	-0.037	0.967
'FL*TPO'	-0.020	0.690	'SCF*FL*TPO*GFS'	0.015	0.903	'SCF*TPO*GFS'	0.016	0.572	'SCF'	0.133	0.880
'FL*GFS'	-0.017	0.739	'SCF*TPO'	0.023	0.845	'SCF*FL*GFS'	0.017	0.544	'SCF*FL*TPO'	0.215	0.809
'TPO'	-0.015	0.760	'SCF*FL'	0.030	0.803	'TPO'	0.018	0.527	'SCF*TPO*GFS'	0.227	0.798
'SCF*TPO*GFS'	-0.012	0.810	'SCF*GFS'	0.049	0.682	'FL*GFS'	0.039	0.174	'FL'	0.266	0.764
'TPO*GFS'	0.012	0.813	'SCF*TPO*GFS'	0.060	0.612	'SCF*TPO'	0.042	0.145	'SCF*GFS'	0.454	0.610
'SCF*GFS'	0.026	0.599	'TPO*GFS'	0.068	0.570	'FL*TPO'	0.060	0.043	'FL*TPO*GFS'	0.715	0.424
'FL'	0.050	0.317	'TPO'	0.109	0.364	'TPO*GFS'	0.063	0.035	'TPO'	1.102	0.224
Plate B (100 versus 500 mcg/ml GFS)										
total	**Effect**	**P**	**CD34+**	**Effect**	**P**	**CD41+CD14-**	**Effect**	**P**	**CD15+**	**Effect**	**P**
'FL*GFS'	-6.265	0.555	'SCF*TPO'	-0.257	0.270	'FL*GFS'	-0.294	0.115	'GFS'	-5.903	0.374
'FL*TPO'	-5.952	0.575	'TPO*GFS'	-0.190	0.409	'TPO*GFS'	-0.279	0.134	'TPO*GFS'	-5.573	0.400
'TPO*GFS'	-5.225	0.622	'FL*GFS'	-0.189	0.413	'SCF*FL'	-0.162	0.373	'FL*TPO'	-5.072	0.443
'GFS'	-3.745	0.723	'SCF*FL'	-0.177	0.442	'FL*TPO'	-0.162	0.373	'FL*GFS'	-4.158	0.528
'SCF*TPO'	-3.259	0.758	'SCF*TPO*GFS'	-0.161	0.485	'TPO'	-0.095	0.599	'SCF*GFS'	-2.802	0.670
'SCF*GFS'	-2.305	0.827	'SCF*FL*GFS'	-0.090	0.693	'SCF*TPO'	-0.052	0.774	'SCF*TPO'	-1.610	0.806
'SCF*FL*TPO*GFS'	-0.756	0.943	'FL*TPO'	-0.063	0.783	'SCF'	-0.035	0.844	'SCF*FL*TPO*GFS'	-1.333	0.839
'SCF*FL'	-0.737	0.944	'TPO'	-0.062	0.785	'SCF*GFS'	-0.031	0.863	'SCF*TPO*GFS'	0.156	0.981
'SCF*TPO*GFS'	-0.213	0.984	'SCF*GFS'	0.018	0.936	'GFS'	-0.014	0.940	'SCF*FL'	0.251	0.969
'FL*TPO*GFS'	1.266	0.905	'FL'	0.024	0.917	'SCF*TPO*GFS'	-0.002	0.991	'SCF*FL*GFS'	0.723	0.912
'SCF*FL*TPO'	1.657	0.875	'SCF*FL*TPO*GFS'	0.078	0.731	'SCF*FL*GFS'	0.051	0.774	'SCF*FL*TPO'	1.431	0.827
'SCF*FL*GFS'	2.184	0.836	'SCF*FL*TPO'	0.102	0.655	'FL'	0.067	0.710	'FL'	1.519	0.817
'FL'	3.413	0.747	'SCF'	0.115	0.614	'FL*TPO*GFS'	0.076	0.671	'FL*TPO*GFS'	1.923	0.770
'SCF'	5.676	0.592	'FL*TPO*GFS'	0.118	0.607	'SCF*FL*TPO'	0.077	0.667	'SCF'	3.646	0.580
'TPO'	9.251	0.386	'GFS'	0.156	0.497	'SCF*FL*TPO*GFS'	0.116	0.521	'TPO'	4.261	0.518
CXCF4+CD34+	**Effect**	**P**	**CD14+**	**Effect**	**P**	**CD34+CXCR4+/CD34+**	**Effect**	**P**	**CXCR4+CD34-**	**Effect**	**P**
'SCF*TPO'	-0.172	0.352	'SCF*TPO'	-0.490	0.566	'FL*TPO'	-0.155	0.317	'SCF*TPO'	-0.111	0.259
'SCF*FL'	-0.118	0.520	'SCF*TPO*GFS'	-0.313	0.713	'FL*GFS'	-0.147	0.343	'TPO*GFS'	-0.075	0.442
'SCF*TPO*GFS'	-0.101	0.580	'TPO*GFS'	-0.291	0.733	'TPO*GFS'	-0.125	0.417	'FL*TPO'	-0.070	0.468
'SCF*FL*GFS'	-0.074	0.686	'SCF*FL*TPO*GFS'	-0.115	0.893	'SCF*TPO'	-0.076	0.622	'FL*GFS'	-0.062	0.520
'TPO*GFS'	-0.050	0.784	'SCF*GFS'	-0.074	0.931	'SCF*GFS'	-0.059	0.702	'SCF*FL'	-0.062	0.522
'FL*GFS'	-0.047	0.796	'SCF*FL'	-0.039	0.964	'SCF*FL'	-0.054	0.726	'SCF*GFS'	-0.035	0.717
'TPO'	-0.027	0.880	'FL'	-0.007	0.993	'SCF*FL*TPO*GFS'	-0.051	0.738	'SCF*TPO*GFS'	-0.034	0.723
'SCF*GFS'	-0.011	0.953	'GFS'	0.002	0.998	'GFS'	-0.049	0.749	'TPO'	0.002	0.984
'SCF*FL*TPO*GFS'	-0.006	0.974	'FL*GFS'	0.066	0.938	'SCF*TPO*GFS'	0.012	0.940	'SCF*FL*GFS'	0.003	0.973
'FL*TPO'	0.022	0.906	'FL*TPO*GFS'	0.173	0.839	'SCF*FL*GFS'	0.033	0.828	'SCF*FL*TPO*GFS'	0.014	0.886
'SCF*FL*TPO'	0.037	0.841	'SCF*FL*GFS'	0.229	0.788	'SCF*FL*TPO'	0.038	0.806	'SCF'	0.020	0.838
'FL'	0.043	0.812	'SCF'	0.266	0.754	'FL*TPO*GFS'	0.043	0.777	'GFS'	0.041	0.674
'SCF'	0.062	0.735	'SCF*FL*TPO'	0.314	0.713	'FL'	0.081	0.599	'SCF*FL*TPO'	0.047	0.629
'FL*TPO*GFS'	0.076	0.679	'FL*TPO'	0.426	0.618	'SCF'	0.102	0.508	'FL*TPO*GFS'	0.051	0.600
'GFS'	0.085	0.644	'TPO'	1.268	0.149	'TPO'	0.155	0.318	'FL'	0.053	0.581