



UNIVERSITY *of*
TASMANIA

MENZIES 
Institute for Medical Research

Haemodynamics and Biochemistry of a Hypertensive Response to Exercise

By

Sonja B. Nikolic

BMedRes (Hons), BPharm

A thesis submitted in fulfilment of the degree of Doctor of Philosophy

November 2014

Menzies Institute for Medical Research

University of Tasmania

Hobart, Tasmania, Australia

Statements and Declarations

Declaration of Originality

This thesis contains no material which has been accepted for a degree or diploma by the University of Tasmania or any other institution, except by way of background information and of which is duly acknowledged in the thesis. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright. I have also acknowledged, where appropriate, the specific contributions made by co-authors of published and submitted manuscripts.

Authority of Access

The publishers of the manuscripts comprising *Chapter 2 (Review of Literature – Part II)*, *Chapter 5* and *Appendix 1* hold the copyright for that content, and access to the material should be sought from the respective journals. The remaining non-published content of the thesis may be made available for loan and limited copying and communication in accordance with the Copyright Act 1968.

Statement of Ethical Conduct

All research associated with this thesis abides by the International and Australian codes on human and animal experimentation, and full ethical approval from the relevant institutions was obtained for all studies outlined in this thesis. All individual participants provided written informed consent for involvement in the respective research studies.

Sonja B. Nikolic

November 2014

Publications by the Author and Statement of Co-Author

Contributions to Papers Contained Within This Thesis

The following papers are incorporated into Chapters of this thesis and were either published or submitted for publication in peer reviewed scientific journals during the course of candidature.

Chapter 2 – Review of Literature – Part II

Nikolic SB, Sharman JE, Adams MJ, Edwards LM. Metabolomics in hypertension. *J Hypertens*. 2014; 32:1159-1169

Author contributions:

| | |
|--------------------|--|
| Nikolic B. Sonja | Conception and design, literature review, manuscript preparation |
| Sharman E. James | Conception and design, critical manuscript revision |
| Adams J. Murray | Critical manuscript revision |
| Edwards M. Lindsay | Conception and design, critical manuscript revision |

Chapter 4

Nikolic SB, Adams MJ, Otahal P, Edwards LM, Sharman JE. Association of von Willebrand factor blood levels with exercise hypertension – Submitted to *European Journal of Applied Physiology*, July 2014

Author contributions:

| | |
|--------------------|--|
| Nikolic B. Sonja | Conception and design, data collection, data analysis and interpretation, manuscript preparation |
| Adams J. Murray | Study conception and design, data interpretation, critical manuscript revision |
| Otahal Peter | Data analysis, critical manuscript revision |
| Edwards M. Lindsay | Critical manuscript revision |
| Sharman E. James | Study conception and design, data interpretation, critical manuscript revision |

Chapter 5

Nikolic SB, Wilson R, Hare JL, Adams MJ, Edwards LM, Sharman JE. Spironolactone reduces aortic stiffness via blood pressure-dependent effects of canrenoate. *Metabolomics*. 2014, 10:105-113

Author contributions:

| | |
|--------------------|--|
| Nikolic B. Sonja | Conception and design, data collection, data analysis and interpretation, manuscript preparation |
| Wilson Richard | Data collection, critical manuscript revision |
| Hare L. James | Data collection, critical manuscript revision |
| Adams J. Murray | Critical manuscript revision |
| Edwards M. Lindsay | Study conception and design, data interpretation, critical manuscript revision |
| Sharman E. James | Study conception and design, data collection, data interpretation, critical manuscript revision |

Chapter 6

Nikolic SB, Edwards LM, Karpievitch YV, Wilson R, Horne J, Adams MJ, Sharman JE. Serum metabolic profile predicts adverse central haemodynamics in patients with type 2 diabetes mellitus – Submitted to *Metabolomics*, August 2014

Author contributions:

| | |
|-----------------------|--|
| Nikolic B. Sonja | Study conception and design, data collection, data analysis and interpretation, manuscript preparation |
| Edwards M. Lindsay | Study conception and design, data interpretation, critical manuscript revision |
| Karpievitch V. Yuliya | Data analysis, critical manuscript revision |
| Wilson Richard | Data collection, critical manuscript revision |
| Horne James | Data collection, critical manuscript revision |
| Adams J. Murray | Critical manuscript revision |
| Sharman E. James | Study conception and design, data interpretation, critical manuscript revision |

Appendix 1

Nikolic SB, Abhayaratna WP, Leano R, Stowasser M, Sharman JE. Waiting a few extra minutes before measuring blood pressure has potentially important clinical and research ramifications. *Journal of Human Hypertension*. 2014 Jan; 28(1):56-61

Author contributions:

| | |
|-----------------------|--|
| Nikolic B. Sonja | Study conception and design, data collection, data analysis and interpretation, manuscript preparation |
| Abhayaratna P. Walter | Critical manuscript revision |
| Leano Rodel | Data collection, critical manuscript revision |
| Michael Stowasser | Critical manuscript revision |
| Sharman E. James | Study conception and design, data interpretation, critical manuscript revision |

Appendix 2

Karpievitch YV, Nikolic SB, Wilson R, Sharman JE, Edwards LM: Metabolomics data normalisation with EigenMS. Submitted to *PLoS ONE*, July 2014

Author contributions:

| | |
|-----------------------|---|
| Karpievitch V. Yuliya | Study conception and design, data analysis and interpretation, manuscript preparation |
| Nikolic B. Sonja | Study conception and design, data collection, critical manuscript revision |
| Wilson Richard | Data collection, critical manuscript revision |
| Sharman E. James | Critical manuscript revision |
| Edwards M. Lindsay | Study conception and design, data interpretation, critical manuscript revision |

We, the undersigned agree with the above stated contributions for each of the above published (or submitted) peer-reviewed manuscripts contained within this thesis:

Associate Professor James E. Sharman

Primary candidate supervisor

Menzies Institute for Medical Research

University of Tasmania

Professor Thomas H. Marwick

Director

Menzies Institute for Medical Research

University of Tasmania

Abstracts and Presentations at Scientific Conferences that Relate to This Thesis

The following abstracts relate specifically to this thesis and were presented at national and/or international scientific conferences during the period of candidature.

Nikolic SB, Wilson R, Hare JL, Adams MJ, Edwards LM, Sharman JE. Spironolactone reduces aortic stiffness in people with a hypertensive response to exercise via the blood pressure-dependent effects of canrenoate. ARTERY 12 (Association for Research into Arterial Structure and Physiology). **Young Investigator Oral presentation - student finalist**. Vienna, Austria. October 2012

Nikolic SB, Wilson R, Hare JL, Adams MJ, Edwards LM, Sharman JE. Effects of downstream drug metabolites of spironolactone on aortic stiffness and ambulatory blood pressure. The 2nd ISH New Investigators Symposium. **Oral presentation**. Sydney, Australia. September 2012

Nikolic SB, Abhayaratna WP, Leano R, Stowasser M, Sharman JE. Waiting a few extra minutes before measuring central blood pressure has potentially important clinical and research ramifications. High Blood Pressure Research Council of Australia (HBPRCA) annual scientific meeting. **Oral presentation - student finalist**. Perth, Australia. December 2011

Nikolic SB, Otahal P, Adams MJ, Edwards LM, Sharman JE. Exercise systemic vascular resistance and raised triglycerides independently predict a hypertensive response to exercise. HBPRCA (High Blood Pressure Research Council of Australia) annual scientific meeting. **Early Career Moderated Poster presentation**. Melbourne, Australia. December 2013

Nikolic SB, Edwards LM, Karpievitch YV, Wilson R, Horne J, Adams MJ, Sharman JE. Serum metabolic profile predicts adverse central haemodynamics in patients with type 2 diabetes mellitus. Joint meeting ESH-ISH, Hypertension. **Poster presentation**. Athens, Greece, June 2014

Nikolic SB, Otahal P, Adams MJ, Edwards LM, Sharman JE. Increased systemic vascular resistance during moderate exercise independently predicts a hypertensive response to exercise. ARTERY 13 (Association for Research into Arterial Structure and Physiology). **Poster presentation**. London, UK. October 2013

Nikolic SB, Wilson R, Hare JL, Adams MJ, Edwards LM, Sharman JE. Effects of downstream drug metabolites of spironolactone on aortic stiffness and ambulatory blood pressure. Omics Australasia Symposium. **Poster presentation**. Perth, Australia. December 2012

Nikolic SB, Wilson R, Hare JL, Adams MJ, Edwards LM, Sharman JE. Effects of downstream drug metabolites of spironolactone on aortic stiffness and ambulatory blood pressure. ISH 2012 - 24th Scientific Meeting of the International Society of Hypertension. **Poster presentation**. Sydney, Australia. October 2012

Nikolic SB, Wilson R, Hare JL, Adams MJ, Edwards LM, Sharman JE. Effects of downstream drug metabolites of spironolactone on aortic stiffness and ambulatory blood pressure. Central Haemodynamics Satellite Meeting: The 4th Pulse of Asia. **Poster presentation**. Sydney, Australia. October 2012

Nikolic SB, Abhayaratna WP, Leano R, Stowasser M, Sharman JE. Waiting a few extra minutes before measuring central blood pressure potentially has important clinical and research ramifications. ARTERY 11 (Association for Research into Arterial Structure and Physiology). **Poster presentation**. Paris, France. October 2011

Nikolic SB, Abhayaratna WP, Leano R, Stowasser M, Sharman JE. Waiting a few extra minutes before measuring central blood pressure potentially has important clinical and research ramifications. Sharing Excellence in Research Conference (UTAS Graduate Research). **Poster presentation**. Hobart, Australia. August 2011

Abstract

A hypertensive response to exercise (HRE) at moderate intensity is associated with an increased cardiovascular (CV) risk, irrespective of a normal resting BP. This is an important observation, as moderate intensity exercise blood pressure (BP) is akin to the condition experienced during normal daily life activities and may better represent a chronic BP load that people experience on a daily basis when compared to resting office BP. However, the pathophysiological mechanisms of an HRE are unclear. Therefore, the overall aim of this thesis was to explore the haemodynamic and biochemical (haemostatic and metabolic) correlates of an HRE.

In study 1 (Chapter 4), haemodynamic and haemostatic factors were examined in 64 participants (aged 57 ± 10 years, 71% male) with a clinical indication for exercise stress testing. This study showed for the first time that people with an HRE have higher blood levels of the von Willebrand factor (vWf; a haemostatic marker of endothelial dysfunction), as well as a significantly different response of vWf to exercise compared to people with normal exercise BP. Moreover, vWf was associated with moderate intensity exercise systolic BP, independent of resting office BP and other CV risk factors, whereas haemodynamic factors (including increased aortic stiffness) were not independently related to exercise systolic BP. This study suggests that haemostatic abnormalities reflecting endothelial dysfunction, rather than haemodynamic irregularities, may contribute to an HRE at moderate intensity.

In study 2 (Chapter 5), retrospective metabolomics analysis of data collected in a clinical trial of people with an HRE was conducted in 115 participants (aged 55 ± 1 years, 58% male) in order to develop a metabolomics technique, as well as to investigate the underlying mechanisms of spironolactone's action on exercise BP and other haemodynamics. This study showed that spironolactone reduced exercise BP, as well as aortic stiffness, via BP-dependent effects of canrenoate, a downstream drug metabolite of spironolactone. Importantly, this study also showed that a reduction in exercise BP was not associated with the decrease in aortic stiffness, giving further support that aortic stiffness may not be as relevant to an HRE as is widely believed.

In study 3 (Chapter 6), untargeted metabolomics analysis was used to explore possible metabolic factors related to an HRE in 39 participants with type 2 diabetes mellitus (T2DM; 62 ± 9 years; 51% male; a population with the high prevalence of an HRE) compared with 39 non-diabetic controls (52 ± 10 years; 46% male). Metabolomics analysis demonstrated that a metabolic pattern of disordered carbohydrate metabolism in T2DM may be a possible metabolic mechanism explaining central (but not peripheral) exercise hypertension. These findings have clinical relevance as central haemodynamics have shown a greater pathophysiological importance when compared with peripheral haemodynamics. Also, inosine levels (a metabolite with anti-inflammatory actions) were found to be decreased in people with T2DM and were also inversely associated with the peripheral moderate intensity exercise systolic BP. This indicates that inflammation may be a contributing factor to an HRE in people with T2DM.

This research program also involved publication of a review article on the application of metabolomics analysis in hypertension research (*Journal of Hypertension*, 2014; *Chapter 2- Review of Literature – Part II*), which will allow especially the non-experts in this field to better understand and interpret studies utilising metabolomics techniques. Importantly, the research program contained in this thesis demonstrates how metabolomics analysis could be used for exploring new insights into the underlying pathophysiological processes associated with high BP (which could be easily applied to different disease conditions). Also, the research projects included the development of methodology regarding metabolomics analysis (manuscript in submission - *Appendix 2*), as well as the development of a protocol for resting BP measurements (*Journal of Human Hypertension*, 2014; *Appendix 1*).

Overall, the work contained in this thesis has found that people with an HRE have abnormal haemodynamics, but these do not explain exercise hypertension. However, people with an HRE have abnormal blood biochemistry (haemostasis and metabolic markers related to carbohydrate metabolism and inflammation in T2DM) and these explain exercise hypertension independent of resting BP and other CV risk factors. Taken altogether, this thesis provides novel information, and represents a significant advancement to the understanding of the pathophysiology of an HRE at moderate intensity.

Dedication

This thesis is dedicated to my husband Dusan Nikolic, for his love, endless support and eternal patience.

Acknowledgements

I feel particularly honoured to have been able to work with many world-class academics and clinicians during the course of this research project. I owe gratitude to many people for their contribution and support over the past three and a half years, and would like to specifically thank the following.

Associate Professor James Sharman, my primary supervisor, for giving me the opportunity to undertake this research project, for providing a constant source of scientific inspiration, and for many hours of guidance, support and patience along the way. Dr Murray Adams, for teaching me laboratory techniques related to haemostasis and for a great deal of support. Also, I would like to thank to Dr Lindsay Edwards for teaching me metabolomics techniques, and for his encouragement and patience during the challenging learning process.

I owe thanks to all past and present members of the Blood Pressure Research Group, Dr Martin Schultz, Rachel Climie, Penny Veloudi, Dianna Marston and Dean Picone for the abundance of friendly assistance in the research process and my friends Harbeer Ahedi, Laura Laslett and Heidi Laugesen for the great deal of support.

I would like to thank to Dr Richard Wilson, Dr James Horne and Associate Professor Noel Davies from the Central Science Laboratory for their scientific input and practical assistance with the metabolomics analysis. Also, special thanks to Dr Yuliya Karpievitch and Petr Otahal for providing statistical support.

I am grateful to Dr Warrick Bishop and clinical staff at the Calvary Cardiac Centre for helping to recruit study participants. Also, I would like to thank all my study participants, for their willingness and time in participating in my study. I truly enjoyed working with you all.

My family has always been a pillar of enormous support and encouragement throughout my entire education for which I am very grateful. My deepest gratitude is owed to my husband Dusan Nikolic, for always being supportive and believing in me.

Thank you.

Table of Contents

| | |
|---|-----|
| Statements and Declarations | i |
| Publications by the Author and Statement of Co-Author Contributions to Papers Contained Within This Thesis..... | ii |
| Abstracts and Presentations at Scientific Conferences that Relate to This Thesis | v |
| Abstract | vii |
| Dedication | ix |
| Acknowledgements..... | x |
| Table of Contents..... | xi |
| List of Figures | xv |
| List of Tables | xvi |
| Chapter 1. Introduction..... | 1 |
| Chapter 2. Review of Literature | 6 |
| 2.1 Part I. Hypertensive response to exercise: reviewing the clinical importance and physiology | 7 |
| 2.1.1 Hypertension and cardiovascular risk..... | 7 |
| 2.1.2 A hypertensive response to exercise – definition and prevalence | 8 |
| 2.1.3 The prognostic significance of a hypertensive response to exercise | 9 |
| 2.1.4 A hypertensive response to exercise at moderate intensity | 9 |
| 2.1.5 Central haemodynamics and exercise..... | 11 |
| 2.1.6 Mechanisms of a hypertensive response to exercise | 12 |
| 2.1.7 Metabolomics profiling - potential for new discoveries..... | 18 |
| 2.2 Part II. Metabolomics in hypertension | 20 |
| 2.2.1 Abstract..... | 20 |
| 2.2.2 Introduction | 21 |
| 2.2.3 Metabolomics: analytical tools, preanalytical steps and study design | 22 |
| 2.2.4 Metabolomics in hypertension research | 31 |
| 2.2.5 Future directions | 37 |
| 2.2.6 Conclusions | 39 |

| | |
|---|----|
| 2.3 Review of literature summary and overall thesis aim | 40 |
| Chapter 3. Methodology | 42 |
| 3.1 Introduction | 43 |
| 3.2 Haemodynamic variables | 43 |
| 3.2.1 Office brachial blood pressure | 43 |
| 3.2.2 Exercise brachial blood pressure | 44 |
| 3.2.3 Out-of-office blood pressure | 45 |
| 3.2.4 Office central blood pressure | 45 |
| 3.2.5 Arterial stiffness | 46 |
| 3.2.6 Bioimpedance cardiography | 47 |
| 3.3 Haemostatic and clinical biochemistry markers | 48 |
| 3.4 Metabolomics | 48 |
| 3.4.1 Liquid chromatography-mass spectrometry (LC-MS) | 48 |
| 3.4.2 Proton nuclear magnetic resonance (¹ H NMR) spectroscopy | 50 |
| Chapter 4. Association of Von Willebrand Factor Blood Levels with Exercise Hypertension | 52 |
| 4.2 Introduction | 54 |
| 4.3 Materials and Methods | 55 |
| 4.3.1 Study participants | 55 |
| 4.3.2 Study protocol | 55 |
| 4.3.3 Haemostatic markers | 56 |
| 4.3.4 Haemodynamic variables | 57 |
| 4.3.5 Biochemical analysis | 58 |
| 4.3.6 Statistical analysis | 58 |
| 4.4 Results | 58 |
| 4.4.1 Clinical characteristics | 58 |
| 4.4.2 Haemostatic markers | 59 |
| 4.4.3 Haemodynamic variables | 60 |
| 4.4.4 Correlates of moderate intensity exercise systolic BP | 61 |
| 4.4.5 Multiple regression analysis | 61 |
| 4.5 Discussion | 66 |

| | |
|--|----|
| 4.6 Contribution of Chapter 4 to thesis aims..... | 68 |
| Chapter 5. Spironolactone Reduces Aortic Stiffness via Blood Pressure-Dependent Effects of Canrenoate | 70 |
| 5.1 Abstract | 71 |
| 5.2 Introduction | 72 |
| 5.3 Methods..... | 74 |
| 5.3.1 Study participants | 74 |
| 5.3.2 Study protocol..... | 74 |
| 5.3.3 Liquid chromatography-mass spectrometry (LC-MS) | 75 |
| 5.3.4 Nuclear magnetic resonance (NMR) spectroscopy | 75 |
| 5.3.5 Aortic stiffness, blood pressure and clinical biochemistry..... | 76 |
| 5.3.6 Data analysis, statistics and feature annotation | 76 |
| 5.4 Results | 77 |
| 5.4.1 Clinical characteristics..... | 77 |
| 5.4.2 Metabolomics (LC-MS) | 79 |
| 5.4.3 Metabolomics (NMR)..... | 80 |
| 5.4.4 Hemodynamic response to spironolactone | 80 |
| 5.4.5 Associations between hemodynamic parameters and spironolactone metabolites .. | 81 |
| 5.4.6 Multiple regression analysis | 81 |
| 5.5 Discussion | 83 |
| 5.6 Contribution of chapter 5 to thesis aims..... | 85 |
| Chapter 6. Serum Metabolic Profile Predicts Adverse Central Haemodynamics in Patients with Type 2 Diabetes Mellitus..... | 86 |
| 6.1 Abstract | 87 |
| 6.2 Introduction | 88 |
| 6.3 Methods..... | 89 |
| 6.3.1 Study participants | 89 |
| 6.3.2 Study protocol..... | 89 |
| 6.3.3 Clinical chemistry..... | 90 |
| 6.3.4 Haemodynamic variables | 90 |
| 6.3.5 Metabolomics analysis | 91 |
| 6.3.6 Data analysis, statistics and feature annotation | 92 |

| | |
|--|-----|
| 6.4 Results | 93 |
| 6.4.1 Clinical characteristics..... | 93 |
| 6.4.2 Haemodynamic variables | 93 |
| 6.4.3 Metabolomics analysis | 95 |
| 6.4.4 Associations between metabolomics principal components and haemodynamic variables..... | 98 |
| 6.5 Discussion | 101 |
| 6.6 Contribution of chapter 6 to thesis aims..... | 104 |
| Chapter 7. Summary and Future Directions | 106 |
| Appendix 1..... | 113 |
| Appendix 2..... | 128 |
| Appendix 3..... | 139 |
| References..... | 143 |
| Publications Arising From This Thesis..... | 172 |

List of Figures

| | |
|--|----|
| Figure 2.1 The association of systolic BP (left) and diastolic BP (right) with mortality due to stroke in each age category increasing by decade. ² | 7 |
| Figure 2.2 Forest plots for categorical exercise BP (maximal and moderate intensity) | 10 |
| Figure 2.3 Pulse pressure amplification..... | 11 |
| Figure 3.1 Aortic pressure waveform | 46 |
| Figure 3.2 Estimation of aortic stiffness using pulse wave velocity..... | 47 |
| Figure 4.1 Von Willebrand factor levels at three different conditions | 63 |
| Figure 4.2 Correlations between moderate intensity exercise systolic blood pressure and von Willebrand factor. | 65 |
| Figure 5.1 Metabolic pathway of spironolactone | 73 |
| Figure 5.2 Spironolactone downstream metabolites | 79 |
| Figure 5.3 Correlations with the spironolactone metabolite canrenoate..... | 82 |
| Figure 5.4 Correlations with the change in aortic pulse wave velocity | 82 |
| Figure 6.1 Score plot..... | 96 |
| Figure 6.2 Difference in inosine in participants with type 2 diabetes mellitus (T2DM) relative to non-diabetic controls, measured in arbitrary units (a.u.). | 97 |
| Figure 6.3 Heat plot | 99 |

List of Tables

| | |
|---|-----|
| Table 2.1 Strengths and weaknesses of example sample types used in metabolomics analysis | 27 |
| Table 4.1 Clinical characteristics of study participants | 59 |
| Table 4.2 Haemostatic markers measured across three different conditions in participants with normotensive and hypertensive response to exercise | 62 |
| Table 4.3 Haemodynamic variables measured during treadmill exercise stress testing (Bruce protocol), and at rest and during moderate intensity exercise at the research clinic in participants with normotensive and hypertensive response to exercise..... | 64 |
| Table 5.1 Clinical characteristics of study participants at baseline | 78 |
| Table 5.2 Changes in hemodynamic variables in placebo compared to spironolactone group after three months of treatment | 80 |
| Table 6.1 Clinical characteristics of study participants | 93 |
| Table 6.2 Peripheral and central haemodynamic variables measured at rest and during exercise in participants with type 2 diabetes mellitus compared with non-diabetic controls.. | 94 |
| Table 6.3 Summary of identified serum metabolites with significantly different concentrations in participants with type 2 diabetes mellitus versus non-diabetic controls | 97 |
| Table 6.4 Multiple regression analysis assessing the associations of negative ion mass spectrometry principal component 1 (NIMS PC1) with peripheral and central haemodynamic variables | 100 |
| Table 6.5 Multiple regression analysis assessing the associations of nuclear magnetic resonance principal component 2 (NMR PC2) with peripheral and central haemodynamic variables | 101 |

‘Any large-scale accomplishment is achieved only when tackled little by little. Be patient and tackle only one thing at a time. Then watch the big picture unfold.’

By Penelope Sac

Chapter 1. Introduction

Hypertension or high blood pressure (BP) is associated with adverse cardiovascular (CV) outcomes^{1, 2} and is a leading risk factor for global disease burden.³ The current recommendations for identifying individuals with hypertension and estimating the overall CV risk is based on resting office peripheral BP measurements.⁴ However, some individuals, despite having normal resting office peripheral BP, experience an exaggerated BP during exercise, a condition defined as a ‘hypertensive response to exercise’ (HRE). A recent meta-analysis in people with a clinical indication for exercise stress testing showed that those with an HRE at moderate intensity (akin to the condition experienced during normal daily life activities) were at greater risk for CV events and mortality, independent from resting office peripheral BP and other CV risk factors.⁵ This may support the notion that physiological stress during exercise may be a useful tool to reveal CV abnormalities that fail detection by conventional BP screening.⁶ The mechanisms of an HRE are unclear, but may be due to haemodynamic, haemostatic and/or metabolic factors. The overall aim of this thesis was to explore the haemodynamic and biochemical (haemostatic and metabolic) correlates of an HRE.

Chapter 2 – Review of Literature - Part I of this thesis provides an overview of the literature regarding the clinical relevance of an HRE and potential mechanisms underlying an HRE. Previous studies examining metabolic factors associated with high peripheral BP (rest and exercise) involved the detection and analysis of already known ‘targeted’ metabolites. This approach reduces the possibility for new discoveries. The recent development of metabolomics analysis has allowed the detection of a large number of unknown ‘untargeted’ metabolites and the potential discovery of new pathophysiological mechanisms associated with diseases. Metabolomics analysis has never been used before to reveal the underlying metabolic mechanisms of an HRE, and *Chapter 5 and Chapter 6* of this thesis represent the first studies of this kind. Due to limited information available regarding the application of metabolomics analysis in hypertension research, *Review of Literature – Part II* represents the synthesis of the literature available in this field. Analytical techniques, pre-analytical steps and study designs used in metabolomics studies, as well as the emerging role for metabolomics in gaining mechanistic insights into the development of hypertension have been summarised. Also, suggestions as to the future directions for metabolomics research in the field of hypertension are proposed. This literature summary has been published as a

review article in the *Journal of Hypertension*, 2014⁷ and it represents a separate presentation of the review of literature in this thesis.

In *Chapter 4*, haemodynamic and haemostatic factors of an HRE have been examined in 64 participants (aged 57 ± 10 years, 71% male) with a clinical indication for exercise stress testing. Study participants underwent CV assessment at rest and during moderate intensity exercise, aligning with the intensity of exercise BP shown to best predict CV events.⁵ Twenty participants with an HRE had higher von Willebrand factor levels (vWf; a haemostatic marker of endothelial dysfunction),⁸ as well as a significantly different response of vWf to exercise compared to people with normal exercise BP. Moreover, vWf was associated with exercise peripheral systolic BP, independent of resting office peripheral BP and other CV risk factors. Although haemodynamic factors such as aortic stiffness or exercise systemic vascular resistance were higher in people with an HRE and related to exercise peripheral systolic BP, these associations were not independent of other CV risk factors. These findings suggested that haemostatic abnormalities reflecting endothelial dysfunction, rather than haemodynamic irregularities, may contribute to an HRE.

To investigate possible metabolic mechanisms of an HRE in an exploratory and ‘untargeted’ way using a metabolomics technique (applied in *Chapter 5* and *Chapter 6*), it was first necessary to develop methodology regarding the metabolomics analysis. *Chapter 5* represents a retrospective analysis of data (including haemodynamic measures and serum samples for metabolomics analysis) collected in a clinical trial of 115 participants with an HRE (aged 55 ± 1 years, 58% male).⁹ Serum samples collected in this clinical trial were primarily used to develop metabolomics protocols and methodology. This clinical trial also represented a convenient sample to explore the metabolic actions of the aldosterone antagonist spironolactone on exercise BP, and other haemodynamics, in people with an HRE. Downstream spironolactone metabolites were identified using metabolomics techniques, and exercise peripheral BP (as well as aortic stiffness) was reduced by active spironolactone metabolite canrenoate via BP-dependent effects. Also, there was no significant relationship between the reduction in exercise peripheral BP and aortic stiffness by spironolactone treatment, giving further support that aortic stiffness is less relevant to an HRE than is widely believed. The results of this study were published in *Metabolomics*, 2014.¹⁰

In *Chapter 6*, metabolomics analysis was used to explore the metabolic factors that may contribute to an HRE in a population with the high prevalence of exercise hypertension, such

as those with type 2 diabetes mellitus (T2DM).¹¹ Serum samples from 39 participants with T2DM (62 ± 9 years; 54% female) and 39 non-diabetic controls (52 ± 10 years; 49% female) were analysed using untargeted metabolomics to investigate the relationships between metabolic profiles and haemodynamic variables measured at rest and during moderate intensity exercise. The metabolomics analysis demonstrated that a signature metabolic pattern of disordered carbohydrate metabolism in T2DM was independently associated with exercise central, but not peripheral, BP haemodynamic indices in patients with T2DM. These findings have clinical relevance as central haemodynamic indices have been shown to have greater pathophysiological importance compared with conventional peripheral BP indices.¹² Metabolic perturbations with the decreased inosine levels in people with T2DM (naturally occurring purine with anti-inflammatory properties) were inversely and independently associated with the peripheral exercise systolic BP. This may suggest that inflammation may be a contributing factor of an HRE in people with T2DM.

In *Appendix 1*, a retrospective analysis of data collected in 250 people with treated hypertension (64 ± 8 years; 52% male) was conducted to define the appropriate timing (protocol) for resting peripheral and central BP measurements. Current guidelines for peripheral BP measurements recommend that office resting peripheral BP should be measured after five minutes of seated rest,⁴ but peripheral BP may decrease for up to 10 minutes. This drop in peripheral BP over time may have significant implications when assessing BP-related risk and managing patients with hypertension. Therefore, this retrospective analysis of data aimed to determine the change and its clinical relevance in peripheral, as well as in central, BP from five to 10 minutes of seated rest. Office peripheral and central BP were significantly lower at 10 minutes compared with five minutes peripheral and central BP. But importantly, peripheral and central BP measured at 10 minutes better correlated with end-organ damage (left ventricular [LV] mass index), and was a better representative of true BP control compared to five minutes BP. Therefore, a 10 minute waiting period was used before the resting office peripheral and central BP was measured in all studies within this thesis. These findings have relevance for the appropriate diagnosis of hypertension, as well as the design of research studies in which resting office peripheral and central BP is measured. The results were published in the *Journal of Human Hypertension*, 2014.¹³

Appendix 2 represents a separate study conducted in the same study population as in *Chapter 6* (people with T2DM and non-diabetic controls) to investigate a method for the normalisation of metabolomics data. Normalisation of serum spectral data is an important step in metabolomics analysis due to variations in instrument sensitivity (mass spectrometer) and signal intensity loss, but it is complicated by the high complexity of biases. A novel singular value decomposition-based normalisation method was developed through collaboration with Karpievitch et al.¹⁴ The main finding was that normalisation removed systematic bias from the metabolomics data and normalised values better correlated with the corresponding haemodynamic data. Thus, this method proved to be useful and was used for the metabolomics data normalisation in *Chapter 6*, with the details explained in *Appendix 2*. The primary analysis was conducted by Dr Karpievitch, with significant input regarding haemodynamic measures, study conception/design and data collection provided by the author of this thesis.

Overall, this research further supports that exercise hypertension is associated with CV abnormalities and it represents a clinically important entity, irrespective of normal resting peripheral BP. People with an HRE have increased aortic stiffness and exercise systemic vascular resistance, but these do not explain exercise hypertension independent of resting peripheral BP and other CV risk markers. However, people with an HRE have abnormal blood biochemistry (haemostasis, lipids, metabolic markers related to carbohydrate metabolism and inflammation in T2DM) and these explain exercise hypertension independent of resting peripheral BP and other CV risk factors. The research program contained in this thesis also highlights the emerging role of metabolomics analysis for exploring new insights into the underlying pathophysiological processes associated with high BP.

Chapter 2. Review of Literature

This chapter includes two sections: *Part I* reviews the clinical importance and proposed mechanisms of a hypertensive response to exercise; *Part II* contains a review article that has been published in the *Journal of Hypertension*, 2014, and includes a synthesis of the literature on the application of metabolomics analysis in hypertension research. Metabolomics analysis is used in two studies within this thesis to reveal the underlying metabolic contributors of a hypertensive response to exercise.

2.1 Part I. Hypertensive response to exercise: reviewing the clinical importance and physiology

2.1.1 Hypertension and cardiovascular risk

Hypertension or high blood pressure (BP) is associated with adverse cardiovascular (CV) outcomes, including myocardial infarction, stroke, kidney disease and death^{1, 2} (Figure 2.1), and is a leading risk factor for global disease burden.³ There are approximately one billion individuals with hypertension worldwide, with an annual death rate associated with the disease of around seven million people.¹⁵ In 2003, up to 29% of the adult population in Australia were diagnosed with hypertension.¹⁶ Due to the increasing prevalence of contributing factors of hypertension including obesity, unhealthy diet and physical inactivity, the number of people with hypertension is predicted to rise, and is estimated that the total number of hypertensive individuals in 2025 will reach approximately 1.56 billion worldwide.¹⁷ The pathogenesis of hypertension is greatly influenced by genetic, lifestyle and environmental factors, but the exact underlying pathophysiological mechanisms of the development of hypertension are still unclear, except in only a small number of cases such as in those with renovascular disease, aldosteronism or over-activity of the sympathetic nervous system.¹⁸

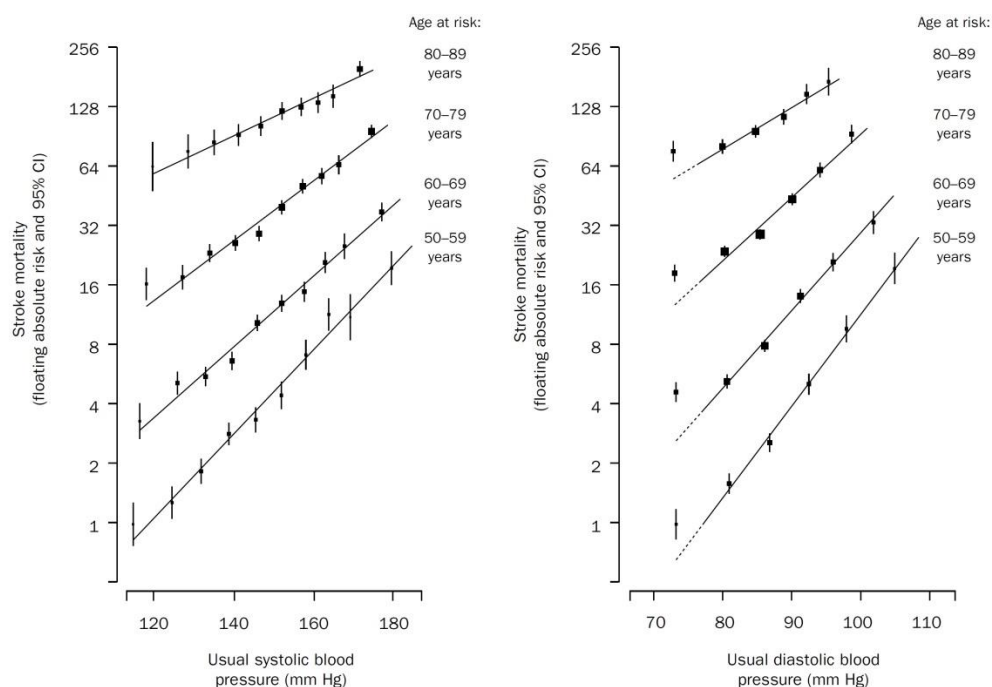


Figure 2.1 The association of systolic BP (left) and diastolic BP (right) with mortality due to stroke in each age category increasing by decade.²

The general recommendation for identifying individuals with hypertension is based on resting office peripheral BP measurements, usually taken in a clinical environment.⁴ However, this conventional type of BP measurement has some limitations that may interfere with the correct diagnosis of hypertension and estimation of the overall CV risk. Relying solely on resting office peripheral BP may lead to the incorrect diagnosis of hypertension in individuals who have white coat hypertension or ‘isolated clinic hypertension’.¹⁹ More importantly, office peripheral BP measurement may miss a significant number of people who have normal resting office peripheral BP, but who are hypertensive ‘out-of-office’ or during normal daily life activities.²⁰ It appears that these individuals have uncontrolled BP that is not easily identified at rest. These individuals have masked hypertension and have almost equal CV risk to those having sustained hypertension.²¹ If we consider the high prevalence of masked hypertension in the general community (up to 19%),²² the consideration of other means of BP measures including out-of-office BP, or even exercise BP as recently suggested,⁶ may be important in identifying these individuals at higher risk and appropriately evaluating their ‘true BP’.²³

2.1.2 A hypertensive response to exercise – definition and prevalence

Irrespective of apparently normal resting office peripheral BP, some people may have exaggerated peripheral BP during exercise, a condition defined as a ‘hypertensive response to exercise’ (HRE). Although there is no specific threshold to define an HRE, most studies have widely used values of peripheral systolic BP exceeding the 90th to 95th percentile of the studied population²⁴ or in most cases a systolic BP of ≥ 210 mmHg for men and ≥ 190 mmHg for women.^{25, 26} Due to the inconsistent definition of an HRE and different study populations examined, the prevalence of an HRE varies between studies. A prevalence of 3% to 4% across most of cohort-based studies was identified in a recent systematic review, from which one third had normal resting peripheral BP.²⁷ However, the prevalence of an HRE in a cohort of consecutive 2216 men and 1229 women with known or suspected coronary artery disease referred for exercise stress testing was 39% (a total of 1319 individuals).²⁸ An HRE is also highly prevalent in people with type 2 diabetes mellitus (T2DM), reported to be more than 50% of the studied population.¹¹ Given the high prevalence of an HRE in people with masked hypertension (up to 58%),²⁹ exercise BP may be a useful tool to identify individuals with masked hypertension or to ‘unmask’ those with uncontrolled BP.⁶

2.1.3 The prognostic significance of a hypertensive response to exercise

There is a growing evidence that BP recorded during exercise may be more useful than conventional office resting BP measurements with respect to determining an individual's risk related to BP.^{5, 30} To determine the prognostic significance of an HRE, Allison et al²⁴ conducted a follow-up study in 150 healthy, normotensive individuals with exercise systolic BP over 90th percentile (equating to ≥ 214 mmHg) and 150 age- and gender-matched subjects with normal BP response to exercise. Investigators found that exaggerated exercise systolic BP was a significant independent predictor of total CV events and future onset of essential hypertension, suggesting that an HRE carries an additional CV risk irrespective of normal resting office BP.²⁴ Similarly, in a study of Kohl et al³¹ maximal exercise systolic BP of 20387 men and 6234 women followed-up for an average of 8.1 years was associated with the increased risk of all-cause, CV and coronary heart disease mortality. Relative risk of CV mortality increased with the increase in exercise systolic BP and the change in systolic BP from rest to exercise was associated with total, CV and non-CV mortality in the study of Filipovsky et al.³² A population-based follow-up study in 1731 middle-aged men without prior history of coronary heart disease showed that exercise systolic BP ≥ 230 mmHg was associated with a 2.47 fold risk of acute myocardial infarction, even after adjustment for resting systolic BP and other CV risk factors.³³

2.1.4 A hypertensive response to exercise at moderate intensity

Exercise BP in previous studies was measured during both moderate and maximal exercise intensity. However, the prognostic value of maximal exercise BP for predicting adverse CV outcomes is less consistent and less powerful when compared to moderate intensity exercise BP. Indeed, an increase of 10 mmHg in moderate intensity exercise systolic BP recorded during stage 2 Bruce protocol had a stronger association with the risk of CV mortality than systolic BP measured at maximal exercise intensity.³⁴ A follow-up study in 1999 apparently healthy individuals that performed a bicycle ergometer exercise test at baseline and were followed for 21 years demonstrated that exercise systolic BP taken at an early moderate workload provided independent prognostic information on CV mortality, whereas maximal exercise systolic BP failed to show this independent association.³⁵

Schultz et al⁵ have recently conducted a systematic review and meta-analysis to investigate the prognostic value of exercise systolic BP at maximal and moderate intensity exercise in

people with a clinical indication for exercise stress testing. The authors found that an HRE at moderate intensity, but not maximal, was associated with a 36% increased risk of CV events and mortality when compared to those having a normotensive BP response to exercise, independently of resting office BP (Figure 2.2).⁵ An increase of 10 mmHg in moderate intensity exercise systolic BP was associated with a 4% increased risk for CV events and mortality.⁵ The stronger association of moderate intensity exercise may partially be attributed to difficulties associated with measuring BP especially during maximal intensity exercise BP due to movement artefacts and measurement errors.

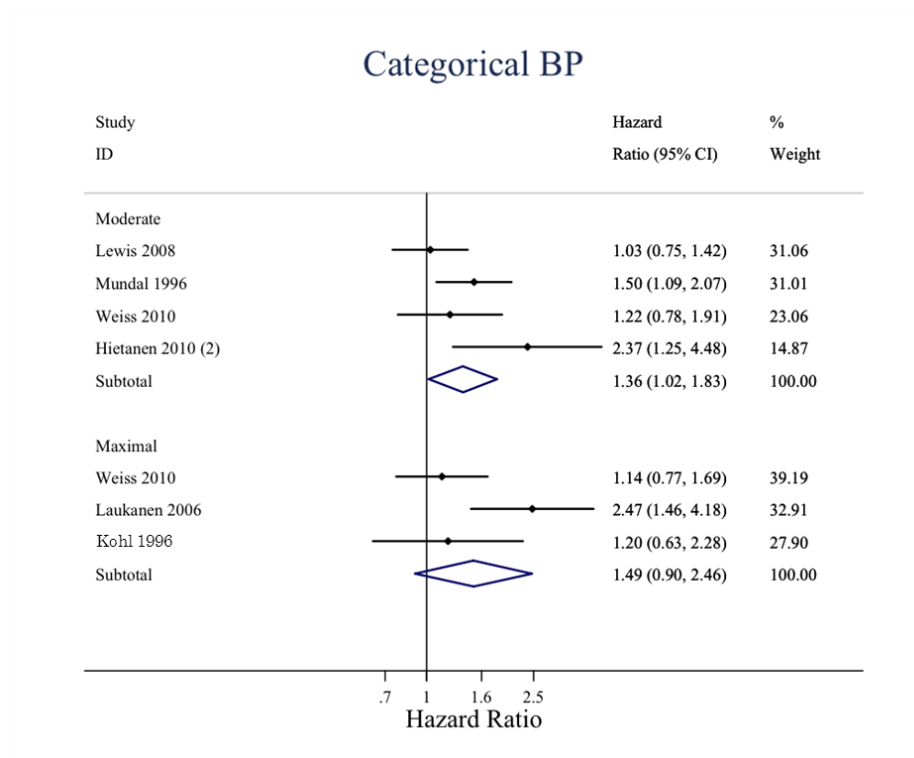


Figure 2.2 Forest plots for categorical exercise BP (maximal and moderate intensity)
Pooled hazard ratio (95% confidence intervals) of an HRE at moderate and maximal intensity for predicting CV outcomes after adjusting for resting BP and other CV risk factors. Moderate intensity: $p = 0.039$, $I^2 = 51.8\%$; maximal intensity: $p = 0.118$, $I^2 = 65.0\%$.⁵

Besides having the higher prognostic values compared to maximal intensity exercise systolic BP, moderate intensity exercise systolic BP may also have an important clinical significance. Exercise BP at moderate intensity is akin to the ambulatory condition experienced during normal daily life activities. As people spend most of a day in ambulatory condition, moderate intensity exercise BP may represent a chronic BP load that people experience on a daily basis and may provide better representation of an individual's true BP when compared to resting or

maximal exercise BP.⁶ Nevertheless, the underlying pathophysiological mechanisms that link an exaggerated moderate intensity exercise BP and adverse CV outcomes are still unclear.

2.1.5 Central haemodynamics and exercise

Although current recommendations for identifying individuals with hypertension and increased CV risk is based on peripheral BP measurements, recent research findings have placed an emphasis on central BP, the pressure to which the vital organs (heart, brain and kidneys) are directly exposed. It has been shown that central haemodynamic indices (including augmentation index [AIx]; a marker of left ventricular afterload) predict CV events independently of brachial BP.¹² Therefore, measuring central BP (at rest and during exercise) may add clinically relevant information to the overall assessment of CV risk associated with hypertension. However, central BP may not be truly represented by BP measured at the periphery,³⁶ as peripheral systolic BP is not the same as central systolic BP, due to pulse pressure (PP) amplification from large central elastic arteries towards the smaller, more muscular peripheral arteries (Figure 2.3).³⁷ Indeed, central systolic BP is normally lower than brachial systolic BP (up to 30 mmHg), and this difference is highly variable between individuals even with similar brachial systolic BP.³⁸⁻⁴⁰ With advancing age or CV diseases, the central large arteries undergo changes and become stiffer, due to a greater recruitment of collagen fibres, resulting in an increase in BP in the predominantly central arteries when compared to peripheral, and a decrease in PP amplification.⁴¹

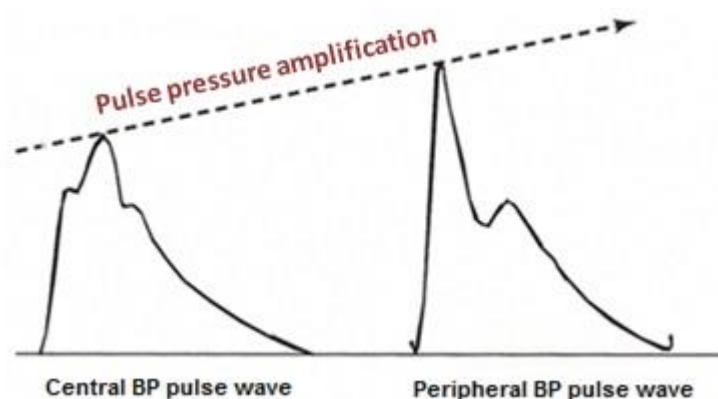


Figure 2.3 Pulse pressure amplification

The amplitude of the pressure wave and systolic BP (the highest point of the pressure wave) increases as the pressure wave propagates away from the large central arteries towards the smaller, peripheral muscular arteries.

The role of exercise central BP has been rarely investigated. Invasive measurements of central BP during exercise demonstrated that central systolic BP increases to a lesser extent than peripheral systolic BP, leading to the increased PP amplification.^{42, 43} Indeed, a study of Sharman et al⁴⁴ showed that PP amplification increased from rest to moderate intensity exercise in healthy individuals.⁴⁴ However, in older individuals with hypercholesterolemia and increased CV risk, the PP amplification is blunted due to a higher central systolic BP relative to peripheral systolic BP when compared to healthy controls.⁴⁵ These findings also support the fact that risk related to BP may be more evident from moderate intensity exercise central BP, rather than resting peripheral BP. Central BP can be readily estimated via a non-invasive measurement of the radial pulse, in a technique that is both valid and reproducible at rest and during exercise.⁴⁶⁻⁴⁹ Future research should be directed towards gaining further insight into the pathophysiological role of central BP during exercise, as this may likely add significant information to the clinical understanding of an HRE.

2.1.6 Mechanisms of a hypertensive response to exercise

Normal physiological response to exercise

With exercise onset, the metabolic needs of skeletal muscles increase which requires the body to simultaneously coordinate multiple physiological responses in order to meet this demand and maintain blood flow to vital organs, such as heart and brain.²⁷ Heart rate and cardiac output rise as a result of elevated sympathetic activity, and blood flow is diverted from low-priority vascular areas to the activated muscles.²⁷ An increase in peripheral vascular dilatation allows blood to be supplied to the periphery which consequently decreases the systemic vascular resistance.²⁷ As workload of exercise increases, the rise in cardiac output predominates over the decrease in systemic vascular resistance leading to an elevation of mean arterial pressure by approximately 40%.²⁷ Systolic BP rises gradually with exercise intensity, whereas diastolic BP goes down slightly.²⁷ However, in some cases, exercise systolic BP increases excessively, although the underlying pathophysiological mechanisms of this rise are unclear.²⁷ This excessive rise in exercise BP (irrespective of normal BP at rest) is associated with adverse CV outcomes (as previously described), and as such, the BP response to exercise may help reveal CV abnormalities that are not otherwise detected at rest.⁶

Haemodynamic contributors to a hypertensive response to exercise

Although the mechanisms of an HRE remain unclear, abnormalities in haemodynamics (which represent BP and flow within the arterial system) are thought to play a key role. Appropriate vascular function is essential in maintaining BP, especially during exercise. Previous studies have suggested that possible causative factors of an HRE might be increased large arterial stiffness, as this may fail to buffer the rise in BP that will occur with increased cardiac output and blood flow during exercise.⁵⁰ Indeed, increased aortic stiffness (as measured by carotid-to-femoral pulse wave velocity; PWV) has been shown to be significantly associated with moderate intensity exercise systolic BP in Framingham Offspring cohort study in 2115 participants even after adjusting for known CV risk factors.⁵⁰ Tsioufis et al⁵¹ also found a significant positive association between aortic stiffness (measured by the same method) and maximal exercise systolic BP, although study participants included those with established essential hypertension, which may have influenced the results. Increased aortic stiffness may also lead to raised central BP and left ventricular (LV) afterload, and ultimately affect cardiac structural and functional characteristics.^{11, 25} A cross-sectional study showed that people with an HRE have a higher prevalence of LV hypertrophy and diastolic dysfunction than those with normal systolic BP during exercise.⁵²

Aortic PWV is an independent predictor of CV risk, observed in different populations, including people with hypertension, T2DM, and in older adults.⁵³ The prognostic importance of aortic PWV is most likely reflected by being a measure of the effect of different CV risk factors (including aging and high BP) on the arterial wall. Indeed, with advancing age, arteries undergo major structural and functional changes, predominantly in large central and elastic arteries and least in muscular arteries, such as those of the upper limb.⁵⁴ Large arteries become stiff and dilated due to a degeneration of elastin fibres and greater recruitment of inelastic collagen, progressively affecting the aortic cushioning function.⁵⁵ Previous studies also reported a close association between aortic PWV and BP.⁵⁶ It is possible that high BP (and its pulsatile load over time) further contributes to structural alterations within the wall, and increase age-related changes and arterial stiffness, leading to ‘premature arterial stiffening.’ Furthermore, a number of metabolic CV risk factors including dyslipidaemia, insulin resistance and altered carbohydrate metabolism are also associated with increased large artery stiffness, and this is described in more details below. Thus, arterial stiffness

appears to be an important marker of CV risk; and therefore, represents a potential therapeutic target for the prevention of increased CV morbidity and mortality.

Aldosterone has also been implicated as an important mediator of increased arterial stiffening by acting through the mineralocorticoid receptors in vascular walls.⁵⁷ Recent studies have indicated that BP-lowering drugs that interfere with the renin-angiotensin-aldosterone system, such as spironolactone, exert potentially beneficial effects on vascular structure (may prevent, or lessen, arterial stiffening) beyond the reduction in BP, probably due to blocking the adverse effects of aldosterone.⁵⁸⁻⁶⁰ Indeed, Edwards et al⁶⁰ showed that 25mg of spironolactone daily for 40 weeks, reduced aortic stiffness (measured by aortic PWV) in 56 patients with early-stage chronic kidney disease when compared to controls, even after correcting for mean BP. Aortic stiffness was also reduced by 50mg of spironolactone in 24 previously untreated patients with essential hypertension, which remained significant after correcting for the reduction in mean BP.⁶¹ As increased aortic stiffness has been suggested as a possible causative factor of an HRE, a recent clinical trial has been conducted in order to investigate the role of aldosterone antagonism with spironolactone on the reduction in aortic stiffness and exercise BP in 115 subjects with an HRE. Spironolactone has been found to decrease aortic stiffness, as well as exercise BP in these people;⁶² however, the exact mechanism of its action on these haemodynamic measures remained unclear. To explore this in more detail, *Chapter 5* of this thesis represents a retrospective analysis of data (haemodynamic and metabolic) collected in this clinical trial.

Several previous studies have suggested that the condition of the peripheral vasculature (or endothelium function) may also significantly contribute to an HRE.^{63, 64} Failure of the endothelium to regulate endothelial-dependent vasoactivity, also referred as endothelial dysfunction, will disable peripheral vasculature to appropriately dilate and compensate for an increase in blood flow. An impaired capacity for exercise-induced vasodilatation will lead to an excessive rise in systemic vascular resistance and consequently exercise BP.⁶⁵ Indeed, a study of healthy untreated subjects with high normal BP or mild hypertension showed that exaggerated exercise systolic BP was associated with reduced endothelial-dependent vasodilatation (assessed by brachial artery reactive hyperaemia).⁶³ Also, flow-mediated vasodilation was negatively associated with exercise systolic BP at moderate intensity in a large community-based population cohort from the Framingham Heart Study.⁵⁰ However, most of the previous studies examined the haemodynamic variables at rest to uncover the

underlying mechanisms of exercise hypertension. Therefore, studies contained in this thesis include examination of haemodynamics measured during resting and exercise conditions.

Arterial stiffness has been traditionally viewed as a measure of vessel wall structural elements and mean BP. However, the increasing body of evidence suggest that arterial stiffness is also regulated by the vascular endothelium,⁶⁶⁻⁶⁹ and these two should not be studied independently. Indeed, besides the distribution of elastic and collagen fibres that change with arterial 'aging',⁵⁵ arterial walls also have a layer of smooth muscle cells that can be modified by a number of vasoactive modulators including nitric oxide. The changes in nitric oxide levels may alter vascular smooth muscle tone and potentially regulate arterial stiffness.⁷⁰ Endothelial dysfunction is generally characterized by the reduction in nitric oxide bioavailability, and numerous studies utilizing therapeutic interventions to improve endothelial function (or nitric oxide bioavailability) also demonstrated the reduction in arterial stiffness. A glyceryl trinitrate (drug metabolized to nitric oxide within the vascular wall) reduced brachial artery stiffness⁶⁶ and decreased the AIx,⁶⁸ a composite measure of arterial stiffness, without any effects on mean BP in humans. Furthermore, intra-arterial infusion of glycerol trinitrate also decreased PWV in the common iliac artery in sheep, without any observed changes in mean BP,⁶⁷ whereas intra-arterial infusion of the nitric oxide synthase inhibitor (*N*^G-monomethyl-L-arginine) increased brachial artery compliance.⁶⁹ Together, this data suggests that nitric oxide may act to reduce large artery stiffness, independent from BP changes. Therefore, therapeutic intervention aimed at increasing the nitric oxide bioavailability may potentially be useful in conditions associated with endothelial dysfunction, as well as increased arterial stiffening, such as an HRE.

The potential relation of haemostatic factors with an HRE

Haemostasis represents the body's physiological system that involves an integrated effort between platelets, vascular endothelial cells and haemostatic clotting factors to ensure normal blood fluidity and blood vessel integrity. Under normal physiological conditions, the vascular endothelium plays a crucial role in regulating normal haemostasis by expressing various membrane molecules and synthesising/releasing a spectrum of regulatory (procoagulant and anticoagulant) substances of coagulation. The endothelium is constantly exposed to different CV risk factors (e.g. high shear pressure, hyperglycaemia, dyslipidaemia, inflammation), and under pathological conditions, a delicate balance of endothelium-derived factors may be disturbed in favour of procoagulant substances. Furthermore, sub-endothelial cells in vascular

vessel walls (e.g. smooth muscle cells) express haemostatic markers, including tissue factor (TF)⁷¹ leading to the rapid initiation of the coagulation cascade and an increase in markers of thrombin generation when the vessel walls are damaged. Indeed, it has been suggested that high BP confers a prothrombotic state, which may have considerable importance in the pathogenesis of target-organ damage and CV disease.⁷² Abnormalities of haemostasis, characterised by endothelial dysfunction and an activation of procoagulant haemostatic markers, including TF,⁷³⁻⁷⁵ are present in hypertension and are related to target organ damage and adverse CV prognosis.⁷⁶⁻⁷⁸

Von Willebrand factor (vWf) is a pro-coagulant haemostatic marker synthesized/stored in endothelial cells with an important role in mediating platelet aggregation and adhesion to the vascular endothelium.⁷⁹ Since vWf is released into the circulation by secretion from endothelial cells when cells are damaged, the concentrations of vWf has been proposed as an indicator of endothelial damage or dysfunction.⁷⁹ Increased plasma vWf levels may lead to thrombin generation and eventually adverse CV events. It has been demonstrated that people with hypertension (a state of endothelial dysfunction)⁸⁰ have increased vWf levels that are positively associated with both systolic and diastolic BP.⁸ Also, elevated plasma vWf levels are associated with CV events in the general population,^{8, 81} as well as in those with established CV disease.⁸² Given that an HRE is also a state of endothelial dysfunction,⁶³ this may suggest that vWf levels may also be raised in people with an HRE. In support of this statement, Lee et al⁸³ have found that vWf was increased in people with high ambulatory BP indices measured during normal daily-life activities (akin to moderate intensity exercise) in 73 patients with stable coronary artery disease, even after adjustment for mean BP. Therefore, endothelial dysfunction may represent a platform on which impaired haemostasis might be contributing to an HRE and associated CV complications.

Other haemostatic markers, such as fibrinogen (an acute phase haemostatic marker that is converted to insoluble fibrin during the clotting process) has also been found to be elevated in people with high BP (rest and ambulatory)^{83, 84} and significantly associated with the presence of hypertension-related target organ damage.⁸⁵ Importantly, fibrinogen is highly predictive of CV risk progression,⁷⁷ where fibrinogen levels over 3.5 g/L were reported to be associated with a 12-fold higher CV risk than those with normal levels.⁸⁶ Whilst there are few studies exploring haemostatic markers in people with an HRE, better understanding of potential

haemostatic abnormalities in this population would provide a significant advance in knowledge regarding HRE development and its associated CV risk.

Metabolic influences to a hypertensive response to exercise

An HRE is associated with various metabolic CV risk factors including increased total cholesterol and triglyceride levels. The Framingham Offspring cohort study of 2115 participants has demonstrated that the total cholesterol-to-high-density cholesterol ratio was independently associated with moderate intensity exercise systolic BP in multiple regression analysis, independent of other CV risk factors.⁵⁰ Men with high levels of total cholesterol had significantly higher levels of mean BP during exercise than age-matched men with normal total cholesterol levels in the study of Sharman et al.⁴⁵ Also, increased triglyceride levels were significantly associated with central exercise systolic BP.⁷⁸ Some of the mechanisms proposed for these associations include the effects of lipids on the arterial wall leading to arterial stiffening.⁸⁷ However, a recent systematic review failed to show these strong associations between aortic stiffness and lipids.⁵⁶ On the other hand, dyslipidaemia is also associated with endothelial dysfunction and reduced nitric oxide bioavailability.⁸⁸ This has been evidenced by impaired endothelium-dependent vasodilatation of forearm resistance vessels in response to acetylcholine in patients with hypercholesterolemia⁸⁸ as well as hypertriglyceridemia⁸⁹ compared with controls.

Insulin resistance and altered carbohydrate metabolism may be involved in haemodynamic abnormalities that lead to an HRE, given the high prevalence of an HRE in people with T2DM.¹¹ Indeed, insulin resistance assessed by HOMR_{IR} (homeostasis model of insulin resistance) index was found to be significantly higher in subjects with an HRE and independently related to the magnitude of elevation in systolic BP from rest to exercise, even after adjusting for age, sex, body mass index and resting systolic BP.⁹⁰ Insulin is a vasodilator of peripheral resistance arteries due to stimulation of endothelial nitric oxide synthesis.⁹¹ In the presence of insulin resistance, endothelial vasodilatation is impaired leading to increased peripheral vascular resistance and increased BP.

The large Hoorn Study⁹² reported reduced compliance of large arteries in individuals with impaired glucose metabolism compared to non-diabetic controls, suggesting that changes in vascular structure may occur even before the onset of T2DM.⁹² Cameron et al⁹³ reported higher values of PWV in all segments of large arteries in individuals with T2DM compared with non-diabetic age-matched controls,⁹³ most likely due to formation of advanced glycation

end-products by non-enzymatic crosslinks between sugars and amino acids.⁹⁴ These alterations in vascular structure may possibly lead to increased large artery stiffness, although the strength of the association between arterial stiffness and the presence of T2DM has recently been found to be rather weak.⁵⁶ Hyperglycaemia may also cause increased production of oxidative reactive species and lead to a generalized state of increased oxidative stress,⁹⁵ which can further damage vascular wall properties. Nevertheless, the underlying mechanisms linking the high prevalence of an HRE in people with T2DM are still unclear.

2.1.7 Metabolomics profiling - potential for new discoveries

Previous studies examining the metabolic abnormalities associated with high BP involved the detection and analysis of already known ‘targeted’ metabolites (e.g. triglycerides, total cholesterol). In recent years, development of analytical techniques and bioinformatics has allowed the ‘untargeted’ systematic detection of 100s to 1000s of low-molecular weight metabolites in a single sample and this new approach, called metabolomics profiling, is an ideal platform for new discoveries, as it may provide a metabolic ‘fingerprints’ for different disease states.⁹⁶ Since metabolic irregularities appear to play a significant part in abnormal BP control and also occur even before appreciable increases in office resting BP are recognised, metabolomics has increasingly been used in hypertension research to gain mechanistic insights into hypertension development.

Importantly, no study has conducted metabolomics profiling in people with an HRE, and it appears that metabolomics analysis may have potential for uncovering the mechanisms of this condition. Indeed, metabolomics profiling or mapping of all endogenous metabolites in individuals with an HRE and their healthy-matched controls, and identifying metabolites (and associated metabolic pathways) that discriminate these two groups may provide unique information on underlying mechanisms of an HRE. This type of analysis is able to identify completely novel metabolites associated with the disease, and is ideal for discovery-based investigations. Since an HRE may represent a ‘warning signal’ of uncontrolled BP,⁶ identified new metabolic perturbations of an HRE may elucidate early stage development of essential hypertension and these discoveries would represent a major clinical advance. Metabolomics-based studies in an HRE population, however, should also include a comprehensive haemodynamic assessment of BP (rest and exercise), alongside measures of arterial stiffness, endothelial dysfunction, as well as haemostatic markers, in order to provide a broad picture of possible mechanisms of an HRE.

Metabolomics analysis in hypertension research is still in its early stages. Previous metabolomics studies in hypertension research have had many limitations, including a poor characterisation of the hypertensive phenotype (e.g. relying solely on office resting BP measurements) which may have led to spurious results and inappropriate conclusions. Being a powerful technique for new discoveries and due to the limited information available regarding the application of metabolomics analysis in the hypertension research, *Review of Literature – Part II* represents the synthesis of the literature available in this field. This literature summary has been published as a review article in the *Journal of Hypertension*, 2014 and it is included as a separate part of the review of literature.

2.2 Part II. Metabolomics in hypertension

2.2.1 Abstract

Hypertension is the most prevalent chronic medical condition and a major risk factor for cardiovascular (CV) morbidity and mortality. In the majority of hypertensive cases, the underlying cause of hypertension cannot be easily identified due to the heterogeneous, polygenic and multifactorial nature of hypertension. Metabolomics is a relatively new field of research that has been used to evaluate metabolic perturbations associated with disease, identify disease biomarkers and to both assess and predict drug safety and efficacy. Metabolomics has been increasingly used to characterise risk factors for CV disease, including hypertension, and appears to have significant potential for uncovering mechanisms of this complex disease. This review details the analytical techniques, pre-analytical steps and study designs used in metabolomics studies, as well as the emerging role for metabolomics in gaining mechanistic insights into the development of hypertension. Suggestions as to the future direction for metabolomics research in the field of hypertension are also proposed.

Review of Literature - Part II has been previously published:

Nikolic SB, Sharman JE, Adams MJ, Edwards LM. Metabolomics in hypertension. *J Hypertens*. 2014; 32:1159-1169

2.2.2 Introduction

Hypertension is a complex, multifactorial disease that has a significant positive association with adverse cardiovascular (CV) outcomes, including myocardial infarction, stroke, kidney disease and death.^{1, 2} There are approximately one billion individuals with hypertension worldwide, with an annual death rate associated with the disease of around seven million people.¹⁵ Hypertension can occur as a consequence of renovascular disease, renal failure, aldosteronism or over-activity of the sympathetic nervous system, but these forms of hypertension account for only a small number of cases.¹⁸ In the majority of cases hypertension occurs without evidence of other disease. Although the pathogenesis of hypertension is greatly influenced by genetic, lifestyle and environmental factors, the underlying physiological and metabolic drivers remain to be fully elucidated.

The traditional method for identifying hypertension is via blood pressure (BP) measurements at the upper arm, usually taken in a clinic environment.⁹⁷ However, pathophysiological changes may occur during the development of hypertension before appreciable increases in clinic upper arm BP are recognised.⁹⁸ The identification of these early perturbations - whether physiological or biochemical - is expected to lead to a better understanding of the pathogenesis of hypertension and, consequently, new opportunities for the development of novel therapies and improved diagnostic methods.

In recent years, technical developments have allowed the unbiased detection, identification and semi-quantification of an increasing range of low-molecular weight compounds (metabolites) that are present within cells, tissues, and body fluids.⁹⁶ This component of systems biology called metabolomics (i.e. the study of the metabolic phenotype or metabolome) is often thought to have emerged quite recently (following genomics, transcriptomics and proteomics);⁹⁹ however, its true roots emerged some decades ago.¹⁰⁰ Since metabolites represent the final products of cellular processes including genes, mRNA, protein activity and bidirectional, complex interactions between these system biology components,¹⁰¹ metabolomics offers an unique view of the metabolic phenotype and phenotypic perturbations associated with diseases as well as the influence of environmental factors (e.g. diet, activity, behaviour, disease and medical/surgical treatment).¹⁰²⁻¹⁰⁴ To date, however, relatively few studies have employed metabolomics in the study of hypertension in animals or humans.^{10, 105-114}

In this review, the analytical tools, pre-analytical steps and study designs used in metabolomics studies are described, and the challenges associated with metabolomics analysis are appraised. It is also discussed how metabolomics has been used to study hypertension in animals and humans, provided insights into the potential benefits that this approach offers, and proposed future directions in hypertension metabolomics research.

2.2.3 Metabolomics: analytical tools, preanalytical steps and study design

Analytical tools

Current estimates suggest that the human metabolome comprises many thousands of small molecules, both confirmed¹¹⁵ and predicted.¹¹⁶ However, unlike DNA or peptide chains, where information is encoded via repeating patterns of relatively uniform chemical subunits, the information contained within the metabolome is intrinsic to the metabolites themselves. Metabolites vary profoundly in polarity, size and concentration (e.g. $\mu\text{mol/L}$, nmol/L , pmol/L), ranging from hydrophilic, polar metabolites with a low molecular weight (e.g. amino acids) to hydrophobic, non-polar high molecular weight metabolites (e.g. lipids). This diversity means that the unbiased detection, identification and quantification of the entire metabolome is extremely technically challenging.¹¹⁷ Indeed, there is currently no single analytical approach that can detect or quantify all metabolites present in human samples. However, multiple analytical techniques have been employed to provide complementary coverage of a range of metabolites with considerable success.¹¹⁸ The combination of nuclear magnetic resonance (NMR) spectroscopy with mass spectrometry (MS) has proven particularly effective and has, therefore, gained increasing popularity.¹¹⁹

The principle of NMR spectroscopy is that atomic nuclei with nonzero spin (a quantum property corresponding to angular momentum), when placed in a powerful magnetic field and irradiated with radiofrequency waves, emit a characteristic radiofrequency signal as they relax from the induced state and return to equilibrium.¹²⁰ This signal is a rich source of information regarding the composition of the irradiated sample, including data regarding chemical structure and abundance.¹²⁰ NMR spectroscopy is quantitative in nature and offers precise structural information that enables relatively easy metabolite identification.⁹⁶ Furthermore, this technique is not destructive (i.e. samples can be re-used many times) and does not require extensive sample preparation.¹²⁰ However, it is somewhat disadvantaged by relatively low analytical sensitivity that allows only the detection of high abundance

metabolites (concentration of 100 nmol/L to 1 μ mol/L or higher; with usually less than 100 metabolites in a human sample).^{96, 120} Furthermore, typical one-dimensional NMR results in complex metabolite profiles with many signals lying close to, or directly on top of each other, such that low abundance metabolites can be masked by high abundance metabolites with a similar or identical chemical shift (a technical term for the ‘frequency’ of an NMR signal). Sophisticated and expensive solutions to this latter problem exist, including two-dimensional NMR spectroscopy and hyphenated methods such as liquid chromatography–NMR (LC-NMR). However, complex biological matrices can also be simplified using cost-effective, traditional analytical preparations prior to analysis (e.g. solid-phase extraction or the use of organic solvents to separate hydrophobic and hydrophilic metabolites).

Mass spectrometry, coupled with separation techniques such as LC or gas chromatography (GC), has a higher sensitivity than NMR spectroscopy, and has the potential to detect a significant number of very low abundance metabolites (as low as 1 pmol/L).¹²¹ The initial chromatography step, coupled with the extremely high resolution of modern instruments, such as ThermoFisher’s Orbitrap, means that thousands of species can be resolved in a single experiment. In MS, molecules are charged or ionized in the ionization process, and these charged molecules and their fragments are separated according to their mass-to-charge ratio.¹²¹ In metabolomics experiments, where ions only rarely carry a charge greater than one, the mass-to-charge ratio is equivalent to their mass. Multiple ionization techniques have been used in efforts to increase the number of detected metabolites, with some doubling the number of detected metabolic features (e.g. electrospray ionization in both positive (+) and negative (-) ionization modes).¹²² However, even though it is possible to resolve many thousands of signals in a single experiment using such sophisticated MS techniques, their unambiguous assignment and identification still represents a significant bioinformatics (and experimental) challenge and one that is difficult, slow and can only be partially resolved.¹²³ For a metabolite to be positively identified, a number of orthogonal parameters such as accurate mass, isotope abundance pattern, retention time and/or MS/MS spectrum should match with those of a purified standard under identical conditions.^{123, 124} Clearly this is not plausible for more than a few dozen metabolites. Thus, it is currently broadly accepted that metabolites must be ‘putatively annotated’ unless their unambiguous annotation is absolutely necessary. This specific use of language (e.g. ‘putative annotation’) has been recommended by the Metabolomics Standards Initiative.¹²⁴

An alternative approach to metabolomics using MS is to develop a ‘targeted’ protocol, in which metabolites are unambiguously identified in pilot experiments and methods for their quantitation are developed in parallel. In subsequent experiments, only these targeted metabolites are measured. This approach can yield quantitative information on hundreds of polar and thousands of lipid species in a single run and is immediately attractive (more lipids can be measured simultaneously because they have repeating structural motifs similar to proteins). There are two disadvantages to this approach. First, metabolites that are not targeted are not detected, reducing discovery opportunities. Second, a major development effort is required in each laboratory before the technique can be used. The second remains a major hurdle to many; the first is being addressed through the use of ‘hybrid’ protocols.¹²⁵

Another general limitation of MS is its low reproducibility compared with NMR. Retention times and signal intensity are sample- and instrument-dependent, thus requiring the use of carefully standardized sample handling and the judicious use of internal standards and/or quality control (QC) samples in each analytical run to provide robust quality assurance.¹²⁴ Yet despite these limitations, MS-based metabolomics represents the method of choice for those seeking data on low abundance metabolites or resolution of large numbers of metabolites simultaneously – situations where NMR is poorly suited.^{124, 126} Given their respective strengths and weaknesses, (summarised in Figure 2.4) the complementary and simultaneous use of NMR and MS supplies excellent depth and breadth of metabolome coverage.

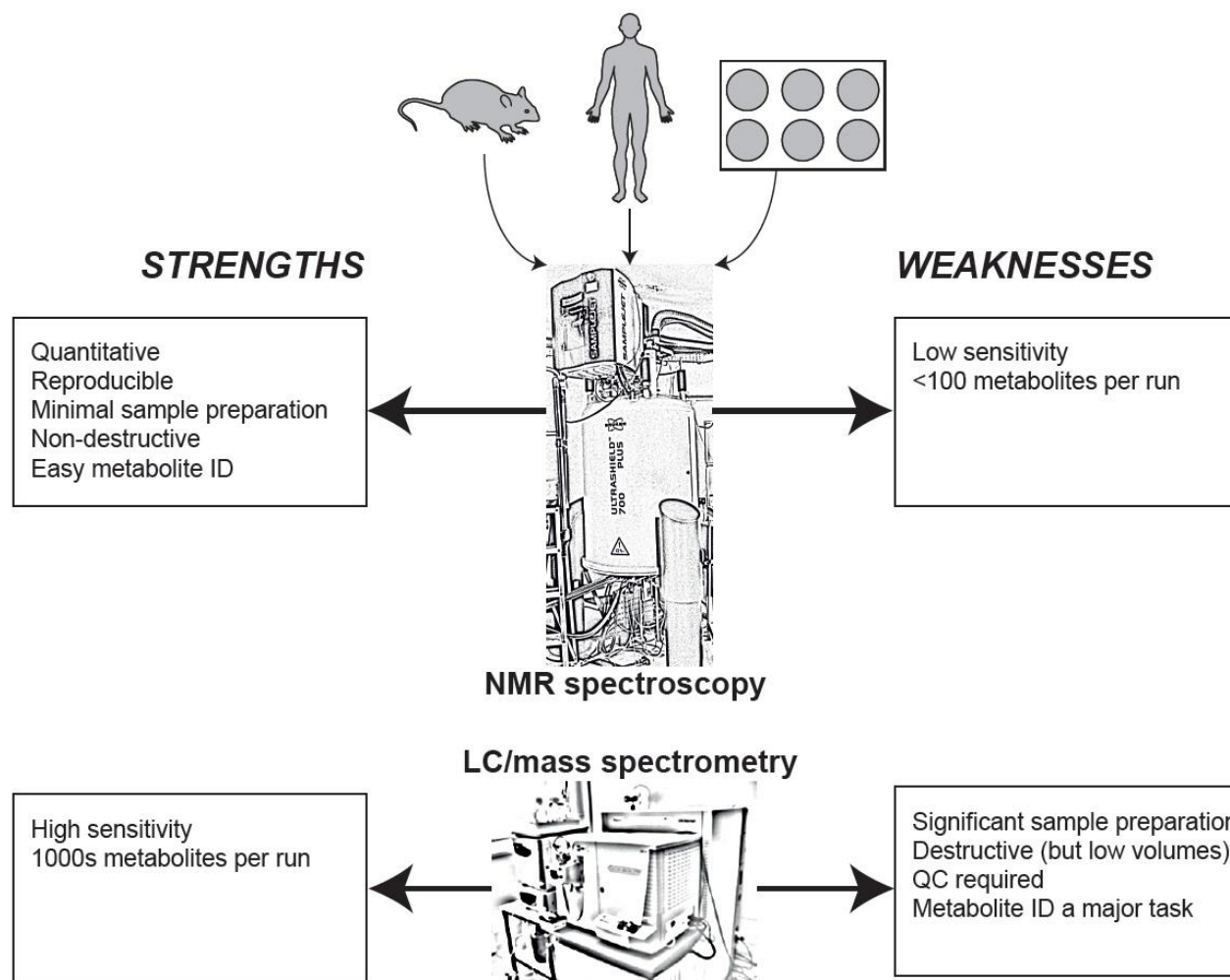


Figure 2.4 Strengths and weaknesses of nuclear magnetic resonance spectroscopy and mass spectrometry.

Pre-analytical steps

Selection of sample types. A huge range of biological samples have been used for metabolomics studies each providing different information and each with particular strengths and weaknesses (Table 2.1). These include extracellular fluids (e.g. blood, urine, cerebrospinal fluid, saliva), tissue extracts from biopsies, or cell extracts (e.g. from primary cultures). The selection of sample source is largely dependent on the study question and sample availability.¹²¹ The most frequently used samples in human metabolomics studies are blood and urine. This is due to the relative easy and minimally invasive nature of collection alongside the widely-held belief that these samples can provide a metabolic overview or ‘footprint’ of a biological system as a whole.¹²⁷ These sample types are commonly described as ‘integrative body fluids’ and represent the net metabolic outflow and uptake of every tissue and organ in the body (and renal function and glomerular filtration, in the case of urine).¹²⁷ Nevertheless, these samples may not accurately reflect pathophysiological changes in specific tissues or cells that could still have important implications in many disease states, including hypertension. For instance, local tissue renin-angiotensin systems (RAS) that are believed to play a role in systemic BP control¹²⁸ may not mediate changes in tissue metabolite exchange at a level that could be detected in serum by current methods. In this case, tissue samples may provide valuable additional information; however, biopsies present ethical and technical challenges that can be difficult to meet.¹²⁹ For example, metabolites that are substrates for enzymes with rapid turnover rates may be significantly degraded in the time taken to extract a biopsy and freeze it.¹³⁰ In addition, many tissues are compositionally heterogeneous (e.g. skeletal muscle is a mix of myocytes, fibroblasts, adipocytes and satellite cells); this complicates interpretation of the metabolic profile.¹²⁹ One strategy to address this is to establish primary cultures of specific cell types from biopsies and profile these.

Sample acquisition and sample handling. Different sample acquisition and subsequent handling can introduce systematic bias into data that may consequently interfere with the metabolomics analysis and possibly produce false discoveries. The process of sampling (e.g. invasive vs. non-invasive techniques) may produce different metabolic patterns as it has been shown that invasive techniques such as venepuncture may increase blood levels of catecholamine due to subject’s anxiety or fear of needles.¹³¹ Many studies have also reported the influence of diurnal variations (daytime vs. night-time) on metabolite profiles, and hence, the sampling time should be consistent; particularly in longitudinal investigations.¹⁰⁶

Consumption of a standard diet before the sample collection may also reduce the inter-subjects variations, as differences in diet may cause metabolic changes (especially in urine metabolites) that may be difficult to differentiate from normal physiological variations.¹³²

Table 2.1 Strengths and weaknesses of example sample types used in metabolomics analysis

| | Strengths | Weaknesses |
|-----------------------------|---|--|
| Blood | Provides a metabolic overview or ‘footprint’ of a biological system as a whole. Easy, minimally invasive sample collection. Reflects changes in tissue metabolism almost immediately. | Requires deproteination for mass spectrometry. May not reflect tissue-level changes. Changes in intra/inter-tissue metabolite flux may not appear. |
| Urine | Provides a metabolic overview or ‘footprint’ of a biological system as a whole. Easy, non-invasive sample collection. Less complex biological fluid composition compared to blood (at least in healthy subjects). | Represents a period of several hours of systemic metabolism, so not able to provide metabolic information at smaller timescales. Contains high urea concentrations harmful to GC-MS instrumentation. Can contain protein in diseased individuals which can interfere with analysis. Variable pH a challenge for NMR analysis. |
| Tissue | Provides highly selective metabolite information. | Technically demanding, time consuming and expensive sample collection. Complex and experimentally difficult metabolite extraction. Usually more than 20 mg of tissue is required. Delays in quenching metabolism can significantly affect results. |
| Primary cell culture | Homogeneous. Can be subjected to further experimentation. | Phenotype can depart from host. Sufficient quantity of cell material for NMR can be challenging. |

Blood requires an additional step of preparation to allow separation of serum or plasma which is recognised as one of the major sources of pre-analytical variation in blood-based metabolomics studies. The selection of tubes containing appropriate anticoagulants (lithium

heparin, potassium ethylenediamine-tetra-acetic acid-EDTA and citrate) for plasma collection is vital as these substances differently affect detection of endogenous metabolites.¹³³ Citrate and EDTA produce various large peaks in the NMR spectrum that selectively mask smaller endogenous metabolite peaks and inhibit information recovery in downstream metabolomics analysis.¹³³ However, lithium heparin (recommended for plasma collection) produces only a weak and broad peak (from polysaccharide metabolites) and does not significantly mask metabolites of importance.¹³³ Also, MS-based metabolomics studies have found that commercially available tubes for serum collection with gel separators shed polymeric substances into blood samples that may cause artificially high levels of analytes and produce false results.¹³⁴

The time interval between blood sample collection and cell separation (centrifugation) can also impact on metabolite composition. The optimal time is considered to be no more than 35 minutes and preferably over ice, as longer waiting time may increase lactate levels due to continued glucose metabolism in blood cells.¹³⁵ Many studies have also examined the impact of the short- and medium-term storage of samples (up to 13 months) and shown minimal impact on metabolic profiles.¹³⁶ However, limited data is available on long-term storage, although a recent pilot study of heparinised bovine plasma samples stored for up to 15 years reported minimal effects.¹³⁷ Nevertheless, experience in our laboratory suggests that some metabolites may degrade rapidly at -20°C. Stability of samples stored for extended periods of time still has to be fully investigated. Differences caused by freeze-thaw cycles were found to be minimal; however, repeated freeze-thaw steps should be avoided.¹³⁸

Metabolite extraction. Serum and plasma are complex biological biofluids consisting of low molecular weight metabolites (organic and inorganic) that are present in a manifold range of concentrations combined with high molecular weight lipids and proteins. Thus, an additional step of metabolite extraction is often required.¹²⁴ The type of metabolite extraction is largely dependent on the metabolites of interest (and budget!), and numerous studies have investigated different methods and organic solvents for extraction and separation of polar and nonpolar metabolites (ethanol, methanol, acetonitrile or acetone). However, we have found that the addition of cold methanol in a ratio of 3:1 (vol/vol) is highly efficient in metabolite extraction and protein depletion, while remaining straightforward to perform (as reported by others).¹²⁴ Urine samples have less complex biological composition compared to blood samples and usually do not require additional metabolite extraction steps due to low protein

content (except for diseased patients that may have high levels of proteins and consequently interfere with the analysis).¹²¹ However, a disadvantage of urine samples is high urea levels which may damage GC-MS instruments, as well as the presence of magnesium and calcium that bind with various metabolites and broaden NMR resonances.¹²¹ Buffers are also typically required to minimize peak-shifting in NMR spectra due to large variations in urinary pH. Traditionally, tissue analysis also requires laborious metabolite extraction including homogenization and lysis of cell walls to release the metabolites.¹²⁹ However, high resolution magic-angle spinning ¹H NMR spectroscopy can be used for analysis of intact tissues and is possible even without any pre-treatment of samples.¹³⁹

Data acquisition and data normalisation. A number of technical issues can introduce systematic bias in metabolomics studies. These mostly relate to mass spectrometry; a great strength of NMR is the relative analytical robustness and reproducibility. In small-scale studies when the number of samples is low (< 100), samples can be analysed in a single batch in a relatively short time period. However, large-scale studies require more care when designing the analytical protocol, especially in MS-based studies, due to low instrument day-to-day reproducibility; several groups (including ours) experience signal attenuation over time as samples directly interact with the instrument. In these cases, samples should be analysed in multiple batches (up to 60 study samples per batch), with three to five study samples bracketed with quality controls (QC) and regular instrument cleaning.¹²⁴ There are three types of QC samples used in metabolomics studies:

1. Pooled QC samples consisting of small aliquots taken from each study sample (good representation of the composition of study samples),
2. Predetermined mixtures of ‘representative’ metabolites,
3. Commercially available samples.

Profiles of QC samples can be analysed later to ensure analytical consistency, and correct for systematic drift where it exists.¹²⁴ Computational methods are also being developed to address these issues, in our lab and elsewhere. Even in small-scale studies, it is critical to distribute experimental and control samples evenly throughout an MS run to reduce the risk of systematic bias.

Study design in metabolomics research

Given that full coverage of the metabolome is not currently possible (and may never be), there are two approaches to metabolomics research, termed untargeted and targeted analysis,

respectively.¹²¹ Targeted analysis was discussed briefly above. Untargeted analysis, or metabolic profiling, comprises an agnostic profile of as wide a range as possible of metabolites (tens to thousands) within the biological system.¹²¹ The advantage of this approach is that it allows the detection of previously unpredicted metabolic perturbations and discriminatory metabolites (potential disease biomarkers) associated with a certain disease (or condition). To conduct an untargeted metabolomics experiment, one designs analytical approaches that allow the unbiased detection of as wide a range of metabolites as possible. Metabolic ‘features’ that discriminate between conditions (e.g. individuals diagnosed with disease, such as hypertension, and their healthy-matched controls)¹¹² are identified, and these features are then annotated (i.e. the corresponding metabolites are unambiguously identified).

Untargeted analysis does not require prior knowledge of biologically relevant metabolites and is usually referred to as hypothesis-generating or discovery-phase experimentation.¹⁴⁰ In contrast to this approach, targeted methods aim to measure a more focused number of previously defined metabolites (e.g. metabolites from a single metabolic pathway or a specific class of small molecule, such as glycerophospholipids) in order to test a previously defined scientific hypothesis (however broad) or perhaps validate a biomarker or biomarkers (usually identified in an earlier untargeted experiment).¹²¹ The advantages of targeted over untargeted methods are: i) that detection and quantification can be robustly validated in advance (particularly if MS is the technical platform) and; ii) they do not require post-hoc metabolite annotation (as this is a component of the initial technical development phase). The advantage of untargeted methods is that they are able to identify completely novel compounds and pathways without recourse to an *a priori* hypothesis.

The sample size required to detect differences between two phenotypes in metabolomics studies varies and is predicated, as ever, on two factors: first, the effect size; and second, the metabolic homogeneity of the biological system under study (e.g. laboratory animals vs. humans).¹⁴¹ Metabolomics analyses in laboratory animals are usually conducted in a small number of samples (up to 20 in total),^{105, 106} as these animals are often homogeneous, are kept under well-controlled conditions (e.g. controlled humidity, temperature, light exposure, diet), and so the influence of genetics, environment and diet on their metabolic profile is (hopefully) minimized. The resulting reduction in inter-individual variability means that orthogonal differences (i.e. those due to treatment or experimental intervention) should be more readily detectable. However, one should always be alert to the law of unintended

consequences: the possibility that other factors related to different animal models (e.g. normotensive versus hypertensive rats) cannot be excluded.¹⁴² On the other hand, considerable variation in genetic, environmental, and dietary factors in humans provides significant diversity in individual phenotypes (large inter- and intra- individual variability) and, consequently, in metabolomic profiles.^{113, 143} That being the case, slight but clinically important effects in disease states may not be detected if studies are underpowered.¹⁴¹ Thus, a significantly larger number of samples are required in human studies if effect sizes are unknown in advance, sometimes to the extent of epidemiological proportions (i.e. 100s to 1000s).¹⁴¹

Besides potentially being underpowered, confounding factors and comorbidities in complex diseases such as hypertension, can also hinder interpretation of results.¹⁴⁴ For example, statin treatment, hormonal status, exercise/cardiorespiratory fitness and gender are all known to change the lipid profile in human serum and, thus, a diagnosis of disease based on lipids may also be affected.¹⁴⁴⁻¹⁴⁷ Furthermore, hypertension is associated with the development of other CV, cerebrovascular and renal complications that create unique signatures in the metabolic profile.^{148, 149} The influence of these potential confounders should be taken into consideration when identifying the study subjects and interpreting the results. Careful characterisation of the phenotype under examination using standardized methods and matching disease-case subjects with healthy controls by known confounders (e.g. age, gender, ethnicity or body mass index) would be the preferred approach in human metabolomics studies to address these limitations.¹⁴¹

2.2.4 Metabolomics in hypertension research

Studies in animals

In hypertension research, metabolomics has mostly been used in animal models such as spontaneously hypertensive rats (SHR), as their pathophysiological processes have been recognised as similar to those of essential hypertension.¹⁵⁰ The BP in SHR (typically measured by the tail-cuff method) gradually rises with aging and becomes significantly increased after approximately 10 weeks of age when compared with healthy-matched normotensive pairs (e.g. Wistar Kyoto; WKY).¹⁵⁰ Akira et al¹⁰⁵ analyzed urine samples of six SHR in the developing stage of hypertension (eight-weeks old) on the assumption that metabolic perturbations during this early-life phase would provide insights to the

pathogenesis of hypertension. Using NMR-based metabolomics and Principal Component Analysis (PCA), urine levels of citrate and α -ketoglutarate were decreased in SHR compared with WKY, presumably resulting from early metabolic perturbations of the citric acid cycle (Krebs cycle) in SHR - a common metabolic pathway for the oxidation of carbohydrates, lipids and proteins in which these two metabolites are intermediates.¹⁰⁵ Metabolic impairments of the citric acid cycle with the increased levels of succinate were also noticed in slightly older male SHR (10 weeks of age) when compared with healthy normotensive control animals.¹⁰⁶ Recent work has confirmed the presence of the succinate receptors GPR91 in macula densa cells of the juxtaglomerular apparatus in kidneys, through which succinate can regulate renin release - an initial step in the activation of RAS.^{151, 152} Thus, increased levels of plasma (and hence urinary) succinate could have an influential role in modulating body fluid homeostasis and BP via the activation of these receptors.

Metabolomics has also been applied to stroke-prone SHR (SHRSP) models that typically develop severe hypertension.¹⁰⁷ Increased levels of taurine and creatine have been found in SHRSP rats at 12 and 26 weeks of age using NMR-based metabolomics.¹⁰⁷ These findings are difficult to place in context with previous studies (although mostly conducted in humans) in which decreased (rather than increased) urinary levels of taurine have been linked with essential hypertension.¹⁵³ Furthermore, an oral administration of taurine attenuated BP of patients with hypertension,¹⁵⁴ as well as SHR and SHRSP rats,¹⁵⁵ possibly via actions on the local RAS in the brain.¹⁵⁶ Differences in the metabolism of taurine between humans and animals, as well as the possibility of altered renal handling of taurine (and creatine) in the SHRSP model may potentially explain these contradictory findings. However, targeted metabolomics analysis using an LC-NMR method in the same study samples identified a novel urinary metabolite, succinyl-aurine, that was only detected in WKY urine.¹⁵⁷ The absence of succinyl-aurine in SHRSP rats, as well as the structural similarity with the hypotensive-acting taurine, may elucidate the involvement of succinyl-aurine in the regulation of BP in these rat models; however, the pathophysiological significance of succinyl-aurine still remains to be examined.

A profound set of metabolic differences between hypertensive and normotensive rat models were also reflected in blood samples. A GC/time-of-flight (TOF)-MS-based metabolomics study investigated age/hypertension-related metabolic changes in blood plasma samples from 10 to 18 weeks of age in SHR compared with WKY controls.¹⁰⁹ Similar age-related changes

in many metabolic compounds (e.g. decrease in amino acids: serine, methionine, ornithine, phenylalanine and an increase in lysine) were detected in both animal models. However, an increase in free fatty acids (FFA) including oleic, linoleic, hexadecanoic and stearic acids were noted in SHR only, along with the rise in BP levels from 10 to 18 weeks of age, indicating a possible role of perturbed FFA metabolism in BP regulation. Indeed, normalised peak areas of increased levels of FFA (oleic, linoleic and palmitic acids) in SHR significantly correlated with systolic BP values on multiple regression analysis in the GC/TOF-MS-based metabolomics study of Aa et al.¹⁰⁸ These investigators have also demonstrated that standard antihypertensive therapy (angiotensin-converting enzyme inhibition [captopril], calcium channel blockade [amlodipine], α 1-adrenoreceptor blockade [terazosin] and diuretic [hydrochlorothiazide]) significantly reduced BP in SHRs, as expected, but treatment had minimal effects on the increased FFA levels.¹⁰⁸ However, total ginsenosides (extracts of the medicinal plant, ginseng, purported to have beneficial antihypertensive properties through unknown mechanisms) caused not only a sustained reduction in BP that persisted even after the withdrawal of therapy, but also down-regulated the increased FFA levels toward normal levels.¹⁰⁸ Therefore, FFA regulation may have some role in maintaining BP control, but this remains to be fully tested.

Studies in humans

As with animal work, the number of metabolomics studies related to hypertension in humans is limited. Among the first was an NMR- and PCA-based metabolomics analysis designed to investigate whether it was possible to distinguish between different BP categories based on metabolic profile.¹¹⁰ Only 64 individuals were studied and these participants were classified into three BP categories (low/normal [≤ 130 mmHg], borderline [131-149 mmHg] and high systolic BP [≥ 150 mmHg]) based on a single BP measurement taken in a hospital environment.¹¹⁰ Investigators used Orthogonal Signal Correction (a filtering method) on the NMR data to minimize/remove the differences between the samples unrelated to BP, and the model was validated against over-fitting using the SIMCA method.¹¹⁰ Interestingly, the PCA scores showed many similarities in serum metabolic profiles between subjects in borderline and high systolic BP categories, whereas regions of NMR spectra attributed to different lipids (in particular lipoproteins associated with very low-density [VLDL] and low-density [LDL] cholesterol) clearly distinguished the borderline/high BP categories from the low/normal BP category (lipids being higher in the higher BP categories).¹¹⁰ This tends to imply that

metabolic changes related to BP (e.g. lipids abnormalities) might be evident in blood samples before overt BP elevation. However, a limitation of this work was reliance on one in-hospital BP reading to classify BP status, which is a low standard of assessment and likely to misclassify true BP. In any case, the findings are generally supportive of many studies that have reported relationships between plasma lipoprotein abnormalities (e.g. increased levels of total cholesterol, high density lipoprotein subfraction HDL3, apolipoprotein B, and lipoprotein a) and raised BP, or other vascular abnormalities related to hypertension, such as increased arterial stiffness.^{87, 98}

Similar to the animal hypertensive models, GC/TOF MS-based metabolomics analysis has showed significantly increased serum FFA levels (heptanoic, oleic, nonanoic, eicosanoic and hexanoic acids) in 34 elderly patients diagnosed with hypertension compared to 29 age-matched normotensive controls; however, limited information was provided as to how BP measures were acquired.¹¹¹ Although still unknown, some explanations for the causal relationship between FFA and the development of hypertension have been proposed. FFA may promote adverse effects on neurovascular tone by increasing the sensitivity of α 1-adrenoreceptors and sympathetic drive or have inhibiting effects on endothelium-dependent vasodilatation and endothelial nitric-oxide synthase.¹⁵⁸ Furthermore, increased levels of FFA may potentially change membrane fluidity by altering the $\text{Na}^+ \text{K}^+$ ATPase pump, Na^+ , K^+ and Ca^{2+} currents, as well as the structure of membrane phospholipids and increase intracellular levels of Na^+ and Ca^{2+} (mechanisms that lead to increased vascular muscle tone and consequently BP).¹⁵⁹ These findings also lend support to epidemiological studies in which FFA have been demonstrated to be independent risk factors for the development of hypertension, even after correcting for known confounders, such as body mass index, weight change, and baseline BP.¹⁶⁰ Besides increased levels of FFA, altered carbohydrate metabolism (including high levels of glucose and galactose, and decreased levels of fructose) was also found in patients with hypertension compared to normotensive controls,¹¹¹ confirming the already known information on the high prevalence of impaired glucose tolerance in patients with essential hypertension.¹⁶¹

People with an already established T2DM have high BP (affecting up to 40% of newly diagnosed T2DM patients)¹⁶² and also develop premature vascular aging and arterial structural remodelling, most likely due to underlying metabolic irregularities.⁹² An increasing number of metabolomics studies have been conducted in people with T2DM to

detect the underlying metabolic disturbances in this population, and to investigate their associations with CV complications.¹⁶³ Besides identifying alterations of carbohydrate and lipid metabolism, metabolomics studies have also revealed abnormalities in tricarboxylic acid, amino acid and bile acid metabolism.¹⁶⁴ These metabolic disturbances may adversely impact on arterial function and possibly contribute to CV complications (including high BP).⁸⁷ Indeed, a metabolomics study in the Framingham Offspring cohort showed that increased levels of three amino acids (isoleucine, tyrosine and phenylalanine) were significantly associated with the future development of CV disease, independent of standard CV disease and diabetes risk factors.¹⁶⁵ Also, a cross-sectional analysis in individuals free of CV disease reported that each increase in the score of the same three amino acids was associated with a higher risk of having subclinical carotid atherosclerosis.¹⁶⁶ A more recent study by Ha et al¹⁶⁷ also demonstrated increased levels of three amino acids in people with T2DM, as well as of lysophosphatidylcholines (lysoPC; intermediates of lipid metabolism), with levels significantly and positively correlated with the brachial pulse wave velocity (PWV; a measure of arterial stiffness). These findings may suggest that the association between metabolic abnormalities and the progression of CV complications may be mediated by irregularities in vascular function, although more studies are needed to investigate the causal mechanisms. Chapter 6 of this thesis represents one of the first studies using untargeted metabolomics analysis to investigate the relationships between abnormal metabolic profile in people with T2DM and arterial/hemodynamic measures under resting and exercise conditions. This was done to provide mechanistic insights of CV complications and high BP in people with T2DM.

Other BP metabolomics studies have also provided some evidence for the role of inflammation as a contributing factor in the development of hypertension. The acute phase inflammatory protein α -1 acid glycoprotein was at an increased level in a group of 40 patients with hypertension (defined as resting BP \geq 140/90 mmHg) compared with 40 healthy controls (defined as resting BP $<$ 130/80 mmHg).¹¹² Inflammation may cause vascular endothelial dysfunction and modifications in the synthesis and degradation of endothelial vasodilators and vasoconstrictors and, consequently, influence the regulation of BP.¹⁶⁸ This was a robust metabolomics study in terms of appropriate methods to define hypertension (triplicate measures of BP on each arm recorded at seated rest) and minimize the effects of potential confounders related to disease (two groups were well-matched by all known risk

factors). There are also some indications that metabolites associated with diet and gut microbial activity might also be linked to BP regulation.¹¹³ A large-scale hypertension-metabolomics study conducted in 4630 subjects across four populations (United Kingdom, United States of America, China and Japan) has reported an inverse association between urinary excretion of formate (a by-product of fermentation of dietary fibre by the gut microbiome) and both systolic and diastolic BP, whereas alanine (being higher in people who consume predominantly a diet high in meat products) had a positive association with BP. Furthermore, hippurate (a normal constituent of urine typically increased with dietary consumption of phenolic compounds) had an inverse association with BP.¹¹³ This finding is in agreement with the previous work in the SHR model in which decreased urine levels of hippurate were observed when compared with control WKY rats, even though both rat strains were fed under the same conditions.¹⁰⁵ The most recent human BP metabolomics study was in a population of 896 normotensive black men and women who were followed over 10 years.¹¹⁴ In this study, a 1SD difference in serum metabolite 4-hydroxyhippurate (also an end-product of polyphenol metabolism by the intestine microflora) was associated with 17% higher risk of developing hypertension, even after correcting for conventional risk factors that included baseline BP.¹¹⁴

Several metabolomics studies have been conducted in preeclampsia.¹⁶⁹⁻¹⁷² This condition is characterised by maternal hypertension and proteinuria. It affects 3-5% of pregnancies annually and is one of the main causes of foetal and maternal morbidity and mortality worldwide.¹⁷³ Essential hypertension and preeclampsia share numerous features including organ damage (renal and endothelial dysfunction), multifactorial origin and risk factors for future development of CV disease.¹⁷³ A UPLC-MS-based metabolomics study showed metabolic perturbations in 60 women at 15 ± 1 weeks of gestation that subsequently developed preeclampsia.¹⁶⁹ Forty five metabolites from 11 metabolic classes were significantly different in the plasma from women with preeclampsia (including amino acids, carnitines, fatty acids, lipids, phospholipids and carbohydrates). A more recent LC-MS-based metabolomics study in 41 women that developed preeclampsia also found carnitine (hydroxyhexanoylcarnitine) and amino acids (alanine, phenylalanine and glutamate) to be elevated compared to healthy controls.¹⁷⁰ Badaho-Singh et al¹⁷¹ used NMR-based metabolomics to identify 20 discriminatory metabolites from which a set of four metabolites (citrate, glycerol, hydroxyisovalerate and methionine) was predictive of preeclampsia.¹⁷¹ The

same group also identified glycerol and carnitine to discriminate late-onset preeclampsia.¹⁷² These findings implicate abnormal lipid metabolism in the pathogenesis of preeclampsia, although much work remains to be done. Taken altogether, the utilisation of metabolomics in hypertension research highlights the potential for revealing the underlying (suspected and possibly unknown) pathophysiological processes of hypertension, but there is still much to be done.

2.2.5 Future directions

Better definition of the hypertensive phenotype

Along with the analytical, technical and study design improvements, future directions in human metabolomics studies should include much stronger characterisation of the hypertensive phenotype than attempted to date. Dissimilar (and in some cases inadequate) methods to assess BP in previous metabolomics studies could have led to inappropriate interpretation of results. If we consider the high prevalence of white coat hypertension and masked hypertension in the general community (15% and 19%, respectively),^{22, 174} accurate office BP as well as the consideration of out-of-office BP measurements are essential to appropriately evaluate BP status.²³ Individuals with masked hypertension have almost equal CV risk²¹ and may have similar metabolic profiles to those having sustained hypertension. On the other hand, individuals with white coat hypertension that experience an exaggerated sympathetic nervous system response to an experimental intervention may be prone to an acute elevation of FFA at the time of BP measurement (due to the activation of a hormone-sensitive lipase by adrenaline),¹⁷⁵ and this could potentially lead to spurious metabolomics conclusions. Importantly, the development of hypertension is usually accompanied by arterial structural remodelling, increased large artery stiffness and central systolic BP.¹⁷⁶ Thus, rigorous BP examination that also includes measures of arterial stiffness and central BP could uncover important mechanistic insights when combined with metabolomics analyses.^{10, 177} Furthermore, metabolomics may also prove to be a powerful tool for the detection of early signs of target organ damage, such as renal, heart or cerebrovascular complications that produce distinctive signatures in the metabolic profiles, even before disease symptoms are observed and medical attention is required.

Metabolomics in clinical trials

Clinical trials conducted even decades ago have produced blood samples for subsequent disease phenotyping or disease risk studies. These remain in storage and may contain clinical information that can be recovered using metabolomics methods. Although the capital cost of instruments such as NMR magnets or high-resolution mass spectrometers is very high (more than \$100000 per instrument), these analytical platforms are often already available to researchers (e.g. in chemistry departments) and can provide NMR or MS analysis at reasonably low cost (approximately \$15-20 per sample). This potentially allows the application of metabolomics profiling to large-scale sets of study samples. A word of caution, however; although metabolomics data is relatively easy to acquire, robust analysis is a far greater technical challenge that requires significant experience in the field. Further, the highly dynamic nature of the metabolome and the significant influences that may arise from different sample acquisition, handling and storage methods may set some limitations for the use of samples acquired for other purposes (as previously described). Only samples collected, handled and stored in standard and identical ways may be directly compared using metabolomics methods, as any variations in sample handling would introduce systematic bias into the data and potentially produce spurious findings.¹²⁴ It is also important that in case-control studies, case and control samples were not collected at different centres, as this may also produce differences in metabolite patterns that should not be assigned to disease exposure.¹²⁴ Finally, citrate and EDTA have been widely used as anticoagulants for plasma collection in most clinical trials but are generally not recommended for metabolomics analysis; yet in this last case, samples can still often be used and significant and valuable biological information recovered effectively.¹³³

Reproducible standard operating procedures that define the protocols for sample collection, handling and storage are important for clinical trials planning on analysis of the samples collected over extended time periods (several months or years for some clinical trials) and at different study centres.¹²⁴ The Human Serum Metabolome (HUSERMET) project is an example in which samples are collected at different study sites in a reproducible and standard way and analysed in multiple analytical experiments and integrated into a single data set.¹²⁴ Quality control samples are implemented and used throughout the course of the study to provide the robust quality assurance.

Biomarker discovery

Biomarkers are widely used diagnostic tools for the evaluation of normal physiological conditions, as well as the presence of pathophysiological processes.¹⁷⁸ Given that metabolic perturbations associated with hypertension are most likely to develop before hypertension is detected by standard methods (rise in upper arm BP), a reliable biomarker and/or several biomarkers/metabolic patterns (due to the complexity of the underlying pathophysiological mechanisms associated with hypertension) that may elucidate early stage development of hypertension would represent a major clinical advance. To date, none of the potential metabolomic biomarkers have transitioned to being clinically useable. Moreover, abnormal metabolic patterns such as that associated with lipid metabolism do not provide significant improvements over well-established risk factors such as standard lipid profiles. This is a problem of all biomarker research,¹⁷⁹ but does not detract from the possibility that useful hypertension biomarkers could yet be discovered to allow detection of early pathological changes beyond conventional risk factors.

Pharmacometabolomics

Pharmacometabolomics is a promising new field in metabolomics that uses the profiling of metabolites in body fluids (blood or urine) before drug treatment to predict patient responses to therapy, including drug metabolism, efficacy and adverse effects.¹⁸⁰⁻¹⁸² Since pharmacometabolomics offers the possibility to optimize treatment more effectively according to the metabolic profile of each individual and avoid adverse drug effects, this approach has major potential for developing personalized drug treatment.¹⁸² As hypertension represents the most prevalent chronic condition worldwide with high costs of prescribed medications and health care services, individual tailored treatment could provide substantial benefits, not only in terms of efficacy and safety of drug treatment, but also in terms of cost savings and treatment efficiency. Further investigations in pharmacometabolomics will also be required to determine the metabolic effects of multiple drug usage, as polypharmacy is common in hypertension management.

2.2.6 Conclusions

Metabolomics in hypertension research has the potential to provide new insights into the underlying pathophysiological processes associated with hypertension, detect early metabolic perturbations (biomarkers) and monitor the efficacy and effects of drug (and other) therapies.

Future metabolomics studies should, however, include a more comprehensive physiological (phenotypic) assessment of BP, alongside more robust determination of BP control category. Although this review focused only on one part of the systems biology paradigm in the study of hypertension, metabolomics should also be interpreted in combination with other system biology methods such as computation, genomics, transcriptomics and proteomics. The genome provide predictive information regarding an individuals' risk for the development of hypertension at any stages of life, as the genetic code itself remains stable and unchanged throughout life. On the other hand, other components of system biology such as transcriptome, proteome and metabolome change noticeably in parallel with the disease developments, as well as therapeutic measures, and therefore would be more appropriate to characterise the disease process while they integrate with environmental factors and genetic predisposition. Up until recently, big emphasis was placed on transcriptomics and proteomics; however, with the further development of analytical techniques and bioinformatics, the role of metabolomics among other system biology fields is expected to continue to grow.

2.3 Review of literature summary and overall thesis aim

An HRE during moderate intensity exercise is associated with an increased CV risk, irrespective of normal resting BP. The incidence of an HRE varies between different populations, but it is highly prevalent in people with masked hypertension and T2DM, reported to be more than 50%. The underlying mechanisms of an HRE and associated increased CV risk are unknown, although previous studies suggested that this may be due to abnormalities in haemodynamic, haemostatic and/or metabolic factors. Increased aortic stiffness and impaired endothelial dysfunction are widely believed haemodynamic contributing factors to an HRE. However, most of the previous studies included people with already developed essential hypertension, which may have led to spurious results. Furthermore, impaired haemostasis is present in people with hypertension, associated with increased CV events and correlated with high BP (rest and ambulatory BP), but still little is known about haemostasis in people with an HRE. Exaggerated exercise BP has been found to be associated with metabolic CV risk factors including elevated lipids and glucose intolerance, and importantly, the recent developments of metabolomics analysis have offered opportunities for exploring new insights into the underlying pathophysiological processes associated with high BP. The underlying contributors of an HRE are still unclear, but it is

important to understand this condition in order to improve detection of individuals with an increased CV risk, reduce the CV risk in these people and potentially develop new treatment strategies; therefore, the overall aim of this thesis was:

- To explore the haemodynamic and biochemical (haemostatic and metabolic) correlates of an HRE.

Each Chapter contains specific aims (outlined below) which contribute to the overall thesis aim. This is specifically outlined at the end of each thesis Chapter.

Aim 1. To investigate the haemostatic and haemodynamic abnormalities in individuals with an HRE and determine their relationships with exercise BP

Hypothesis 1. Individuals with an HRE will have haemostatic abnormalities (in particular raised haemostatic markers that reflect endothelial dysfunction) compared to individuals with normotensive response to exercise and these markers will be significantly associated with exercise BP

Hypothesis 2. Individuals with an HRE will have arterial haemodynamic abnormalities (e.g. increased arterial stiffness and systemic vascular resistance) compared to individuals with normotensive response to exercise and these haemodynamic measures will be significantly associated with exercise BP

Aim 2. To develop the methodology regarding metabolomics analysis and to explore the metabolic actions of the aldosterone antagonist spironolactone on exercise BP and aortic stiffness in individuals with an HRE

Hypothesis 1. Aldosterone antagonist spironolactone will reduce exercise BP and aortic stiffness via BP-independent effects of spironolactone

Hypothesis 2. The reduction in aortic stiffness by spironolactone treatment will be significantly associated with the reduction in exercise BP

Aim 3. To investigate the metabolic abnormalities in a population with the high prevalence of an HRE (people with T2DM) using untargeted metabolomics analysis and determine their relationships with exercise BP (peripheral and central)

Hypothesis 1. Individuals with T2DM will have metabolic abnormalities (including increased levels of glucose, fructose and lactate) compared to non-diabetic individuals and these metabolic markers will be significantly associated with exercise BP

Chapter 3. Methodology

This chapter describes methodology involved in examining the haemodynamic, haemostatic, and metabolic contributors of a hypertensive response of exercise in all studies that comprise this thesis.

3.1 Introduction

Validated and highly reproducible haemodynamic measures were included in most of the chapters in this thesis, as individually described below. Haemostatic markers were measured in the study within *Chapter 4* and included standard haemostatic protocols and measures. Importantly, a significant part of this research program also involved development of the protocol regarding resting blood pressure (BP) measurements (the details described in 3.2.1 section), as well as metabolomics analysis. Metabolomics analysis is still in its infancy, and despite a lot of effort employed worldwide from various research groups, standard protocols and operating procedures involving the sample preparation, sample analysis and data analysis in metabolomics analysis are still not clearly defined. Inappropriate sample preparation and subsequent data analysis can introduce systematic bias into data that may consequently interfere with the metabolomics analysis and possibly produce false discoveries. Therefore, in order to apply metabolomics analysis to explore metabolic contributors of a hypertensive response to exercise (HRE), we first had to define the protocols that would be used for the subsequent analysis of study data (used in *Chapter 5* and *Chapter 6*). In section 3.4, the final protocols for the sample preparation, sample analysis and data analysis are described, and in *Appendix 2*, a novel method for the normalisation of metabolomics data is presented as a separate study.

3.2 Haemodynamic variables

3.2.1 Office brachial blood pressure

Resting office brachial BP was measured using a validated semi-automated oscillometric device¹⁸³ (Omron HEM-907; OMRON Europe B.V. (OMCE), Hoofddorp, The Netherlands) or a validated mercury-free manual sphygmomanometer (UM-101B, A&D Medical, Thebarton, South Australia).¹⁸⁴ Office brachial BP was measured by a trained non-clinician in duplicate and the mean of these two readings was used for subsequent analysis. Brachial pulse pressure (PP) was calculated as the difference between brachial systolic and diastolic BP. As number of factors may influence the BP measurement such as room temperature, background noise, alcohol or nicotine consumption,⁴ participants were examined in a quiet and temperature-controlled room. Participants were also asked to refrain from consuming heavy meals and caffeine or smoking for at least three hours prior to examination and avoid

exercise on a day of examination. Blood pressure was measured in accordance with guidelines, using an appropriately sized cuff with the arm supported at heart level.⁴

Current guidelines for the management of hypertension recommend that office brachial blood pressure (BP) should be optimally measured after five minutes of rest,⁴ but brachial BP may decrease for up to 10 minutes of rest before reaching a plateau level.^{185, 186} In setting up the protocol for BP measurements for our studies, we conducted a retrospective analysis of data collected in the BP GUIDE study (<http://www.anzctr.org.au>; ACTRN12608000041358) in which brachial BP was measured after five and 10 minutes of rest to determine the most appropriate time for BP measurements.¹³ We found that BP recorded after 10 minutes was more representative of true blood pressure control which was assessed by comparison with patients' out-of-office BP (regarded as a 'gold-standard' measure of BP).¹³ Also, BP recorded at 10 minutes but not after the conventional five minute wait was highly associated to BP-related end-organ damage such as left ventricular (LV) mass.¹³ These findings suggest that BP measured after the recommended five minute rest period may not be an appropriate time for BP measurement and using this as the sole method to assess BP control may result in misclassification or inappropriate management of some individuals. Therefore, 10 minutes waiting period before the BP measurement was used in our studies. The results of this study were published in the *Journal of Human Hypertension* and are included in the *Appendix 1*.

3.2.2 Exercise brachial blood pressure

Stress test brachial BP. Exercise stress tests were performed at local cardiology departments where study participants were recruited for the *Chapter 4* study. Brachial BP during the exercise stress testing (standard Bruce protocol) was measured using either a validated mercury-free sphygmomanometer (UM-101B, A&D Medical, Thebarton, South Australia)¹⁸⁴ or automated device (Tango, SunTech Medical Instruments, NC, USA)¹⁸⁷ in the final two minutes of each three-minute exercise stage. The initial speed of the treadmill was set to 2.7 km/h and the inclination was set to 10% (stage 1). At three minute intervals, the inclination of the treadmill was increased by 2% and the speed increased to 4.0 km/h at stage 2; 5.5 km/h at stage 3; 6.8 km/h at stage 4 and; 8.0 km/h at stage 5.

Research clinic exercise brachial BP. Exercise brachial BP at the research clinic (moderate intensity) was measured using a validated mercury-free sphygmomanometer (UM-101B, A&D Medical, Thebarton, South Australia).¹⁸⁴ Exercise was performed on an upright cycle

ergometer (WattBike, Wattbike Ltd, Nottingham, United Kingdom; *Chapter 4*) or on a bicycle ergometer positioned on the bed in a semi-recumbent position (*Chapter 6*), as described in Keith et al¹⁸⁸ (Rehab Trainer 881, MONARK Exercise110 AB, Vansbro, Sweden). The workload of exercise was set for each individual to achieve a heart rate of 60% of age-predicted maximal heart rate calculated as per formula $(220 - \text{age} \times 0.60)$. Exercise brachial BP was measured in duplicate and the mean value was used for analysis. During the BP measurements, the participant's arm was supported at the level of the heart using an adjusted platform, and the participant was asked to keep the arm as still as possible.

3.2.3 Out-of-office blood pressure

24 hour ambulatory BP. A validated device (TM-2430, A&D Mercury, A&D Medical, Thebarton, South Australia)¹⁸⁹ was used for 24 hour ambulatory BP monitoring. Participants were advised to maintain routine daily activities during BP monitoring and to have their arm still and relaxed during each measurement. The device was set to record BP in previously determined intervals (every 20 minutes during the day and every 30 minutes during the night or every 30 minutes during the day and every hour during the night).²³ The mean values of BP measured during daytime (daytime BP), night time (night time BP) and overall 24 hour (24 hour ambulatory BP) were used for the analysis.

Home BP. Seven-day home BP was self-measured using a validated oscillometric device (UA-767, A&D Mercury, A&D Medical, Thebarton, South Australia).¹⁹⁰ Participants were instructed to take BP measurements in a warm and quiet room after at least five minutes of seated rest in a chair enabling back support, with feet flat on the ground and the arm supported at heart level.¹⁹¹ Home BP was measured in duplicate in the morning (between 6am and 10am), midday and evening (between 6pm and 10pm). The first of two BP measurements was discarded and the second BP was recorded and used for analysis.

3.2.4 Office central blood pressure

Office central BP was measured at rest, as well as during exercise, using radial applanation tonometry and validated generalized transfer function (SphygmoCor 8.1, AtCor Medical, Sydney, NSW).^{13, 14} Central BP was measured in duplicate and the mean value was used for the analysis. Acquired pressure waveforms were calibrated by brachial BP values taken immediately prior to applanation tonometry. Central PP was calculated as the difference between systolic and diastolic BP (Figure 3.1). Augmentation index (AIx) was defined as the

difference between the second and first systolic peak of the central pressure waveform (or augmentation pressure [AP]) and expressed as a percentage of the PP. As AIX is influenced by heart rate, AIX was also normalised to a heart rate of 75 bpm. The ratio of brachial and central PP defined the PP amplification.

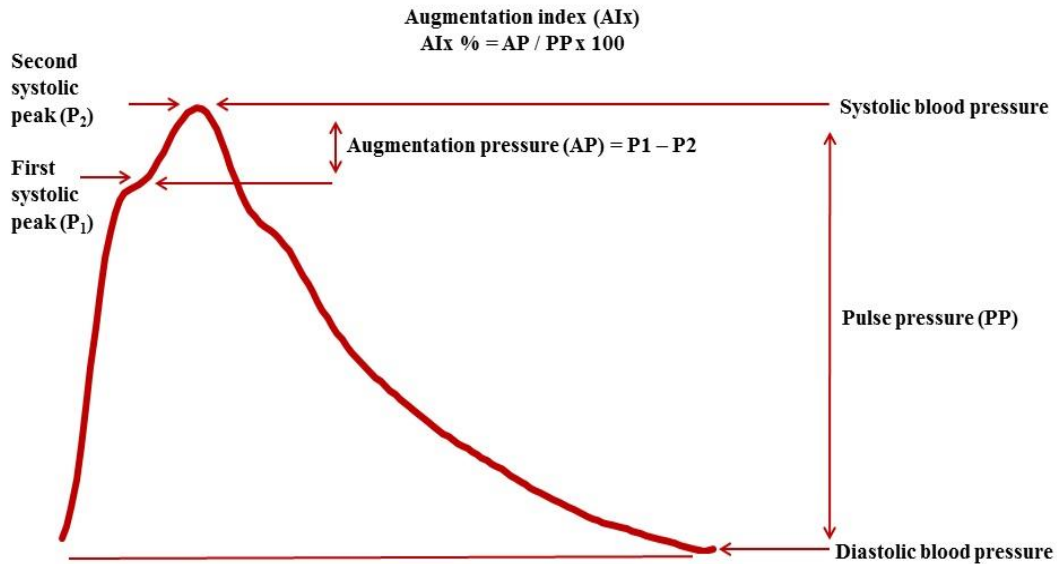


Figure 3.1 Aortic pressure waveform

Central (aortic) pressure waveform derived from the radial pressure waveform by applying generalized transfer function. Central systolic and diastolic blood pressure (BP) represents the highest and the lowest points of the pressure waveform, respectively. Central pulse pressure (PP) is calculated as the difference between systolic and diastolic BP, whereas augmentation pressure (AP) is calculated as the difference between the first and the second systolic peaks. Augmentation index (AIX) is defined as the percentage of AP to PP.

3.2.5 Arterial stiffness

Arterial stiffness was estimated by measuring aortic and brachial pulse wave velocity (PWV) using sequential applanation tonometry in combination with a three-lead electrocardiogram (SphygmoCor 8.1, AtCor Medical, Sydney, NSW).¹⁷⁷ PWV represents the speed (expressed in m/s) at which the pressure waveform travels along the large arteries (aortic or brachial artery), during each cardiac cycle (Figure 3.2). Pressure waveforms were recorded at the common carotid artery and femoral (for aortic PWV) or radial (for brachial PWV) artery, and the ratio of the distance and the time delay between the pressure waves recorded at two sites defines PWV. The time delay of the pressure waveform is calculated by software by the using ‘foot-to-foot’ method - time delay between the foot of each pressure waveform recorded at

two sites. The surface distance between the two recording sites is taken as the distance covered by the waves.

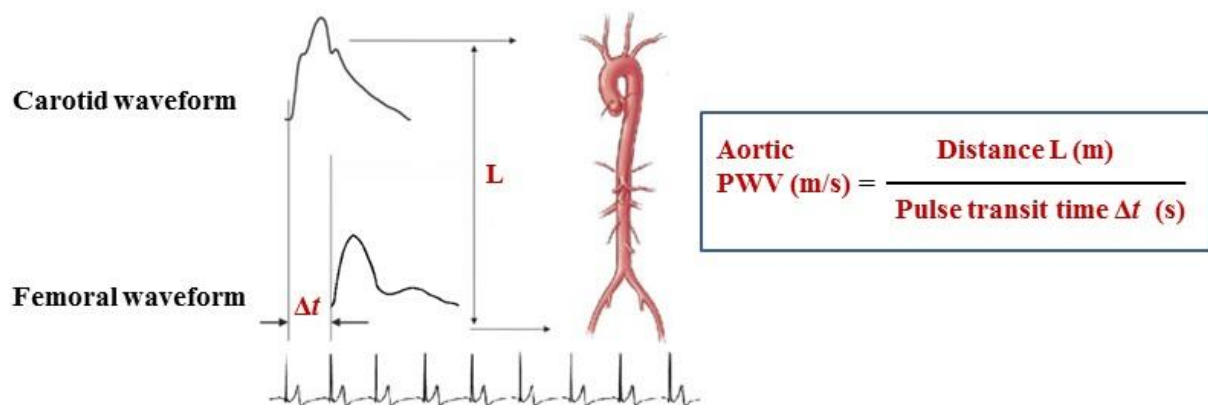


Figure 3.2 Estimation of aortic stiffness using pulse wave velocity

The 'Foot-to-foot' method for calculating the carotid-femoral pulse wave velocity (PWV) which is expressed in m/s. Pressure waveforms are recorded at the common carotid and femoral arteries and the speed of wave is calculated as the ratio of the distance (L) and the time delay (Δt) between the pressure waveforms recorded at two sites.

Aortic PWV during moderate intensity exercise (*Chapter 6*) was measured in the semi-recumbent position by the modified protocol as designed by Keith et al.¹⁸⁸ Participants exercised on a bicycle ergometer (Rehab Trainer 881, MONARK Exercise AB, Vansbro, Sweden) that was placed on the bed and participants were instructed to pedal until they achieved a heart rate of 10 beats per minute (bpm) above the set heart rate target. When the required heart rate was reached, participants were asked to stop pedalling in order for the femoral (or carotid) pressure waveform to be acquired by the operator. The process lasted for approximately 15 seconds, during which time the participants heart rate declined towards the target heart rate. Duplicate measures were acquired and the mean value was used for the analysis.

3.2.6 Bioimpedance cardiography

Stroke volume (SV), cardiac output (CO) and systemic vascular resistance (SVR) were measured at rest and during exercise using a validated and reproducible Physio Flow device (Physio Flow; Manatec Biomedical; Macheren, France).^{192, 193} This was achieved by placing six pre-gelled sensor electrodes on the participant's chest and detecting changes in blood volume and velocity during each cardiac cycle which results in changes in electrical

conductivity and impedance. SV was estimated as the largest change in impedance during systole, whereas CO was defined by multiplying the heart rate (HR) with SV. SVR was defined as the ratio of the sum of mean arterial pressure and CO.

3.3 Haemostatic and clinical biochemistry markers

Vacutainer[®] tubes with 3.8% sodium citrate were used for blood sample collection and analysis of haemostatic markers including von Willebrand factor (vWf), fibrinogen, tissue factor (TF) and thrombin-antithrombin complexes (TAT). Study participants were seated for at least 10 minutes after which blood was drawn by venepuncture from cubital veins via an inserted indwelling cannula to enable direct access for blood sampling during different physiological conditions (at rest, during exercise and post-exercise). Immediately following sample collection, the cannula was filled with heparin solution (to prevent blood clotting within the cannula) and collected blood samples were centrifuged for 20 minutes at 2000 g to obtain citrated plasma, with 0.5 mL aliquots stored at -80°C until analysis. Prior to analysis of haemostatic markers, plasma samples were thawed at 37°C. TF, vWf, and TAT were determined using commercially available immunoassays according to manufacturer's instructions (ELISA; Assaypro, St. Charles, MO, USA). Fibrinogen was measured using a standardised clotting-based Clauss assay. Clotting times were measured using the STart[®]-4 haemostasis analyser (Diagnostica Stago, Asnières-sur-Seine, France) and compared to specialty assayed reference plasma (SARP, Helena Laboratories, Beaumont, TX, USA).

A fasted or non-fasted blood samples were drawn to determine standard metabolic markers (total, low-density and high-density lipoprotein cholesterol, glucose, glycosylated haemoglobin [HbA_{1c}], insulin and triglycerides) and samples were analysed in local hospital pathology laboratory, as per standard pathology procedures.

3.4 Metabolomics

3.4.1 Liquid chromatography-mass spectrometry (LC-MS)

Sample preparation. Fasting serum samples were collected and 0.5 mL aliquots were stored at -80°C until analysis. Samples were thawed and a 100 µL aliquot of each sample was mixed with 300 µL of ice-cold methanol, incubated at 4°C for 20 minutes and centrifuged at 13200 g for 10 minutes. The supernatant was lyophilized in a vacuum centrifuge (Labconco, Kansas City, USA) and re-stored at -80°C. The dried samples were reconstituted in 100 µL of HPLC-

grade water prior to LC-MS analysis. Samples were vortexed and centrifuged for 15 minutes, and supernatant was transferred to vials. Quality control samples were prepared by pooling 10 µL of random samples and analysed throughout the LC-MS runs (four study samples were bracketed by two quality controls) to assess the equipment drift and 'batch-to-batch' variability, as recommended in the paper of Dunn et al.¹²⁴

Sample analysis. Samples were analysed using an LTQ-Orbitrap XL mass spectrometer (ThermoFisher Scientific, Bremen, Germany) controlled by XCalibur v2 software (ThermoFisher Scientific). To prevent systematic bias, samples were randomly distributed throughout the runs. Chromatographic separation was performed on a Waters Nova-Pak 4.0 µm C₁₈ column (3.91x150 mm) with an injection volume of 10 µL. The mobile phases used for the sample elution were A) 0.1% formic acid in water and B) 0.1% formic acid in methanol, with a flow rate of 0.8 mL/minute. Gradient conditions were as follows: 0-4 minutes hold at 10% B, 4-6.5 minutes linear gradient 10-50% B, 6.5-14.5 minutes linear gradient 50-80% B, 14.5-15.5 minutes linear gradient 80-100% B, 15.5-17.5 minutes hold at 100% B, 0.5 minutes re-equilibration in 10% B. Centroid mass spectra was acquired in full scan mode at the resolution of 30,000 across a mass range of 50-1000 mass/charge (m/z). Data were acquired in both positive (+) and negative (-) ionization modes. Ionization conditions were as follows: capillary temperature of 300°C, flow rate of sheath gas of 30 au, auxiliary gas flow rate of 5 au, capillary voltage of 7 V for (+) ion acquisition and capillary voltage of -44 V for (-) ion acquisition. Spectra for tandem mass spectrometry (MS/MS) were acquired using FTMS Mode at an isolation width of two mass units and collision energy of 20 V. Retention times for MS/MS targets were specified according to retention time values acquired by LC-MS. Raw LC-MS data were converted to mzXML format. Data were then processed for peak finding, grouping and retention time alignment using XCMS running in the R environment (R 2.14; <http://cran.r-project.org/>). Karpievitch et al¹⁴ have developed and applied a novel singular value decomposition-based method (EigenMS) for data normalisation to detect and correct for any systematic bias, caused by different batch effects, day-to-day variations in instrument performance, signal intensity loss due to time-dependent effects of the LC-MS column performance, accumulation of the contaminants in the MS ion source and MS sensitivity among others. The EigenMS normalisation technique is described in more details in *Appendix 2*.

LC-MS data analysis. The LC-MS data was analysed by either a repeated-measures ANOVA to compare each feature and then corrected the resulting p values using the Benjamini and Hochberg False Discovery Rate correction¹⁹⁴ or principal component analysis (PCA). The LC-MS data were mean-centred and Pareto-scaled before using PCA to dimensionally reduce the data using PLS Toolbox 6.5 (Eigenvector Research Inc., Wenatchee, US). Visual inspection of a ‘scree’ plot of captured variance versus number of components was used to identify the number of PC comprising each model (prior to any further analysis). Plots of scores on each PC were visually inspected. Those that appeared to separate the groups were compared using an independent t -test. Loading coefficients were used to identify metabolites that significantly contributed to discriminatory PCs. In addition to PCA, individual metabolites were compared across groups by independent t -test with the false discovery rate held at 5% using the method of Benjamini and Hochberg.¹⁹⁵ Features were accepted as being significantly different if the corrected $p < 0.05$. Metabolomics data, when included in an analysis with conventional data, was checked for normality and non-normally distributed data was square-root-transformed. The LC-MS data were checked for artefacts (for example, Fourier-transform ‘shoulders’), de-isotoped and putatively annotated using online databases (Human Metabolome Database [HMDB] and Metlin) and the Taverna workflows of Brown et al.¹⁹⁶ Compounds of interest were further examined by tandem mass spectrometry (MS/MS) to provide additional structural information and to confirm annotation, where possible, by comparison with MS/MS spectra held in public databases.

3.4.2 Proton nuclear magnetic resonance (¹H NMR) spectroscopy

Sample preparation for Bruker spectrometer operating at 800.13 MHz. Fasting serum samples were collected and 0.5 mL aliquots were stored at -80°C until analysis. Samples were thawed and 300 µL was added to 300 µL of D₂O in a 1.5 mL Eppendorf tube. The mixture was briefly vortexed and then centrifuged at 16606 g for five minutes after which 550 µL of the supernatant was transferred to a 5 mm NMR tube for analysis.

Sample analysis. NMR data were acquired on a Bruker Avance III spectrometer operating at 800.13 MHz (Bruker Biospin, Karlsruhe, Germany). Data were acquired with a Carr-Purcell-Meiboom-Gill pulse sequence with presaturation during the recycle delay (2 s) and a total echo time of 51.2 ms. The time domain was 128 k and the sweep width was 20 ppm. Data were Fourier transformed with exponential line broadening of 0.5 Hz in the frequency domain. Spectra were phased and baselined in Topspin 3.0 (Bruker Biospin, Karlsruhe,

Germany) before being imported into Matlab (Mathworks, Natick, MA) at full resolution. Resulting spectra were normalised and bucketed using probabilistic-quotient normalisation¹⁹⁷ and adaptive intelligent binning¹¹² respectively, both in Matlab.

Sample preparation for Agilent/Varian Inova spectrometer operating at 400 MHz. Fasting serum samples were collected and 0.5 mL aliquots were stored at -80 °C until analysis. A 500 µL aliquot of serum was mixed with 1 mL of methanol (2:1 v/v with original sample), incubated in a -20°C freezer for 20 minutes and centrifuged for 10 minutes at 13,200 g. Supernatants were dried in a vacuum centrifuge overnight (Labconco, Kansas City, USA), re-suspended in 700 µL NMR buffer (2 mM 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt in D₂O).

Sample analysis. ¹H NMR spectra were acquired using an Agilent/Varian Inova 400 MHz wide bore spectrometer (Varian Medical Systems, Palo Alto, CA, USA), in a 5mm ID-PFG probe. Spectra were acquired with 90° pulse excitation, sweep width of 4,299 ppm and relaxation delay of 2 seconds at 25°C. A total of 128 transients were collected into 32000 complex data points. The water resonance was set to the centre of the sweep width on the transmitter offset and attenuated using a presaturation pulse of 2 seconds during the relaxation delay and the mixing time (100 ms) of the NOESY pulse sequence. Resulting spectra were normalised and bucketed using probabilistic-quotient normalisation¹⁹⁷ and adaptive intelligent binning¹¹² respectively, both in Matlab.

NMR data analysis. NMR data were mean-centred and Pareto-scaled, before being analyzed using PCA and partial least squares-discriminant analysis (PLSDA), both in PLS Toolbox (PLS Toolbox 6.5, Eigenvector Research Inc., Wenatchee, US). Visual inspection of a ‘scree’ plot of captured variance versus number of components was used to identify the number of PC comprising each model (prior to any further analysis). Plots of scores on each PC were visually inspected. Those that appeared to separate the groups were compared using an independent t-test. Loading coefficients were used to identify metabolites that significantly contributed to discriminatory PCs. Permutation testing was used to assess whether PLSDA models were the result of over-fitting; models were considered significant if the estimated $p < 0.05$. Data processing of metabolomics data were conducted in Matlab. Features of interest were putatively annotated using online databases HMDB and Metlin.

Chapter 4. Association of Von Willebrand Factor Blood Levels with Exercise Hypertension

At the time of thesis submission, this chapter is under resubmission in *European Journal of Applied Physiology*:

Sonja B. Nikolic, Murray J. Adams, Petr Otahal, Lindsay M. Edwards, James E. Sharman

This chapter reports on haemodynamic and haemostatic correlates of a hypertensive response to exercise at moderate intensity, akin to intensity of exercise blood pressure shown to best predict cardiovascular events, in participants with a clinical indication for exercise stress testing.

4.1 Abstract

Background. A hypertensive response to moderate intensity exercise (HRE) is associated with increased cardiovascular (CV) risk. The mechanisms of an HRE are unclear, although previous studies suggest this may be due to haemostatic and/or haemodynamic factors. We investigated the relationships between an HRE with haemostatic and hemodynamic indices.

Methods. Sixty four participants (aged 57 ± 10 years, 71% male) with indication for exercise stress testing underwent CV assessment at rest and during moderate intensity exercise, from which 20 participants developed an HRE (defined as moderate exercise systolic BP ≥ 170 mmHg/men and ≥ 160 mmHg/women). Rest, during exercise and post-exercise blood samples were analysed for haemostatic markers (von Willebrand factor (vWf), fibrinogen, thrombin-antithrombin complexes and tissue factor), as well as haemodynamic measures of brachial and central blood pressure (BP), aortic stiffness and systemic vascular resistance index (SVRi).

Results. HRE participants had higher rest vWf compared with normal BP response to exercise (NRE) participants (1927 mU/mL, 95% CI: 1240-2615, vs. 1129 mU/mL, 95% CI: 871-1386; $p=0.016$). Vwf levels significantly decreased from rest to post-exercise in HRE participants ($p=0.005$), whereas vWf levels significantly increased from rest to exercise in NRE participants ($p=0.030$). HRE participants also had increased triglycerides, aortic stiffness, resting BP and exercise SVRi ($p < 0.05$ for all). vWf at rest (but no other haemostatic marker) predicted exercise brachial systolic BP ($\beta = 0.220$, $p = 0.043$; Adjusted $R^2 = 0.451$, $p < 0.001$) independent of age, sex, body mass index, triglycerides, resting brachial systolic BP and aortic stiffness.

Conclusions: Increased resting blood levels of vWf are independently associated with moderate intensity exercise systolic BP. These findings implicate abnormalities in haemostasis as a possible factor contributing to an HRE at moderate intensity.

4.2 Introduction

A recent meta-analysis in people with a clinical indication for exercise stress testing showed that those with a hypertensive response to exercise (HRE) at moderate intensity were at greater risk for cardiovascular (CV) events and mortality independent of resting blood pressure (BP)⁵. The mechanisms of an HRE are unclear, although abnormal haemostatic and/or haemodynamic factors may play key roles. With respect to haemodynamics, an HRE is associated with endothelial dysfunction⁵⁰ and increased large artery stiffness,⁵¹ which together may lead to raised left ventricular (LV) afterload, and ultimately affect cardiac structural and functional characteristics.^{11, 25} However, little is still known on systemic haemodynamic function (including central BP, stroke volume, cardiac output and peripheral vascular resistance) during moderate intensity exercise in people with an HRE.

Under pathological conditions (e.g. increased shear pressure), the vascular endothelium plays a crucial role in regulating normal haemostasis by expressing/releasing regulatory substances of coagulation including tissue factor (TF).⁷¹ This may lead to an initiation of the coagulation cascade, an increase in thrombin generation, and potentially CV complications. Indeed, fibrinogen (a haemostatic marker that is elevated during coagulation) has been shown to be associated with an increased CV risk.⁷⁷ The von Willebrand factor (vWf) is also one of the haemostatic markers that is released with endothelial damage, and is therefore proposed to be an indicator of endothelial damage/dysfunction.⁷⁹ Importantly, raised vWf is positively associated with both systolic and diastolic BP, and is higher in patients with hypertension compared with controls.⁸ vWf is also an independent correlate of increased 24-hour ambulatory pulse pressure in select patient populations.⁸³ Altogether, the above data raises the possibility that irregularities of haemostasis, as well as arterial haemodynamics, may contribute to an exaggerated exercise BP. In this study, we investigated the relationships between haemostatic and haemodynamic markers with moderate exercise BP. We hypothesized that haemostatic markers (vWf, TF, fibrinogen and markers of thrombin generation) and systemic haemodynamics (including central BP, stroke volume, cardiac output and peripheral vascular resistance) would be significantly different in people with an HRE and significantly associated with moderate intensity exercise BP.

4.3 Materials and Methods

4.3.1 Study participants

The study population consisted of people with an indication for exercise stress testing who were free from coronary artery disease. The recruitment was conducted through local hospital cardiology departments, but all haemodynamic and haemostatic measurements (under rest and moderate intensity exercise conditions) were collected at the research clinic on another occasion. Participants were excluded if they were under 18 years of age, had a hypotensive response to exercise (failure of systolic BP to increase with increasing exercise intensity) or BP reading during exercise was not of good quality, had resting BP $\geq 140/90$ mmHg when tested at the research clinic or were unable to attend the research clinic for comprehensive cardiovascular (CV) examination under resting and moderate intensity exercise conditions. From 109 potential participants, several were excluded due to coronary artery disease ($n = 18$), exercise hypotension ($n = 1$), poor quality exercise BP (cardiac nurses were not confident with the BP reading during exercise due to movement and noise; $n=15$) or were hypertensive at rest ($n = 11$). This left 64 participants who completed the study. The study was approved by the Human Research Ethics Committee of the University of Tasmania. Written informed consent was obtained from all participants and procedures were in agreement with the Declaration of Helsinki.

4.3.2 Study protocol

All participants underwent a standard maximal intensity treadmill exercise stress test (Bruce protocol) at local hospital cardiology departments. The initial speed of the treadmill was set to 2.7 km/h and the inclination was set to 10% (stage 1). At three minute intervals, the inclination of the treadmill was increased by 2% and the speed increased to 4.0 km/h at stage 2; 5.5 km/h at stage 3; 6.8 km/h at stage 4 and; 8.0 km/h at stage 5. Those meeting inclusion criteria were invited to attend the research clinic on another occasion in the morning after an overnight fast (scheduled as soon as possible after exercise stress testing) for cardiovascular examination at rest and during moderate intensity exercise. An indwelling cannula was placed in the participant's arm to enable direct access for blood sampling of haemostatic markers at three different conditions: 1) at rest, 2) during exercise, and 3) post-exercise. Samples used to measure standard clinical biochemistry markers were collected at rest only. Participants were examined in a quiet, temperature-controlled room that included

haemodynamic measurements of office brachial and central BP, arterial stiffness, cardiac output, stroke volume and systemic vascular resistance index (SVRI). After resting measurements, participants performed exercise at moderate intensity on an upright cycle ergometer (WattBike, Wattbike Ltd, Nottingham, United Kingdom). The workload of exercise was set for each individual to achieve a heart rate of 60% of age-predicted maximal heart rate, simulating the moderate intensity levels achieved during exercise stress testing (approximately stage 2, Bruce protocol), in keeping with the intensity of exercise BP shown to best predict CV events.⁵ Heart rate was calculated as per the formula $(220 - \text{age} \times 0.60)$. When steady-state heart rate was achieved, haemodynamic measurements were repeated. Exercise brachial systolic BP levels measured at this stage were used to identify those with an HRE. Blood samples 'during moderate intensity exercise' were collected immediately on stopping exercise, and this was completed within 30 seconds. Post-exercise samples were collected after 15 minutes of seated rest.

4.3.3 Haemostatic markers

Vacutainer[®] tubes with 3.8% sodium citrate were used for blood sample collection and analysis of haemostatic markers that best reflect the coagulation activation and thrombin generation (TF and thrombin-antithrombin complexes; TAT), abnormal vascular function (vWf), as well as a marker previously reported to be significantly associated with adverse cardiovascular outcomes (fibrinogen). Tubes were centrifuged immediately following sample collection for 20 minutes at 2000 g to obtain citrated platelet-poor plasma, with 0.5 mL aliquots stored at -80°C until analysis until batched analysis at the end of the study. Prior to analysis of haemostatic markers, plasma samples from all participants were thawed at 37°C. TF, vWf, and TAT were determined using commercially available immunoassays according to manufacturer's instructions (ELISA; Assaypro, St. Charles, MO, USA). Fibrinogen was measured using a standardised clotting-based Clauss assay. Clotting times were measured using the STart®-4 haemostasis analyser (Diagnostica Stago, Asnières-sur-Seine, France) and compared to specialty assayed reference plasma (SARP, Helena Laboratories, Beaumont, TX, USA). All standards and controls were measured in duplicate, with participants' samples measured singularly. All controls for each assay were within acceptable limits. Intra- and inter-assay coefficient of variations (CV) for immunoassays were 4.8% and 7.2% for TAT; 4.9% and 7.0% for TF; 5.0% and 7.1% for vWF, and 1.4% and <5.0% for fibrinogen.

4.3.4 Haemodynamic variables

A validated manual mercury-free sphygmomanometer (UM-101B, A&D Medical, Thebarton, South Australia)¹⁸⁴ or automated device (Tango, SunTech Medical Instruments, NC, USA)¹⁸⁷ were used for brachial BP measurements during exercise stress testing (in the final two minutes of each three-minute exercise stage) at hospital cardiology departments. Seated resting brachial BP at the research clinic was measured in duplicate after 10 minutes using a validated semi-automated oscillometric device (Omron HEM-907; OMRON Europe B.V. (OMCE), Hoofddorp, The Netherlands).¹⁸³ BP was measured by a trained non-clinician in accordance with guidelines.^{4, 198} Exercise BP at the research clinic was measured in duplicate using a validated mercury-free sphygmomanometer (UM-101B, A&D Medical, Thebarton, South Australia).¹⁸⁴ A previous study in a large sample of apparently healthy subjects, including both men and women undertaking exercise stress testing, has defined an HRE as the upper limits of exercise BP responses.¹⁹⁹ The same convention was followed in this current study conducted in a similar study population where an HRE was defined as moderate intensity exercise systolic BP in the highest tertile of the study population (this equated to systolic BP ≥ 170 mmHg for men and ≥ 160 mmHg for women). However, the criteria used in this study to define an HRE may not be applicable to different study populations, such as younger healthy individuals. Accordingly, a normotensive response to exercise (NRE) was defined as moderate intensity exercise systolic BP in the first and second tertiles of the study population. Central BP at rest and during moderate intensity exercise was recorded using radial applanation tonometry and calibrated by brachial BP values taken immediately prior to applanation tonometry (SphygmoCor 8.1, AtCor Medical, Sydney, NSW).^{47, 48} Pulse pressure (PP) was defined as the difference between systolic and diastolic BP. Augmentation index was defined as the difference between the second and first systolic peak of the central pressure waveform (or augmentation pressure) and expressed as a percentage of the PP. The ratio of brachial and central PP defined the PP amplification. Aortic and brachial pulse wave velocities (measures of arterial stiffness) were measured using applanation tonometry (SphygmoCor 8.1, AtCor Medical, Sydney, NSW).¹⁷⁷ Cardiac output, stroke volume and SVRi were measured using a validated and reproducible Physio Flow device (Physio Flow; Manatec Biomedical; Macheren, France).¹⁹³

4.3.5 Biochemical analysis

Standard pathology laboratory procedures were performed using serum samples to determine levels of glucose, triglycerides, total, low-density lipoprotein (LDL) and high-density lipoprotein (HDL).

4.3.6 Statistical analysis

Data are presented as mean and standard deviation unless otherwise stated. The unequal-variance independent *t*-test was used to compare clinical characteristics between HRE and NRE groups. Comparisons between categorical variables were conducted by chi-squared tests. Pearson correlation was used to determine the associations between continuous variables (after appropriate transformations, see below).

Repeated measures regression analysis for haemostatic outcome variables was used to compare groups (HRE vs. NRE) over time (including an interaction of group with time). Residuals were found to be non-normally distributed and this was rectified by transformations; natural logarithm for vWf, square root transformation for TAT and TF, and square transformation for fibrinogen. Models were adjusted for age, sex, body mass index, triglycerides and resting systolic BP. Model contrasts were used to compare between groups at each time point and to compare time within each group. Multiple linear regression analysis was used to examine predictors (resting) of moderate intensity exercise brachial systolic BP. Regression diagnostics were examined in all models to ensure assumptions were not violated. A value of $p < 0.05$ was taken as statistically significant. Statistical analysis was conducted using SPSS 20.0 (IBM SPSS Statistics for Windows, Armonk, NY: IBM Corp) and Stata 12.1 (StataCorp. 2011. Stata Statistical Software: Release 12. College Station, TX: StataCorp LP).

4.4 Results

4.4.1 Clinical characteristics

Table 4.1 summarises the clinical characteristics of study participants. Out of 64 participants, 20 were identified with an HRE. The majority of study participants were male and there were no significant differences in age, sex or use of antihypertensive medications between HRE and NRE participants. Serum triglycerides were significantly higher in HRE participants compared with NRE participants.

Table 4.1 Clinical characteristics of study participants

| Characteristics | NRE (n = 44) | HRE (n = 20) | <i>p</i> value |
|--|-----------------|-----------------|----------------|
| Age (years) | 56 ± 10 | 60 ± 10 | 0.140 |
| Sex, male (%) | 31 (71) | 14 (70) | 1.000 |
| Body mass index (kg/m ²) | 26.6 ± 3.4 | 28.9 ± 5.1 | 0.075 |
| Antihypertensive medications (%) | 10 (23) | 8 (40) | 0.261 |
| Angiotensin converting enzyme inhibitors (%) | 2 (10) | 6 (14) | 1.000 |
| Calcium channel blocks (%) | 3 (7) | 4 (20) | 0.257 |
| Angiotensin receptor inhibitors (%) | 3 (7) | 4 (20) | 0.257 |
| Statins (%) | 8 (18) | 6 (30) | 0.463 |
| Glucose (mmol/L) | 5.9 ± 2.7 | 5.2 ± 0.5 | 0.138 |
| Triglycerides (mmol/L) | 1.28 ± 0.56 | 1.62 ± 0.47 | 0.017 |
| Total cholesterol (mmol/L) | 5.19 ± 0.89 | 5.38 ± 1.29 | 0.547 |
| High density lipoprotein (mmol/L) | 1.40 ± 0.37 | 1.32 ± 0.26 | 0.348 |
| Low density lipoprotein (mmol/L) | 3.18 ± 0.76 | 3.39 ± 1.19 | 0.478 |

Data are mean ± standard deviation or n (%); NRE, normotensive response to exercise; HRE, hypertensive response to exercise; ACEi, angiotensin converting enzyme inhibitors

4.4.2 Haemostatic markers

Repeated measures regression analysis of vWf showed significant between groups effect ($p = 0.016$), no significant effect of time ($p = 0.080$) and a significant interaction effect for group*time ($p = 0.003$). Also, there was a significant effect of time ($p < 0.001$) for TF, but no significant interaction effect for group*time ($p = 0.434$) or between groups effect ($p = 0.699$). Fibrinogen and TAT had no significant main effects or interactions for time and/or group.

Table 4.2 shows back-transformed predicted marginal means and 95% confidence intervals (CI) from the repeated measures regression and shows the tests for between group effects at each time point for the haemostatic markers in HRE and NRE participants across three conditions (rest, during exercise and post-exercise) after adjusting for age, sex, body mass index, triglycerides and resting systolic BP. vWf at rest was significantly higher in HRE participants compared to NRE participants, but there were no significant differences between groups in vWf during or post-exercise (also shown in Figure 4.1).

Examining within each group, vWf significantly increased from rest to exercise for the NRE group (355 mU/mL, 95% CI: 35 – 676, $p = 0.030$), whereas vWf significantly decreased from rest to post-exercise for the HRE group (-882 mU/mL, 95% CI: -1494 – -269, $p = 0.005$). Further, the NRE group showed significant changes for TF from rest to post-exercise (-16.3 pg/mL, 95% CI: -28.0 – -4.6, $p = 0.006$) and from exercise to post-exercise (-22.6 pg/mL, 95% CI: -35.2 – -10.1, $p < 0.001$). No further significant changes were observed within groups.

4.4.3 Haemodynamic variables

There were no significant differences between the groups in the duration of hospital exercise stress testing (NRE: 10.5 ± 2.6 minutes vs. HRE: 9.7 ± 2.6 minutes, $p = 0.252$), or in maximal exercise metabolic equivalents units (METs; NRE: 12.4 ± 2.5 METs vs. HRE 11.6 ± 2.7 METs, $p = 0.277$). Table 4.3 summarises the haemodynamic variables measured during the hospital exercise stress testing (maximal and moderate intensity - stage 2 levels, Bruce protocol), and during rest and moderate intensity exercise at the research clinic. There was no significant differences between hospital stage 2 exercise systolic BP and clinic moderate intensity exercise systolic BP in NRE or HRE groups (mean difference 4.4 ± 23.7 , $p = 0.233$; and -0.2 ± 23.2 , $p = 0.970$; respectively). Although all participants were normotensive at rest, brachial systolic and central systolic BP variables were significantly higher in HRE participants compared to NRE participants. Also, aortic pulse wave velocity was significantly higher in HRE participants even after correcting for mean BP ($p = 0.003$). The change in brachial systolic BP from rest to moderate intensity exercise was significantly different between groups (NRE: 29 ± 10 vs. HRE: 47 ± 11 ; $p < 0.001$), as well as the change in central systolic BP (NRE: 12 ± 8 vs. HRE: 20 ± 11 ; $p = 0.010$). Augmentation pressure, augmentation index and PP amplification, and changes from rest to during moderate intensity exercise in these variables, were not significantly different between the groups. There were also no significant differences in heart rate, cardiac output or stroke volume at rest or during moderate intensity exercise between the two groups, although HRE participants had significantly increased SVR_i at moderate intensity exercise compared to NRE participants. The duration of exercise was similar in both groups (NRE: 18 ± 7 minutes vs. HRE: 19 ± 8 minutes, $p = 0.630$), and there were no significant differences in workloads between groups (NRE: 69 ± 23 watts vs. HRE: 76 ± 29 watts, $p = 0.373$).

4.4.4 Correlates of moderate intensity exercise systolic BP

Moderate intensity exercise systolic BP was significantly, positively correlated with rest vWf but inversely correlated with the change in vWf from rest to exercise. However, the correlation with the change in vWf from rest to post-exercise was not significant (Figure 4.2). Furthermore, triglycerides ($r = 0.411$, $p = 0.001$), body mass index ($r = 0.256$, $p = 0.046$) and aortic pulse wave velocity ($r = 0.325$, $p = 0.011$) were also associated with the moderate intensity exercise systolic BP. During moderate intensity exercise, SVRi was significantly and positively correlated with exercise systolic BP ($r = 0.385$, $p = 0.002$).

4.4.5 Multiple regression analysis

A multiple regression model was constructed with moderate intensity exercise systolic BP as the dependent variable and age, sex, body mass index, vWf, triglycerides, resting brachial systolic BP and aortic pulse wave velocity as independent variables. Rest vWf ($\beta = 0.220$, $p = 0.043$) was a predictor of moderate intensity exercise systolic BP, independent of age ($\beta = 0.024$, $p = 0.847$), sex ($\beta = 0.089$, $p = 0.432$), body mass index ($\beta = 0.109$, $p = 0.334$), triglycerides ($\beta = 0.228$, $p = 0.035$), resting brachial systolic BP ($\beta = 0.458$, $p < 0.001$) and aortic pulse wave velocity ($\beta = 0.064$, $p = 0.643$). Adjusted R^2 for the model = 0.451; $p < 0.001$. When we constructed the same model with exercise SVRi entered in the model instead of aortic pulse wave velocity, rest vWF ($\beta = 0.225$, $p = 0.041$) was still a predictor of moderate intensity exercise systolic BP, independent of age ($\beta = 0.052$, $p = 0.632$), sex ($\beta = 0.073$, $p = 0.506$), body mass index ($\beta = 0.118$, $p = 0.290$), triglycerides ($\beta = 0.220$, $p = 0.048$), resting brachial systolic BP ($\beta = 0.462$, $p < 0.001$) and exercise SVRi ($\beta = 0.038$, $p = 0.643$). Adjusted R^2 for the model = 0.447; $p < 0.001$. When aortic pulse wave velocity and exercise SVRi were included in the same model, the association with vWf changed to the borderline of statistical significance but with no major shift in the standardised beta coefficient ($\beta = 0.219$, $p = 0.050$; adjusted R^2 for the model = 0.437; $p < 0.001$).

Table 4.2 Haemostatic markers measured across three different conditions in participants with normotensive and hypertensive response to exercise

| Characteristics | | NRE (n = 44) | HRE (n = 20) | <i>p</i> value |
|---------------------------------------|---------------|---------------------|--------------------|----------------|
| Von Willebrand factor (mU/mL) | Rest | 1129 (871-1386) | 1927 (1240-2615) | 0.016 |
| | Exercise | 1484 (1143-1825)* | 1488 (945-2030) | 0.992 |
| | Post-exercise | 1286 (980-1592) | 1045 (658-1433)* | 0.369 |
| Fibrinogen (g/L) | Rest | 3.59 (3.41-3.78) | 3.78 (3.52-4.04) | 0.265 |
| | Exercise | 3.63 (3.44-3.82) | 3.76 (3.49-4.03) | 0.448 |
| | Post-exercise | 3.47 (3.27-3.67) | 3.80 (3.53-4.08) | 0.067 |
| Tissue factor (pg/mL) | Rest | 104.8 (51.0-158.5) | 130.0 (15.2-244.8) | 0.669 |
| | Exercise | 111.1 (55.7-166.5) | 126.7 (13.4-239.9) | 0.812 |
| | Post-exercise | 88.5 (38.9-138.0)*+ | 118.0 (8.3-227.7) | 0.629 |
| Thrombin-antithrombin complex (ng/mL) | Rest | 1.22 (0.94-1.51) | 1.22 (0.80-1.65) | 0.997 |
| | Exercise | 1.21 (0.92-1.49) | 1.01 (0.62-1.39) | 0.438 |
| | Post-exercise | 1.17 (0.89-1.45) | 1.08 (0.68-1.48) | 0.726 |

NRE, normotensive response to exercise; HRE, hypertensive response to exercise. Values represent means (95% confidence interval) adjusted for age, sex, body mass index, triglycerides and resting systolic blood pressure. The *p* values are for the difference between NRE and HRE at each of the three time points. **p* < 0.05 for the comparison with the rest haemostatic marker within group; +*p* < 0.05 for the comparison with the exercise haemostatic marker within group.

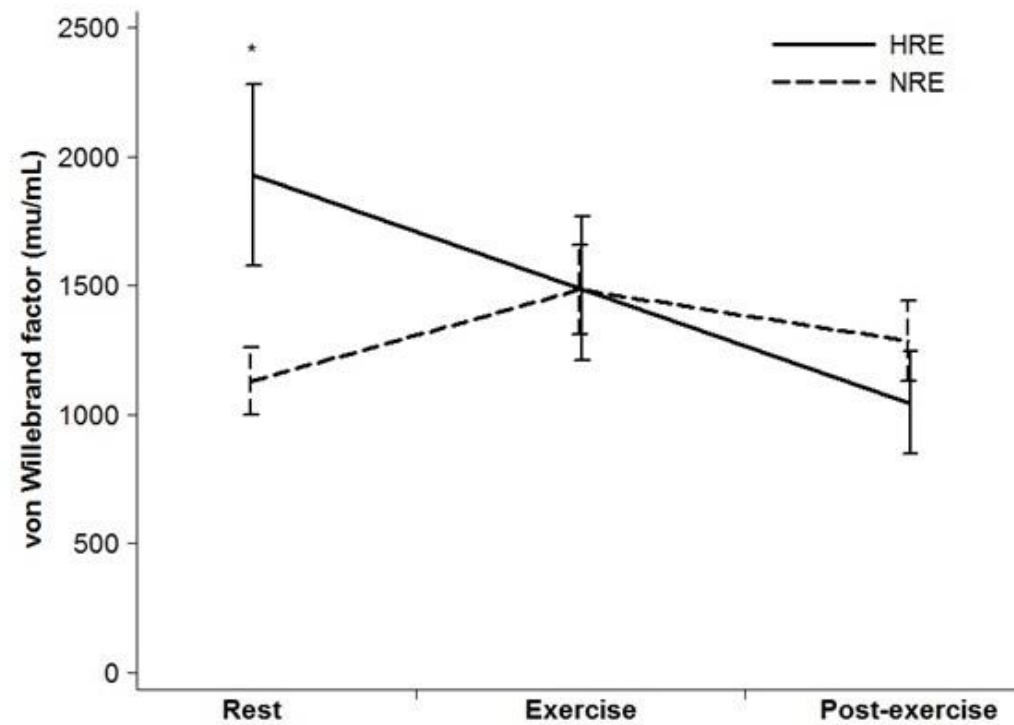


Figure 4.1 Von Willebrand factor levels at three different conditions

Von Willebrand factor (vWf) at rest, during exercise and post-exercise in participants with normotensive response to exercise (NRE) and hypertensive response to exercise (HRE). There was a significant difference in vWf at rest between groups (* $p = 0.016$). Error bars represent means and standard error of the mean after adjusting for age, sex, body mass index, triglycerides and resting systolic blood pressure.

Table 4.3 Haemodynamic variables measured during treadmill exercise stress testing (Bruce protocol), and at rest and during moderate intensity exercise at the research clinic in participants with normotensive and hypertensive response to exercise

| Characteristics | NRE (n=44) | HRE (n=20) | <i>p</i> value |
|--|---------------|---------------|----------------|
| Treadmill exercise stress testing | | | |
| Max. ex brachial systolic BP (mmHg) | 172 ± 24 | 191 ± 23 | 0.006 |
| Max. ex brachial diastolic BP (mmHg) | 81 ± 11 | 82 ± 11 | 0.752 |
| Max. ex brachial PP (mmHg) | 92 ± 20 | 109 ± 23 | 0.007 |
| Max. ex heart rate (bpm) | 157 ± 15 | 149 ± 20 | 0.106 |
| Stage 2 ex brachial systolic BP (mmHg) | 154 ± 26 | 174 ± 24 | 0.004 |
| Stage 2 ex brachial diastolic BP (mmHg) | 77 ± 11 | 80 ± 11 | 0.422 |
| Stage 2 ex brachial PP (mmHg) | 77 ± 22 | 95 ± 22 | 0.005 |
| Stage 2 heart rate (bpm) | 129 ± 17 | 129 ± 20 | 0.992 |
| Research clinic rest conditions | | | |
| Office brachial systolic BP (mmHg) | 120 ± 12 | 128 ± 12 | 0.021 |
| Office brachial diastolic BP (mmHg) | 73 ± 9 | 74 ± 8 | 0.517 |
| Office brachial PP (mmHg) | 47 ± 8 | 54 ± 9 | 0.009 |
| Central systolic BP (mmHg) | 110 ± 12 | 117 ± 13 | 0.043 |
| Central diastolic BP (mmHg) | 73 ± 9 | 75 ± 8 | 0.518 |
| Central PP (mmHg) | 37 ± 9 | 43 ± 10 | 0.029 |
| Aortic pulse wave velocity (m/s) | 7.7 ± 1.6 | 9.0 ± 1.3 | 0.001 |
| Brachial pulse wave velocity (m/s) | 8.5 ± 1.0 | 8.2 ± 0.9 | 0.215 |
| Heart rate (bpm) | 62 ± 10 | 61 ± 7 | 0.549 |
| Cardiac output (L/min) | 4.8 ± 1.2 | 4.8 ± 1.0 | 0.815 |
| Stroke volume (mL) | 77 ± 14 | 81 ± 17 | 0.468 |
| SVRi (dyne/s/cm ⁵) | 3086 ± 624 | 3250 ± 781 | 0.412 |
| Research clinic moderate intensity exercise | | | |
| Ex brachial systolic BP (mmHg) | 150 ± 12 | 175 ± 8 | <0.001 |
| Ex brachial diastolic BP (mmHg) | 76 ± 6 | 76 ± 7 | 0.855 |
| Ex brachial PP (mmHg) | 74 ± 12 | 98 ± 9 | <0.001 |
| Ex central systolic BP (mmHg) | 123 ± 9 | 137 ± 7 | <0.001 |
| Ex central diastolic BP (mmHg) | 79 ± 6 | 79 ± 7 | 0.723 |
| Ex central PP (mmHg) | 44 ± 8 | 58 ± 7 | <0.001 |
| Ex heart rate (bpm) | 102 ± 9 | 101 ± 9 | 0.862 |
| Ex cardiac output (L/min) | 10.3 ± 1.2 | 10.7 ± 1.8 | 0.405 |
| Ex stroke volume (mL) | 102 ± 13 | 106 ± 17 | 0.409 |
| Ex SVRi (dyne/s/cm ⁵) | 1573 ± 231 | 1725 ± 222 | 0.019 |

Data are mean ± standard deviation ; NRE, normotensive response to exercise; HRE, hypertensive response to exercise; max, maximal; ex, exercise; BP, blood pressure; PP, pulse pressure; SVRi, systemic vascular resistance index

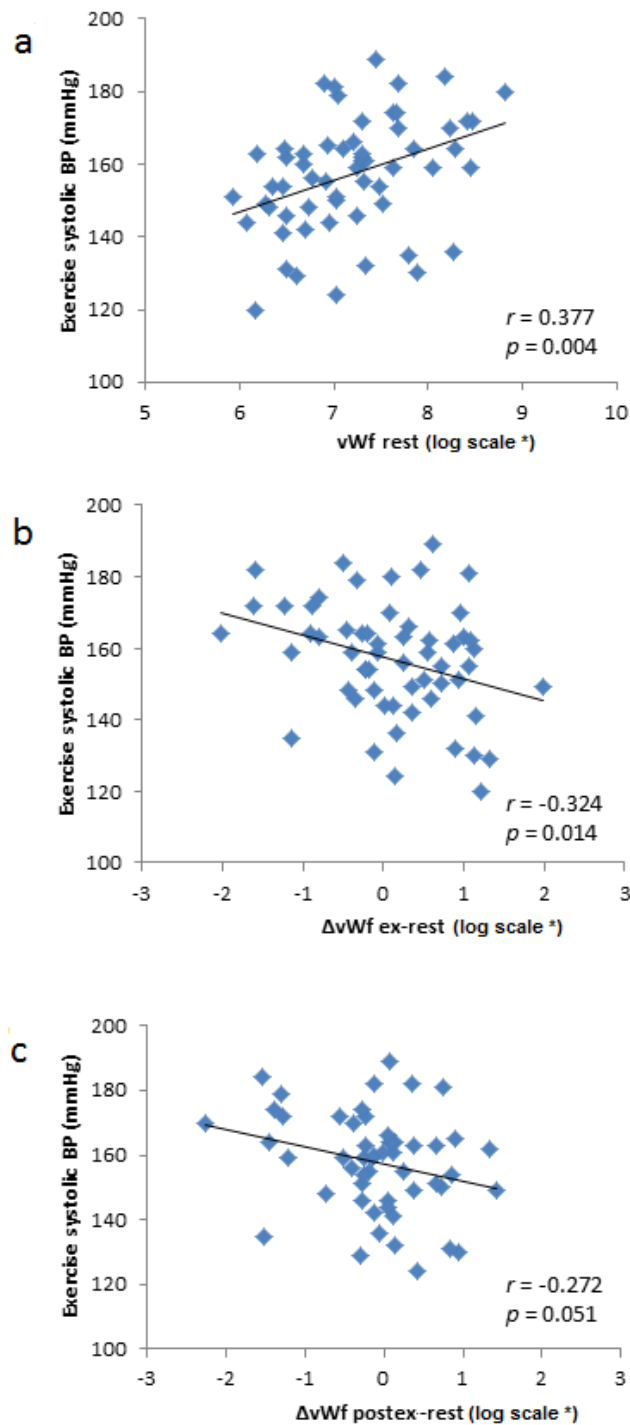


Figure 4.2 Correlations between moderate intensity exercise systolic blood pressure and von Willebrand factor.

Correlations between moderate intensity exercise systolic blood pressure (BP) and (A) rest von Willebrand factor (vWf); (B) change in vWf from rest to exercise (ex); and (C) change in vWf from rest to post-exercise (post ex). *VWf and changes in vWf are represented on the natural logarithm scale.

4.5 Discussion

An HRE at moderate intensity is associated with increased CV risk.⁵ In a sample of 64 people undergoing routine exercise stress testing, we identified an independent relationship between resting vWf levels and moderate intensity exercise systolic BP, as well as higher resting vWf levels in those with an HRE. Furthermore, participants with an HRE had significantly different responses of vWf to moderate exercise compared to people with normal exercise systolic BP. Measures of arterial haemodynamics (large artery stiffness and SVR_i during moderate intensity exercise) were also higher in HRE individuals. These findings implicate haemostatic abnormalities as a possible factor contributing to an HRE at moderate intensity.

The vascular endothelium is involved in production of many vasoactive substances, including haemostatic factors that are released with endothelial damage/dysfunction. Our study showed that vWf was increased in participants with an HRE, suggesting the presence of endothelial dysfunction.⁷⁹ Interestingly, vWf was significantly correlated with the moderate intensity exercise systolic BP. This positive association may reflect the interaction between endothelial dysfunction and high BP, due to the dysregulation of endothelial vasoactive substances (including vWf) and an altered capacity of the endothelium to maintain homeostasis and vascular tone. Indeed, the association between endothelial dysfunction and high BP could be a failure of the peripheral vasculature to appropriately dilate in order to mitigate pressure rises, particularly during exercise. These findings are in agreement with previous studies that also found the association between endothelial dysfunction (although measured by different methods) and exercise systolic BP. Indeed, in a large community-based population cohort of 2115 participants from the Framingham Heart Study, flow-mediated vasodilation was negatively associated with exercise systolic BP at moderate intensity.⁵⁰ Similarly, a study of healthy subjects with high normal BP or mild hypertension showed that reduced endothelial-dependent vasodilatation (assessed by the same method) was inversely associated with exaggerated exercise systolic BP, but only in men.⁶³

vWf facilitates platelet adhesion and aggregation, and also contributes to blood coagulation by acting as a carrier for factor VIII. In this current study, the observed elevation of vWF in the absence of a significant increase in TAT complexes suggests low grade activation of the coagulation system rather than extensive amplification and propagation that would be reflected by increased thrombin generation. Nevertheless, increased levels of vWf may be clinically significant in patients with an HRE, especially in those with other CV risk

factors.²⁰⁰ It was also interesting to observe that vWf levels significantly decreased in HRE individuals 15 minutes following moderate intensity exercise, despite having elevated exercise BP levels. This finding may possibly suggest that moderate intensity exercise could act to suppress coagulation and/or platelet activation and potentially have beneficial effects that may be more pronounced in those with excessive initial vWf levels (such as HRE subjects). Moderate intensity exercise (in contrast to strenuous exercise) has been found to suppress agonist-and shear-induced platelet aggregation by reducing vWF binding to platelets and down-regulation of platelet activation (GPIIb/IIIa) in sixteen sedentary men.²⁰¹ Another possible contributing factor to lower vWf levels in HRE subjects following the exercise may be disturbed nitric oxide bioavailability from endothelial dysfunction.⁶³ Indeed, the study of Jilma et al²⁰² showed that vWf levels were significantly attenuated during exercise when systemic nitric oxide was blocked. However, further studies are needed to explore these postulates.

A reduction in large central artery compliance could contribute to BP elevation during exercise. Indeed, increased aortic stiffness (measured by carotid-to-femoral pulse wave velocity) has been shown to be associated with peak exercise systolic BP at maximal intensity in 171 untreated essential hypertension subjects.⁵¹ Similarly, the study by Thanassoulis et al⁵⁰ found that aortic stiffness (in addition to endothelial dysfunction, as mentioned above) was independently associated with moderate intensity exercise systolic BP, measured during the second stage of an exercise stress test. Consistent with these data, this current study found that aortic stiffness, measured by the same technique as Thanassoulis et al⁵⁰, was associated with the exercise systolic BP at moderate intensity, and was higher in those with an HRE. Furthermore, we also found that SVR_i during moderate intensity exercise (but not at rest) was increased in HRE participants. The combination of increased central artery stiffness with increased vascular resistance would reasonably expect to increase exercise BP from the ejection of higher cardiac output into a stiff arterial system together with impaired peripheral run-off of blood. Interestingly, one study has shown that an excessive rise in systemic vascular resistance measured invasively during submaximal or maximal exercise independently predicted incident CV events and total mortality.²⁰³ This tends to support the notion that some of the risk attributable to an HRE may be due to altered vascular function although we did not find an independent relationship in this current study.

Our study was a cross-sectional analysis of data from a selected study population. The findings do not establish causality and may not be generalizable to other populations. Endothelial function of specific arterial beds, such as the brachial artery, was not measured in this study and would have been a valuable addition. Also, different devices were used to measure resting and exercise BP. Although both devices have been validated, the use of separate machines might have affected the study results. Furthermore, the use of a standard formula to estimate heart rate might have underestimated heart rate and the intensity of exercise, as per Tanaka et al.²⁰⁴ However, it should be noted that the same formula was used to determine the heart rate in all study participants (HRE and NRE groups), and the possible underestimation of heart rate would not significantly affect the results of this study. Future studies may be worth directing attention towards the role of vWf and endothelial dysfunction as contributors to an HRE at moderate intensity, as well as therapeutic/exercise interventions targeting haemostatic markers and endothelial function with benefit to vascular function and BP control.

In summary, this study has demonstrated that abnormal haemostasis as reflected by higher resting vWf, as well as structural and functional vascular abnormalities (such as increased aortic stiffness and systemic vascular resistance during exercise) were present in subjects with an HRE. Although endothelial dysfunction was not estimated with standard measures in this study (e.g. flow-mediated vasodilation), an increased vWf (also a marker of endothelial dysfunction) may provide some indications that endothelial dysfunction may be present in people with an HRE and may be a contributing factor of an HRE. For the first time, we show that resting blood levels of vWf were significantly associated with moderate intensity exercise systolic BP, independent of resting brachial systolic BP and other CV risk factors.

4.6 Contribution of Chapter 4 to thesis aims

Previously published literature highlights the potential clinical relevance of an HRE at moderate intensity. *Chapter 4* represents the first comprehensive CV assessment at rest and during moderate intensity exercise to explore the haemodynamic and haemostatic correlates of an HRE in people with a clinical indication for exercise stress testing. This study showed for the first time that haemostatic abnormalities reflecting endothelial dysfunction (increased vWf levels) were present in people with an HRE, and were significantly and independently correlated with moderate intensity exercise systolic BP. Endothelial function is not only

required for appropriate vascular tone and BP, but also for the regulation of normal haemostasis. Therefore, endothelial dysfunction may be a platform on which abnormal haemostasis might be contributing to an HRE and associated CV complications. These findings may have significant clinical relevance in people with an HRE, especially in those with other (possibly metabolic) co-morbidities. Furthermore, haemodynamic factors, including increased aortic stiffness, appeared to have a minimal role in an HRE at moderate intensity, as there were no independent significant associations with exercise systolic BP. On the other hand, there were some indications that metabolic factors may be contributing to an HRE, evidenced by increased triglyceride levels in people with an HRE, and their independent associations with moderate intensity exercise systolic BP. In order to explore other metabolic contributors of an HRE in an exploratory and ‘untargeted’ way using metabolomics analysis, next chapter (*Chapter 5*) represents the study that was primarily used for the development of methodology regarding metabolomics analysis (utilised in *Chapter 5* and *Chapter 6*), but it was also a convenient sample to explore the metabolic actions of the aldosterone antagonist spironolactone on exercise BP, as well as other haemodynamic measures, in people with an HRE.

Chapter 5. Spironolactone Reduces Aortic Stiffness via Blood Pressure-Dependent Effects of Canrenoate

This thesis chapter has been previously published:

Nikolic SB, Wilson R, Hare JL, Adams MJ, Edwards LM, Sharman JE. Spironolactone reduces aortic stiffness via blood pressure-dependent effects of canrenoate. *Metabolomics*. 2014;10:105-113

This chapter represents the first application of the metabolomics analysis in people with a hypertensive response of exercise and was primarily used to develop methodology regarding metabolomics analysis (also utilised in *Chapter 6*). However, this study was also a convenient sample to explore the metabolic actions of the aldosterone antagonist spironolactone on exercise blood pressure and aortic stiffness in people with a hypertensive response to exercise. The final published version of this paper (as presented in *Chapter 5*) included the exploration of the underlying metabolic effects of spironolactone on aortic stiffness only (a proposed contributor of a hypertensive response to exercise), whereas additional analysis and results regarding the spironolactone effects on exercise blood pressure are presented in *Appendix 3*.

5.1 Abstract

Background. Spironolactone is thought to improve aortic stiffness via blood pressure (BP) independent (antifibrotic) effects, but the exact mechanism is unknown. We used metabolomics and hemodynamic measures to reveal the underlying actions of spironolactone in people with a hypertensive response to exercise (HRE).

Methods. Baseline and follow-up serum samples from 115 participants randomized to three months spironolactone (25 mg/day) or placebo were analysed using liquid chromatography/mass spectrometry and nuclear magnetic resonance spectroscopy. Hemodynamic measures recorded at baseline and follow-up included aortic pulse wave velocity (stiffness) and 24 hour ambulatory BP.

Results. Aortic stiffness was significantly reduced by spironolactone compared with placebo (-0.18 ± 0.17 m/s versus 0.30 ± 0.16 m/s; $p < 0.05$), but this was no longer significant after adjustment for the change in daytime systolic BP ($p = 0.132$). Further, the change in aortic stiffness was correlated with the change in daytime and 24 hour systolic BP ($p < 0.05$). Metabolomics detected 42 features that were candidate downstream metabolites of spironolactone (no endogenous metabolites), although none were correlated with changes in aortic stiffness ($p > 0.05$ for all). However, the spironolactone metabolite canrenoate was associated with the change in daytime systolic BP ($r = -0.355$, $p = 0.017$) and 24 hour pulse pressure ($r = -0.332$, $p = 0.026$). This remained highly significant on multiple regression and was independent of age, body mass index and sex.

Conclusion. Canrenoate appears to be an active metabolite with BP-dependent effects on the attenuation of aortic stiffness in people with an HRE. This finding, together with the lack of change in endogenous metabolites, suggests that the antifibrotic effects of spironolactone could be BP-dependent.

5.2 Introduction

Spironolactone is a diuretic that acts as a competitive aldosterone antagonist at the mineralocorticoid receptors of the epithelial cells in the kidneys.²⁰⁵ Studies in animals and humans have indicated that spironolactone exerts broad metabolic, structural and functional effects via mineralocorticoid receptors outside the kidneys, although the details of these actions remain unclear.⁵⁸⁻⁶⁰ Recently, mineralocorticoid receptors have been implicated in the function of the vasculature and it has been suggested that blockade by spironolactone may prevent, or lessen, arterial fibrosis and aortic stiffness independent of blood pressure (BP) changes.^{58, 60} Indeed, spironolactone has been shown to prevent aortic collagen accumulation, improve carotid artery distensibility, and lower aortic stiffness despite no significant changes in resting or office BP.^{58, 61} However, some of these studies did not consider the ambulatory or central BP-lowering effects of spironolactone, which are likely to be different to spironolactone's effect on resting BP.⁶¹ Thus, the magnitude of the association between the BP-lowering effect of spironolactone and arterial stiffness might have been underestimated.

Metabolomics is the systematic profiling of metabolites in biological systems and their changes in response to physiological stimuli. It may be used to characterise metabolic pathways associated with cardiovascular (CV) diseases and therapy.²⁰⁶ Indeed, mapping of endogenous metabolites pre- and post-drug treatment, and identifying perturbed metabolic pathways, may provide unique information on underlying drug mechanisms of action.²⁰⁶ To our knowledge, this approach has never been used to characterise the effects of spironolactone. Given that this drug undergoes extensive metabolism, and its activity is mainly attributed to its breakdown products (e.g. canrenoate and canrenone; Figure 5.1),²⁰⁷ the identification of these products and consequent correlation with arterial/hemodynamic changes induced by spironolactone, may shed new light on the underlying physiological actions of the drug. This study sought to achieve this by conducting a metabolomic analysis of serum from subjects in a convenience sample from our recently completed randomized clinical trial where aortic stiffness and BP measures were acquired at baseline and after three months of spironolactone treatment in patients with a hypertensive response to exercise (HRE).⁹ We hypothesized that metabolite drug responses would be associated with changes in aortic stiffness and BP.

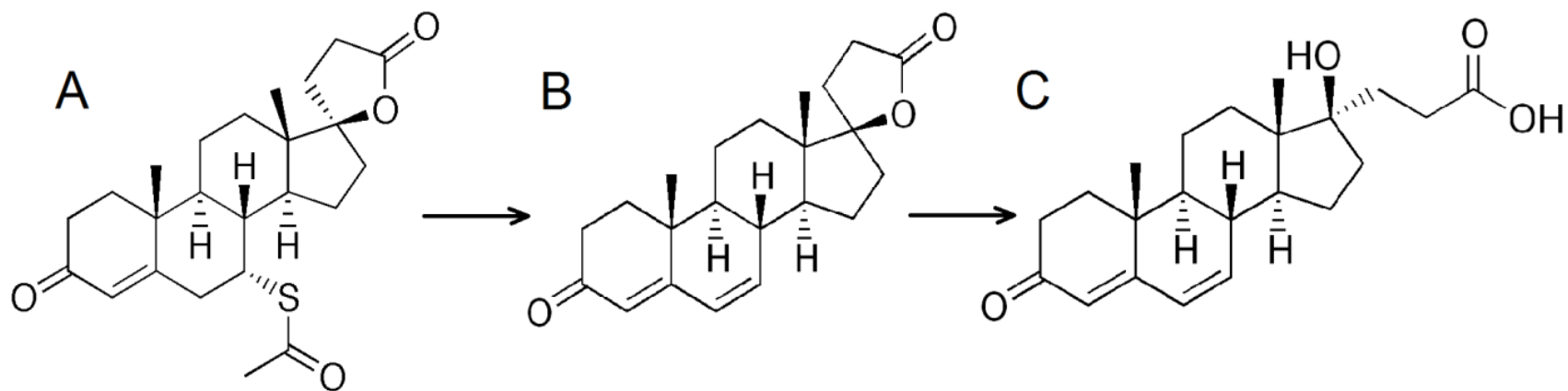


Figure 5.1 Metabolic pathway of spironolactone

Metabolic pathway of spironolactone (A) forming two main downstream metabolites: canrenone (B) and canrenoate (C). Spironolactone is dethioacetylated to canrenone that further undergoes hydrolysis of its lactone ring to canrenoate.

5.3 Methods

5.3.1 Study participants

A total of 115 participants with an HRE were recruited into this randomized placebo-controlled, double-blind clinical trial (<http://www.anzctr.org.au>; ACTRN12609000835246). HRE was defined as resting BP < 140/90 mmHg with a peak exercise BP \geq 210/110 mmHg for men and \geq 190/105 mmHg for women.³⁰ Exclusion criteria for the principal clinical trial were: age over 70 years, a prior history of hypertension or taking antihypertensive medications, a history of known coronary artery disease or renal dysfunction (serum creatinine > 80 μ mol/L), gynecomastia and pregnancy. Eligible participants were selected from hospital and community cardiology clinic records of exercise stress tests that were conducted in a period between 2004 and 2008. The study was approved by the Human Research Ethics Committee of the University of Queensland and all procedures conformed to the Declaration of Helsinki. Study participants gave written informed consent prior to participation.

5.3.2 Study protocol

Study participants attended the clinic at two different occasions. First clinic baseline visit was conducted in the morning where fasting blood samples were collected for metabolomics analysis (pre-samples) and standard clinical biochemistry. Resulting serum samples were stored at -80°C for later analysis. Following this, participants were examined with hemodynamic measurements including 24 hour ambulatory BP, office brachial BP, office central BP and aortic stiffness. After the baseline visit, participants were randomly assigned either 25 mg of spironolactone or placebo daily for three months. Participants and researchers were blinded to treatment allocations. Subjects then returned to the clinic for their follow-up examination after the three months of treatment. All participants were advised to take their last dose of spironolactone on the day of the examination. This maintained the steady-state concentration of long half-life metabolites of spironolactone (e.g. canrenoate and canrenone) that was achieved after daily drug intake. Blood samples were collected as before (post-samples) and all hemodynamic measurements were repeated at three months.

5.3.3 Liquid chromatography-mass spectrometry (LC-MS)

Serum samples were thawed and a 100 μ L aliquot of each sample was mixed with 300 μ L of ice-cold methanol. The sample was then incubated at 4°C for 20 minutes and centrifuged at 13200 g for 10 minutes. The supernatant was collected and lyophilized in a vacuum centrifuge (Labconco, Kansas City, USA) and re-stored at -80°C. The dried samples were rehydrated in 100 μ L of HPLC-grade water immediately prior to LC-MS analysis. Quality control samples were also prepared by pooling 10 μ L of 40 random samples (placebo and spironolactone) and analysed throughout the LC-MS runs to assess the equipment drift and ‘batch-to-batch’ variability, as recommended by Dunn et al.¹²⁴ Samples were analysed using an LTQ-Orbitrap XL mass spectrometer (ThermoFisher Scientific, Bremen, Germany) controlled by XCalibur v2 software (ThermoFisher Scientific). To prevent systematic bias, samples from participants on spironolactone and placebo samples were randomly distributed throughout the runs. Each participant’s pre- and post-treatment samples were analysed together. Data were acquired in both positive (+) and negative (-) ionization modes. Retention times for tandem mass spectrometry (MS/MS) targets were specified according to retention time values acquired by LC-MS. Data were processed for peak finding, grouping and retention time alignment using XCMS running in the R environment (R 2.14; <http://cran.r-project.org/>).

5.3.4 Nuclear magnetic resonance (NMR) spectroscopy

Serum samples were thawed and 300 μ L was added to 300 μ L of D₂O in a 1.5 mL Eppendorf tube. The mixture was briefly vortexed and then centrifuged at 16606 g for five minutes after which 550 μ L of the supernatant was transferred to a 5 mm NMR tube for analysis. NMR data were acquired on a Bruker Avance III spectrometer operating at 800.13 MHz (Bruker Biospin, Karlsruhe, Germany). Data were acquired with a Carr-Purcell-Meiboom-Gill pulse sequence with presaturation during the recycle delay (2 s) and a total echo time of 51.2 ms. The time domain was 128 k and the sweep width was 20 ppm. Data were Fourier transformed with exponential line broadening of 0.5 Hz in the frequency domain. Spectra were phased and baselined in Topspin 3.0 (Bruker Biospin, Karlsruhe, Germany) before being imported into Matlab (Mathworks, Natick, MA) at full resolution. Resulting spectra were normalised and bucketed using probabilistic-quotient normalisation¹⁹⁷ and adaptive intelligent binning¹¹² respectively, both in Matlab.

5.3.5 Aortic stiffness, blood pressure and clinical biochemistry

Aortic stiffness: ECG-gated sequential applanation tonometry of the carotid and femoral arteries using the SphygmoCor system (SphygmoCor 8.1, AtCor Medical, Sydney, NSW) was used to measure aortic pulse wave velocity (PWV), a measure of aortic stiffness.¹⁷⁷ *24 hour ambulatory BP:* A validated device (TM-2430, A&D Mercury, A&D Medical, Thebarton, South Australia)¹⁸⁹ - which was set to take measurements every 30 minutes during the day (between 6 am and 10 pm) and every hour during night (between 10 pm and 6 am) - recorded 24 hour ambulatory BP.²³ The mean 24 hour pulse pressure (PP) was calculated as the difference between 24 hour systolic and diastolic BP. *Office brachial BP:* After at least 10 minutes of rest in a supine position, office brachial BP was measured by trained technician in duplicate using a validated manual sphygmomanometer with suitable sized cuff, as per recommendations.^{4, 198} The mean of these two readings was used for subsequent analysis. *Office central BP:* A validated and highly reproducible radial applanation tonometry^{47, 48} using the SphygmoCor system (SphygmoCor 8.1, AtCor Medical, Sydney, NSW) was used to measure office central BP. The mean of the office brachial BP measurements, measured before the radial applanation tonometry, was used to calibrate the radial pressure waveform. *Clinical biochemistry:* Standard clinical biochemistry included analysis of glucose, glycosylated haemoglobin (HbA_{1c}), triglycerides, total, low-density and high-density lipoprotein cholesterol, as per routine pathology laboratory procedures.

5.3.6 Data analysis, statistics and feature annotation

As previously reported,¹⁹⁴ we found that principle components analysis (PCA; and its variants) was ineffective for identifying differences in the LC-MS data. Thus, as before, we used a repeated-measures ANOVA to compare each feature and then corrected the resulting *p* values using the Benjamini and Hochberg False Discovery Rate correction.¹⁹⁴ Features were accepted as being significantly different if the corrected $p < 0.05$. The LC-MS data were hand-checked for artefacts (for example, Fourier-transform ‘shoulders’) before being de-isotoped and putatively annotated using the Human Metabolome Database (HMDB) and the Taverna workflows.¹⁹⁶ Compounds of interest were further examined by MS/MS to provide additional structural information and to confirm annotation, where possible, by comparison with MS/MS spectra held in public databases.

Post-intervention NMR data were mean-centred and Pareto-scaled, before being analyzed using PCA and partial least squares-discriminant analysis (PLSDA), both in PLS Toolbox (PLS Toolbox 6.5, Eigenvector Research Inc., Wenatchee, US). Permutation testing was used to assess whether PLSDA models were the result of over-fitting; models were considered significant if the estimated $p < 0.05$.

Data processing of metabolomics data and subsequent statistical analysis were all conducted in Matlab. All additional statistical analysis was conducted using SPSS 17.0 (SPSS Inc., Chicago, Illinois, USA). Clinical characteristics of study participants are presented as mean and standard error of the mean (SEM). Comparisons between placebo and spironolactone groups were performed by independent t -tests. Comparisons between categorical variables were assessed by chi-squared tests. Changes in hemodynamic variables were adjusted by analysis of covariance (ANCOVA) for potential confounders. Metabolomics data, when included in an analysis with conventional data, was checked for normality and non-normally distributed data was square-root-transformed. Associations between metabolites of interest and physiological variables were assessed by linear correlation. Multiple regression was used for the predictors of change in physiological parameters after treatment. In all cases, a value of $p < 0.05$ was taken as statistically significant.

5.4 Results

5.4.1 Clinical characteristics

Of the 115 participants included in the clinical trial, 58 were randomized to spironolactone treatment and 57 to placebo. Of these, serum samples were available from 92 participants for the metabolomics analysis (43 in placebo group and 49 in spironolactone group). Two study participants were taking antihypertensive drugs (one in the placebo and one in the spironolactone group), but these were not excluded from the metabolomics analysis. Table 5.1 represents the baseline clinical characteristics of study participants, which was not significantly different from the complete study population.

Table 5.1 Clinical characteristics of study participants at baseline

| Variable | Placebo (n = 43) | Spirolactone (n = 49) | <i>p</i> value |
|------------------------------|-----------------------------|----------------------------------|-----------------------|
| Age (years) | 55 ± 1 | 55 ± 1 | 0.961 |
| Sex (% male) | 55.8 | 59.2 | 0.833 |
| BMI (kg/m ²) | 29.1 ± 0.6 | 29.2 ± 0.7 | 0.993 |
| Type 2 diabetes mellitus (%) | 4 (9.3) | 9 (18.4) | 0.344 |
| 24 hour SBP (mmHg) | 133 ± 2 | 133 ± 1 | 0.894 |
| 24 hour PP (mmHg) | 53 ± 1 | 54 ± 1 | 0.288 |
| Day SBP (mmHg) | 137 ± 2 | 137 ± 1 | 0.978 |
| Day PP (mmHg) | 54 ± 1 | 55 ± 1 | 0.575 |
| Night SBP (mmHg) | 117 ± 2 | 118 ± 2 | 0.694 |
| Night PP (mmHg) | 47 ± 1 | 50 ± 1 | 0.050 |
| Office brachial SBP (mmHg) | 125 ± 2 | 126 ± 2 | 0.724 |
| Office brachial PP (mmHg) | 52 ± 2 | 52 ± 1 | 0.802 |
| Office central SBP (mmHg) | 114 ± 2 | 114 ± 2 | 0.795 |
| Office central PP (mmHg) | 40 ± 1 | 39 ± 1 | 0.954 |
| Aortic PWV (m/s) | 8.2 ± 0.3 | 8.4 ± 0.3 | 0.588 |
| Glucose (mmol/L) | 5.7 ± 0.2 | 5.9 ± 0.3 | 0.461 |
| HbA _{1c} (%) | 5.9 ± 0.1 | 6.0 ± 0.2 | 0.531 |
| Triglycerides (mmol/L) | 1.5 ± 0.2 | 1.5 ± 0.2 | 0.790 |
| Total cholesterol (mmol/L) | 5.3 ± 0.1 | 5.1 ± 0.1 | 0.368 |
| LDL cholesterol (mmol/L) | 3.3 ± 0.1 | 3.2 ± 0.1 | 0.608 |
| HDL cholesterol (mmol/L) | 1.4 ± 0.1 | 1.4 ± 0.1 | 0.965 |
| ACE inhibitors (%) | 1 (2.3) | 1 (2.0) | 1.000 |
| Statins (%) | 9 (20.9) | 7 (14.3) | 0.573 |

BMI, body mass index; SBP, systolic blood pressure; PP, pulse pressure; PWV, pulse wave velocity; HbA_{1c}, glycosylated haemoglobin; LDL, low density lipoprotein; HDL, high density lipoprotein; ACE, angiotensin converting enzyme; values are mean ± SEM or %; *p* < 0.05 was considered significant

5.4.2 Metabolomics (LC-MS)

All data (pre/post and spironolactone/placebo) were combined and pre-processed together using XCMS. We identified 7363 common features across the combined mass spectra acquired in negative ionization mode and 9185 features from the spectra acquired in positive ionization mode. Of these, 42 features (34 from negative and eight from positive ionization mode) were significantly different in the spironolactone group compared with placebo ($p < 0.05$ after the Benjamini and Hochberg False Discovery Rate correction). Nine isotopes and 17 adducts were subsequently identified and removed, leaving 16 features for further investigation. However, we were not able to putatively annotate any of these as being endogenous (i.e. non-xenobiotic) metabolites. Since these 16 features were undetectable in any but the post-spironolactone group and none could be annotated using any public human metabolomics repositories, we concluded that these features were almost certainly downstream breakdown products of spironolactone itself. As a result, several were immediately identified (from accurate mass and MS/MS) as signals from the most commonly-reported breakdown products of spironolactone: canrenoate (CHEBI: 50159; 357.2071 m/z in negative ion mode) and canrenone (ChEMBL42874: 341.2111 m/z in positive ion mode; Figure 5.2).

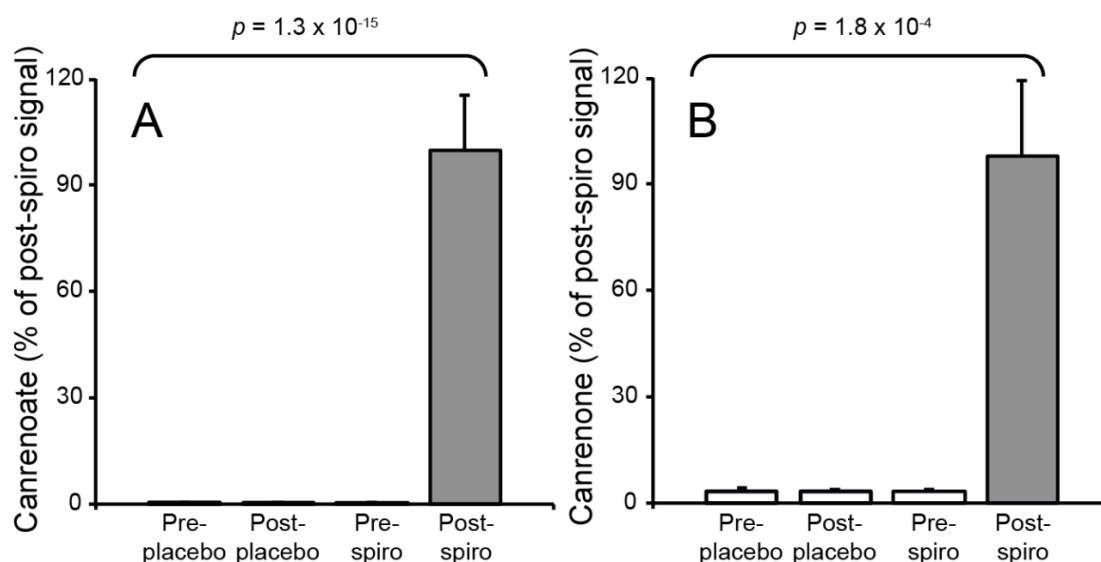


Figure 5.2 Spironolactone downstream metabolites

Spironolactone downstream metabolites (A) canrenoate and (B) canrenone measured by LC-MS (n = 49).

5.4.3 Metabolomics (NMR)

The post-spirolactone and post-placebo groups were normalised and pre-processed together. The adaptive intelligent binning algorithm identified 336 spectral regions of interest. Yet despite outstanding spectral quality resulting from the high field strengths employed, subsequent analysis of the NMR data using both principal component analysis and partial least squares-discriminant analysis was unable to identify any significant effect of spironolactone treatment ($p > 0.05$ for all models).

5.4.4 Hemodynamic response to spironolactone

As per Table 5.2, the group that received spironolactone for three months had a significant reduction in all ambulatory BP values, except for night systolic BP where the reduction was of borderline significance. There was a larger decrease in office brachial and central BP in the spironolactone treatment group compared to placebo, but these changes were also of borderline statistical significance. Aortic PWV decreased significantly in the spironolactone group compared with placebo group. However, the reduction in aortic PWV was no longer significant after adjustment for the change in daytime systolic BP ($p = 0.132$).

Table 5.2 Changes in hemodynamic variables in placebo compared to spironolactone group after three months of treatment

| Variable | Placebo (n = 43) | Spirolactone (n = 49) | p value |
|------------------------------|---------------------|--------------------------|---------|
| Δ 24 hour SBP (mmHg) | 0.8 ± 1.3 | -3.5 ± 1.0 | 0.010 |
| Δ 24 hour PP (mmHg) | 2.3 ± 1.1 | -2.3 ± 0.7 | <0.001 |
| Δ Day SBP (mmHg) | 1.1 ± 1.4 | -3.3 ± 1.1 | 0.014 |
| Δ Day PP (mmHg) | 2.6 ± 1.5 | -2.0 ± 0.8 | 0.007 |
| Δ Night SBP (mmHg) | 0.5 ± 1.9 | -3.9 ± 1.5 | 0.067 |
| Δ Night PP (mmHg) | 1.4 ± 1.2 | -2.6 ± 0.9 | 0.010 |
| Δ Office brachial SBP (mmHg) | -0.4 ± 1.3 | -3.6 ± 1.2 | 0.070 |
| Δ Office brachial PP (mmHg) | -0.9 ± 1.3 | -1.9 ± 1.0 | 0.560 |
| Δ Office central SBP (mmHg) | -0.7 ± 1.3 | -3.9 ± 1.1 | 0.063 |
| Δ Office central PP (mmHg) | -1.3 ± 1.1 | -2.2 ± 0.8 | 0.488 |
| Δ Aortic PWV (m/s) | 0.30 ± 0.16 | -0.18 ± 0.17 | 0.047 |

SBP, systolic blood pressure; PP, pulse pressure; PWV, pulse wave velocity; values are mean ± SEM; $p < 0.05$ was considered significant

5.4.5 Associations between hemodynamic parameters and spironolactone metabolites

Changes in hemodynamic parameters were correlated with the post-spironolactone signal intensity for each of the metabolomic features of interest. There were no significant correlations between the change in aortic PWV and any metabolic features, including canrenoate and canrenone ($p > 0.05$ for all). However, canrenoate was associated with the change in daytime systolic BP and the change in 24 hour PP (Figure 5.3). On the other hand, there were no significant associations between the changes in canrenone and ambulatory BP values after treatment ($p < 0.05$ for all). The change in aortic PWV was significantly related to the change in daytime systolic BP and the change in 24 hour systolic BP (Figure 5.4).

5.4.6 Multiple regression analysis

The change in daytime systolic BP was significantly correlated with canrenoate ($r = -0.355$, $p = 0.017$), the change in aortic PWV ($r = 0.333$, $p = 0.024$), body mass index ($r = -0.325$, $p = 0.023$), change in 24 hour systolic BP ($r = 0.875$, $p < 0.001$), change in 24 hour PP ($r = 0.527$, $p < 0.001$), change in 24 hour diastolic BP ($r = 0.583$, $p < 0.001$) and change in daytime diastolic BP ($r = 0.610$, $p < 0.001$). A multiple regression model for predictors of the change in daytime systolic BP was constructed, with the following variables entered as covariates; age, sex, body mass index, the use of statins and angiotensin converting enzyme inhibitors, canrenoate and the change in aortic PWV. Ambulatory BP variables were not included in the model due to collinearity or non-significance. Canrenoate ($\beta = -0.394$, $p = 0.012$) and the change in aortic PWV ($\beta = 0.445$, $p = 0.011$) were independent predictors of the change in daytime systolic BP (Adjusted $R^2 = 0.212$, $p = 0.031$). A multiple regression model for predictors of the change in aortic PWV was also constructed, with the age, sex, body mass index, the use of statins and angiotensin converting enzyme inhibitors, canrenoate, the change in office brachial systolic BP and the change in daytime systolic BP entered as independent variables. The change in daytime systolic BP ($\beta = 0.344$, $p = 0.029$) and female gender ($\beta = -0.410$, $p = 0.006$) were independent predictors of the change in aortic PWV (Adjusted $R^2 = 0.315$, $p = 0.006$), whereas canrenoate was of borderline significance ($\beta = 0.293$, $p = 0.058$) and the change in office brachial systolic BP was not significant ($\beta = 0.167$, $p = 0.230$).

Further data analysis concerning the effects of spironolactone treatment on exercise BP is presented in Appendix 3 of the thesis.

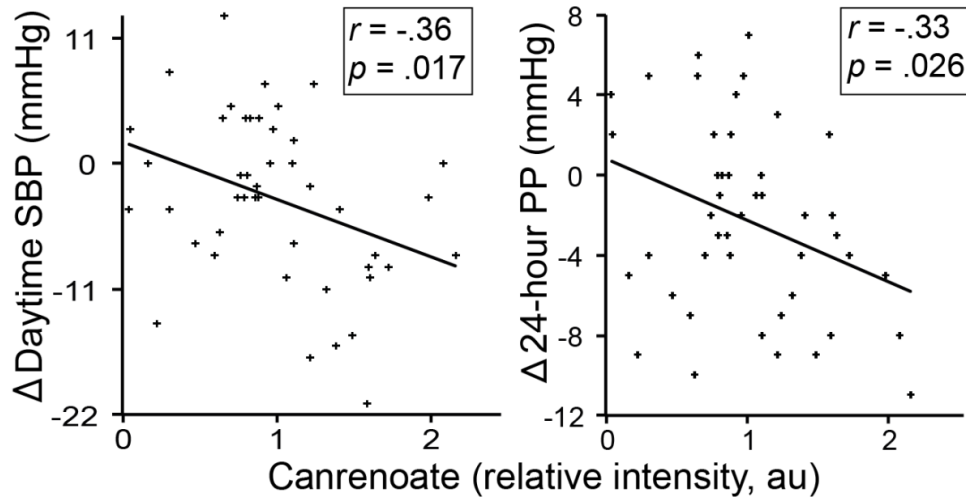


Figure 5.3 Correlations with the spironolactone metabolite canrenoate

Correlations with the spironolactone metabolite canrenoate (expressed relative to the group mean signal intensity). LEFT: the change in daytime systolic blood pressure (SBP); RIGHT: the change in 24 hour pulse pressure (PP), both after three months of spironolactone treatment (n = 49).

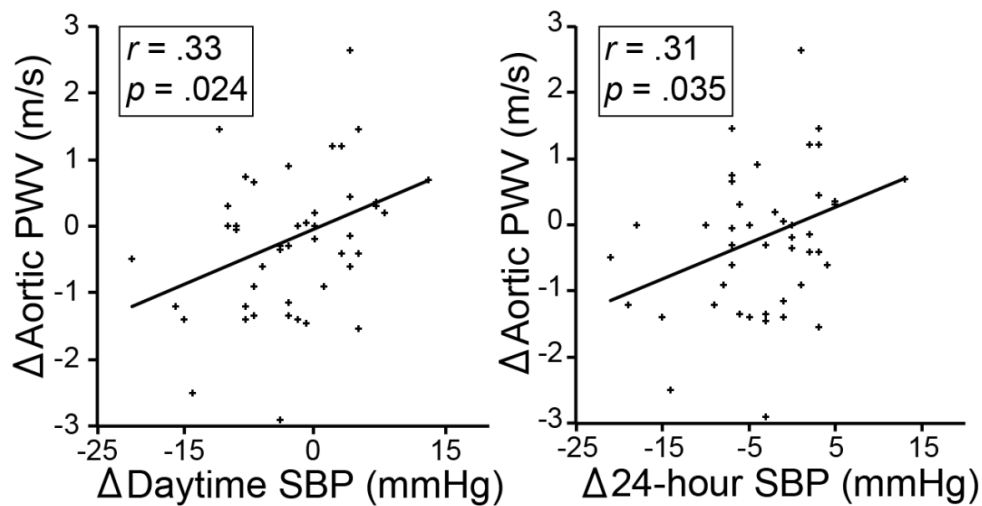


Figure 5.4 Correlations with the change in aortic pulse wave velocity

Correlations with the change in aortic pulse wave velocity (PWV). LEFT: the change in daytime systolic blood pressure (SBP); RIGHT: the change in 24 hour pulse pressure (PP), both after three months of spironolactone treatment (n = 49).

5.5 Discussion

To our knowledge, this is the first study that has used the combination of metabolomics and hemodynamics to examine the underlying physiological actions of spironolactone. The principal findings were that: i) spironolactone did not induce any detectable changes in the endogenous metabolite profile, ii) the changes in aortic stiffness were due to a reduction in ambulatory BP over three months of daily spironolactone treatment and iii) canrenoate may be responsible for the ambulatory BP-lowering effect but not for the reduction in aortic stiffness. These findings suggest that spironolactone has a BP-dependent effect on the attenuation of aortic stiffness, with canrenoate being the major active metabolite.

Spironolactone has been found to be effective in people with heart failure, a condition associated with advanced cardiac fibrosis.²⁰⁸ Indeed, in the Randomized Aldactone Evaluation Study (RALES), spironolactone significantly reduced mortality by 30 %, together with a reduction in cardiac fibrosis (markers of cardiac collagen turnover), compared with placebo.^{59, 208} Although the mechanism is not clear, beneficial effects of spironolactone in these individuals has been attributed to the antagonising effects of aldosterone on cardiac mineralocorticoid receptors, thus preventing the progression of cardiac fibrosis.⁵⁹ Recent studies have also confirmed the presence of mineralocorticoid receptors in vascular smooth muscle cells, endothelial cells, and fibroblasts.²⁰⁹⁻²¹¹ It has been suggested that, irrespective of BP control, blocking vascular mineralocorticoid receptors may prevent or attenuate negative effects of aldosterone on the structure and function of the vascular wall.^{58, 61, 212} Thus, spironolactone treatment has been purported to improve vascular function via BP-independent, antifibrotic mechanisms.^{58, 212}

We used metabolomics to study metabolites in an unbiased, untargeted manner and investigate whether spironolactone causes metabolic perturbations that might explain arterial wall effects of spironolactone in individuals with an HRE. Despite using state-of-the-art analytical platforms, and subject numbers within recommended ranges to achieve sufficient power,¹²¹ systematic changes in endogenous metabolism were not detected. Confidence in the sensitivity of the methods is provided by identification of known xenobiotic metabolites. However, it should be noted that our study cohort was reasonably healthy without established hypertension at rest and, therefore, we cannot exclude the possibility that significant metabolic effects of spironolactone may have only been detectable in individuals with more advanced arterial stiffness and sustained hypertension, or those with other (possibly

metabolic) co-morbidities. Indeed, under conditions of severe aortic stiffness the effects of spironolactone may become BP-independent through mineralocorticoid/antifibrotic mechanisms of action, but this need to be tested in other studies. Although previous studies report on the possible BP-independent effects of spironolactone within periods less than three months, it could also be argued that this was an insufficient treatment period in the current work.

Previous studies^{60, 61} have reported that the effect of spironolactone in reducing arterial stiffness was independent of mechanical factors (e.g. decrease in pressure load). Even if one accepts the possibility that spironolactone has a BP-independent effect on the arterial wall (at least in individuals with more advanced arterial stiffness, as suggested above), overall BP-dependent effects of spironolactone on arterial function could have been more thoroughly estimated by taking into account all measures of BP that spironolactone is known to affect (i.e. brachial, central and ambulatory BP). For example, treatment with spironolactone for 40 weeks reduced measures of arterial stiffness, which remained significant after correcting for the reduction in mean office BP, and, thus, interpreted as a BP-independent effect of spironolactone.⁶⁰ However, the authors also reported a significant reduction in ambulatory BP but this was not statistically accounted for.⁶⁰ Mahmud *et al*⁶¹ in a yet smaller study (n=24), reported that the change in aortic PWV after spironolactone treatment was no longer significant after correcting for resting mean BP, again suggesting a BP-independent effect, but no account was made for possible changes in ambulatory BP. On the other hand, lack of a change in aortic PWV observed in hypertensive people after six months of spironolactone treatment could also be explained by the lack of change in ambulatory BP values.²¹³ Taken all together, the effect of the change in BP induced by spironolactone treatment on arterial properties in previous studies might have been underestimated.

Our study supports the notion that the overall BP reduction induced by spironolactone could have a significant beneficial effect on arterial physiology, producing an effect that is mostly, if not entirely, BP-dependent. Indeed, we found that spironolactone significantly reduced aortic PWV, but this reduction was no longer significant when corrected for the reduction in ambulatory BP, despite the absence of a significant change in resting office brachial or central BP. Importantly, the change in daytime systolic BP was a significant predictor of the change in aortic PWV on multiple regression analysis independently of the change in resting office brachial BP. As spironolactone has a very short half-life once it is absorbed, major

effects of spironolactone are mainly attributed to its active metabolites with a longer half-life (up to 22 hours), such as canrenoate and canrenone.²⁰⁷ Notably, the metabolite canrenoate independently correlated with the change in ambulatory BP suggesting that this is an active metabolite of spironolactone with regard to its BP-lowering effect.

In conclusion, spironolactone seems to improve aortic stiffness via BP-dependent effects. In this current study, we show that in people with an HRE, spironolactone had no significant metabolic effects, and all improvements in aortic stiffness were related to ambulatory BP reduction by spironolactone. These findings, together with the association of canrenoate with ambulatory BP reduction, suggest that the reduction in aortic stiffness by spironolactone was due to BP-dependent, rather than the BP-independent or antifibrotic, effects of spironolactone. This new information highlights the importance of using out-of-office BP measures when assessing the clinical, and so-called BP-independent, effects of antihypertensive medications such as spironolactone.

5.6 Contribution of chapter 5 to thesis aims

Chapter 5 represents the first application of the metabolomics analysis in people with an HRE. This study was primarily used to develop a methodology regarding metabolomics analysis, but it was also a convenient sample to explore the metabolic actions of the aldosterone antagonist spironolactone on exercise BP and aortic stiffness in people with an HRE. Utilising the metabolomics technique, this study showed that spironolactone did not have any significant endogenous metabolic effects in people with an HRE, but reduced the exercise BP, as well as aortic stiffness, by BP-lowering effects. Importantly, *Chapter 5* further supports the results from *Chapter 4* that vascular haemodynamic factors, such as increased aortic stiffness, are less relevant to exercise BP than what is widely thought, given the lack of a significant relationship between the reductions in aortic stiffness and exercise BP by spironolactone treatment. Whilst this study itself represents an important contribution to knowledge surrounding treatment strategies to improve an HRE, this study also demonstrates how the application of metabolomics combined with haemodynamic measures could be a powerful method to characterise metabolic pathways associated with CV diseases.

Once the technique was developed, metabolomics analysis was used to explore the metabolic factors that might be contributing to an HRE in a population with the high prevalence of an HRE, such as those with type 2 diabetes mellitus (presented in *Chapter 6*).

Chapter 6. Serum Metabolic Profile Predicts Adverse Central Haemodynamics in Patients with Type 2 Diabetes Mellitus

At the time of thesis submission, this chapter is under peer review in *Acta Diabetologica*.

Sonja B. Nikolic, Lindsay M. Edwards, Yuliya V. Karpievitch, Richard Wilson, James Horne, Murray J. Adams, James E. Sharman

This chapter reports on possible metabolic factors that may be contributing to a hypertensive response to exercise at moderate intensity in participants with type 2 diabetes mellitus (a population with the high prevalence of a hypertensive response to exercise). This was achieved by exploring metabolic perturbations (utilising untargeted metabolomics analysis) that were related to both peripheral and central haemodynamic variables recorded during resting, as well as exercise conditions.

6.1 Abstract

Background. People with type 2 diabetes mellitus (T2DM) have abnormal peripheral and central haemodynamics at rest and during exercise, probably due to metabolic perturbations, but mechanisms are unknown. We used untargeted metabolomics to determine the relationships between metabolic perturbations and haemodynamics (peripheral and central) measured at rest and during exercise.

Methods. Serum samples from 39 participants with T2DM (62 ± 9 years; 46% male) and 39 controls (52 ± 10 years; 51% male) were analysed by liquid chromatography-mass spectrometry, nuclear magnetic resonance spectroscopy and principal component analysis. Scores on principal components (PC) were used to assess relationships with haemodynamics including peripheral and central BP, central augmentation index (AIx) and central augmentation pressure (AP).

Results. Participants with T2DM had higher resting and exercise haemodynamics (peripheral and central BP, central AIx and central AP) compared to controls ($p < 0.05$). PC comprised of a signature metabolic pattern of disordered carbohydrate metabolism in T2DM was independently associated with resting and exercise central AIx and central AP ($p < 0.05$). Glucose alone independently predicted central BP, rest and exercise central AIx and exercise AP ($p < 0.05$).

Conclusion. Serum metabolic profile was associated with resting and exercise central, but not peripheral, haemodynamics in T2DM participants, suggesting that metabolic irregularities may explain abnormal central haemodynamics in T2DM patients.

6.2 Introduction

Type 2 diabetes mellitus (T2DM) is associated with increased risk of cardiovascular (CV) morbidity and mortality.²¹⁴ High brachial blood pressure (BP) is common in T2DM, affecting up to 40% of newly diagnosed T2DM patients and significantly contributing to CV complications.¹⁶² T2DM is also associated with premature vascular aging and arterial structural remodelling, leading to increased arterial stiffness.⁹² However, T2DM has a greater impact on central arterial stiffness - and central BP indices - than the stiffness of peripheral arterial segments (measured by pulse wave velocity, PWV).²¹⁵ This is an important observation, as central arterial stiffness (or aortic PWV) and central BP indices such as augmentation index (AIx) are significant independent predictors of CV risk.¹²

Interestingly, increased central BP (including central systolic BP and AIx) are independent predictors of the onset of T2DM, even after correcting for mean BP and baseline glucose concentrations.²¹⁶ However, these early CV changes are not always apparent when subjects are tested under resting conditions. Indeed, the stress of exercise may reveal BP abnormalities that fail to be detected by conventional BP screening.⁶ People with T2DM who are normotensive at rest have a higher prevalence of exercise hypertension both peripherally and centrally (also referred as a hypertensive response to exercise [HRE]) than age-matched controls.¹¹ An HRE at moderate intensity has been associated with an increased risk of CV events and mortality when compared to those having a normotensive BP response to exercise.⁵ The pathophysiological mechanisms underlying abnormal peripheral and central haemodynamics at rest and during exercise in T2DM are yet to be elucidated.

The pathogenesis of T2DM is complex, involving genetic, environmental and lifestyle factors such as diet, lack of physical activity and obesity. Together, these lead to progressive decline in metabolic control and ultimately diabetes.¹⁶³ These metabolic disturbances may also adversely impact on central arterial haemodynamics and contribute to CV complications,⁸⁷ but the exact mechanisms are unclear. Metabolomics (the comprehensive analysis of low-molecular weight molecules in biological systems) has been increasingly used to evaluate perturbations associated with T2DM and have revealed alterations in the metabolism of carbohydrates, lipids, amino acids and bile acids.¹⁶⁴ Understanding how modification of the metabolic profile may influence peripheral or central haemodynamics (at rest and during exercise) could provide mechanistic insights of disease progression in people with T2DM.

In this study, we used untargeted metabolomics analysis and pattern recognition (principal component analysis; PCA) with the aim of identifying metabolic perturbations in people with T2DM compared with healthy non-diabetics. We aimed to determine the relationships between metabolic patterns (principal components; PC) and both peripheral and central haemodynamics recorded during resting and exercise conditions. Given the central haemodynamic disturbances in patients with T2DM, and also that exercise provides a means to reveal haemodynamic irregularity that may be obscured at rest, we hypothesised that metabolomic profiles would be more closely associated with central haemodynamics (and exercise) than with peripheral haemodynamics.

6.3 Methods

6.3.1 Study participants

Forty participants with T2DM and 40 sex-matched non-diabetic controls were recruited for this study through local community advertisements. Participants were excluded if they were below 18 years of age, pregnant or had a clinical history of cardiovascular complications including myocardial infarction, stroke or sustained arrhythmia. Of 80 participants, two participants were additionally excluded due to a missing serum sample (control group) and haemodynamic data (diabetic group). T2DM was diagnosed by the participants' physician according to the accepted clinical criteria for hyperglycemia (fasting or two hours post glucose load).²¹⁷ The study was approved by the Human Research Ethics Committee of the University of Tasmania. All procedures were performed in accordance with the Declaration of Helsinki and all participants provided informed consent.

6.3.2 Study protocol

Resting serum blood samples were acquired in the morning after over-night fasting for metabolomics analysis and clinical chemistry measures, and participants were fitted with an ambulatory BP device to measure 24 hour BP. On another occasion scheduled within seven days (also in the morning), participants were examined for peripheral and central haemodynamics at rest and during exercise in a quiet and temperature-controlled room. Participants refrained from consuming heavy meals and caffeine or smoking for at least three hours prior to examination and avoided exercise on the day of examination. Brachial BP was measured after 10 minutes of rest on a bed in semi-recumbent position, followed by

measurements of central BP, AIx, augmentation pressure (AP), aortic stiffness and systemic vascular resistance index (SVRi). When resting measures were completed, participants were asked to perform moderate intensity semi-recumbent cycling exercise (60% of age predicted maximal heart rate) on a bicycle ergometer positioned on the bed (Rehab Trainer 881, MONARK Exercise110 AB, Vansbro, Sweden), and all measures were repeated during exercise, as previously described.¹⁸⁸

6.3.3 Clinical chemistry

Serum samples were analysed using standard laboratory procedures to determine glucose, glycated haemoglobin (HbA_{1c}), insulin, triglycerides, total, low-density lipoprotein (LDL) and high-density lipoprotein (HDL).

6.3.4 Haemodynamic variables

Peripheral haemodynamics. Brachial BP was measured in duplicate by a trained non clinician using a validated semi-automated device (Omron HEM-907; OMRON Europe B.V. (OMCE), Hoofddorp, The Netherlands),¹⁸³ in accordance with guidelines.⁴ Pulse pressure (PP) was defined as the difference between systolic and diastolic BP defined Brachial BP during exercise was acquired in duplicate using a validated mercury-free sphygmomanometer (UM-101B, A&D Medical, Thebarton, South Australia).¹⁸⁴ An HRE was defined as exercise peripheral systolic BP ≥ 170 mmHg for men and ≥ 160 mmHg for women (or the highest tertile of the study population, as previously described).¹⁹⁹ Ambulatory BP was measured every 20 minutes during the day and every 30 minutes during the night using a TM-2430 monitor (A&D Medical, Sydney, Australia). Cardioimpedance was used to measure SVRi (Physio Flow; Manatec Biomedical; Macheren, France).¹⁹³ *Central haemodynamics.* Duplicate measures of central BP were recorded at rest and during exercise using validated and reproducible radial applanation tonometry (SphygmoCor 8.1, AtCor Medical, Sydney, NSW).^{47, 48} The pressure waveforms were calibrated using the brachial systolic and diastolic BP values measured prior to applanation tonometry. AP was calculated as the difference between the second and first systolic peak of the central pressure waveform, and expressed as a percentage of the PP for definition of AIx (also normalised to heart rate of 75 bpm). Resting and exercise aortic stiffness was measured by pulse wave velocity (PWV) and applanation tonometry (SphygmoCor 8.1, AtCor Medical, Sydney, NSW).¹⁷⁷

6.3.5 Metabolomics analysis

Liquid chromatography-mass spectrometry (LC-MS) - Collected blood samples for metabolomics analysis were allowed to clot at room temperature for 30 minutes before being centrifuged at 3000 rpm for 15 minutes. Serum was transferred into aliquot tubes and stored at -80°C until analysis. Samples were thawed and a 100 µL aliquot of each sample was mixed with 300 µL of ice-cold methanol, incubated at 4°C for 20 minutes and centrifuged at 13200 g for 10 minutes. The supernatant was lyophilized in a vacuum centrifuge (Labconco, Kansas City, USA) and re-stored at -80°C. The dried samples were reconstituted in 100 µL of HPLC-grade water prior to LC-MS analysis. Samples were vortexed and centrifuged for 15 minutes, and supernatant was transferred to vials.¹⁰ Quality control (QC) samples were prepared by mixing 10 µL aliquots taken from each study sample and analysed throughout the LC-MS runs to ensure analytical consistency and correct for systematic drifts (four study samples were bracketed by two QCs). Samples from participants with T2DM were matched with samples from non-diabetic participants by age, sex, and anthropometric measures and were analysed together to protect against systematic bias. An LTQ-OrbitrapXL mass spectrometer (ThermoFisher Scientific, Bremen, Germany) controlled by XCalibur v2 software (ThermoFisher Scientific) was used for sample analysis. Chromatographic separation was performed on a Waters Nova-Pak 4.0 µm C₁₈ column (3.91x150 mm) with an injection volume of 10 µL. The mobile phases used for the sample elution were A) 0.1% formic acid in water and B) 0.1% formic acid in methanol, with a flow rate of 0.8 mL/minute. Both positive (PIMS) and negative (NIMS) ionization modes were used for data acquisition. XCMS software running in R (R 2.14; <http://cran.r-project.org/>) was used for data processing - peak finding, grouping and retention time alignment. The parameters for deconvolution were: method='centWave', ppm=3, peakwidth=5-30, snthresh=6, mzdiff=0.01; and for retention time correction: method="obiwarp" and profStep=0.1. A singular value decomposition-based method (EigenMS) was used for data normalisation.

Proton nuclear magnetic resonance (¹H NMR) spectroscopy – A 500 µL aliquot of serum was mixed with 1 mL of methanol (2:1 v/v with original sample), incubated in a -20°C freezer for 20 minutes and centrifuged for 10 minutes at 13,200 g. Supernatants were dried in a vacuum centrifuge overnight (Labconco, Kansas City, USA), re-suspended in 700 µL NMR buffer (2 mM 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt in D₂O) and stored at -80°C until the analysis. ¹H NMR spectra were acquired using an Agilent/Varian Inova

400 MHz wide bore spectrometer (Varian Medical Systems, Palo Alto, CA, USA), in a 5mm ID-PFG probe, as described previously.²¹⁸ Briefly, spectra were acquired with 90° pulse excitation, sweep width of 4,299 ppm and relaxation delay of 2 seconds at 25°C. A total of 128 transients were collected into 32000 complex data points. The water resonance was set to the centre of the sweep width on the transmitter offset and attenuated using a pre-saturation pulse of 2 seconds during the relaxation delay and the mixing time (100 ms) of the NOESY pulse sequence. Data were Fourier-transformed, probabilistic-quotient normalised and ‘binned’ using an adaptive-intelligent algorithm described elsewhere.¹¹²

6.3.6 Data analysis, statistics and feature annotation

The ¹H NMR and LC-MS data were mean-centred and Pareto-scaled before using PCA to dimensionally reduce the data using PLS Toolbox 6.5 (Eigenvector Research Inc., Wenatchee, US). Visual inspection of a ‘scree’ plot of captured variance versus number of components was used to identify the number of PCs comprising each model (prior to any further analysis). Plots of scores on each PC were visually inspected. Those that appeared to separate the groups were compared using an independent *t*-test. Loading coefficients were used to identify metabolites that significantly contributed to discriminatory PCs. In addition, individual metabolites were compared across groups by independent *t*-test with significance cut-off *p* = 0.05 after Benjamini-Hochberg multiple testing adjustment.¹⁹⁵ The LC-MS data were checked for artefacts, de-isotoped and putatively annotated using online databases and the Taverna workflows.¹⁹⁶ Unambiguous identification of LC-MS metabolites was confirmed by tandem mass spectrometry (MS/MS), ¹H NMR spectroscopy and online databases. All identified metabolites listed in results are accompanied by the METLIN identification (MID) number.

The rest of the standard statistical analyses were conducted using SPSS 20.0 (IBM SPSS Statistics for Windows, Armonk, NY: IBM Corp). Clinical characteristics of study participants were presented as mean and standard deviation. Comparisons between participants with T2DM and non-diabetic controls were performed by independent *t*-tests, whereas comparisons between categorical variables were assessed by chi-squared tests. Analysis of covariance was used to explore the difference in aortic PWV between groups while adjusting for resting peripheral BP. Associations between PCs, individual identified metabolites and physiological variables were assessed by Pearson correlations. Multiple linear regression was used to assess the independent associations between PCs and

haemodynamic variables, while correcting for age, sex, body mass index, the use of antihypertensive medications and statins. A *p*-value of 0.05 was used as a significance cut-off.

6.4 Results

6.4.1 Clinical characteristics

Table 6.1 represents the clinical characteristics of study participants. There were no differences in sex between groups, but participants with T2DM were older, had higher waist-to-hip ratio, body mass index, and were taking more medications (statins and antihypertensives) compared to non-diabetic controls. As expected, T2DM participants had significantly higher fasting serum glucose, HbA_{1C}, triglycerides and insulin concentrations than controls. However, total cholesterol, HDL and LDL cholesterol were significantly lower in participants with T2DM, probably due to lipid lowering medications.

Table 6.1 Clinical characteristics of study participants

| Characteristics | Control (n = 39) | T2DM (n = 39) | <i>p</i> value |
|--------------------------------------|---------------------|------------------|----------------|
| Age (years) | 52 ± 10 | 62 ± 9 | <0.001 |
| Sex, male (%) | 46 | 51 | 0.821 |
| Body mass index (kg/m ²) | 24.7 ± 3.1 | 31.1 ± 5.5 | <0.001 |
| Antihypertensive medications (%) | 0 | 61 | <0.001 |
| Statins (%) | 0 | 64 | <0.001 |
| Diabetic medications (%) | 0 | 67 | <0.001 |
| Glucose (mmol/L) | 4.7 ± 0.4 | 7.6 ± 1.8 | <0.001 |
| HbA _{1C} (%) | 5.5 ± 0.4 | 7.2 ± 0.8 | <0.001 |
| Insulin (mU/L) | 2.4 ± 4.6 | 10.3 ± 8.8 | <0.001 |
| Triglycerides (mmol/L) | 1.0 ± 0.5 | 1.5 ± 0.7 | <0.001 |
| Total cholesterol (mmol/L) | 5.4 ± 1.0 | 4.4 ± 1.0 | <0.001 |
| HDL cholesterol (mmol/L) | 1.7 ± 0.4 | 1.3 ± 0.4 | <0.001 |
| LDL cholesterol (mmol/L) | 3.3 ± 0.9 | 2.7 ± 0.8 | <0.001 |

Glycated haemoglobin HDL, high density lipoprotein; LDL, low density lipoprotein.

6.4.2 Haemodynamic variables

Table 6.2 summarises the peripheral and central haemodynamic variables measured at rest and during exercise.

Table 6.2 Peripheral and central haemodynamic variables measured at rest and during exercise in participants with type 2 diabetes mellitus compared with non-diabetic controls

| Characteristics | Control (n = 39) | T2DM (n = 39) | <i>p</i> value |
|-------------------------------------|------------------|---------------|----------------|
| Peripheral haemodynamics | | | |
| Brachial systolic BP (mmHg) | 114 ± 9 | 125 ± 13 | <0.001 |
| Brachial pulse pressure (mmHg) | 49 ± 4 | 56 ± 11 | <0.001 |
| Brachial diastolic BP (mmHg) | 65 ± 6 | 69 ± 8 | 0.011 |
| SVRi (dyne/s/cm ⁵) | 2780 ± 361 | 2750 ± 471 | 0.754 |
| Ex brachial systolic BP (mmHg) | 135 ± 14 | 156 ± 17 | <0.001 |
| Ex brachial pulse pressure (mmHg) | 62 ± 11 | 79 ± 15 | <0.001 |
| Ex brachial diastolic BP (mmHg) | 73 ± 8 | 78 ± 8 | 0.017 |
| Ex SVRi (dyne/s/cm ⁵) | 1843 ± 323 | 2028 ± 352 | 0.019 |
| 24h systolic BP (mmHg) | 130 ± 12 | 135 ± 13 | 0.095 |
| 24h pulse pressure (mmHg) | 51 ± 12 | 59 ± 10 | <0.001 |
| Daytime systolic BP (mmHg) | 136 ± 13 | 139 ± 15 | 0.331 |
| Daytime pulse pressure (mmHg) | 52 ± 8 | 61 ± 10 | <0.001 |
| Night systolic BP (mmHg) | 113 ± 11 | 121 ± 13 | 0.002 |
| Night pulse pressure (mmHg) | 47 ± 7 | 54 ± 10 | <0.001 |
| Central haemodynamics | | | |
| Central systolic BP (mmHg) | 102 ± 9 | 115 ± 12 | <0.001 |
| Central pulse pressure (mmHg) | 36 ± 5 | 45 ± 9 | <0.001 |
| Central diastolic BP (mmHg) | 66 ± 6 | 70 ± 8 | 0.013 |
| Augmentation pressure (mmHg) | 8 ± 5 | 13 ± 5 | <0.001 |
| Augmentation index (%) | 13 ± 11 | 23 ± 6 | <0.001 |
| Aortic pulse wave velocity (m/s) | 6.4 ± 1.4 | 8.0 ± 2.1 | <0.001 |
| Ex central systolic BP (mmHg) | 114 ± 12 | 133 ± 14 | <0.001 |
| Ex central pulse pressure (mmHg) | 39 ± 7 | 53 ± 12 | <0.001 |
| Ex central diastolic BP (mmHg) | 75 ± 9 | 79 ± 9 | 0.010 |
| Ex augmentation pressure (mmHg) | 8 ± 5 | 13 ± 5 | <0.001 |
| Ex augmentation index (%) | 13 ± 13 | 25 ± 8 | <0.001 |
| Ex aortic pulse wave velocity (m/s) | 7.1 ± 1.4 | 9.6 ± 2.0 | <0.001 |

BP, blood pressure; SVRi, systemic vascular resistance index; ex, exercise.

Peripheral haemodynamics. Patients with T2DM had higher resting and exercise brachial BP compared to non-diabetic controls ($p < 0.001$). The prevalence of an HRE in T2DM group was 31%, and in non-diabetic controls 5%. There were no significant differences in SVRi between groups at rest, but during exercise, SVRi was significantly higher in participants with T2DM. Ambulatory BP variables were also significantly higher in participants with T2DM, except for 24 hour systolic BP and daytime systolic BP. *Central haemodynamics.* Patients with T2DM had higher resting and exercise central BP, AP and AIx compared to non-diabetic controls. Resting aortic PWV was also significantly increased, even after correcting for the brachial systolic BP ($p = 0.029$). The change in brachial systolic BP from rest to exercise was significantly higher in participants with T2DM compared to non-diabetic controls (31 ± 13 mmHg vs. 21 ± 13 mmHg; $p = 0.001$), and similar for the change in central systolic BP (18 ± 12 mmHg vs. 12 ± 11 mmHg; $p = 0.026$).

6.4.3 Metabolomics analysis

The metabolic profiles of participants with T2DM acquired by LC-MS were compared to those of non-diabetic controls. PCA models were built for both NIMS and PIMS data. For the NIMS, a six-component PCA model accounted for 62% of the total variance. NIMS PC1 (27% of variance explained; Figure 6.1) and NIMS PC3 (8% of variance explained) gave good discrimination between groups ($p < 0.001$ and $p < 0.002$, respectively). Examination of the loading coefficients revealed metabolites that significantly contributed to NIMS PC1 and included glucose (MID 133), fructose (MID 135), lactate (MID 63094), α -N-phenylacetyl-L-glutamine (MID 58397), glycochenodeoxycholic acid 3-glucuronide and 2-hydroxy-3-(4-methoxyethylphenoxy)-propanoic acid (MID 1255). The most significant contributor to NIMS PC3 was inosine (MID 84), and the relative difference of inosine in T2DM participants relative to non-diabetic controls is presented in Figure 6.2. Also, there were no significant differences in inosine levels between T2DM participants that were taking antihypertensive medications and/or statins compared to those that were not using the medications ($p > 0.05$). For the PIMS data, a three-component PCA model was selected and accounted for 79% of the total variance in the data. PIMS PC1 (64% of total variance) gave good separation between groups ($p < 0.001$). Loading coefficients showed that amino acids including tyrosine (MID 34), proline (MID 29) and leucine (MID 24) were the significant contributors to PIMS PC1. The ^1H NMR metabolic profiles showed metabolic patterns that were similar and complementary to the metabolic profiles acquired using LC-MS. For the ^1H

NMR data, a three-component PCA accounted for 65% of the total variance. NMR PC1 (33% of variance explained) and NMR PC2 (21% of variance explained) produced good discrimination between groups ($p < 0.001$ for both). Major NMR PC1 contributors were again glucose (MID 133), fructose (MID 135) and lactate (MID 63094), whereas significant contributors to NMR PC2 were creatinine (MID 8) and 3-hydroxybutyric acid (MID 85159). All metabolites presented were increased in T2DM participants, except for inosine and creatinine that were decreased. Identified metabolites were summarised in Table 6.3.

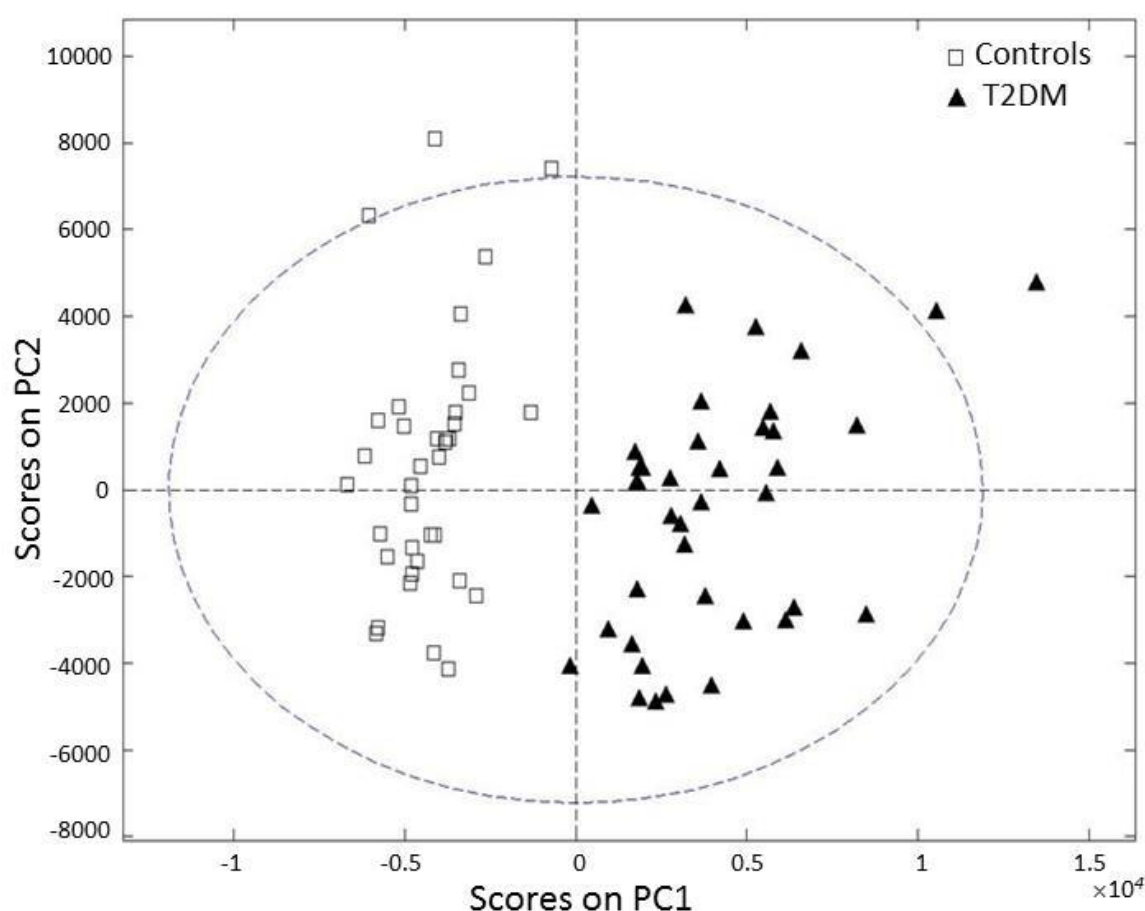


Figure 6.1 Score plot

The score plot from the principal component analysis of negative ion mass spectrometry spectra of serum from participants with type 2 diabetes mellitus (T2DM) and non-diabetic controls; groups are different on PC1 ($p < 0.001$). Metabolites that significantly contributed to negative ion mass spectrometry spectra principal component 1 included glucose, fructose, lactate, α -N-phenylacetyl-L-glutamine, glycochenodeoxycholic acid 3-glucuronide and 2-hydroxy-3-(4-methoxyethylphenoxy)-propanoic acid.

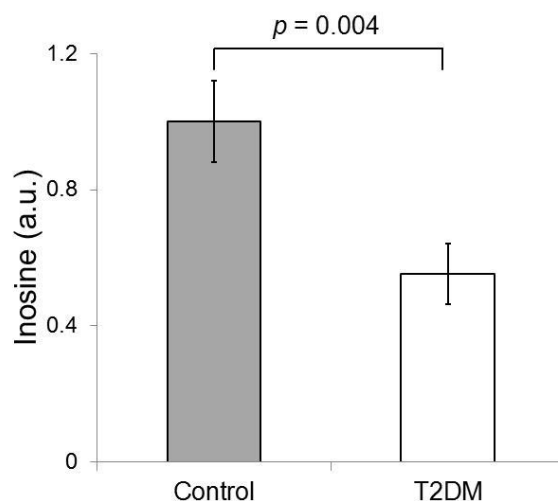


Figure 6.2 Difference in inosine in participants with type 2 diabetes mellitus (T2DM) relative to non-diabetic controls, measured in arbitrary units (a.u.).

Table 6.3 Summary of identified serum metabolites with significantly different concentrations in participants with type 2 diabetes mellitus versus non-diabetic controls

| Metabolite | Formula | Platform | * <i>p</i> |
|--|---|----------|---------------------|
| Glucose | C ₆ H ₁₂ O ₆ | NIMS/NMR | 3×10 ⁻¹³ |
| Fructose | C ₆ H ₁₂ O ₆ | NIMS/NMR | 4×10 ⁻⁷ |
| Lactate | C ₃ H ₆ O ₃ | NIMS/NMR | 4×10 ⁻⁵ |
| α-N-Phenylacetyl-L-glutamine | C ₁₃ H ₁₆ N ₂ O ₄ | NIMS | 8×10 ⁻⁴ |
| Glycochenodeoxycholic acid 3-glucuronide | C ₃₂ H ₅₁ NO ₁₁ | NIMS | 2×10 ⁻⁶ |
| 2-Hydroxy-3-(4-methoxyethylphenoxy)-propanoic acid | C ₁₂ H ₁₆ O ₅ | NIMS | 4×10 ⁻⁴ |
| Inosine | C ₁₀ H ₁₂ N ₄ O ₅ | NIMS | 4×10 ⁻³ |
| Tyrosine | C ₉ H ₁₁ NO ₃ | PIMS | 1×10 ⁻⁴ |
| Proline | C ₅ H ₉ NO ₂ | PIMS | 3×10 ⁻⁴ |
| Leucine | C ₆ H ₁₃ NO ₂ | PIMS | 8×10 ⁻⁶ |
| Creatinine | C ₄ N ₇ H ₃ O | NMR | 1×10 ⁻⁴ |
| 3-Hydroxybutyric acid | C ₄ H ₈ O ₃ | NMR | 1×10 ⁻² |

NIMS, negative ion mass spectrometry; NMR, nuclear magnetic resonance; PIMS, positive ion mass spectrometry; **p* value after Benjamini and Hochberg correction. Inosine and creatinine were decreased, whereas all other metabolites were increased in participants with T2DM compared to non-diabetic controls

6.4.4 Associations between metabolomics principal components and haemodynamic variables.

Figure 6.3 illustrates the correlations between PCs (Pearson's r coefficient, absolute values), identified metabolites and peripheral and central haemodynamic variables. Among all PCs, NIMS PC1 was the strongest correlate of peripheral and central haemodynamics being the highest for rest and exercise central systolic BP, AP and AIx, as well as for exercise brachial systolic BP, 24 hour and daytime PP (all $r > 0.400$). Also, mean univariable correlations of NIMS PC1 were highest for exercise central haemodynamics ($r = 0.457$), followed by rest central haemodynamics ($r = 0.405$), exercise peripheral haemodynamics ($r = 0.395$), and the lowest were for rest peripheral haemodynamics ($r = 0.225$). Table 6.4 shows the associations of NIMS PC1 on multiple regression analysis with individual peripheral and central haemodynamics after adjusting for age, sex and body mass index, the use of antihypertensive medications and statins. NIMS PC1 was significantly associated with exercise SVRI, 24 hour and daytime PP. Among central haemodynamics, NIMS PC1 was significantly associated with central rest and exercise AP and AIx. When the same regression models were constructed with the addition of statins, the results did not change significantly.

NIMS PC3 was significantly inversely associated with brachial resting ($\beta = -0.231$, $p = 0.032$) and exercise ($\beta = -0.212$, $p = 0.033$) systolic BP, even after correcting for age, sex, body mass index, the use of antihypertensive medications and statins. NMR PC2 scores were significantly, inversely associated with exercise brachial systolic BP and PP, 24 hour and daytime PP. Among central haemodynamics, NMR PC2 scores were negatively associated with central both resting and exercise BP, exercise central PP, and resting AP and AIx (Table 6.5) after correction for age, sex, body mass index, the use of antihypertensive medications and statins.

Among individual metabolites, glucose was the strongest independent predictor of central rest systolic BP ($\beta = 0.274$, $p = 0.032$), exercise AP ($\beta = 0.233$, $p = 0.016$) and rest and exercise AIx ($\beta = 0.254$, $p = 0.019$ and $\beta = 0.272$, $p = 0.007$; respectively) after correcting for age, sex, body mass index, the use of antihypertensive medications and statins.

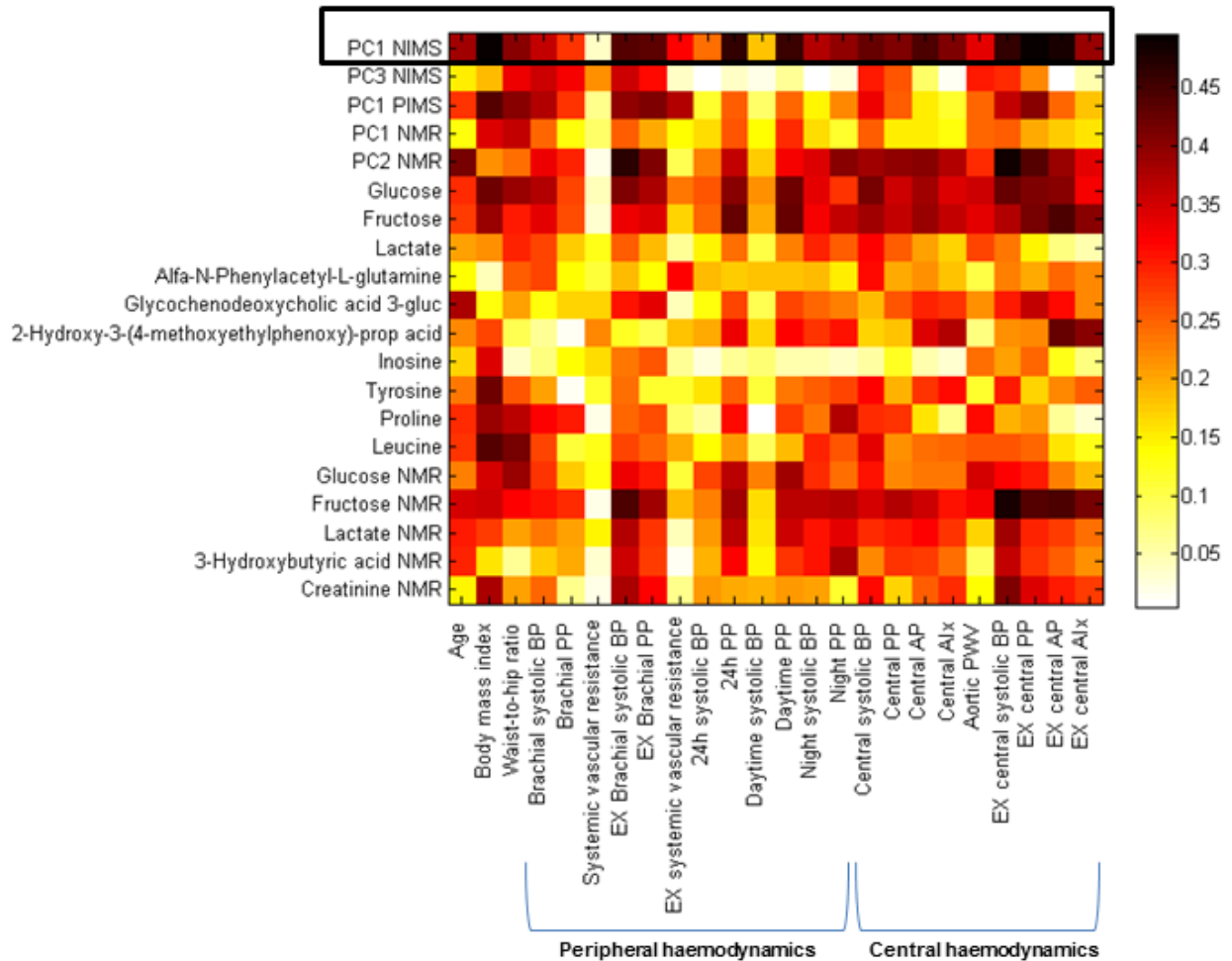


Figure 6.3 Heat plot

Correlations (Pearson's r coefficient, absolute values) between principal components, identified metabolites and peripheral and central haemodynamic variables measured at rest and during exercise. PC, principal component; NIMS, negative ion mass spectrometry; PIMS, positive ion mass spectrometry; NMR, nuclear magnetic resonance; BP, blood pressure; PP, pulse pressure, AP, augmentation pressure; AIx, augmentation index; PWV, pulse wave velocity. Among all PCs, NIMS PC1 was the strongest correlate of peripheral and central haemodynamics (highlighted by rectangular box) being the highest for rest and exercise central systolic BP, AP and AIx, as well as for exercise brachial systolic BP, 24 hour and daytime PP (all $r > 0.400$).

Table 6.4 Multiple regression analysis assessing the associations of negative ion mass spectrometry principal component 1 (NIMS PC1) with peripheral and central haemodynamic variables

| Dependent variable | Standardized β | <i>p</i> value | Adjusted R^2 |
|---------------------------------|--|-----------------------|----------------------------------|
| Peripheral haemodynamics | | | |
| Brachial systolic BP | 0.118 | 0.399 | 0.202 |
| Brachial pulse pressure | 0.056 | 0.677 | 0.270 |
| SVRi | 0.110 | 0.490 | 0.067 |
| Ex brachial systolic BP | 0.102 | 0.430 | 0.313 |
| Ex brachial pulse pressure | 0.019 | 0.875 | 0.402 |
| Ex SVRi | 0.303 | 0.041 | 0.140 |
| 24h pulse pressure | 0.389 | 0.009 | 0.156 |
| Daytime pulse pressure | 0.401 | 0.007 | 0.140 |
| Central haemodynamics | | | |
| Central systolic BP | 0.241 | 0.085 | 0.210 |
| Central pulse pressure | 0.108 | 0.392 | 0.350 |
| Augmentation pressure | 0.276 | 0.022 | 0.428 |
| Augmentation index | 0.275 | 0.020 | 0.462 |
| Aortic pulse wave velocity | 0.085 | 0.524 | 0.294 |
| Ex central systolic BP | 0.158 | 0.218 | 0.335 |
| Ex central pulse pressure | 0.085 | 0.470 | 0.434 |
| Ex augmentation pressure | 0.243 | 0.029 | 0.510 |
| Ex augmentation index | 0.270 | 0.015 | 0.535 |

BP, blood pressure; SVRi, systemic vascular resistance index; ex, exercise; Adjusted for age, sex, body mass index, the use of antihypertensive medications and statins

Table 6.5 Multiple regression analysis assessing the associations of nuclear magnetic resonance principal component 2 (NMR PC2) with peripheral and central haemodynamic variables

| Dependent variable | Standardized β | <i>p</i> value | Adjusted R^2 |
|---------------------------------|--|-----------------------|----------------------------------|
| Peripheral haemodynamics | | | |
| Brachial systolic BP | -0.214 | 0.055 | 0.236 |
| Brachial pulse pressure | -0.111 | 0.299 | 0.280 |
| SVR _i | 0.031 | 0.808 | 0.022 |
| Ex brachial systolic BP | -0.289 | 0.005 | 0.384 |
| Ex brachial pulse pressure | -0.203 | 0.034 | 0.439 |
| Ex SVR _i | -0.079 | 0.512 | 0.091 |
| 24hour pulse pressure | -0.280 | 0.020 | 0.138 |
| Daytime pulse pressure | -0.252 | 0.037 | 0.102 |
| Central haemodynamics | | | |
| Central systolic BP | -0.240 | 0.033 | 0.228 |
| Central pulse pressure | -0.188 | 0.063 | 0.375 |
| Augmentation pressure | -0.235 | 0.015 | 0.433 |
| Augmentation index | -0.202 | 0.036 | 0.454 |
| Aortic pulse wave velocity | -0.103 | 0.338 | 0.300 |
| Ex central systolic BP | -0.280 | 0.006 | 0.392 |
| Ex central pulse pressure | -0.209 | 0.025 | 0.470 |
| Ex augmentation pressure | -0.123 | 0.176 | 0.488 |
| Ex augmentation index | -0.125 | 0.170 | 0.505 |

BP, blood pressure; ex, exercise; Adjusted for age, sex, body mass index, the use of antihypertensive medications and statins

6.5 Discussion

To our knowledge, this is the first study that has used untargeted metabolomics to investigate the relationships between resting serum metabolic profile and underlying haemodynamic abnormalities in T2DM measured at rest and during exercise. We have found that a PC consisting of common metabolites of impaired carbohydrate metabolism was more strongly associated with increased central (and exercise) haemodynamics than with peripheral

haemodynamics, suggesting that the metabolic irregularities in glucose metabolism, the hallmark of T2DM, may be mechanistically involved in abnormal central haemodynamics. Furthermore, inosine was decreased in T2DM participants (implicated as an anti-inflammatory agent), and inversely associated with the increased BP values (rest and exercise), this finding suggests a possible role of inflammation as a contributing factor in hypertension development and exaggerated exercise BP.

The PCA of NIMS data (PC1) identified major contributors to the separation between T2DM participants and non-diabetic controls including expected markers of dysfunctional carbohydrate metabolism. Increased lactate, as a variable within this PC, suggests not only 'overflow metabolism' but may also signify a systemic decrease in oxidative capacity in insulin-resistant skeletal muscle²¹⁹ or adipose tissue.²²⁰ This signature metabolic pattern underlying T2DM was significantly associated with central BP indices (rest and exercise AP and AIX; markers of left ventricular [LV] afterload). Also, glucose levels alone independently predicted rest central systolic BP, exercise AP and rest and exercise AIX. These observations suggest that disordered carbohydrate metabolism may be directly involved in haemodynamic abnormalities in diabetics. The underlying mechanisms for closer association with central haemodynamics may be due to formation of advanced glycation end-products by non-enzymatic crosslinks between sugars and amino acids. This occurs preferentially in central elastic arterial walls compared to more muscular peripheral arteries, causing alterations in elastin and collagen fibres, and increased large artery stiffness. Indeed, high concentrations of advanced glycation end-products have been found in post-mortem aorta samples from diabetic patients compared to non-diabetic controls and were significantly correlated with the aortic stiffness.⁹⁴ Central BP is pathophysiologically more important than brachial BP because it closely represents the pressure to which the vital organs (heart, brain and kidneys) are exposed. Hence central BP more strongly relates to CV events than brachial BP.¹² However, the present study was cross-sectional in nature and we cannot demonstrate causality. Also, we cannot exclude the possibility that the metabolic triad of glucose, fructose and lactate is simply a surrogate for disease severity with which central haemodynamic dysfunction and other pathogenic variables may correlate. NIMS PC1 was also significantly associated with exercise (but not resting) SVRI, which was significantly increased in T2DM participants, suggesting that the changes of peripheral vasculature may be evident during physiological stress, such as exercise.

We observed decreased levels of inosine (a major contributor of NIMS PC3) in participants with T2DM. Inosine is a naturally occurring purine formed from the conversion of adenosine by adenosine deaminase and it has been shown to be an anti-inflammatory agent.²²¹ Treatment with inosine had a protective effect against the development of diabetes in two murine models of diabetes (streptozotocin-induced diabetes and spontaneous non-obese diabetic mouse model) by decreasing pancreatic leukocyte infiltration and oxidative stress.²²² Since inosine was decreased in T2DM participants in our study, and inversely associated with the increased rest and exercise BP values, this finding suggests a possible role of inflammation as a contributing factor in hypertension development and exaggerated exercise BP. Indeed, C-reactive protein (a sensitive marker of inflammation) was independently associated with insulin sensitivity and systolic BP in a large, population-based study,²²³ as well as with exercise systolic BP during moderate intensity cycling in healthy men and women.²²⁴ Inflammation may be a triggering factor for insulin resistance leading to T2DM, but also the cause of vascular endothelial dysfunction (altered vasodilator/vasoconstrictor balance) and consequent BP regulation.¹⁶⁸ Further studies are warranted to establish whether inosine supplementation exerts anti-inflammatory effects with benefit to vascular function and BP control.

Consistent with previous studies which reported increased amino acid levels in T2DM individuals,^{35, 36} the PCA of PIMS in the current study also revealed higher levels of amino acids including leucine, tyrosine and proline that discriminated T2DM participants from non-diabetic controls. However, increased levels of amino acids, and in particular branched-chain amino acids (identified as modulators of insulin secretion),²²⁵ may promote insulin resistance even in non-diabetic individuals most likely via disturbance of skeletal muscle insulin signalling.²²⁶ A recent metabolomics study in the Framingham Offspring cohort has showed that the amino acids isoleucine, leucine, valine, tyrosine and phenylalanine were increased 12 years before the onset of T2DM and were significantly associated with the future incidence of T2DM.¹⁶⁵ Importantly, increased levels of three amino acids (isoleucine, tyrosine and phenylalanine) were also significantly associated with the future development of CV disease, independently of standard CV disease and diabetes risk factors (including measures of insulin resistance), suggesting that disturbed amino acid metabolism may represent a novel link between CV disease development and T2DM. A cross-sectional analysis in individuals free of CV disease reported that each increase in the score of the same three amino acids was

associated with higher risk of having subclinical carotid atherosclerosis, as well as with approximately 80% increased risk of exercise-induced myocardial ischemia in individuals referred for diagnostic exercise stress testing.¹⁶⁶ Also, amino acids levels were significantly associated with the presence of coronary artery disease in individuals undergoing catheterization.²²⁷ Overall, these findings may suggest that the association between amino acids and the development of CV disease may be mediated by irregularities in vascular function, although further studies are required to investigate causal mechanisms.

Although lipid metabolism may also play a significant role in the development of haemodynamic abnormalities, metabolomics analysis in this study failed to detect any lipid-related metabolites, as many study participants were on lipid-lowering medications as part of their standard BP management. Therefore, future studies including people with newly diagnosed T2DM that are still free from lipid-lowering medications may provide additional information regarding metabolic disorders and their connections to abnormal haemodynamic variables. Also, resting and exercise BP were measured by different devices; however, both devices have been validated according to the standard criteria for BP measurements and shown to be highly reproducible. The use of different devices might have affected the study results, including the noticed increase in diastolic BP during exercise.^{183, 184}

In conclusion, our findings show that a signature metabolic pattern of disordered carbohydrate metabolism was independently associated with central haemodynamics in patients with T2DM under resting and exercise conditions. Furthermore, decreased inosine levels in subjects with T2DM and inverse correlation with BP values (rest and exercise) suggest that inflammation may contribute to haemodynamic abnormalities in T2DM and potentially offer a novel therapeutic route.

6.6 Contribution of chapter 6 to thesis aims

People with T2DM have a higher prevalence of an HRE compared to those without T2DM, but the underlying mechanisms are unknown. *Chapter 6* represents the first study that used the combination of untargeted metabolomics analysis, along with the haemodynamic measures, to help explaining possible metabolic factors that may be contributing to an HRE at moderate intensity in people with T2DM. This study showed that a signature metabolic pattern of disordered carbohydrate metabolism in T2DM (including increased levels of glucose, fructose and lactate) was independently associated with the exercise central BP

haemodynamic indices, suggesting that impaired carbohydrate metabolism may be a possible metabolic mechanism explaining central (but not peripheral) exercise hypertension. These findings are aligned with the observations from previous studies reporting that T2DM has a greater impact on central, rather than peripheral, arterial segments. Given the independent prognostic value of central BP measures and their higher pathophysiological significance compared to peripheral BP variables, these findings have clinical relevance. On the other hand, utilisation of exploratory metabolomics analysis has provided some evidence for the role of inflammation as a contributing factor of abnormal peripheral BP and an HRE in people with T2DM.

Chapter 7. Summary and Future Directions

Hypertension is a leading risk factor for global disease burden,³ affecting approximately one billion individuals worldwide, with this number expected to rise.¹⁵ Resting office blood pressure (BP) is used for the diagnosis of hypertension;⁴ however, despite apparently normal BP at rest, some individuals experience a hypertensive response to exercise (HRE). An HRE at moderate intensity (akin to the condition experienced during normal daily life activities) is associated with an increased risk for adverse cardiovascular (CV) events.⁵ It may provide better representation of an individual's true BP when compared to their resting office BP,⁶ but, little is known about the underlying pathophysiological contributors of an HRE. Therefore, this research program represented the first comprehensive CV examination of people with an HRE at moderate intensity and broad exploration of haemodynamic and biochemical (haemostatic and metabolic) correlates of an HRE.

Chapter 4 makes an important contribution to the understanding of haemodynamic and haemostatic correlates of an HRE at moderate intensity in people with apparently normal resting office BP. This study demonstrated that a haemostatic marker, von Willebrand factor (vWf),⁷⁹ was significantly increased in people with an HRE and positively associated with moderate intensity exercise systolic BP. Although endothelial dysfunction was not estimated with standard measures in this study (e.g. flow-mediated vasodilation), an increased vWf (also a marker of endothelial dysfunction) may provide some indications that endothelial dysfunction may be present in people with an HRE and may be a contributing factor of an HRE. Indeed, the association between vWf and moderate intensity exercise systolic BP may be a reflection of the relationship between endothelial dysfunction and high exercise BP, due to the altered capacity of the endothelium to appropriately dilate in order to mitigate pressure rises (particularly during exercise) and maintain normal vascular tone. Future studies may direct attention towards establishing the role of endothelial in an HRE at moderate intensity.

Despite being a marker of endothelial dysfunction, vWf is also a haemostatic contributor to blood coagulation, thrombosis and possible CV events, which may possibly explain the link between an increased CV risk and an HRE. Indeed, increased shear pressure (or in the case of an HRE, a greater ambulatory cyclic BP load over time) would have a pronounced effects on vascular breakdown and increased pro-coagulation capacity, leading to the initiation of the coagulation cascade and an increase in thrombin generation. However, in this study population, a vWF was increased in the absence of a significant rise in other haemostatic (pro-coagulant) markers, which included tissue factor, thrombin-antithrombin complex and

fibrinogen. These findings suggest that apparently healthy people with an HRE experience a low grade (rather than exaggerated) activation of the coagulation system. Nevertheless, this finding has potential clinical significance, especially in those who also have other CV risk factors that could possibly act as promoters of blood coagulation.

Interestingly, people with an HRE had a significantly different response in vWf following moderate intensity exercise compared with those with a normal BP response (having lower vWf levels compared to baseline), despite having elevated exercise BP levels. Although a limited number of studies have examined the influence of moderate intensity exercise (in contrast to strenuous exercise) on haemostatic markers, our study is in agreement with the study of Wang et al²⁰¹ who also demonstrated that moderate intensity exercise suppresses platelet activation and coagulation in sedentary men (sedentary behaviour significantly associated with increased CV risk).²²⁸ It appears that moderate intensity exercise in HRE subjects could potentially have beneficial effects that may be more pronounced in those with excessive initial vWf levels and potentially decrease the risk for, at least pro-thrombotic, CV events. However, future studies should investigate these potential beneficial effects of moderate intensity exercise in this population.

This study also included the collection of serum samples for exploring metabolic correlates of an HRE at moderate intensity, as an HRE is also found to be associated with various metabolic CV risk factors including increased total cholesterol and triglyceride levels. Therefore, future steps will include the metabolomics analysis of these serum samples (collected at rest and following the exercise) in people with an HRE and their matched controls to investigate the metabolic correlates of an HRE, as well as their changes induced by moderate intensity exercise. Due to the complexity of the metabolomics analysis, this study will be pursued following the completion of this thesis.

Current beliefs ascribe abnormalities in haemodynamic components, in particular aortic stiffness, to play a significant role in an HRE, as increased aortic stiffness may fail to buffer the rise in BP occurring with increased cardiac output and blood flow during exercise.⁵⁰ However, the results of this study outlined a less crucial role of aortic stiffness in the development of an HRE. Indeed, although aortic stiffness was increased in people with an HRE, an independent relationship with exercise systolic BP at moderate intensity was not observed. The lack of an independent association between arterial stiffness and an HRE may suggest that the endothelial dysfunction may play a key role in an HRE, given that arterial

stiffness is closely regulated by the endothelial function and nitric oxide bioavailability.⁶⁶⁻⁶⁹ Therefore, future studies should explore whether therapeutic intervention targeting endothelial dysfunction and nitric oxide bioavailability may potentially prove to have benefits to vascular function and BP control in an HRE population.

It should be noted, however, that this study included reasonably healthy subjects with an HRE at moderate intensity without an established hypertension at rest, compared to previous studies that included mostly hypertensive individuals.⁵¹ Therefore, the possibility that more advanced arterial stiffness (noticed in people with already established essential hypertension) may have a greater influence on exercise hypertension cannot be excluded. Also, the findings were limited to a small population of people with an HRE, and further verification of these results in a larger sample cohort is needed. Furthermore, the findings of this study also lend some support that physiological stress, such as exercise, may represent a useful tool to reveal underlying CV abnormalities that are not evident at rest. This is evidenced by finding that systemic vascular resistance was increased in people with an HRE only when measured during exercise conditions, but not at rest.

Chapter 5 represents the first study that used the metabolomics analysis, along with haemodynamic measures, in people with an HRE, primarily for developing metabolomics analysis, as well as to explore metabolic actions of spironolactone on exercise BP and aortic stiffness. The results of *Chapter 5* further supported that aortic stiffness may not be as relevant to an HRE as is widely believed. Indeed, spironolactone reduced exercise systolic BP and aortic stiffness by purely BP-dependent effects, and there was a lack of a significant relationship between the reduction in exercise BP and the decrease in aortic stiffness by spironolactone treatment. Another important observation of this study was that the reduction in exercise systolic BP by spironolactone treatment was not independent of the use of statins, angiotensin converting enzyme inhibitors and other CV risk factors, suggesting that other metabolic mechanisms (possibly those involved with the statins or angiotensin converting enzyme inhibitors treatment) may be involved in the development of an HRE. Thus, future studies exploring the metabolic effects of statins and/or angiotensin converting enzyme inhibitors treatment in people with an HRE using metabolomics techniques would represent a significant advancement to the understanding of possible underlying mechanisms associated with an HRE. Nevertheless, this study itself represents an important scientific contribution, as it shows for the first time how metabolomics analysis when combined with extensive

haemodynamic examination (including aortic stiffness and exercise BP measures) could help uncover important mechanistic insights of different conditions (including an HRE) and discovering possible treatment strategies.

Investigations of the metabolic correlates of an HRE at moderate intensity (including peripheral and central) were investigated in *Chapter 6* in people with a high prevalence of an HRE, such as those with type 2 diabetes mellitus (T2DM).¹¹ Using a combination of the metabolomics and haemodynamic analysis, the results suggested that metabolites associated with the disordered carbohydrate metabolism in T2DM, might have a greater impact on central (rather than peripheral) exercise BP and an HRE. Although this study cannot demonstrate causality due to its cross-sectional nature, this closer association with central BP may potentially be due to the formation of advanced glycation end-products preferably in the central elastic arterial walls compared to the more muscular peripheral arteries, causing alterations in elastin and collagen fibres. Also, altered carbohydrate metabolism may also cause an increased production of oxidative reactive species that may lead to further damage of vascular structure and function.

The findings that metabolic impairments underlying T2DM have a greater impact on the preferably central arterial system may be clinically significant, given that central BP indices (including the augmentation index [AIx]) more strongly relate to CV events than peripheral,¹² and that the central BP reflects the pressure to which the vital organs (heart, kidneys and brain) are directly exposed. These findings may further highlight the importance of including central BP measurements (resting and ambulatory) when evaluating someone's BP control, especially in those with T2DM. However, central BP is still not established in routine clinical practise, but given that central BP cannot be truly represented by conventional BP measured at the periphery (brachial artery),³⁶ and 'true' BP treatment may not be guided from peripheral BP measurements, there is a need for standardization of central BP measurement techniques and introducing the measurement of central BP into clinical practise.

The pathophysiology of an HRE seems to be multifactorial, where inflammatory factors may also contribute to its development and associated complications. Indeed, the utilisation of exploratory metabolomics analysis found that the metabolic pattern with the decreased inosine levels (identified as anti-inflammatory metabolite)²²¹ in T2DM participants were inversely and independently associated with peripheral exercise BP. Inflammation may possibly cause a vasodilator/vasoconstrictor imbalance in the vascular endothelial, leading to

endothelial dysfunction and BP deregulation.¹⁶⁸ Further studies are needed to establish the role of inosine and inflammation in people with an HRE, and whether inosine supplementation exerts anti-inflammatory effects with benefits to vascular function and an HRE. Although lipid metabolism may also play a significant role in the development of an HRE, it is worth noting that metabolomics analysis in this study population (people with advanced T2DM) failed to detect any lipid-related metabolites and their associations with an HRE, as many study participants were on lipid-lowering medications as part of their standard BP management. Therefore, future studies including people with newly diagnosed T2DM that are still free from lipid-lowering medications may provide additional information regarding metabolic disorders and their connections to an HRE. However, an additional metabolomics analysis of serum samples from *Chapter 4* should provide more evidence for the role of lipid metabolism in non-diabetic people with an HRE.

Chapter 2 – Review of Literature - Part II represents the first comprehensive review of the literature explaining the clinical application of metabolomics analysis in hypertension research, as well as the basic principles and challenges of metabolomics. This review article will especially help the non-experts in this field to better understand and interpret studies utilising metabolomics techniques. In addition, the research program contained in this thesis demonstrates how metabolomics analysis could be used for exploring new insights into the underlying pathophysiological processes associated with high BP. In the future, methodology regarding the metabolomics analysis developed in these studies (including the normalisation method presented in *Appendix 2*) could be applied to further understand the pathophysiological mechanisms, not only in people with an HRE, but also in other diseases. Also, the findings in *Appendix 1* reporting the clinical significance of measuring BP a few minutes after the recommended five minutes of rest,⁴ may have relevance to an appropriate diagnosis of hypertension and the design of future clinical trials.

Overall, this research further supported evidence that an HRE at moderate intensity, irrespective of normal resting BP, represents a clinically important entity. Vascular abnormalities were present in people with an HRE, reflected by an increased aortic stiffness, but these do not explain an HRE independent of resting BP and other CV risk factors. Importantly, abnormal blood biochemistry including haemostatic irregularities (also reflecting endothelial dysfunction), increased metabolic markers related to carbohydrate metabolism and inflammation in T2DM, explain an HRE independent of resting BP and other

CV risk factors. Taken altogether, this thesis provides novel information, and represents a significant advancement to the understanding of the pathophysiology of an HRE at moderate intensity.

Appendix 1

Waiting a Few Extra Minutes Before Measuring Blood Pressure Has Potentially Important Clinical and Research Ramifications

This thesis section has been previously published:

Nikolic SB, Abhayaratna WP, Leano R, Stowasser M, Sharman JE. Waiting a few extra minutes before measuring blood pressure has potentially important clinical and research ramifications. *Journal of human hypertension*. 2014;28:56-61

In *Appendix 1*, a retrospective analysis of data was conducted to define the appropriate timing (protocol) for resting blood pressure measurements used in all studies that comprise this thesis. Importantly, the findings of this study have also relevance for the appropriate diagnosis of hypertension, as well as the design of research studies in which resting office blood pressure is measured.

A1.1 Abstract

Background. Office blood pressure (BP) is recommended to be measured after five minutes of seated rest, but it may decrease for 10 minutes. This study aimed to determine the change (and its clinical relevance) in brachial and central BP from five to 10 minutes of seated rest.

Methods. Office brachial and central BP (measured after five and 10 minutes), left ventricular (LV) mass index, seven-day home and ambulatory BP were measured in 250 participants with treated hypertension.

Results. Office brachial and central BP were significantly lower at 10 compared with five minutes BP ($p < 0.001$). Seven-day home systolic BP was significantly lower than office systolic BP measured at five minutes ($p < 0.001$), but was similar to office systolic BP at 10 minutes ($p = 0.511$). From five to 10 minutes, the percentage of participants with controlled BP increased and the percentage of participants with high central pulse pressure (PP) decreased ($p < 0.001$). Moreover, brachial and central PP were significantly correlated with LV mass index measured at 10 ($r = 0.171, p = 0.006$ and $r = 0.139, p = 0.027$, respectively), but not at five minutes ($r = 0.115, p = 0.068$ and $r = 0.084, p = 0.185$, respectively).

Conclusion. Blood pressure recorded after 10 minutes is more representative of true BP control. These findings have relevance to appropriate diagnosis of hypertension and design of clinical trials.

A1.2 Introduction

Current guidelines for the management of hypertension recommend that office brachial blood pressure (BP) should be optimally measured after five minutes of seated rest.^{4, 198} However, brachial BP may decrease for up to 10 minutes of seated rest, after which reaching a plateau level.^{185, 186} Furthermore, office brachial BP averaged over 10 minutes seated rest in the absence of an observer has been shown to closely relate to out-of-office BP (daytime ambulatory BP).²²⁹ This is an important finding because out-of-office BP measures (e.g. seven-day home BP and daytime ambulatory BP) have been shown to be stronger predictors of cardiovascular (CV) risk when compared with office BP values.^{230, 231} Consequently, office brachial BP measured after the recommended five minutes rest period may not be a good representative of true BP, and using this as the sole method to assess BP control may result in misclassification or inappropriate management of some individuals.²³² The first aim of this study was to determine the change in seated brachial BP when measured after five and 10 minutes of seated rest. This study also sought to determine the clinical relevance of the change in brachial BP over time by assessing: 1) the change in BP classification; 2) the comparison with out-of-office BP and; 3) associations with BP-related end-organ damage (left ventricular [LV] mass).

Central BP may be significantly different from the brachial BP measured at the same time.³⁹ However, central BP is pathophysiologically more important than brachial BP with respect to CV disease and has a stronger relation to LV mass than brachial BP.²³³ In recent years, large longitudinal studies have indicated that central BP has more power for predicting future CV events and mortality when compared with brachial BP.¹² Furthermore, central BP may also change differently from brachial BP in response to physiological stimuli (e.g. posture, medications and exercise).^{44, 234} To our knowledge, there have been no studies examining how central BP acutely changes over five compared with 10 minutes, and this was an additional aim of this study. As with the change in brachial BP over time, we also sought to determine the clinical relevance of central BP changes after five compared with 10 minutes of seated rest.

A1.3 Methods

A1.3.1 Study population

Data was analysed from baseline examination of 250 participants, aged 18 to 75 years, with treated essential hypertension who were enrolled in the BP GUIDE study (<http://www.anzctr.org.au>; ACTRN12608000041358). See Table A1.1 for participant characteristics. Exclusion criteria included taking more than three antihypertensive medications or office BP > 180/100 mmHg, a history of coronary artery or renal disease, secondary hypertension, aortic valve stenosis or obstructive atherosclerosis at the upper limb (difference of > 20 mmHg in systolic BP between both arms), severe LV hypertrophy (LV mass index, indexed to height^{2.7}, in women $\geq 59\text{g/m}^{2.7}$ and men $\geq 64\text{g/m}^{2.7}$ measured by echocardiography) and pregnant females. The study was approved by the local Human Research Ethics Committees of attending study sites. All participants gave informed consent and all the procedures were performed in accordance with the Declaration of Helsinki (2000).

Table A1.1 Characteristics of study participants

| Variable | Mean \pm SD or % |
|---|--------------------------------------|
| Age (years) | 64 \pm 8 |
| Gender (% male) | 52 |
| Body mass index (kg/m ²) | 29.3 \pm 4.8 |
| Waist-to-hip ratio | 0.94 \pm 0.51 |
| Left ventricular mass index (g/m ^{2.7}) | 32.7 \pm 8.9 |
| Heart rate (bpm) | 68 \pm 10 |
| Diabetes (%) | 7.6 |
| Glucose (mmol/L) | 5.9 \pm 6.1 |
| Total cholesterol (mmol/L) | 5.13 \pm 1.02 |
| Low density lipoprotein (mmol/L) | 3.04 \pm 0.94 |
| High density lipoprotein (mmol/L) | 1.39 \pm 0.47 |
| Triglycerides (mmol/L) | 1.65 \pm 1.11 |
| Medications | |
| Angiotensin converting enzyme inhibitors (%) | 30 |
| Angiotensin receptor inhibitors (%) | 63 |
| Beta blockers (%) | 9 |
| Statins (%) | 30 |
| Diuretics (%) | 36 |

A1.3.2 Protocol

Study participants attended the research clinic at only one occasion in the morning where all measurements were acquired. Participants were asked to avoid exercise on the day of examination, as well as caffeine-containing beverages, heavy meals and smoking at least three hours prior to the visit. All BP measures were recorded by a trained (non-clinician) research technician in a quiet, temperature-controlled room. Brachial BP was recorded as the average of duplicate measures taken one minute apart after five minutes of seated rest, as per recommendations. Duplicate measures of central BP were then recorded and calibrated to the averaged brachial BP obtained previously (approximately five minutes of seated rest). Tonometric measures took approximately two to four minutes. Following this, two measures of brachial BP recorded one minute apart were acquired again, and the average values were used to recalibrate the central BP (approximately 10 minutes of seated rest). After acquiring the BP measures, a blood sample was collected for standard clinical biochemistry and two-dimensional echocardiography measures of LV mass were recorded. At the end of the clinic visit, each participant was fitted with a 24 hour ambulatory BP device. They were also provided with a BP device for seven-day home BP monitoring together with instructions on how to measure this, commencing on the day after the clinical visit.

A1.3.3 Office brachial BP

Upper arm BP was recorded using a validated semi-automated oscillometric device (Omron HEM-907; OMRON Europe B.V. (OMCE), Hoofddorp, The Netherlands).¹⁸³ Blood pressure was measured in accordance with guidelines, using an appropriately sized cuff with the arm supported at heart level.^{4, 198} Participants were seated quietly in a chair with feet flat on the floor and back supported.^{4, 198} Uncontrolled BP was defined as $\geq 140/90$ mmHg as per accepted criteria.¹⁹⁸ Brachial pulse pressure (PP) was calculated as the difference between brachial systolic and diastolic BP.

A1.3.4 Office central BP

Seated central BP was measured by radial applanation tonometry using validated and highly reproducible generalized transfer function (SphygmoCor 8.1; AtCor Medical, Sydney, Australia).^{47, 48} Central BP was calibrated by two methods: calibration 1) with the brachial systolic and diastolic BP; and calibration 2) with mean ($1/3$ PP + diastolic BP) and diastolic BP; and both were used for the analysis. Central PP was calculated as the difference between

central systolic and diastolic BP. The Strong Heart Study ²³⁵ showed that central PP ≥ 50 mmHg independently predicted adverse CV outcomes. Accordingly, for the purpose of this study, we defined high central PP as ≥ 50 mmHg.

A1.3.5 Home BP

Seven-day home BP was self-measured using a validated oscillometric device (UA-767, A&D Mercury, A&D Medical, Thebarton, South Australia).¹⁹⁰ Participants were instructed to take BP measures in a warm and quite room after at least five minutes of seated rest in a chair enabling back support, with feet flat on the ground and the arm with the cuff supported at heart level. Home BP was measured in duplicate in the morning (between 6am and 10am), midday and evening (between 6pm and 10pm). The first of two BP measurements was discarded and the second BP was recorded and used for analysis. Home BP was compared with office BP values to identify individuals with white coat hypertension (increased office and normal home BP) and masked hypertension (normal office and increased home BP). Raised home BP was defined as $\geq 135/85$ mmHg.¹⁹¹

A1.3.6 24 hour ambulatory BP

A validated device (TM-2430, A&D Mercury, A&D Medical, Thebarton, South Australia)¹⁸⁹ was used for ambulatory BP monitoring. Participants were advised to maintain routine daily life activities and the device was set to record BP every 30 minutes during the day (6am to 10pm) and every hour during the night (10pm to 6am).²³ Daytime BP was also compared with office BP values to identify individuals with white coat and masked hypertension. Raised daytime BP was defined as $\geq 135/85$ mmHg.²³

A1.3.7 Echocardiography

LV mass was measured by two-dimensional echocardiography (Vivid 7; GE Medical Systems, Milwaukee, WI), calculated by method of Devereux²³⁶ and indexed to height ^{2.7} (LV mass index).

A1.3.8 Blood biochemistry

A fasted or non-fasted blood sample was drawn to determine total, low-density and high-density lipoprotein cholesterol, glucose and triglycerides. Blood was analysed as per standard hospital pathology laboratory procedures.

A1.3.9 Statistical analysis

Statistical analyses were performed using SPSS for windows software version 17.0 (SPSS Inc., Chicago, Illinois, USA). Comparison of BP variables measured at five and 10 minutes were determined by independent *t*-tests. Between-group categorical variables were assessed by the chi-squared test. Correlations between continuous variables were assessed by Pearson's correlation. Difference in the strengths of associations between central and brachial PP with LV mass index were compared by calculation of Z statistic scores. Multiple regression was used to identify predictors of the change in brachial systolic BP from five to 10 minutes seated rest. A value of $p < 0.05$ was taken as statistically significant.

A1.4 Results

A1.4.1 BP changes over time

There were no significant differences in central BP values when calibrated using brachial systolic and diastolic BP compared with calibration using mean and diastolic BP. As shown in Table A1.2, both brachial and central BPs measured at 10 minutes were significantly lower than the BP values recorded at five minutes. Brachial PP was also significantly lower after 10 minutes compared with five minutes of seated rest. Central PP also declined over time, but this was of borderline significance.

Table A1.2 Blood pressure values measured at five and 10 minutes of seated rest

| BP variable (mmHg) | 5 minutes | 10 minutes | Change | <i>p</i> value |
|-----------------------------|--------------|--------------|------------|----------------|
| Brachial SBP | 131.3 ± 14.0 | 127.1 ± 13.5 | -4.2 ± 6.3 | <0.001 |
| Brachial DBP | 77.1 ± 9.7 | 75.4 ± 9.6 | -1.8 ± 4.2 | 0.041 |
| Brachial PP | 54.2 ± 11.3 | 51.7 ± 11.2 | -2.5 ± 5.8 | 0.015 |
| Central SBP (calibration 1) | 119.1 ± 13.7 | 115.5 ± 13.1 | -3.7 ± 5.4 | 0.002 |
| Central DBP (calibration 1) | 78.1 ± 9.8 | 76.3 ± 9.7 | -1.8 ± 4.1 | 0.035 |
| Central PP (calibration 1) | 41.0 ± 10.7 | 39.2 ± 10.4 | -1.8 ± 4.4 | 0.054 |
| Central SBP (calibration 2) | 118.8 ± 13.1 | 115.2 ± 12.6 | -3.6 ± 5.5 | 0.003 |
| Central PP (calibration 2) | 40.7 ± 10.7 | 38.9 ± 10.7 | -1.7 ± 4.5 | 0.080 |

SBP, systolic blood pressure; DBP, diastolic BP; Calibration 1 – calibration by systolic and diastolic BP; calibration 2 – calibration by mean and diastolic BP

A1.4.2 Clinical relevance of the change in BP

Figure A1.1 shows the change in the distribution of participants with uncontrolled BP after five and 10 minutes of seated rest. There was a significant decrease over time in the number of participants regarded as having uncontrolled BP (from $n = 72$ to $n = 49$; $p < 0.001$). Also, when seven-day home BP was used to identify participants with white coat or masked hypertension, in the time period from five to 10 minutes of seated rest, there were 6% ($n = 15$) fewer participants classified as having white coat hypertension and 4% ($n = 10$) more participants classified as having masked hypertension (Figure A1.2). Furthermore, when we used daytime BP to identify these individuals, there were 4% ($n = 10$) less participants classified as having white coat hypertension and 8% ($n = 19$) more participants classified as having masked hypertension.

The change in the percentage of participants with high central PP after five and 10 minutes of seated rest is presented in Figure A1.3. There was a significant decrease of 6.4% ($n=16$) in the number of participants with high central PP ($p < 0.001$) at 10 minutes compared with five minutes BP classification. When central PP calibrated with mean and diastolic BP was used, a significant decrease of 4.9% ($n = 13$) in the number of participants with high central PP ($p < 0.001$) was noticed.

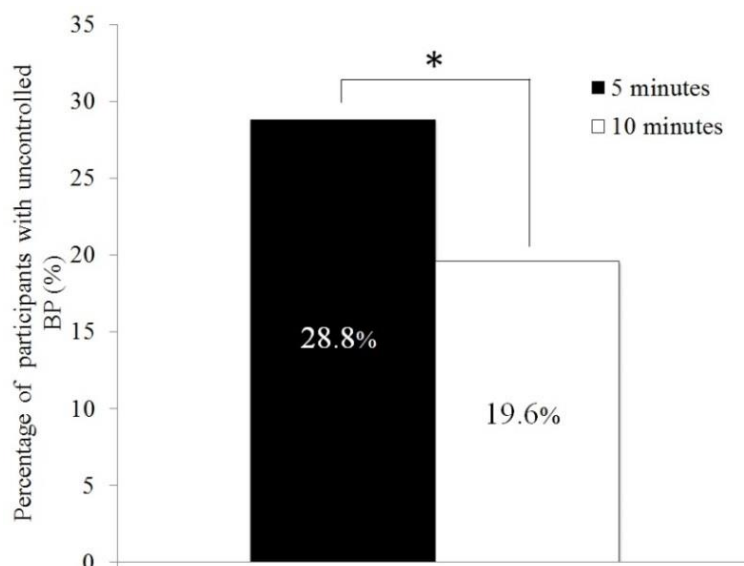


Figure A1.1 Percentage of study participants with uncontrolled blood pressure when measured after five minutes and 10 minutes of seated rest; $*p < 0.001$ and $n = 250$. From five to 10 minutes, there was a significant decrease in the percentage of individuals regarded as having uncontrolled blood pressure.

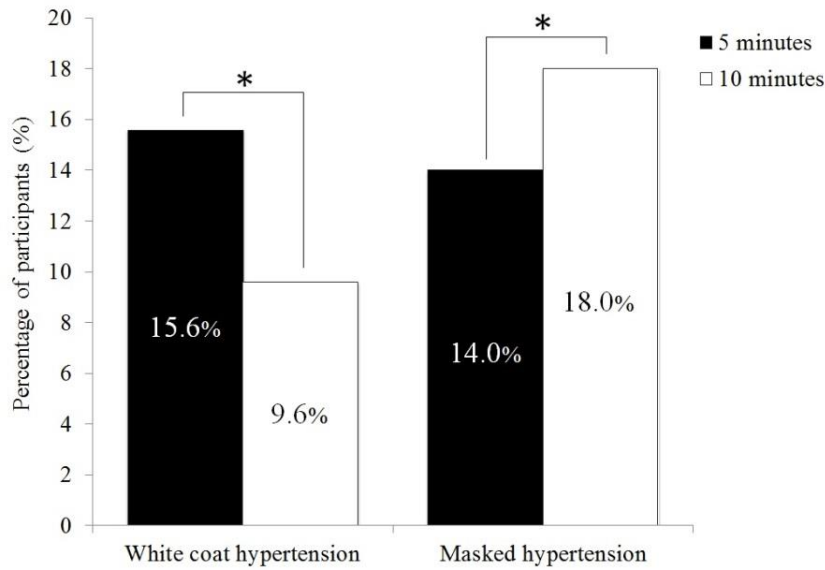


Figure A1.2 Percentage of study participants with white coat hypertension and masked hypertension when home BP was compared with five and 10 minutes brachial BP. From five to 10 minutes, there was a significant decrease (39 vs. 24; $p < 0.001$) in the number of participants who were classified as having white coat hypertension and a significant increase (35 vs. 45; $p < 0.001$) in the number of participants who were identified as having masked hypertension.

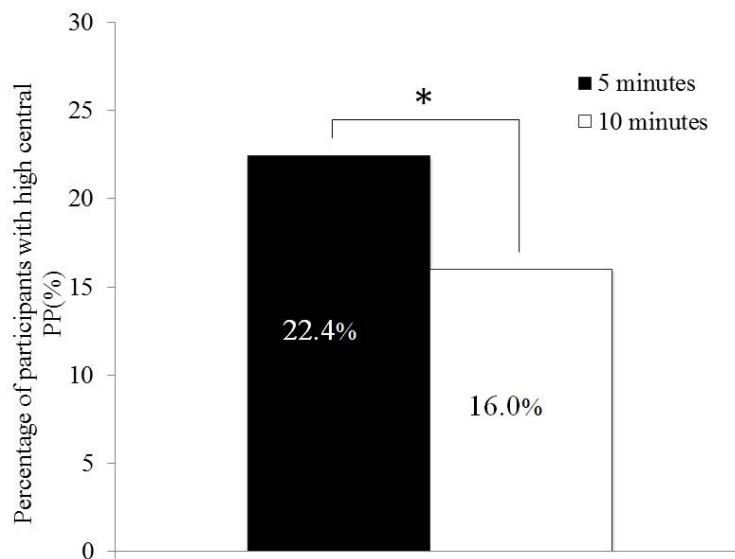


Figure A1.3 Percentage of study participants with high central pulse pressure when measured after five and 10 minutes of seated rest; $*p < 0.001$ and $n = 250$. Note a significant decrease in the percentage of participants with high central PP at 10 minutes compared with five minutes.

Table A1.3 shows the correspondence between out-of-office BPs and the BPs recorded at different times. Brachial systolic BP values measured after five and 10 minutes rest were significantly lower than daytime ambulatory systolic BP. However, the brachial systolic BP at 10 minutes was similar to seven-day home systolic BP. On the other hand, brachial systolic BP measured at five minutes was significantly higher than seven-day home systolic BP. The average number of blood pressure readings taken over the 24 hour period for the study population was 41.7. From these, 1.5 readings (on average) were excluded due to measurement error, leaving 96.4% valid readings.

Table A1.4 shows the correlations between LV mass index and PP values. Brachial PP measured at 10 minutes seated rest was significantly correlated with LV mass index, whereas brachial PP measured at five minutes did not correlate with LV mass index. Similarly, ten minutes central PP (calibrated by both methods) was significantly correlated with LV mass index, whereas there was no significant correlation between five minutes central PP and LV mass index. The correlations between central systolic BP and PP with LV mass index (at both times) were slightly improved when calibration 2 was used and compared with calibration 1 method, but the difference in slopes were not of statistical significance ($p > 0.05$ for all). Similarly, the slope of relationships between central PP and brachial PP (at 10 minutes) with the LV mass index were not significantly different (calibration 1 central PP vs. brachial PP, $Z = 0.520$, $p = 0.607$ and calibration 2 central PP vs. brachial PP, $Z = 0.340$, $p = 0.734$). Neither brachial nor central systolic BPs were correlated with LV mass index at either time points, whereas brachial and central diastolic BPs were significantly correlated only when measured at 10 minutes ($r = -0.173$, $p = 0.006$ and $r = -0.171$, $p = 0.006$), similar to PP. On the other hand, LV mass index was significantly correlated with seven-day home systolic BP ($r = 0.140$, $p = 0.027$), seven-day home PP ($r = 0.145$, $p = 0.022$), mean 24 hour systolic BP ($r = 0.175$, $p = 0.005$) and mean 24 hour PP ($r = 0.259$, $p < 0.001$).

The change in brachial systolic BP from five to 10 minutes was significantly correlated with the use of angiotensin converting enzyme inhibitors (ACEi; $r = 0.212$, $p < 0.001$), brachial systolic BP at five minutes ($r = 0.302$, $p < 0.001$), brachial PP at five minutes ($r = 0.282$, $p < 0.001$), central systolic BP at five minutes ($r = 0.279$, $p < 0.001$) and central PP at five minutes ($r = 0.260$, $p < 0.001$). Due to significant collinearity between BP variables, only the use of ACEi and brachial systolic BP at five minutes were entered into a multiple regression model as independent predictors of the change in brachial systolic BP over time. The use of

ACEi ($\beta = 0.161, p = 0.008$) and brachial systolic BP at five minutes ($\beta = 0.273, p < 0.001$) were significant predictors of the change in brachial BP, but this was not a strong model (Adjusted $R^2 = 0.109, p < 0.001$).

Table A1.3 Daytime ambulatory and home systolic blood pressure and their differences from office blood pressure measured after five and 10 minutes of seated rest

| BP variable | BP (mmHg) | Mean difference from 5 mins BP | <i>p</i> value | Mean difference from 10 mins BP | <i>p</i> value |
|--------------------|----------------------|---|-----------------------|--|-----------------------|
| Daytime SBP | 135.3 ± 11.7 | -3.9 ± 14.1 | < 0.001 | -8.2 ± 13.5 | < 0.001 |
| Home SBP | 127.7 ± 12.3 | 3.7 ± 14.6 | < 0.001 | -0.6 ± 13.7 | 0.511 |

Data are mean ± standard deviation. BP, blood pressure; SBP, systolic blood pressure

Table A1.4 The correlation coefficients of left ventricular mass index with brachial and central BP values at five and 10 minutes of seated rest

| BP variable | 5 minutes | | 10 minutes | |
|-----------------------------|------------------|-----------------------|-------------------|-----------------------|
| | r value | <i>p</i> value | r value | <i>p</i> value |
| Brachial SBP | 0.012 | 0.851 | 0.020 | 0.752 |
| Brachial DBP | -0.118 | 0.061 | -0.172 | 0.006 |
| Brachial PP | 0.115 | 0.068 | 0.171 | 0.006 |
| Central SBP (calibration 1) | 0.017 | 0.789 | 0.016 | 0.803 |
| Central PP (calibration 1) | 0.084 | 0.185 | 0.139 | 0.027 |
| Central SBP (calibration 2) | 0.011 | 0.862 | 0.012 | 0.876 |
| Central PP (calibration 2) | 0.120 | 0.057 | 0.165 | 0.009 |

SBP, systolic blood pressure; DBP, diastolic BP; PP, pulse pressure; calibration 1 – calibration by systolic and diastolic BP; calibration 2 – calibration by mean and diastolic BP

Discussion

Office brachial BP is recommended to be measured after five minutes of seated rest.¹⁹⁸ The principal findings of this study were, firstly, that significant falls in office brachial and central BP were observed in the time period from five to 10 minutes of seated rest. Secondly, this drop in BP over time would have resulted in significant reclassification of BP control, and this was evident for both brachial and central BP values. Thirdly, we observed similar values for out-of-office systolic BP and the brachial systolic BP recorded after 10 minutes, but not after the recommended five minutes of seated rest. Finally, evidence of end-organ damage was correlated with the BP (brachial and central) values recorded after 10 minutes, but not five minutes of seated rest. Thus, waiting a few minutes longer than the recommended five minutes before measuring BP appears to provide a better representation of true BP. These findings have relevance to appropriate diagnosis of hypertension as well as the design of clinical trials in which brachial and central BP are measured.

Office brachial BP changes over time. Numerous studies have reported a time-dependent decrease in seated brachial BP.^{185, 186, 229, 237-240} The exact mechanisms of this BP drop are yet to be elucidated, but may be explained by a gradual decrease in systemic vascular resistance caused by hemodynamic modifications to the seated position.¹⁸⁵ Relaxation of the patient and sympathetic withdrawal over time may also be a potential cause of BP reduction.¹⁸⁵ The magnitude of the brachial BP change over time has been consistently described in the literature. A large population study in 5999 participants reported a significant decrease (10.3 mmHg in men and 10.4 mmHg in women) in brachial systolic BP (but no change in diastolic BP) over 25 minutes of seated rest.²³⁷ This change resulted in a decrease in the prevalence of isolated systolic hypertension. Sala et al¹⁸⁵ reported a similar decrease in systolic BP (10.7 ± 1.0 mmHg) and also a decrease in diastolic BP (3.4 ± 0.6 mmHg) in the period from two to 16 minutes of seated rest. These investigators also estimated that the bulk of the fall in systolic BP (75.3%) and diastolic BP (71.7%) occurred in the initial 10 minutes of seated rest. This finding has been repeated in a recent study by van der Wel et al.,¹⁸⁶ who also showed that the plateau level of the systolic BP decline occurred after about 10 minutes of seated rest. Our findings are in agreement with these previous studies but have also focused on the potential clinical impact of the change in BP from five to 10 minutes of seated rest. Taken together, these studies suggest that five minutes is not a long enough period to wait before office BP measurements are acquired.

Clinical implications of BP changes over time. The change in brachial BP over time has potential clinical relevance as it may affect the evaluation and diagnosis of hypertension. Our study showed that if only the BP after five minutes rest was considered, more participants would be classified as having uncontrolled BP compared with the BP at 10 minutes. Indeed, 9.2% (n = 23) were reclassified from uncontrolled office BP to controlled office BP when the BP at 10 minutes was taken into account. In the clinical environment, this differential diagnosis could conceivably result in different (and possibly inappropriate) management of some individuals. A possible reason contributing to this change in classification could be related to a ‘white coat effect’²⁰ given that this was 6% more likely if only the BP at five minutes was considered. Our finding on the reclassification regarding prevalence of masked hypertension is also of potential clinical relevance.²⁴¹ An argument may be raised that it would be better to overestimate BP in these patients, as this may increase the chances for more appropriate diagnosis and treatment. Even though only a small number of participants were reclassified to having masked hypertension according to the 10 minute BP level (4 to 8% depending on whether home or 24 hour ambulatory BP was used to determine out-of-office BP), this finding provides additional support for routinely undertaking out-of-office BP to diagnose hypertension,²³¹ which may be particularly relevant in patients with type 2 diabetes.²²

Out-of-office BP recordings (home and daytime ambulatory BP) have been shown to be stronger predictors of CV risk than office brachial BP.^{230, 231} The lower prognostic value of office BP compared with out-of-office BP readings could potentially be attributed to inadequate time allowed before taking office BP recordings. Importantly, Myers et al²²⁹ have showed that office brachial BP, recorded by an automated device and averaged over 10 minutes with the patient resting alone in a clinic room, was more similar with ambulatory daytime BP compared with standard five minutes BP measurements taken by either physician or technician.²²⁹ Although the 10 minutes automated office BP was more similar to ambulatory daytime BP, the automated BP was significantly lower even after correction for multiple comparisons.²²⁹ While the additional amount of time spent waiting may have contributed to the lower automated office BP readings at 10 minutes, the absence of the observer in itself may independently contribute to lower automated office BP readings. Our study is in agreement with the study of Myers et al,²²⁹ as we have also found that our 10 minutes office BP was lower than daytime ambulatory BP. Although the difference between

these two BP measurements is greater in our study, this could be explained by different settings in which BP measurements were taken (patient's physician referred ambulatory BP unit versus research setting) and different study population (hypertensive participants with nearly half untreated versus all treated hypertensive participants) and this may have contributed to our lower office BP values. Furthermore, we have also showed that brachial BP measured at 10 minutes (but not five minutes) were more similar to the average of seven-day resting home BP readings than BP measurements taken during daytime ambulatory condition. Thus, waiting for extra time, taking more readings and bringing the office BP closer to out-of-office BP values may potentially improve the utility of office BP measurements.²²⁹

Central BP indices have been shown to independently correlate with severe CV events and mortality.^{12, 233} While yet to be incorporated into general medicine, the consideration of central BP may improve identification and management of patients with hypertension or increased CV risk.^{12, 38, 39, 242} Indeed, two individuals with similar brachial systolic BP may have significantly different central systolic BP and, accordingly, may be classified into different risk groups related to BP.³⁹ Furthermore, different BP-lowering drugs can differentially affect central BP compared with brachial BP and, looking to the future, this should be taken into account when assessing and deciding appropriate treatment options.^{234, 243, 244} In our study, we have showed for the first time that central BP decreased over time, similarly to brachial BP. Our data indicates the potential for overestimating risk related to BP when based on five minutes central PP readings, since 6.4% less individuals would have been identified with high central PP on the basis of the 10 minute readings.²³⁵

Limitations. Our study represents a cross-sectional analysis of data from patients with treated hypertension, and the findings may not be generalizable to other populations. Different oscillometric devices were used to measure office and home BP. Although both devices have been validated,^{183, 190} the use of separate machines might have affected study results. Central BP was measured by radial applanation tonometry using a generalized transfer function. Although this method has been validated and shown to be highly reproducible,^{47, 48} calibration of the waveform using brachial BP may have resulted in error of central BP estimation.²⁴⁵ Furthermore, radial pressure waveforms were acquired only at one time point (after the BP measurements at five minutes) and the BP recorded at approximately 10 minutes was used to recalibrate the radial pressure waveforms. Since another tonometry

reading was not acquired after 10 minutes, we may expect an under-estimation of the change in central BP from five to 10 minutes of seated rest, but this need to be tested in another study. In contrast to some previous studies,²⁴⁶ the home BP values in our study were lower than ambulatory BP measurements. This might be explained by the protocol used for home BP measurements, whereby the first BP reading was discarded from each duplicate reading. This is not entirely in keeping with European guidelines;¹⁹¹ however, the reason for this approach was based on the evidence that the first reading (morning and evening) has been shown to be persistently higher than any subsequent BP measurement, and the average of later measurements is a stronger correlate of ambulatory BP.²³⁸

In conclusion, our study showed that both brachial and central BP decreased significantly when measured a few minutes after the recommended five minutes of seated rest in individuals treated for hypertension. This change in BP over time may have significant implications when assessing the BP-related risk and managing patients with hypertension. Given the increasing body of evidence indicating the superiority of out-of-office BP values in predicting CV risk and total mortality,^{230, 231} any advances that enable measurement of an office BP value that is closer to out-of-office BPs would be a valuable advancement in the clinical assessment of BP control. Although studies will be required to confirm, our finding that 10 minutes office BP was similar to seven-day home BP measurements may suggest that office BP measured after 10 minutes may provide better prognostic information about CV risk than the conventional five minutes wait period before measuring BP. In any case, this current study tends to support the work by Myers et al²²⁹ that multiple, automated office BP measurements with the patient sitting alone in a quiet examining room could be the most appropriate method to measure office BP.

Appendix 2

Metabolomics Data Normalisation with EigenMS

At the time of thesis submission, this section is under resubmission in *PLoS ONE*

Yuliya V Karpievitch¹, Sonja B Nikolic², Richard Wilson³, James E Sharman³ and Lindsay M Edwards⁴

Appendix 2 represents a separate study conducted in the same study population as in *Chapter 6* to investigate a novel method for the normalisation of metabolomics data, which is a crucial step in metabolomics analysis. As this method proved useful, this normalisation method was used for metabolomics data normalisation in *Chapter 6*, with the details explained in this chapter. The primary analysis was conducted by Dr Karpievitch, but a significant input in regard to haemodynamic measures, study conception/design and data collection was provided by the author of this thesis.

A2.1 Abstract

Background. Liquid chromatography mass spectrometry (LC-MS) has become one of the analytical platforms of choice for metabolomics studies. However, LC-MS metabolomics data can suffer from the effects of various systematic biases. These include batch effects, day-to-day variations in instrument performance, signal intensity loss due to time-dependent effects of the LC column performance, accumulation of the contaminants in the MS ion source and MS sensitivity among others. We therefore propose the use of a singular value decomposition-based normalisation method (EigenMS) for metabolomics data.

Methods. We analyzed a clinical human dataset where LC-MS serum metabolomics data and physiological measurements were collected from forty healthy subjects and forty with overt type 2 diabetes and applied EigenMS to detect and correct for any systematic bias. EigenMS works in several stages. First, it preserves the treatment group differences in the metabolomics data by estimating treatment effects with an ANOVA model (multiple fixed effects can be estimated and preserved). Singular value decomposition of the residuals matrix then determines the bias trends in the data. The number of bias trends is estimated via a permutation test and the effects of the bias trends are eliminated.

Results. EigenMS removed bias of unknown complexity from the LC-MS metabolomics data, allowing for increased sensitivity in differential analysis. Moreover, normalised samples better correlated with both other normalised samples and corresponding physiological data.

Conclusion. We thus advocate the use of singular value decomposition-based normalisation for metabolomics data.

A2.2 Introduction

Along with nuclear magnetic resonance, liquid chromatography coupled to mass spectrometry (LC-MS) has become one of the most common analytical platforms for studying cell, tissue or body fluid metabolomes.²⁴⁷⁻²⁵³ Advantages of the method include high sensitivity and the ability to discriminate thousands of features in a single experiment (particularly when using a high mass resolution instrument such as the ThermoFisher Orbitrap). Yet, as with any high-throughput technology, systematic biases are often observed in LC-MS metabolomics data.²⁵⁴ As the number of samples in the dataset increases so does the possibility of a time-dependent variation in the resulting metabolite data. Time-dependent trends in LC-MS metabolomics datasets typically result from analyte retention time drift due to changes in LC column performance or variations in signal intensity caused by fluctuations in MS sensitivity. While these issues can be addressed in part by careful experimental design and the use of quality control samples, there remains a need for robust post-acquisition data normalisation. Normalisation methods need to be flexible enough to capture biases of arbitrary complexity, while avoiding overfitting that would invalidate downstream statistical inference. Careful normalisation of metabolite peak intensities enables greater accuracy in quantitative comparisons between disease groups as well as better correlation of metabolite signals to any physiological or phenotypic data collected in tandem. We report here the application of a singular value decomposition-based method (SVD), called EigenMS, to remove systematic biases from metabolomics data in the presence of missing observations.²⁵⁵ This normalisation method, previously shown to be effective in normalising LC-MS proteomics data,²⁵⁵ improved downstream differential analysis and increased correlation of the metabolite peak intensities with corresponding physiological measurements of what we call clinical biochemistry.

A2.3 Methods

To demonstrate the utility of our approach, we used a recently-acquired metabolomic dataset examining the serum of patients with type 2 diabetes ($n = 40$) and control subjects without diabetes ($n = 39$). Biological sample preparation and data acquisition followed the same protocol as reported in Nikolic et al.¹⁰ LC-MS data were acquired using an Orbitrap XL mass spectrometer (ThermoFisher Scientific) with XCalibur v2 software. Chromatographic separation was carried out using C18 reverse-phase HPLC.

We generated a pooled quality control (QC) sample in order to monitor LC and MS performance across sample runs by combining small aliquots (10 uL) of every sample in the study, as recommended by Sangster et al.²⁵⁶ This pooled QC sample was then used throughout the experiment as a process control as shown in. Because all QC injections originated from the same mixture and thus should be chemically identical, QC samples allow one to detect variations in the observed intensities that may affect downstream statistical analyses.

We monitored system performance using blocks of four experimental samples flanked by the QC samples between MS source and inlet cleaning. Thus our basic experimental running order included a cleanup of the ion spray cone and exterior surface of the ion transfer capillary with 50:50 methanol and water mixture between each block. At the end of each day of operation we used a more extensive cleaning protocol using 50:50 methanol and water to flush the sample transfer tube and atmospheric pressure ionization probe, according to the guidelines for daily operation of LTQ XL specified by the manufacturer. Mass spectral peak deconvolution and retention time correction were carried out in R using XCMS. The parameters for deconvolution were: `method='centWave'`, `ppm=3`, `peakwidth=5-30`, `snthresh=6`, `mzdiff=0.01`; and for RT correction: `method="obiwarp"` and `profStep=0.1`, yielding ~7000 peaks.

EigenMS uses a combination of ANOVA and singular value decomposition to capture and remove biases from LC-MS metabolomic peak intensity measurements while preserving the variation of interest. ANOVA is used first to capture and preserve the variation attributable to the treatment effect(s) under study. SVD is then applied to a matrix of residuals to find any systematic trends attributable to bias. The number of bias trends is determined by a permutation test and the effects of the bias trends are then removed from the data. EigenMS is based on the surrogate variable analysis of Leek and Storey,²⁵⁷ with modifications including analysis of data with missing values that are typical in LC-MS experiments and an approach to prevent overfitting such that EigenMS could be integrated into any existing omics analysis pipeline. EigenMS is available as a stand-alone set of two functions implemented in R.

A2.4 Results and discussion

A characteristic of biofluid metabolite analysis via LC-MS is progressive signal intensity loss due to the accumulation of contaminants within the MS ion source, sample transfer lines and the heated ion transfer capillary.²⁵⁸ We designed our experiment based on the guidelines outlined in Lai et al²⁵⁸ as well as based on in-house experimentation to establish the number of samples that could be run without large signal intensity loss on our instrumentation. We conditioned the LC column by running several QC samples as suggested by Want *et al* and others prior to running any experimental samples to avoid high signal variation during the first few runs.^{254, 256}

Within each block different treatment groups were matched and run order randomized. However, even using frequent cleaning, some signal loss was observed as is evident from the declining abundance profile within each day (Figure A2.1). These data were acquired on the instrument operating in positive ion mode. We did not observe similar intensity loss throughout the data when the instrument was operating in the negative ion mode. However, we still observed variation that we could not easily explain. While careful experimental design (within-block treatment group matching) alleviated the influence of some of the biases and signal intensity loss that we and others have observed, we suggest that normalisation is needed to correct for any remaining intensity loss as well as any other known and unknown systematic biases. Further, if one wishes to identify relationships between LC-MS metabolomics data and other variables, normalisation becomes essential.

Sample intensity loss makes comparisons between experimental groups more difficult, it also leads to an increased number of missing values in subsequent samples. Thus careful experimental design and sample run order randomization are required to minimize the introduction of systematic biases and any possible confounding of the results. Our experience leads us to strongly endorse others' recommendation of QC samples to monitor the performance of an LC-MS instrument.

We normalised the data with EigenMS. EigenMS identified 12 systematic bias trends and eliminated their effects from the data. Figure A2.2 shows boxplots of the intensities for the disease and control groups before (top panel) and after (bottom panel) normalisation for the same date as in Figure A2.1. Samples in Figure A2.2 are grouped by disease group (red vs. green) and within each group they appear in the run order on the instrument, such that the

first sample in red was run right next to the first sample in green and so on. Even with regular cleaning of the inlet we encountered some signal loss as evident from the downward trend in the means (middle bars) of the boxplots. Figure A2.2 bottom panel shows that normalisation successfully adjusted for the signal loss and any other systematic biases and placed the means of each sample almost on a straight line.

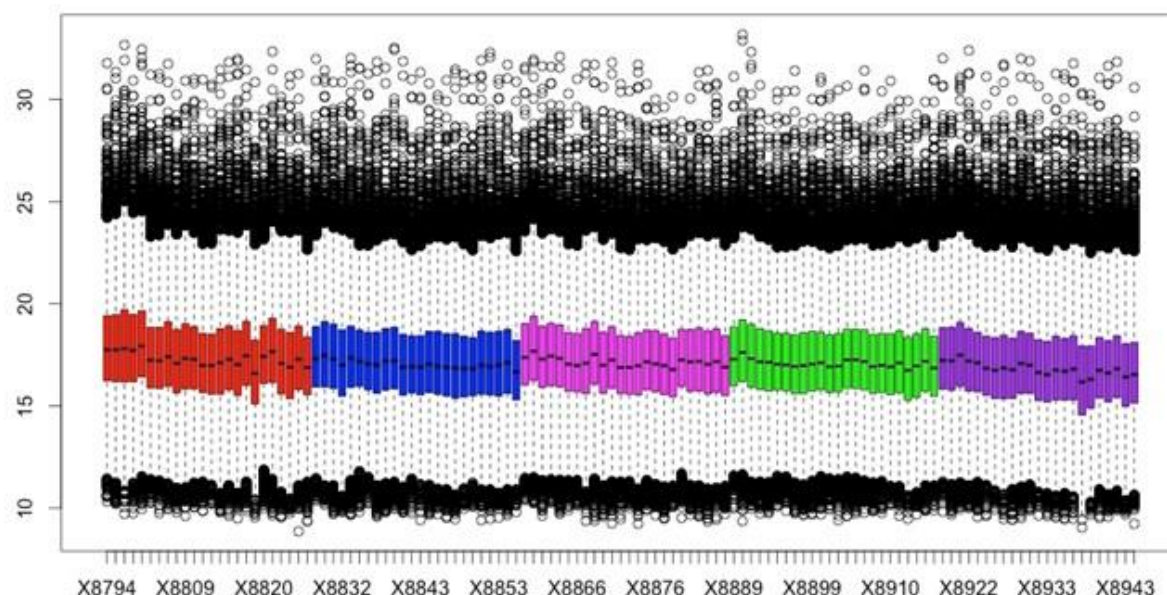


Figure A2.1. Boxplots of sample intensities.

Boxplots of sample intensities in run order on the instrument for positive ion mode. Each box represents a sample. Each of five days is presented in different color.

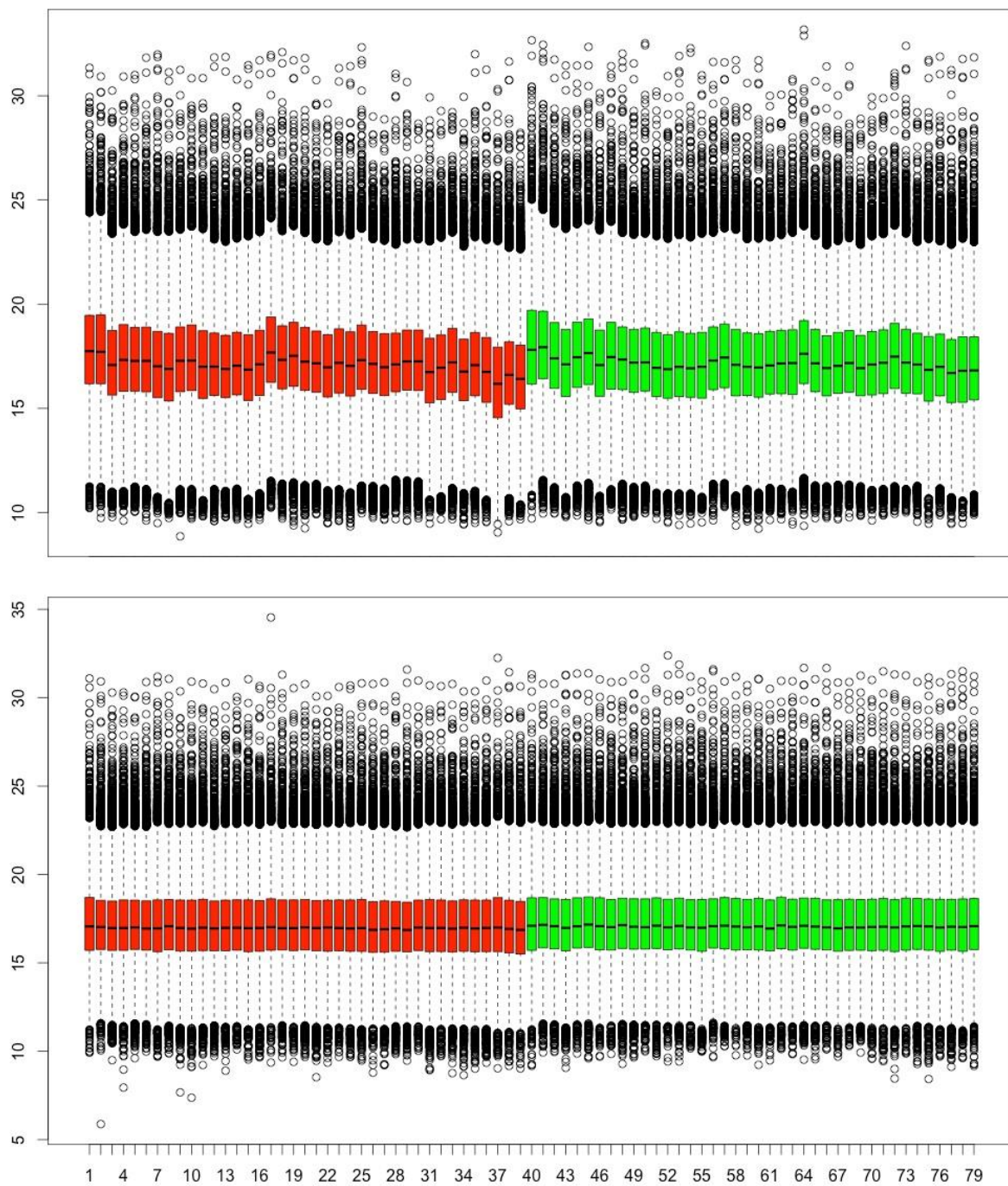


Figure A2.2 Boxplots of the raw and normalised intensities

Boxplots of the intensities before (top panel) and after (bottom panel) normalisation. Each box represents a sample. Samples are grouped by disease group (red vs. green) and are in chronological run order on the instrument within each group.

Figure A2.3 shows SVD trends in raw (left 3 panels) and normalised (right 3 panels) diabetes data. Samples are ordered by group and appear in chronological run order within each group in the same way as Figure A2.2. Trends are ordered from top to bottom by the decreasing amount of variation explained by each trend. Due to the nature of SVD, each trend is orthogonal to every other. Notably, 20 percent of the variation in the raw data is attributable to the signal loss that appears as the top trend. Note that SVD trends can be rotated around the x-axis, thus the top trend in the raw data represents signal intensity loss. The top trend also shows a jump in each group which occurred between days 3 and 4 of the experiment. All the processing was done following the same protocol, but we still observed a variation due to the day effect where samples run on days 4 and 5 were affected differently than samples run on days 1-3. The rest of the trends in the raw data are not easily interpretable and are attributed to unknown systematic biases. Figure A2.3 (right panel) shows the normalised data. The top trend is representative of the differences between the disease and control groups. Only 3.7% of variation is attributable to the differences between the disease groups, but nonetheless this trend is a major trend in the normalised data as compared to the signal intensity loss trend in the raw data. Most importantly we were able to report 2578 discriminatory metabolite peaks in the normalised ($p < 0.05$) data as compared to only 1840 metabolite signals in the raw data (compared using an unpaired t -test; $p < 0.05$ with after Benjamini-Hochberg multiple testing adjustment).²⁵⁹

We saw an improvement in correlations of normalised metabolite intensities to the physiology variables we measured for each subject. We selected 1100 peaks that we found to be significantly different between two groups with p -value below 0.001 in normalised data. We correlated these 1100 peaks to clinical measurements we obtained for each subject, such as: blood glucose level, glycated haemoglobin (HbA_{1c}), exercise central augmentation pressure normalised to heart rate of 75, and total cholesterol. The first four of these are usually elevated in diabetics while cholesterol is typically higher in controls. If EigenMS works well we expect to see increased correlation between normalised metabolites and physiological variables, providing an easy test of EigenMS' performance.

Figure A2.4 shows correlations of raw (x-axis) and normalised (y-axis) metabolite intensities to glucose (top left), HbA_{1c} (top right), exercise central augmentation pressure (bottom left) and cholesterol (bottom right). The blue lines show correlations of zero. The red lines indicate no difference between correlations obtained from normalised and raw data. The

scatter plots show that we obtained higher correlations of normalised metabolite peak intensities to the variables measured in the laboratory. For glucose, for example, correlations for most of the positively correlated peaks increased as is evident from the dots falling above the diagonal line. For negatively correlated metabolites most of the correlations fall near the diagonal line still producing more scatter below the diagonal line indicating increased correlations. Similar patterns are observed for the rest of the physiological variables.

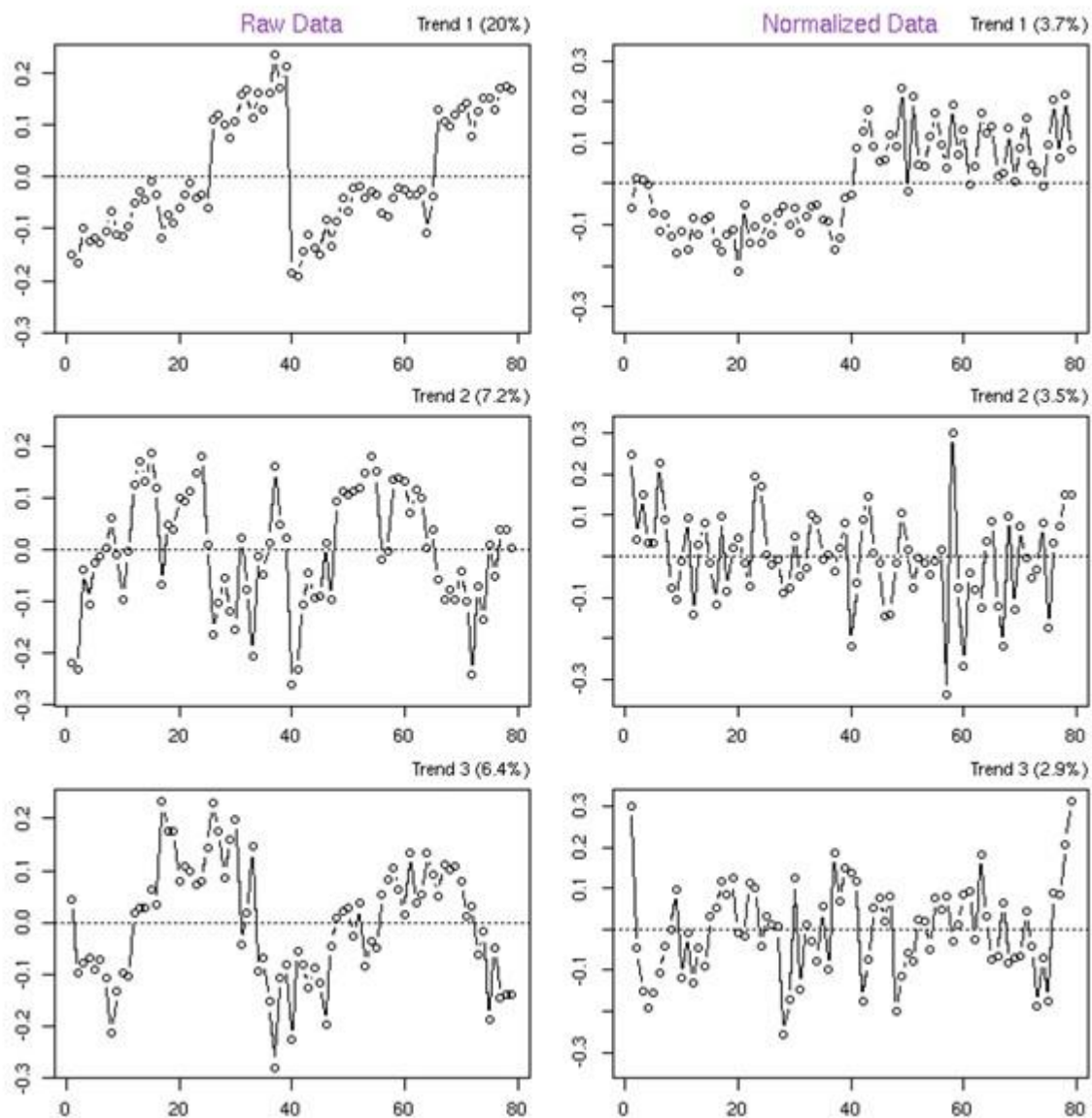


Figure A2.3 SVD trends in raw and normalised data

SVD trends in raw (left panel) and normalised (right panel) clinical study data. Percentage at the top of each subplot shows the percent of the variation in the data explained by each trend.

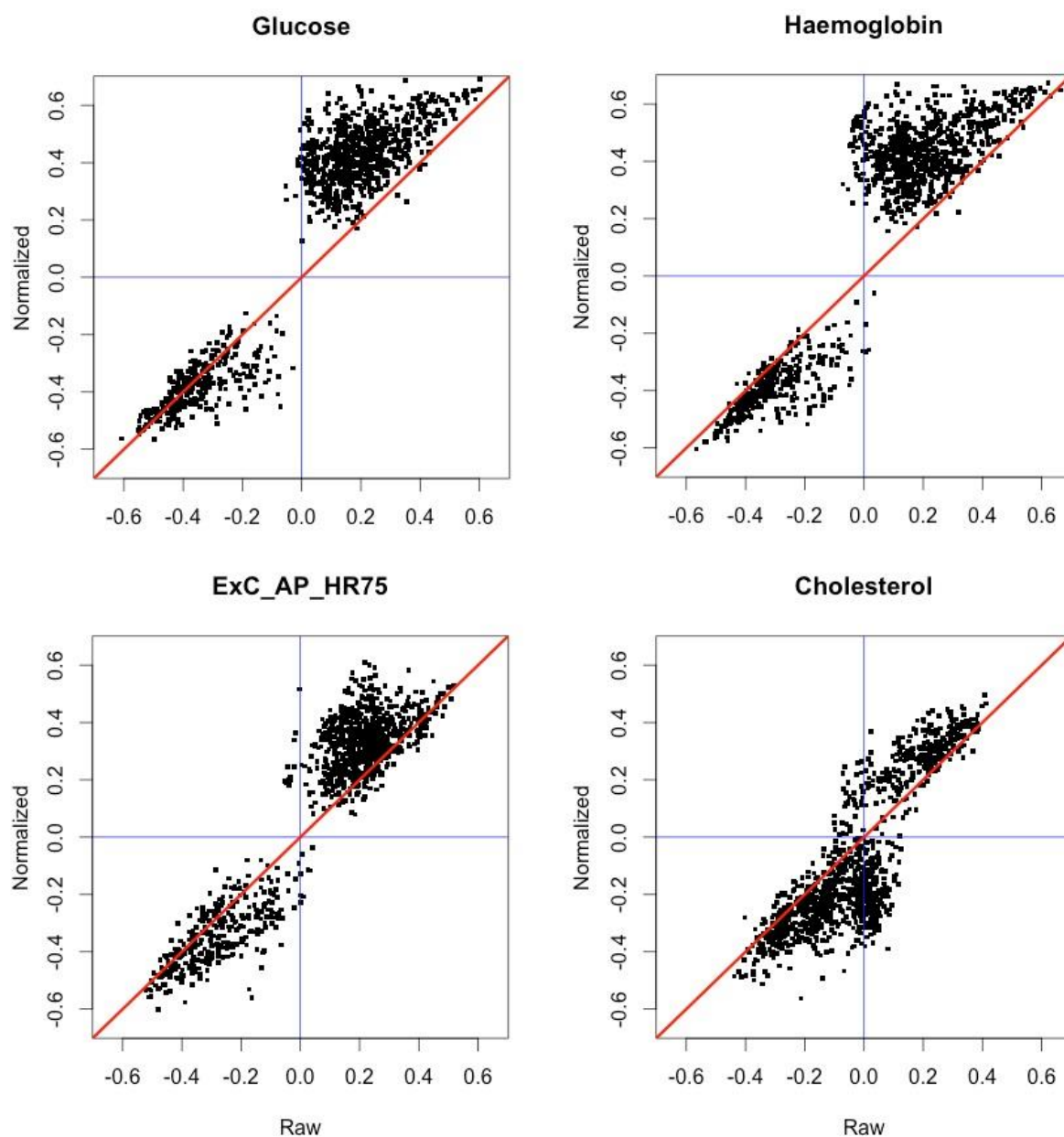


Figure A2.4 Correlations to physiology data

Correlations of raw (x-axis) and normalised (y-axis) metabolite intensities to physiology data. Blue lines represent correlation of zero. Red diagonal line shows the line on which all dots would fall if there was not difference between correlations of raw and normalised data. Observed counter-clockwise shift of points indicates better correlation with physiological data.

A2.5 Conclusions

Normalisation is an important step in MS data analysis, but it is complicated by the high complexity of biases. EigenMS has been shown to remove biases of arbitrary complexity from proteomics data.²⁵⁵ Here we show that it works equally well for metabolomics data. By extension, we believe that the method should work equally well for any omics data where the variation of interest can be preserved via the fixed effects in an ANOVA model and the matrix of residuals analyzed for the presence of bias trends. The ability of EigenMS to capture complex biases and eliminate them preserves the validity of any downstream statistical analysis. The software is implemented in R and is freely available from the authors' website and SourceForge.

Appendix 3

Additional analysis for *Chapter 5*

Appendix 3 provides information and data analysis that was not included in the final published version of the study contained in *Chapter 5*. Data analysis involves the exploration of the effects of the aldosterone antagonist spironolactone on exercise blood pressure in people with a hypertensive response to exercise.

A3.1 Background

The exact mechanisms of the effects of spironolactone on exercise blood pressure (BP) in people with a hypertensive response to exercise (HRE) are unclear, but there are two possible pathways: a) by a direct BP-dependent effect of spironolactone on exercise BP or b) by BP-independent effects of spironolactone to reduce aortic stiffness (widely proposed contributor of an HRE),⁵⁸⁻⁶⁰ and consequently decrease the exercise BP. However, the underlying mechanisms are unknown, and this was the additional aim of the study presented in *Chapter 5* of this thesis.

A3.2 Methods

Exercise BP. Brachial exercise BP was measured every 1-2 minutes during the treadmill exercise stress testing using electrocardiographic monitoring (CASE 14; GE Medical Systems). Maximal exercise BP was recorded at the moment of maximal exhaustion.

A3.3 Results

A3.3.1 Exercise BP variables

There were no statistically significant differences in exercise BP variables between placebo and spironolactone groups at either baseline or after three months of treatment (Table A3.1). However, the group that received spironolactone had a significant reduction in exercise systolic BP compared to the group that receive placebo.

Table A3.1 Blood pressure variables in placebo compared to spironolactone group at baseline and at follow-up (after three months of treatment)

| BP variable (mmHg) | Placebo (n = 43) | Spironolactone (n = 49) | <i>p</i> value |
|---------------------------------|---------------------|----------------------------|----------------|
| Baseline exercise systolic BP | 216 ± 17 | 222 ± 17 | 0.113 |
| Baseline exercise diastolic BP | 94 ± 12 | 93 ± 13 | 0.589 |
| Follow-up exercise diastolic BP | 215 ± 17 | 216 ± 17 | 0.805 |
| Follow-up exercise diastolic BP | 91 ± 13 | 91 ± 13 | 0.965 |
| Δ systolic BP | -1 ± 11 | -6 ± 11 | 0.029 |
| Δ diastolic BP | -3 ± 8 | -1 ± 12 | 0.522 |

BP, blood pressure; Δ, change

A3.3.2 Univariable correlates of the reduction in exercise BP after three months of treatment

Changes in exercise BP were correlated with the post-spirolactone signal intensity for each of the metabolomic features of interest. There were no significant correlations between the change in exercise systolic BP and any metabolic features, including canrenoate and canrenone ($p > 0.05$ for all). Also, there were no significant associations between the reduction in exercise systolic BP and the decrease in aortic stiffness after three months of treatment with spironolactone. However, the change in exercise systolic BP was significantly associated with the reduction in 24 hour systolic BP (Figure A3.1).

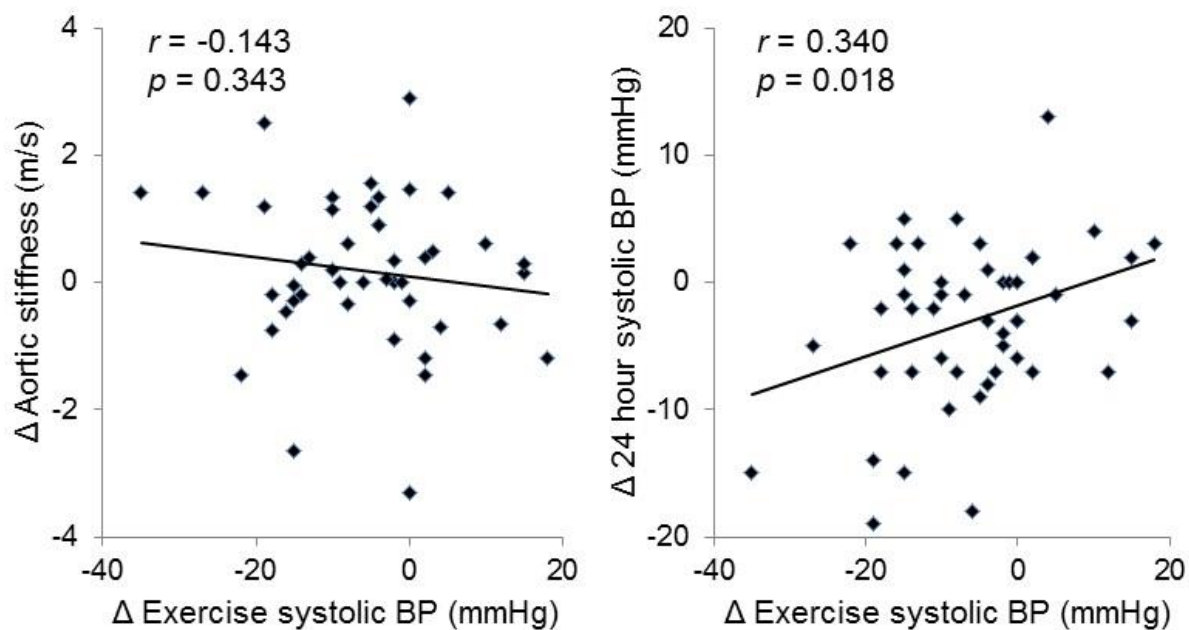


Figure A3.1 Correlations with the change in exercise systolic BP; LEFT: the change in aortic stiffness; RIGHT: the change in 24 hour systolic BP, both after three months of spironolactone treatment ($n = 49$).

A3.3.3 Multiple regression analysis

A multiple regression model for predictors of the change in exercise systolic BP was constructed, with the age, sex, body mass index, the use of statins and angiotensin converting enzyme inhibitors, canrenoate and the change in 24 hour systolic BP entered as independent variables. There were no significant independent predictors of the change in exercise systolic BP (Adjusted $R^2 = 0.139$, $p = 0.093$).

A3.4 Conclusion

Three months of spironolactone treatment reduced the exercise systolic BP most likely due to a BP-dependent effect on the reduction in ambulatory 24 hour BP; however, this reduction in exercise systolic BP was not independent of other cardiovascular risk factors and the use of statins and angiotensin converting enzyme inhibitors. This may suggest that other metabolic mechanisms (involved with the statins or angiotensin converting enzyme inhibitors treatment) may be contributing to an HRE, and future studies should explore the metabolic effects of other medications on exercise BP in more details. Importantly, the reduction of exercise systolic BP was not associated with the decrease in aortic stiffness, giving support to aortic stiffness not being as relevant to exercise hypertension as is widely believed.

References

1. Psaty BM, Furberg CD, Kuller LH, Cushman M, Savage PJ, Levine D, O'Leary DH, Bryan RN, Anderson M, Lumley T. Association between blood pressure level and the risk of myocardial infarction, stroke, and total mortality: The cardiovascular health study. *Arch Intern Med*. 2001;161:1183-1192
2. Lewington S, Clarke R, Qizilbash N, Peto R, Collins R. Age-specific relevance of usual blood pressure to vascular mortality: A meta-analysis of individual data for one million adults in 61 prospective studies. *Lancet*. 2002;360:1903-1913
3. Lim SS, Vos T, Flaxman AD, Danaei G, Shibuya K, Adair-Rohani H, Amann M, et al. A comparative risk assessment of burden of disease and injury attributable to 67 risk factors and risk factor clusters in 21 regions, 1990-2010: A systematic analysis for the global burden of disease study 2010. *Lancet*. 2012;380:2224-2260
4. Pickering TG, Hall JE, Appel LJ, Falkner BE, Graves J, Hill MN, Jones DW, Kurtz T, Sheps SG, Roccella EJ. Recommendations for blood pressure measurement in humans and experimental animals: Part 1: Blood pressure measurement in humans: A statement for professionals from the subcommittee of professional and public education of the american heart association council on high blood pressure research. *Circulation*. 2005;111:697-716
5. Schultz MG, Otahal P, Cleland VJ, Blizzard L, Marwick TH, Sharman JE. Exercise-induced hypertension, cardiovascular events, and mortality in patients undergoing exercise stress testing: A systematic review and meta-analysis. *Am J Hypertens*. 2013;26:357-366
6. Schultz MG, Hare JL, Marwick TH, Stowasser M, Sharman JE. Masked hypertension is "unmasked" by low-intensity exercise blood pressure. *Blood pressure*. 2011;20:284-289
7. Nikolic SB, Sharman JE, Adams MJ, Edwards LM. Metabolomics in hypertension. *J Hypertens*. 2014;32:1159-1169
8. Blann AD, Naqvi T, Waite M, McCollum CN. Von willebrand factor and endothelial damage in essential hypertension. *Journal of human hypertension*. 1993;7:107-111

9. Hare JL, Sharman JE, Leano R, Jenkins C, Wright L, Marwick TH. Impact of spironolactone on vascular, myocardial, and functional parameters in untreated patients with a hypertensive response to exercise. *American Journal of Hypertension*. 2013;[Epub ahead of print]
10. Nikolic SB, Wilson R, Hare JL, Adams MJ, Edwards LM, Sharman JE. Spironolactone reduces aortic stiffness via blood pressure-dependent effects of canrenoate. *Metabolomics*. 2014;10:105-113
11. Scott JA, Coombes JS, Prins JB, Leano RL, Marwick TH, Sharman JE. Patients with type 2 diabetes have exaggerated brachial and central exercise blood pressure: Relation to left ventricular relative wall thickness. *Am J Hypertens*. 2008;21:715-721
12. Vlachopoulos C, Aznaouridis K, O'Rourke MF, Safar ME, Baou K, Stefanadis C. Prediction of cardiovascular events and all-cause mortality with central haemodynamics: A systematic review and meta-analysis. *Eur Heart J*. 2010
13. Nikolic SB, Abhayaratna WP, Leano R, Stowasser M, Sharman JE. Waiting a few extra minutes before measuring blood pressure has potentially important clinical and research ramifications. *Journal of human hypertension*. 2014;28:56-61
14. Karpievitch YV, Taverner T, Adkins JN, Callister SJ, Anderson GA, Smith RD, Dabney AR. Normalization of peak intensities in bottom-up ms-based proteomics using singular value decomposition. *Bioinformatics (Oxford, England)*. 2009;25:2573-2580
15. Whitworth JA. 2003 world health organization (who)/international society of hypertension (ish) statement on management of hypertension. *J Hypertens*. 2003;21:1983-1992
16. Briganti EM, Shaw JE, Chadban SJ, Zimmet PZ, Welborn TA, McNeil JJ, Atkins RC, Australian Diabetes O, Lifestyle S. Untreated hypertension among australian adults: The 1999-2000 australian diabetes, obesity and lifestyle study (ausdiab). *The Medical journal of Australia*. 2003;179:135-139
17. Kearney PM, Whelton M, Reynolds K, Muntner P, Whelton PK, He J. Global burden of hypertension: Analysis of worldwide data. *Lancet*. 2005;365:217-223

18. Carretero OA, Oparil S. Essential hypertension. Part i: Definition and etiology. *Circulation*. 2000;101:329-335
19. Pickering TG, Coats A, Mallion JM, Mancia G, Verdecchia P. Blood pressure monitoring. Task force v: White-coat hypertension. *Blood pressure monitoring*. 1999;4:333-341
20. Pickering TG, Gerin W, Schwartz JE, Spruill TM, Davidson KW. Franz volhard lecture: Should doctors still measure blood pressure? The missing patients with masked hypertension. *J Hypertens*. 2008;26:2259-2267
21. Bobrie G, Chatellier G, Genes N, Clerson P, Vaur L, Vaisse B, Menard J, Mallion JM. Cardiovascular prognosis of "masked hypertension" detected by blood pressure self-measurement in elderly treated hypertensive patients. *JAMA*. 2004;291:1342-1349
22. Franklin SS, Thijs L, Li Y, Hansen TW, Boggia J, Liu Y, Asayama K, Bjorklund-Bodegard K, Ohkubo T, Jeppesen J, Torp-Pedersen C, Dolan E, Kuznetsova T, Stolarz-Skrzypek K, Tikhonoff V, Malyutina S, Casiglia E, Nikitin Y, Lind L, Sandoya E, Kawecka-Jaszcz K, Filipovsky J, Imai Y, Wang J, Ibsen H, O'Brien E, Staessen JA, on behalf of the International Database on Ambulatory blood pressure in relation to Cardiovascular Outcomes I. Masked hypertension in diabetes mellitus: Treatment implications for clinical practice. *Hypertension*. 2013
23. Head GA, McGrath BP, Mihailidou AS, Nelson MR, Schlaich MP, Stowasser M, Mangoni AA, Cowley D, Brown MA, Ruta LA, Wilson A. Ambulatory blood pressure monitoring in australia: 2011 consensus position statement. *J Hypertens*. 2012;30:253-266
24. Allison TG, Cordeiro MA, Miller TD, Daida H, Squires RW, Gau GT. Prognostic significance of exercise-induced systemic hypertension in healthy subjects. *The American journal of cardiology*. 1999;83:371-375
25. Mottram PM, Haluska B, Yuda S, Leano R, Marwick TH. Patients with a hypertensive response to exercise have impaired systolic function without diastolic dysfunction or left ventricular hypertrophy. *Journal of the American College of Cardiology*. 2004;43:848-853

26. Lauer MS, Pashkow FJ, Harvey SA, Marwick TH, Thomas JD. Angiographic and prognostic implications of an exaggerated exercise systolic blood pressure response and rest systolic blood pressure in adults undergoing evaluation for suspected coronary artery disease. *Journal of the American College of Cardiology*. 1995;26:1630-1636
27. Le VV, Mitiku T, Sungar G, Myers J, Froelicher V. The blood pressure response to dynamic exercise testing: A systematic review. *Progress in cardiovascular diseases*. 2008;51:135-160
28. Campbell L, Marwick TH, Pashkow FJ, Snader CE, Lauer MS. Usefulness of an exaggerated systolic blood pressure response to exercise in predicting myocardial perfusion defects in known or suspected coronary artery disease. *The American journal of cardiology*. 1999;84:1304-1310
29. Sharman JE, Hare JL, Thomas S, Davies JE, Leano R, Jenkins C, Marwick TH. Association of masked hypertension and left ventricular remodeling with the hypertensive response to exercise. *Am J Hypertens*. 2011;24:898-903
30. Mundal R, Kjeldsen SE, Sandvik L, Erikssen G, Thaulow E, Erikssen J. Exercise blood pressure predicts cardiovascular mortality in middle-aged men. *Hypertension*. 1994;24:56-62
31. Kohl HW, 3rd, Nichaman MZ, Frankowski RF, Blair SN. Maximal exercise hemodynamics and risk of mortality in apparently healthy men and women. *Medicine and science in sports and exercise*. 1996;28:601-609
32. Filipovsky J, Ducimetiere P, Safar ME. Prognostic significance of exercise blood pressure and heart rate in middle-aged men. *Hypertension*. 1992;20:333-339
33. Laukkanen JA, Kurl S, Rauramaa R, Lakka TA, Venalainen JM, Salonen JT. Systolic blood pressure response to exercise testing is related to the risk of acute myocardial infarction in middle-aged men. *European journal of cardiovascular prevention and rehabilitation : official journal of the European Society of Cardiology, Working Groups on Epidemiology & Prevention and Cardiac Rehabilitation and Exercise Physiology*. 2006;13:421-428

34. Weiss SA, Blumenthal RS, Sharrett AR, Redberg RF, Mora S. Exercise blood pressure and future cardiovascular death in asymptomatic individuals. *Circulation*. 2010;121:2109-2116
35. Kjeldsen SE, Mundal R, Sandvik L, Erikssen G, Thaulow E, Erikssen J. Supine and exercise systolic blood pressure predict cardiovascular death in middle-aged men. *J Hypertens*. 2001;19:1343-1348
36. McEniery CM, Cockcroft JR, Roman MJ, Franklin SS, Wilkinson IB. Central blood pressure: Current evidence and clinical importance. *Eur Heart J*. 2014
37. Nichols WW, O'Rourke MF. *McDonald's blood flow in arteries: Theoretical, experimental and clinical principles*. London: Hodder Arnold; 2005.
38. Schultz MG, Gilroy D, Wright L, Bishop WL, Abhayaratna WP, Stowasser M, Sharman JE. Out-of-office and central blood pressure for risk stratification: A cross-sectional study in patients treated for hypertension. *European journal of clinical investigation*. 2012;42:393-401
39. Sharman J, Stowasser M, Fassett R, Marwick T, Franklin S. Central blood pressure measurement may improve risk stratification. *Journal of human hypertension*. 2008;22:838-844
40. McEniery CM, Yasmin, McDonnell B, Munnery M, Wallace SM, Rowe CV, Cockcroft JR, Wilkinson IB, Anglo-Cardiff Collaborative Trial I. Central pressure: Variability and impact of cardiovascular risk factors: The anglo-cardiff collaborative trial ii. *Hypertension*. 2008;51:1476-1482
41. McEniery CM, Yasmin, Hall IR, Qasem A, Wilkinson IB, Cockcroft JR. Normal vascular aging: Differential effects on wave reflection and aortic pulse wave velocity: The anglo-cardiff collaborative trial (acct). *Journal of the American College of Cardiology*. 2005;46:1753-1760
42. Kroeker EJ, Wood EH. Comparison of simultaneously recorded central and peripheral arterial pressure pulses during rest, exercise and tilted position in man. *Circulation research*. 1955;3:623-632

43. Rowell LB, Brengelmann GL, Blackmon JR, Bruce RA, Murray JA. Disparities between aortic and peripheral pulse pressures induced by upright exercise and vasomotor changes in man. *Circulation*. 1968;37:954-964
44. Sharman JE, McEniery CM, Campbell RI, Coombes JS, Wilkinson IB, Cockcroft JR. The effect of exercise on large artery haemodynamics in healthy young men. *European journal of clinical investigation*. 2005;35:738-744
45. Sharman JE, McEniery CM, Dhakam ZR, Coombes JS, Wilkinson IB, Cockcroft JR. Pulse pressure amplification during exercise is significantly reduced with age and hypercholesterolemia. *J Hypertens*. 2007;25:1249-1254
46. Chen CH, Nevo E, Fetis B, Pak PH, Yin FC, Maughan WL, Kass DA. Estimation of central aortic pressure waveform by mathematical transformation of radial tonometry pressure. Validation of generalized transfer function. *Circulation*. 1997;95:1827-1836
47. Holland DJ, Sacre JW, McFarlane SJ, Coombes JS, Sharman JE. Pulse wave analysis is a reproducible technique for measuring central blood pressure during hemodynamic perturbations induced by exercise. *Am J Hypertens*. 2008;21:1100-1106
48. Sharman JE, Lim R, Qasem AM, Coombes JS, Burgess MI, Franco J, Garrahy P, Wilkinson IB, Marwick TH. Validation of a generalized transfer function to noninvasively derive central blood pressure during exercise. *Hypertension*. 2006;47:1203-1208
49. Wilkinson IB, Fuchs SA, Jansen IM, Spratt JC, Murray GD, Cockcroft JR, Webb DJ. Reproducibility of pulse wave velocity and augmentation index measured by pulse wave analysis. *J Hypertens*. 1998;16:2079-2084
50. Thanassoulis G, Lyass A, Benjamin EJ, Larson MG, Vita JA, Levy D, Hamburg NM, Widlansky ME, O'Donnell CJ, Mitchell GF, Vasan RS. Relations of exercise blood pressure response to cardiovascular risk factors and vascular function in the framingham heart study. *Circulation*. 2012;125:2836-2843
51. Tsioufis C, Dimitriadis K, Thomopoulos C, Tsiachris D, Selima M, Stefanadi E, Tousoulis D, Kallikazaros I, Stefanadis C. Exercise blood pressure response, albuminuria, and arterial stiffness in hypertension. *The American journal of medicine*. 2008;121:894-902

52. Lauer MS, Levy D, Anderson KM, Plehn JF. Is there a relationship between exercise systolic blood pressure response and left ventricular mass? The framingham heart study. *Annals of internal medicine*. 1992;116:203-210
53. Vlachopoulos C, Aznaouridis K, Stefanadis C. Prediction of cardiovascular events and all-cause mortality with arterial stiffness: A systematic review and meta-analysis. *Journal of the American College of Cardiology*. 2010;55:1318-1327
54. O'Rourke MF, Hashimoto J. Mechanical factors in arterial aging: A clinical perspective. *Journal of the American College of Cardiology*. 2007;50:1-13
55. Greenwald SE. Ageing of the conduit arteries. *J Pathol*. 2007;211:157-172
56. Cecelja M, Chowienczyk P. Dissociation of aortic pulse wave velocity with risk factors for cardiovascular disease other than hypertension: A systematic review. *Hypertension*. 2009;54:1328-1336
57. Magni P, Motta M. Aldosterone receptor antagonists: Biology and novel therapeutic applications. *Current hypertension reports*. 2005;7:206-211
58. Lacolley P, Safar ME, Lucet B, Ledudal K, Labat C, Benetos A. Prevention of aortic and cardiac fibrosis by spironolactone in old normotensive rats. *Journal of the American College of Cardiology*. 2001;37:662-667
59. Zannad F, Alla F, Dousset B, Perez A, Pitt B. Limitation of excessive extracellular matrix turnover may contribute to survival benefit of spironolactone therapy in patients with congestive heart failure: Insights from the randomized aldactone evaluation study (rales). Rales investigators. *Circulation*. 2000;102:2700-2706
60. Edwards NC, Steeds RP, Stewart PM, Ferro CJ, Townend JN. Effect of spironolactone on left ventricular mass and aortic stiffness in early-stage chronic kidney disease: A randomized controlled trial. *Journal of the American College of Cardiology*. 2009;54:505-512
61. Mahmud A, Feely J. Aldosterone-to-renin ratio, arterial stiffness, and the response to aldosterone antagonism in essential hypertension. *Am J Hypertens*. 2005;18:50-55
62. Hare JL, Sharman JE, Leano R, Jenkins C, Wright L, Marwick TH. Impact of spironolactone on vascular, myocardial, and functional parameters in untreated patients with a hypertensive response to exercise. *Am J Hypertens*. 2013

63. Stewart KJ, Sung J, Silber HA, Fleg JL, Kelemen MD, Turner KL, Bacher AC, Dobrosielski DA, DeRegis JR, Shapiro EP, Ouyang P. Exaggerated exercise blood pressure is related to impaired endothelial vasodilator function. *Am J Hypertens.* 2004;17:314-320
64. Tzemos N, Lim PO, MacDonald TM. Exercise blood pressure and endothelial dysfunction in hypertension. *International journal of clinical practice.* 2009;63:202-206
65. Wilson MF, Sung BH, Pincomb GA, Lovallo WR. Exaggerated pressure response to exercise in men at risk for systemic hypertension. *The American journal of cardiology.* 1990;66:731-736
66. Bank AJ, Kaiser DR, Rajala S, Cheng A. In vivo human brachial artery elastic mechanics: Effects of smooth muscle relaxation. *Circulation.* 1999;100:41-47
67. Wilkinson IB, Qasem A, McEniery CM, Webb DJ, Avolio AP, Cockcroft JR. Nitric oxide regulates local arterial distensibility in vivo. *Circulation.* 2002;105:213-217
68. Wilkinson IB, Hall IR, MacCallum H, Mackenzie IS, McEniery CM, van der Arend BJ, Shu YE, MacKay LS, Webb DJ, Cockcroft JR. Pulse-wave analysis: Clinical evaluation of a noninvasive, widely applicable method for assessing endothelial function. *Arteriosclerosis, thrombosis, and vascular biology.* 2002;22:147-152
69. Joannides R, Richard V, Haefeli WE, Benoist A, Linder L, Luscher TF, Thuillez C. Role of nitric oxide in the regulation of the mechanical properties of peripheral conduit arteries in humans. *Hypertension.* 1997;30:1465-1470
70. Gow BS. The influence of vascular smooth muscle on the viscoelastic properties of blood vessels. In: Bergel DH, ed. *Cardiovascular Fluid Dynamics.* London, UK: Academic Press. 1972:66 –97
71. Wilcox JN, Smith KM, Schwartz SM, Gordon D. Localization of tissue factor in the normal vessel wall and in the atherosclerotic plaque. *Proceedings of the National Academy of Sciences of the United States of America.* 1989;86:2839-2843
72. Kakar P, Lip GY. Hypertension: Endothelial dysfunction, the prothrombotic state and antithrombotic therapy. *Expert review of cardiovascular therapy.* 2007;5:441-450

73. Vaclavik J, Sedlak R, Plachy M, Navratil K, Plasek J, Jarkovsky J, Vaclavik T, Husar R, Kocianova E, Taborsky M. Addition of spironolactone in patients with resistant arterial hypertension (aspirant): A randomized, double-blind, placebo-controlled trial. *Hypertension*. 2011;57:1069-1075
74. Gomez R, Nunez L, Caballero R, Vaquero M, Tamargo J, Delpon E. Spironolactone and its main metabolite canrenoic acid block hkv1.5, kv4.3 and kv7.1 + mink channels. *British journal of pharmacology*. 2005;146:146-161
75. Steffel J, Luscher TF, Tanner FC. Tissue factor in cardiovascular diseases: Molecular mechanisms and clinical implications. *Circulation*. 2006;113:722-731
76. Poli KA, Tofler GH, Larson MG, Evans JC, Sutherland PA, Lipinska I, Mittleman MA, Muller JE, D'Agostino RB, Wilson PW, Levy D. Association of blood pressure with fibrinolytic potential in the framingham offspring population. *Circulation*. 2000;101:264-269
77. Blann AD, Waite MA. Von willebrand factor and soluble e-selectin in hypertension: Influence of treatment and value in predicting the progression of atherosclerosis. *Coronary artery disease*. 1996;7:143-147
78. Sharman JE, Brown J, Holland DJ, Macdonald G, Kostner K, Marwick TH. Influence of altered blood rheology on ventricular-vascular response to exercise. *Hypertension*. 2009;54:1092-1098
79. Blann A. Von willebrand factor and the endothelium in vascular disease. *BR J Biomed Sci*. 1993;50:125-134
80. Spieker LE, Noll G, Ruschitzka FT, Maier W, Luscher TF. Working under pressure: The vascular endothelium in arterial hypertension. *Journal of human hypertension*. 2000;14:617-630
81. Willeit P, Thompson A, Aspelund T, Rumley A, Eiriksdottir G, Lowe G, Gudnason V, Di Angelantonio E. Hemostatic factors and risk of coronary heart disease in general populations: New prospective study and updated meta-analyses. *PloS one*. 2013;8:e55175
82. Thompson SG, Kienast J, Pyke SD, Haverkate F, van de Loo JC. Hemostatic factors and the risk of myocardial infarction or sudden death in patients with angina pectoris.

- European concerted action on thrombosis and disabilities angina pectoris study group. *N Engl J Med.* 1995;332:635-641
83. Lee KW, Blann AD, Lip GY. High pulse pressure and nondipping circadian blood pressure in patients with coronary artery disease: Relationship to thrombogenesis and endothelial damage/dysfunction. *Am J Hypertens.* 2005;18:104-115
 84. Spencer CG, Martin SC, Felmeden DC, Blann AD, Beevers GD, Lip GY. Relationship of homocysteine to markers of platelet and endothelial activation in "high risk" hypertensives: A substudy of the anglo-scandinavian cardiac outcomes trial. *International journal of cardiology.* 2004;94:293-300
 85. Sechi LA, Zingaro L, Catena C, Casaccio D, De Marchi S. Relationship of fibrinogen levels and hemostatic abnormalities with organ damage in hypertension. *Hypertension.* 2000;36:978-985
 86. Stone MC, Thorp JM. Plasma fibrinogen--a major coronary risk factor. *The Journal of the Royal College of General Practitioners.* 1985;35:565-569
 87. Wilkinson I, Cockcroft JR. Cholesterol, lipids and arterial stiffness. *Advances in cardiology.* 2007;44:261-277
 88. Chowienzyk PJ, Watts GF, Cockcroft JR, Ritter JM. Impaired endothelium-dependent vasodilation of forearm resistance vessels in hypercholesterolaemia. *Lancet.* 1992;340:1430-1432
 89. Lewis TV, Dart AM, Chin-Dusting JP. Endothelium-dependent relaxation by acetylcholine is impaired in hypertriglyceridemic humans with normal levels of plasma ldl cholesterol. *Journal of the American College of Cardiology.* 1999;33:805-812
 90. Park S, Shim J, Kim JB, Ko YG, Choi D, Ha JW, Rim SJ, Jang Y, Chung N. Insulin resistance is associated with hypertensive response to exercise in non-diabetic hypertensive patients. *Diabetes research and clinical practice.* 2006;73:65-69
 91. Steinberg HO, Brechtel G, Johnson A, Fineberg N, Baron AD. Insulin-mediated skeletal muscle vasodilation is nitric oxide dependent. A novel action of insulin to increase nitric oxide release. *The Journal of clinical investigation.* 1994;94:1172-1179

92. Henry RM, Kostense PJ, Spijkerman AM, Dekker JM, Nijpels G, Heine RJ, Kamp O, Westerhof N, Bouter LM, Stehouwer CD, Hoorn S. Arterial stiffness increases with deteriorating glucose tolerance status: The hoorn study. *Circulation*. 2003;107:2089-2095
93. Cameron JD, Bulpitt CJ, Pinto ES, Rajkumar C. The aging of elastic and muscular arteries: A comparison of diabetic and nondiabetic subjects. *Diabetes care*. 2003;26:2133-2138
94. Sims TJ, Rasmussen LM, Oxlund H, Bailey AJ. The role of glycation cross-links in diabetic vascular stiffening. *Diabetologia*. 1996;39:946-951
95. Yan LJ. Pathogenesis of chronic hyperglycemia: From reductive stress to oxidative stress. *Journal of diabetes research*. 2014;2014:137919
96. Dunn WB, Bailey NJ, Johnson HE. Measuring the metabolome: Current analytical technologies. *Analyst*. 2005;130:606-625
97. Mancia G, De Backer G, Dominiczak A, Cifkova R, Fagard R, Germano G, et al 2007 guidelines for the management of arterial hypertension: The task force for the management of arterial hypertension of the european society of hypertension (esh) and of the european society of cardiology (esc). *J Hypertens*. 2007;25:1105-1187
98. Pannier BM, Cambillau MS, Vellaud V, Atger V, Moatti N, Safar ME. Abnormalities of lipid metabolism and arterial rigidity in young subjects with borderline hypertension. *Clinical and investigative medicine. Medecine clinique et experimentale*. 1994;17:42-51
99. Griffin JL. The cinderella story of metabolic profiling: Does metabolomics get to go to the functional genomics ball? *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*. 2006;361:147-161
100. Nicholson JK, O'Flynn MP, Sadler PJ, Macleod AF, Juul SM, Sonksen PH. Proton-nuclear-magnetic-resonance studies of serum, plasma and urine from fasting normal and diabetic subjects. *The Biochemical journal*. 1984;217:365-375
101. Sauer U, Heinemann M, Zamboni N. Genetics. Getting closer to the whole picture. *Science (New York, N.Y.)*. 2007;316:550-551

102. Jones GL, Sang E, Goddard C, Mortishire-Smith RJ, Sweatman BC, Haselden JN, Davies K, Grace AA, Clarke K, Griffin JL. A functional analysis of mouse models of cardiac disease through metabolic profiling. *The Journal of biological chemistry*. 2005;280:7530-7539
103. Sreekumar A, Poisson LM, Rajendiran TM, Khan AP, Cao Q, Yu J, Laxman B, Mehra R, Lonigro RJ, Li Y, Nyati MK, Ahsan A, Kalyana-Sundaram S, Han B, Cao X, Byun J, Omenn GS, Ghosh D, Pennathur S, Alexander DC, Berger A, Shuster JR, Wei JT, Varambally S, Beecher C, Chinnaiyan AM. Metabolomic profiles delineate potential role for sarcosine in prostate cancer progression. *Nature*. 2009;457:910-914
104. Coen M, Holmes E, Lindon JC, Nicholson JK. Nmr-based metabolic profiling and metabonomic approaches to problems in molecular toxicology. *Chemical research in toxicology*. 2008;21:9-27
105. Akira K, Masu S, Imachi M, Mitome H, Hashimoto M, Hashimoto T. 1h nmr-based metabonomic analysis of urine from young spontaneously hypertensive rats. *J Pharm Biomed Anal*. 2008;46:550-556
106. Fujiwara M, Arifuku K, Ando I, Nemoto T. Pattern recognition analysis for classification of hypertensive model rats and diurnal variation using 1h-nmr spectroscopy of urine. *Analytical sciences : the international journal of the Japan Society for Analytical Chemistry*. 2005;21:1259-1262
107. Akira K, Imachi M, Hashimoto T. Investigations into biochemical changes of genetic hypertensive rats using 1h nuclear magnetic resonance-based metabonomics. *Hypertension research : official journal of the Japanese Society of Hypertension*. 2005;28:425-430
108. Aa JY, Wang GJ, Hao HP, Huang Q, Lu YH, Yan B, Zha WB, Liu LS, Kang A. Differential regulations of blood pressure and perturbed metabolism by total ginsenosides and conventional antihypertensive agents in spontaneously hypertensive rats. *Acta pharmacologica Sinica*. 2010;31:930-937
109. Lu Y, A J, Wang G, Hao H, Huang Q, Yan B, Zha W, Gu S, Ren H, Zhang Y, Fan X, Zhang M, Hao K. Gas chromatography/time-of-flight mass spectrometry based metabonomic approach to differentiating hypertension- and age-related metabolic

- variation in spontaneously hypertensive rats. *Rapid communications in mass spectrometry : RCM*. 2008;22:2882-2888
110. Brindle JT, Nicholson JK, Schofield PM, Grainger DJ, Holmes E. Application of chemometrics to 1h nmr spectroscopic data to investigate a relationship between human serum metabolic profiles and hypertension. *Analyst*. 2003;128:32-36
 111. Liu Y, Chen T, Qiu Y, Cheng Y, Cao Y, Zhao A, Jia W. An ultrasonication-assisted extraction and derivatization protocol for gc/tofms-based metabolite profiling. *Analytical and bioanalytical chemistry*. 2011;400:1405-1417
 112. De Meyer T, Sinnaeve D, Van Gasse B, Tsiorkova E, Rietzschel ER, De Buyzere ML, Gillebert TC, Bekaert S, Martins JC, Van Criekinge W. Nmr-based characterization of metabolic alterations in hypertension using an adaptive, intelligent binning algorithm. *Anal Chem*. 2008;80:3783-3790
 113. Holmes E, Loo RL, Stalder J, Bictash M, Yap IK, Chan Q, Ebbels T, De Iorio M, Brown IJ, Veselkov KA, Daviglus ML, Kesteloot H, Ueshima H, Zhao L, Nicholson JK, Elliott P. Human metabolic phenotype diversity and its association with diet and blood pressure. *Nature*. 2008;453:396-400
 114. Zheng Y, Yu B, Alexander D, Mosley TH, Heiss G, Nettleton JA, Boerwinkle E. Metabolomics and incident hypertension among blacks: The atherosclerosis risk in communities study. *Hypertension*. 2013
 115. Psychogios N, Hau DD, Peng J, Guo AC, Mandal R, Bouatra S, Sinelnikov I, Krishnamurthy R, Eisner R, Gautam B, Young N, Xia J, Knox C, Dong E, Huang P, Hollander Z, Pedersen TL, Smith SR, Bamforth F, Greiner R, McManus B, Newman JW, Goodfriend T, Wishart DS. The human serum metabolome. *PLoS ONE*. 2011;6:e16957
 116. Wishart DS, Jewison T, Guo AC, Wilson M, Knox C, Liu Y, Djoumbou Y, Mandal R, Aziat F, Dong E, Bouatra S, Sinelnikov I, Arndt D, Xia J, Liu P, Yallou F, Bjorn Dahl T, Perez-Pineiro R, Eisner R, Allen F, Neveu V, Greiner R, Scalbert A. Hmdb 3.0--the human metabolome database in 2013. *Nucleic Acids Res*. 2013;41:D801-807

117. Wishart DS, Tzur D, Knox C, Eisner R, Guo AC, Young N, et al. Hmdb: The human metabolome database. *Nucleic Acids Res.* 2007;35:D521-526
118. Lenz EM, Wilson ID. Analytical strategies in metabonomics. *Journal of proteome research.* 2007;6:443-458
119. Atherton HJ, Gulston MK, Bailey NJ, Cheng KK, Zhang W, Clarke K, Griffin JL. Metabolomics of the interaction between ppar-alpha and age in the ppar-alpha-null mouse. *Molecular systems biology.* 2009;5:259
120. Bothwell JH, Griffin JL. An introduction to biological nuclear magnetic resonance spectroscopy. *Biological reviews of the Cambridge Philosophical Society.* 2011;86:493-510
121. Dunn WB, Broadhurst DI, Atherton HJ, Goodacre R, Griffin JL. Systems level studies of mammalian metabolomes: The roles of mass spectrometry and nuclear magnetic resonance spectroscopy. *Chemical Society reviews.* 2011;40:387-426
122. Nordstrom A, Want E, Northen T, Lehtio J, Siuzdak G. Multiple ionization mass spectrometry strategy used to reveal the complexity of metabolomics. *Anal Chem.* 2008;80:421-429
123. Wishart DS. Advances in metabolite identification. *Bioanalysis.* 2011;3:1769-1782
124. Dunn WB, Broadhurst D, Begley P, Zelena E, Francis-McIntyre S, Anderson N, Brown M, Knowles JD, Halsall A, Haselden JN, Nicholls AW, Wilson ID, Kell DB, Goodacre R, Human Serum Metabolome C. Procedures for large-scale metabolic profiling of serum and plasma using gas chromatography and liquid chromatography coupled to mass spectrometry. *Nature protocols.* 2011;6:1060-1083
125. Paglia G, Hrafnisdottir S, Magnusdottir M, Fleming RM, Thorlacius S, Palsson BO, Thiele I. Monitoring metabolites consumption and secretion in cultured cells using ultra-performance liquid chromatography quadrupole-time of flight mass spectrometry (uplc-q-tof-ms). *Anal Bioanal Chem.* 2012;402:1183-1198
126. Theodoridis G, Gika HG, Wilson ID. Mass spectrometry-based holistic analytical approaches for metabolite profiling in systems biology studies. *Mass spectrometry reviews.* 2011

127. Kell DB, Brown M, Davey HM, Dunn WB, Spasic I, Oliver SG. Metabolic footprinting and systems biology: The medium is the message. *Nature reviews. Microbiology*. 2005;3:557-565
128. Bader M. Tissue renin-angiotensin-aldosterone systems: Targets for pharmacological therapy. *Annual review of pharmacology and toxicology*. 2010;50:439-465
129. Wu H, Southam AD, Hines A, Viant MR. High-throughput tissue extraction protocol for nmr- and ms-based metabolomics. *Analytical biochemistry*. 2008;372:204-212
130. Musgrove RE, Horne J, Wilson R, King AE, Edwards LM, Dickson TC. The metabolomics of alpha-synuclein (snca) gene deletion and mutation in mouse brain. *Metabolomics*. June 2013
131. Grouzmann E, Cavadas C, Grand D, Moratel M, Aubert JF, Brunner HR, Mazzolai L. Blood sampling methodology is crucial for precise measurement of plasma catecholamines concentrations in mice. *Pflugers Archiv : European journal of physiology*. 2003;447:254-258
132. Walsh MC, Brennan L, Malthouse JP, Roche HM, Gibney MJ. Effect of acute dietary standardization on the urinary, plasma, and salivary metabolomic profiles of healthy humans. *The American journal of clinical nutrition*. 2006;84:531-539
133. Barton RH, Waterman D, Bonner FW, Holmes E, Clarke R, Procardis C, Nicholson JK, Lindon JC. The influence of edta and citrate anticoagulant addition to human plasma on information recovery from nmr-based metabolic profiling studies. *Molecular bioSystems*. 2010;6:215-224
134. Drake SK, Bowen RA, Remaley AT, Hortin GL. Potential interferences from blood collection tubes in mass spectrometric analyses of serum polypeptides. *Clinical chemistry*. 2004;50:2398-2401
135. Deprez S, Sweatman BC, Connor SC, Haselden JN, Waterfield CJ. Optimisation of collection, storage and preparation of rat plasma for 1h nmr spectroscopic analysis in toxicology studies to determine inherent variation in biochemical profiles. *J Pharm Biomed Anal*. 2002;30:1297-1310

136. Brinc D, Chan MK, Venner AA, Pasic MD, Colantonio D, Kyriakopolou L, Adeli K. Long-term stability of biochemical markers in pediatric serum specimens stored at -80 degrees c: A caliper substudy. *Clinical biochemistry*. 2012;45:816-826
137. Trabi M, Keller MD, Johnsson NN. Nmr-based metabonomics of bovine blood: An investigation into the effects of long term storage on plasma samples. *Metabolomics*. 2013;9:1041-1047
138. Teahan O, Gamble S, Holmes E, Waxman J, Nicholson JK, Bevan C, Keun HC. Impact of analytical bias in metabonomic studies of human blood serum and plasma. *Anal Chem*. 2006;78:4307-4318
139. Moka D, Vorreuther R, Schicha H, Spraul M, Humpfer E, Lipinski M, Foxall PJ, Nicholson JK, Lindon JC. Biochemical classification of kidney carcinoma biopsy samples using magic-angle-spinning 1h nuclear magnetic resonance spectroscopy. *J Pharm Biomed Anal*. 1998;17:125-132
140. Kell DB, Oliver SG. Here is the evidence, now what is the hypothesis? The complementary roles of inductive and hypothesis-driven science in the post-genomic era. *BioEssays : news and reviews in molecular, cellular and developmental biology*. 2004;26:99-105
141. Broadhurst DI, Kell DB. Statistical strategies for avoiding false discoveries in metabolomics and related experiments. *Metabolomics*. 2006;2:171-196
142. Ebbels TM, Holmes E, Lindon JC, Nicholson JK. Evaluation of metabolic variation in normal rat strains from a statistical analysis of 1h nmr spectra of urine. *J Pharm Biomed Anal*. 2004;36:823-833
143. Solanky KS, Bailey NJ, Beckwith-Hall BM, Davis A, Bingham S, Holmes E, Nicholson JK, Cassidy A. Application of biofluid 1h nuclear magnetic resonance-based metabonomic techniques for the analysis of the biochemical effects of dietary isoflavones on human plasma profile. *Analytical biochemistry*. 2003;323:197-204
144. Kirschenlohr HL, Griffin JL, Clarke SC, Rhydwen R, Grace AA, Schofield PM, Brindle KM, Metcalfe JC. Proton nmr analysis of plasma is a weak predictor of coronary artery disease. *Nat Med*. 2006;12:705-710

145. Freedman DS, Otvos JD, Jeyarajah EJ, Shalurova I, Cupples LA, Parise H, D'Agostino RB, Wilson PW, Schaefer EJ. Sex and age differences in lipoprotein subclasses measured by nuclear magnetic resonance spectroscopy: The framingham study. *Clinical chemistry*. 2004;50:1189-1200
146. Bittner V. Lipoprotein abnormalities related to women's health. *The American journal of cardiology*. 2002;90:77i-84i
147. Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: The scandinavian simvastatin survival study (4s). *Lancet*. 1994;344:1383-1389
148. Brindle JT, Antti H, Holmes E, Tranter G, Nicholson JK, Bethell HW, Clarke S, Schofield PM, McKilligin E, Mosedale DE, Grainger DJ. Rapid and noninvasive diagnosis of the presence and severity of coronary heart disease using 1h-nmr-based metabonomics. *Nat Med*. 2002;8:1439-1444
149. Qi S, Ouyang X, Wang L, Peng W, Wen J, Dai Y. A pilot metabolic profiling study in serum of patients with chronic kidney disease based on (1) h-nmr-spectroscopy. *Clinical and translational science*. 2012;5:379-385
150. Pinto YM, Paul M, Ganten D. Lessons from rat models of hypertension: From goldblatt to genetic engineering. *Cardiovascular research*. 1998;39:77-88
151. Vargas SL, Toma I, Kang JJ, Meer EJ, Peti-Peterdi J. Activation of the succinate receptor gpr91 in macula densa cells causes renin release. *Journal of the American Society of Nephrology : JASN*. 2009;20:1002-1011
152. He W, Miao FJ, Lin DC, Schwandner RT, Wang Z, Gao J, Chen JL, Tian H, Ling L. Citric acid cycle intermediates as ligands for orphan g-protein-coupled receptors. *Nature*. 2004;429:188-193
153. Militante JD, Lombardini JB. Treatment of hypertension with oral taurine: Experimental and clinical studies. *Amino acids*. 2002;23:381-393
154. Fujita T, Ando K, Noda H, Ito Y, Sato Y. Effects of increased adrenomedullary activity and taurine in young patients with borderline hypertension. *Circulation*. 1987;75:525-532
155. Nara Y, Yamori Y, Lovenberg W. Effect of dietary taurine on blood pressure in spontaneously hypertensive rats. *Biochemical pharmacology*. 1978;27:2689-2692

156. Muratani H, Ferrario CM, Averill DB. Ventrolateral medulla in spontaneously hypertensive rats: Role of angiotensin ii. *The American journal of physiology*. 1993;264:R388-395
157. Akira K, Mitome H, Imachi M, Shida Y, Miyaoka H, Hashimoto T. Lc-nmr identification of a novel taurine-related metabolite observed in (1)h nmr-based metabonomics of genetically hypertensive rats. *J Pharm Biomed Anal*. 2010;51:1091-1096
158. Egan BM SK. Evidence linking fatty fat acids, the risk factor cluster, and vascular pathophysiology. Implications for the diabetic hypertensive patient. *Endocrinology of the Vasculature, Contemporary Endocrinology*. 1996;1:157-172
159. Ordway RW, Singer JJ, Walsh JV, Jr. Direct regulation of ion channels by fatty acids. *Trends in neurosciences*. 1991;14:96-100
160. Fagot-Campagna A, Balkau B, Simon D, Warnet JM, Claude JR, Ducimetiere P, Eschwege E. High free fatty acid concentration: An independent risk factor for hypertension in the paris prospective study. *International journal of epidemiology*. 1998;27:808-813
161. Tugrul A, Guldiken S, Ugur-Altun B, Arikan E. An evaluation of glucose tolerance in essential hypertension. *Yonsei medical journal*. 2009;50:195-199
162. Turner RC, Holman RR, Matthews DR, Bassett PA, Coster R, Stratton IM, et al. Hypertension in diabetes study (hds) .1. Prevalence of hypertension in newly presenting type-2 diabetic-patients and the association with risk-factors for cardiovascular and diabetic complications. *J. Hypertens*. 1993;11:309-317
163. Friedrich N. Metabolomics in diabetes research. *J Endocrinol*. 2012;215:29-42
164. Lu J, Xie G Fau - Jia W, Jia W Fau - Jia W, Jia W. Metabolomics in human type 2 diabetes research. *Front Med*. 2013;7:4-13
165. Wang TJ, Larson MG, Vasan RS, Cheng S, Rhee EP, McCabe E, Lewis GD, Fox CS, Jacques PF, Fernandez C, O'Donnell CJ, Carr SA, Mootha VK, Florez JC, Souza A, Melander O, Clish CB, Gerszten RE. Metabolite profiles and the risk of developing diabetes. *Nat Med*. 2011;17:448-453

166. Magnusson M, Lewis GD, Ericson U, Orho-Melander M, Hedblad B, Engstrom G, Ostling G, Clish C, Wang TJ, Gerszten RE, Melander O. A diabetes-predictive amino acid score and future cardiovascular disease. *Eur Heart J*. 2013;34:1982-1989
167. Ha CY, Kim JY, Paik JK, Kim OY, Paik YH, Lee EJ, Lee JH. The association of specific metabolites of lipid metabolism with markers of oxidative stress, inflammation and arterial stiffness in men with newly diagnosed type 2 diabetes. *Clinical endocrinology*. 2012;76:674-682
168. Boos CJ, Lip GY. Is hypertension an inflammatory process? *Curr Pharm Des*. 2006;12:1623-1635
169. Kenny LC, Broadhurst DI, Dunn W, Brown M, North RA, McCowan L, Roberts C, Cooper GJ, Kell DB, Baker PN, Screening for Pregnancy Endpoints C. Robust early pregnancy prediction of later preeclampsia using metabolomic biomarkers. *Hypertension*. 2010;56:741-749
170. Odibo AO, Goetzinger KR, Odibo L, Cahill AG, Macones GA, Nelson DM, Dietzen DJ. First-trimester prediction of preeclampsia using metabolomic biomarkers: A discovery phase study. *Prenatal diagnosis*. 2011;31:990-994
171. Bahado-Singh RO, Akolekar R, Mandal R, Dong E, Xia J, Kruger M, Wishart DS, Nicolaides K. Metabolomics and first-trimester prediction of early-onset preeclampsia. *The journal of maternal-fetal & neonatal medicine : the official journal of the European Association of Perinatal Medicine, the Federation of Asia and Oceania Perinatal Societies, the International Society of Perinatal Obstet*. 2012;25:1840-1847
172. Bahado-Singh RO, Akolekar R, Mandal R, Dong E, Xia J, Kruger M, Wishart DS, Nicolaides K. First-trimester metabolomic detection of late-onset preeclampsia. *American journal of obstetrics and gynecology*. 2013;208:58 e51-57
173. Report of the national high blood pressure education program working group on high blood pressure in pregnancy. *American journal of obstetrics and gynecology*. 2000;183:S1-S22
174. Martin CA, McGrath BP. White-coat hypertension. *Clinical and experimental pharmacology & physiology*. 2013

175. Vaughan M, Berger JE, Steinberg D. Hormone-sensitive lipase and monoglyceride lipase activities in adipose tissue. *The Journal of biological chemistry*. 1964;239:401-409
176. Liao D, Arnett DK, Tyroler HA, Riley WA, Chambless LE, Szklo M, Heiss G. Arterial stiffness and the development of hypertension. The ariC study. *Hypertension*. 1999;34:201-206
177. Laurent S, Cockcroft J, Van Bortel L, Boutouyrie P, Giannattasio C, Hayoz D, Pannier B, Vlachopoulos C, Wilkinson I, Struijker-Boudier H. Expert consensus document on arterial stiffness: Methodological issues and clinical applications. *Eur Heart J*. 2006;27:2588-2605
178. Mamas M, Dunn WB, Neyses L, Goodacre R. The role of metabolites and metabolomics in clinically applicable biomarkers of disease. *Archives of toxicology*. 2011;85:5-17
179. Feng Z, Prentice R, Srivastava S. Research issues and strategies for genomic and proteomic biomarker discovery and validation: A statistical perspective. *Pharmacogenomics*. 2004;5:709-719
180. Nicholson JK, Everett JR, Lindon JC. Longitudinal pharmacometabonomics for predicting patient responses to therapy: Drug metabolism, toxicity and efficacy. *Expert opinion on drug metabolism & toxicology*. 2012;8:135-139
181. Corona G, Rizzolio F, Giordano A, Toffoli G. Pharmaco-metabolomics: An emerging "omics" tool for the personalization of anticancer treatments and identification of new valuable therapeutic targets. *Journal of cellular physiology*. 2012;227:2827-2831
182. Clayton TA, Lindon JC, Cloarec O, Antti H, Charuel C, Hanton G, Provost JP, Le Net JL, Baker D, Walley RJ, Everett JR, Nicholson JK. Pharmaco-metabonomic phenotyping and personalized drug treatment. *Nature*. 2006;440:1073-1077
183. El Assaad MA, Topouchian JA, Darne BM, Asmar RG. Validation of the omron hem-907 device for blood pressure measurement. *Blood pressure monitoring*. 2002;7:237-241

184. Stergiou GS, Giovas PP, Gkinos CP, Tzamouranis DG. Validation of the a&d um-101 professional hybrid device for office blood pressure measurement according to the international protocol. *Blood pressure monitoring*. 2008;13:37-42
185. Sala C, Santin E, Rescaldani M, Magrini F. How long shall the patient rest before clinic blood pressure measurement? *Am J Hypertens*. 2006;19:713-717
186. van der Wel MC, Buunk IE, van Weel C, Thien TA, Bakx JC. A novel approach to office blood pressure measurement: 30-minute office blood pressure vs daytime ambulatory blood pressure. *Ann Fam Med*. 2011;9:128-135
187. Cameron JD, Stevenson I, Reed E, McGrath BP, Dart AM, Kingwell BA. Accuracy of automated auscultatory blood pressure measurement during supine exercise and treadmill stress electrocardiogram-testing. *Blood pressure monitoring*. 2004;9:269-275
188. Keith LJ, Rattigan S, Keske MA, Jose M, Sharman JE. Exercise aortic stiffness: Reproducibility and relation to end-organ damage in men. *Journal of human hypertension*. 2013;27:516-522
189. Palatini P, Frigo G, Bertolo O, Roman E, Da Corta R, Winnicki M. Validation of the a&d tm-2430 device for ambulatory blood pressure monitoring and evaluation of performance according to subjects' characteristics. *Blood pressure monitoring*. 1998;3:255-260
190. Rogoza AN, Pavlova TS, Sergeeva MV. Validation of a&d ua-767 device for the self-measurement of blood pressure. *Blood pressure monitoring*. 2000;5:227-231
191. Parati G, Stergiou GS, Asmar R, Bilo G, de Leeuw P, Imai Y, Kario K, Lurbe E, Manolis A, Mengden T, O'Brien E, Ohkubo T, Padfield P, Palatini P, Pickering T, Redon J, Revera M, Ruilope LM, Shennan A, Staessen JA, Tisler A, Waeber B, Zanchetti A, Mancia G, Monitoring ESHWGoBP. European society of hypertension guidelines for blood pressure monitoring at home: A summary report of the second international consensus conference on home blood pressure monitoring. *J Hypertens*. 2008;26:1505-1526
192. Charloux A, Lonsdorfer-Wolf E, Richard R, Lampert E, Oswald-Mammosser M, Mettauer B, Geny B, Lonsdorfer J. A new impedance cardiograph device for the non-

- invasive evaluation of cardiac output at rest and during exercise: Comparison with the "direct" fick method. *Eur J Appl Physiol*. 2000;82:313-320
193. Schultz MG, Climie RE, Nikolic SB, Ahuja KD, Sharman JE. Reproducibility of cardiac output derived by impedance cardiography during postural changes and exercise. *Artery Res*. 2012;78-84
 194. Edwards LM, Lawler NG, Nikolic SB, Peters JM, Horne J, Wilson R, Davies NW, Sharman JE. Metabolomics reveals increased isoleukotoxin diol (12,13-dhome) in human plasma after acute intralipid infusion. *J Lipid Res*. 2012
 195. Benjamini Y, Hochberg Y. Controlling the false discovery rate: A practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society*. 1995;57:289-300
 196. Brown M, Wedge DC, Goodacre R, Kell DB, Baker PN, Kenny LC, Mamas MA, Neyses L, Dunn WB. Automated workflows for accurate mass-based putative metabolite identification in lc/ms-derived metabolomic datasets. *Bioinformatics (Oxford, England)*. 2011;27:1108-1112
 197. Dieterle F, Ross A, Schlotterbeck G, Senn H. Probabilistic quotient normalization as robust method to account for dilution of complex biological mixtures. Application in 1h nmr metabonomics. *Anal Chem*. 2006;78:4281-4290
 198. Chobanian AV, Bakris GL, Black HR, Cushman WC, Green LA, Izzo JL, Jr., Jones DW, Materson BJ, Oparil S, Wright JT, Jr., Roccella EJ. The seventh report of the joint national committee on prevention, detection, evaluation, and treatment of high blood pressure: The jnc 7 report. *JAMA*. 2003;289:2560-2572
 199. Daida H, Allison TG, Squires RW, Miller TD, Gau GT. Peak exercise blood pressure stratified by age and gender in apparently healthy subjects. *Mayo Clinic proceedings*. 1996;71:445-452
 200. Lip GY, Blann AD. Von willebrand factor and its relevance to cardiovascular disorders. *British heart journal*. 1995;74:580-583
 201. Wang JS, Liao CH. Moderate-intensity exercise suppresses platelet activation and polymorphonuclear leukocyte interaction with surface-adherent platelets under shear flow in men. *Thrombosis and haemostasis*. 2004;91:587-594

202. Jilma B, Dirnberger E, Eichler HG, Matulla B, Schmetterer L, Kapiotis S, Speiser W, Wagner OF. Partial blockade of nitric oxide synthase blunts the exercise-induced increase of von willebrand factor antigen and of factor viii in man. *Thrombosis and haemostasis*. 1997;78:1268-1271
203. Fagard RH, Pardaens K, Staessen JA, Thijs L. Prognostic value of invasive hemodynamic measurements at rest and during exercise in hypertensive men. *Hypertension*. 1996;28:31-36
204. Tanaka H, Monahan KD, Seals DR. Age-predicted maximal heart rate revisited. *Journal of the American College of Cardiology*. 2001;37:153-156
205. Wehling M. Effects of aldosterone and mineralocorticoid receptor blockade on intracellular electrolytes. *Heart failure reviews*. 2005;10:39-46
206. Wishart DS. Applications of metabolomics in drug discovery and development. *Drugs in R&D*. 2008;9:307-322
207. Overdiek HW, Merkus FW. The metabolism and biopharmaceutics of spironolactone in man. *Reviews on drug metabolism and drug interactions*. 1987;5:273-302
208. Pitt B, Zannad F, Remme WJ, Cody R, Castaigne A, Perez A, Palensky J, Wittes J. The effect of spironolactone on morbidity and mortality in patients with severe heart failure. Randomized aldactone evaluation study investigators. *N Engl J Med*. 1999;341:709-717
209. Funder JW, Pearce PT, Smith R, Campbell J. Vascular type i aldosterone binding sites are physiological mineralocorticoid receptors. *Endocrinology*. 1989;125:2224-2226
210. Hatakeyama H, Miyamori I, Fujita T, Takeda Y, Takeda R, Yamamoto H. Vascular aldosterone. Biosynthesis and a link to angiotensin ii-induced hypertrophy of vascular smooth muscle cells. *The Journal of biological chemistry*. 1994;269:24316-24320
211. Lombes M, Oblin ME, Gasc JM, Baulieu EE, Farman N, Bonvalet JP. Immunohistochemical and biochemical evidence for a cardiovascular mineralocorticoid receptor. *Circulation research*. 1992;71:503-510
212. Benetos A, Lacolley P, Safar ME. Prevention of aortic fibrosis by spironolactone in spontaneously hypertensive rats. *Arteriosclerosis, thrombosis, and vascular biology*. 1997;17:1152-1156

213. Mottram PM, Haluska B, Leano R, Cowley D, Stowasser M, Marwick TH. Effect of aldosterone antagonism on myocardial dysfunction in hypertensive patients with diastolic heart failure. *Circulation*. 2004;110:558-565
214. de Vegt F, Dekker JM, Ruhe HG, Stehouwer CD, Nijpels G, Bouter LM, Heine RJ. Hyperglycaemia is associated with all-cause and cardiovascular mortality in the hoorn population: The hoorn study. *Diabetologia*. 1999;42:926-931
215. Kimoto E, Shoji T, Shinohara K, Inaba M, Okuno Y, Miki T, Koyama H, Emoto M, Nishizawa Y. Preferential stiffening of central over peripheral arteries in type 2 diabetes. *Diabetes*. 2003;52:448-452
216. Chen JY, Chou Ch Fau - Lee YL, Lee YI Fau - Tsai W-C, Tsai Wc Fau - Lin C-C, Lin Cc Fau - Huang Y-Y, Huang Yy Fau - Chen J-H, Chen JH. Association of central aortic pressures indexes with development of diabetes mellitus in essential hypertension. *Am J Hypertens*. 2010;23:1069-1073
217. Welborn T, De Courten M. Case detection and diagnosis expert working group: National evidence based guidelines for the management of type 2 diabetes mellitus; part 3 case detection and diagnosis of type 2 diabetes. *National Health and Medical Research Council*. 2001
218. Edwards LM, Lawler NG, Nikolic SB, Peters JM, Horne J, Wilson R, Davies NW, Sharman JE. Metabolomics reveals increased isoleukotoxin diol (12,13-dhome) in human plasma after acute intralipid infusion. *J Lipid Res*. 2012;53:1979-1986
219. Robinson BH. Lactic acidemia and mitochondrial disease. *Molecular genetics and metabolism*. 2006;89:3-13
220. DiGirolamo M, Newby FD, Lovejoy J. Lactate production in adipose tissue: A regulated function with extra-adipose implications. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 1992;6:2405-2412
221. Mabley JG, Pacher P, Liaudet L, Soriano FG, Hasko G, Marton A, Szabo C, Salzman AL. Inosine reduces inflammation and improves survival in a murine model of colitis. *American journal of physiology. Gastrointestinal and liver physiology*. 2003;284:G138-144

222. Mabley JG, Rabinovitch A, Suarez-Pinzon W, Hasko G, Pacher P, Power R, Southan G, Salzman A, Szabo C. Inosine protects against the development of diabetes in multiple-low-dose streptozotocin and nonobese diabetic mouse models of type 1 diabetes. *Molecular medicine (Cambridge, Mass.)*. 2003;9:96-104
223. Festa A, D'Agostino R, Jr., Howard G, Mykkanen L, Tracy RP, Haffner SM. Chronic subclinical inflammation as part of the insulin resistance syndrome: The insulin resistance atherosclerosis study (iras). *Circulation*. 2000;102:42-47
224. Hamer M, Steptoe A. Vascular inflammation and blood pressure response to acute exercise. *Eur J Appl Physiol*. 2012;112:2375-2379
225. Floyd JC, Jr., Fajans SS, Conn JW, Knopf RF, Rull J. Stimulation of insulin secretion by amino acids. *The Journal of clinical investigation*. 1966;45:1487-1502
226. Krebs M, Krssak M, Bernroider E, Anderwald C, Brehm A, Meyerspeer M, Nowotny P, Roth E, Waldhausl W, Roden M. Mechanism of amino acid-induced skeletal muscle insulin resistance in humans. *Diabetes*. 2002;51:599-605
227. Shah SH, Bain JR, Muehlbauer MJ, Stevens RD, Crosslin DR, Haynes C, Dungan J, Newby LK, Hauser ER, Ginsburg GS, Newgard CB, Kraus WE. Association of a peripheral blood metabolic profile with coronary artery disease and risk of subsequent cardiovascular events. *Circulation. Cardiovascular genetics*. 2010;3:207-214
228. Kim Y, Wilkens LR, Park SY, Goodman MT, Monroe KR, Kolonel LN. Association between various sedentary behaviours and all-cause, cardiovascular disease and cancer mortality: The multiethnic cohort study. *International journal of epidemiology*. 2013;42:1040-1056
229. Myers MG, Valdivieso M, Kiss A. Use of automated office blood pressure measurement to reduce the white coat response. *J Hypertens*. 2009;27:280-286
230. Niiranen TJ, Hanninen MR, Johansson J, Reunanen A, Jula AM. Home-measured blood pressure is a stronger predictor of cardiovascular risk than office blood pressure: The finn-home study. *Hypertension*. 2010;55:1346-1351
231. Hansen TW, Kikuya M, Thijs L, Bjorklund-Bodegard K, Kuznetsova T, Ohkubo T, Richart T, Torp-Pedersen C, Lind L, Jeppesen J, Ibsen H, Imai Y, Staessen JA. Prognostic superiority of daytime ambulatory over conventional blood pressure in

- four populations: A meta-analysis of 7,030 individuals. *J Hypertens*. 2007;25:1554-1564
232. Verberk WJ, Kroon AA, Lenders JW, Kessels AG, van Montfrans GA, Smit AJ, van der Kuy PH, Nelemans PJ, Rennenberg RJ, Grobbee DE, Beltman FW, Joore MA, Brunenberg DE, Dirksen C, Thien T, de Leeuw PW, Home Versus Office Measurement RoUTSI. Self-measurement of blood pressure at home reduces the need for antihypertensive drugs: A randomized, controlled trial. *Hypertension*. 2007;50:1019-1025
 233. Sharman JE, Fang ZY, Haluska B, Stowasser M, Prins JB, Marwick TH. Left ventricular mass in patients with type 2 diabetes is independently associated with central but not peripheral pulse pressure. *Diabetes care*. 2005;28:937-939
 234. Williams B, Lacy PS, Thom SM, Cruickshank K, Stanton A, Collier D, Hughes AD, Thurston H, O'Rourke M. Differential impact of blood pressure-lowering drugs on central aortic pressure and clinical outcomes: Principal results of the conduit artery function evaluation (cafe) study. *Circulation*. 2006;113:1213-1225
 235. Roman MJ, Devereux RB, Kizer JR, Okin PM, Lee ET, Wang W, Umans JG, Calhoun D, Howard BV. High central pulse pressure is independently associated with adverse cardiovascular outcome the strong heart study. *Journal of the American College of Cardiology*. 2009;54:1730-1734
 236. Devereux RB, Reichek N. Echocardiographic determination of left ventricular mass in man. Anatomic validation of the method. *Circulation*. 1977;55:613-618
 237. van Loo JM, Peer PG, Thien TA. Twenty-five minutes between blood pressure readings: The influence on prevalence rates of isolated systolic hypertension. *J Hypertens*. 1986;4:631-635
 238. Verberk WJ, Kroon AA, Kessels AG, Lenders JW, Thien T, van Montfrans GA, Smit AJ, de Leeuw PW. The optimal scheme of self blood pressure measurement as determined from ambulatory blood pressure recordings. *J Hypertens*. 2006;24:1541-1548
 239. Myers MG, Godwin M, Dawes M, Kiss A, Tobe SW, Grant FC, Kaczorowski J. Conventional versus automated measurement of blood pressure in primary care

- patients with systolic hypertension: Randomised parallel design controlled trial. *BMJ*. 2011;342:d286
240. Mancia G, Bertinieri G, Grassi G, Parati G, Pomidossi G, Ferrari A, Gregorini L, Zanchetti A. Effects of blood-pressure measurement by the doctor on patient's blood pressure and heart rate. *Lancet*. 1983;2:695-698
 241. Ohkubo T, Kikuya M, Metoki H, Asayama K, Obara T, Hashimoto J, Totsune K, Hoshi H, Satoh H, Imai Y. Prognosis of "masked" hypertension and "white-coat" hypertension detected by 24-h ambulatory blood pressure monitoring 10-year follow-up from the ohasama study. *Journal of the American College of Cardiology*. 2005;46:508-515
 242. Wang KL, Cheng HM, Chuang SY, Spurgeon HA, Ting CT, Lakatta EG, Yin FC, Chou P, Chen CH. Central or peripheral systolic or pulse pressure: Which best relates to target organs and future mortality? *J Hypertens*. 2009;27:461-467
 243. Hirata K, Vlachopoulos C, Adji A, O'Rourke MF. Benefits from angiotensin-converting enzyme inhibitor 'beyond blood pressure lowering': Beyond blood pressure or beyond the brachial artery? *J Hypertens*. 2005;23:551-556
 244. Morgan T, Lauri J, Bertram D, Anderson A. Effect of different antihypertensive drug classes on central aortic pressure. *Am J Hypertens*. 2004;17:118-123
 245. Hope SA, Meredith IT, Cameron JD. Effect of non-invasive calibration of radial waveforms on error in transfer-function-derived central aortic waveform characteristics. *Clinical science (London, England : 1979)*. 2004;107:205-211
 246. Saladini F, Benetti E, Malipiero G, Casiglia E, Palatini P. Does home blood pressure allow for a better assessment of the white-coat effect than ambulatory blood pressure? *J Hypertens*. 2012;30:2118-2124
 247. Zelena E, Dunn WB, Broadhurst D, Francis-McIntyre S, Carroll KM, Begley P, O'Hagan S, Knowles JD, Halsall A, Consortium H, Wilson ID, Kell DB. Development of a robust and repeatable uplc-ms method for the long-term metabolomic study of human serum. *Anal Chem*. 2009;81:1357-1364
 248. Gika HG, Theodoridis GA, Wilson ID. Liquid chromatography and ultra-performance liquid chromatography-mass spectrometry fingerprinting of human urine: Sample

- stability under different handling and storage conditions for metabonomics studies. *Journal of chromatography. A*. 2008;1189:314-322
249. Guy PA, Tavazzi I, Bruce SJ, Ramadan Z, Kochhar S. Global metabolic profiling analysis on human urine by uplc-tofms: Issues and method validation in nutritional metabolomics. *Journal of chromatography. B, Analytical technologies in the biomedical and life sciences*. 2008;871:253-260
 250. Bordbar A, Mo ML, Nakayasu ES, Schrimpe-Rutledge AC, Kim YM, Metz TO, Jones MB, Frank BC, Smith RD, Peterson SN, Hyduke DR, Adkins JN, Palsson BO. Model-driven multi-omic data analysis elucidates metabolic immunomodulators of macrophage activation. *Molecular systems biology*. 2012;8:558
 251. Deatherage Kaiser BL, Li J, Sanford JA, Kim YM, Kronewitter SR, Jones MB, Peterson CT, Peterson SN, Frank BC, Purvine SO, Brown JN, Metz TO, Smith RD, Heffron F, Adkins JN. A multi-omic view of host-pathogen-commensal interplay in - mediated intestinal infection. *PloS one*. 2013;8:e67155
 252. Kim YM, Schmidt BJ, Kidwai AS, Jones MB, Deatherage Kaiser BL, Brewer HM, Mitchell HD, Palsson BO, McDermott JE, Heffron F, Smith RD, Peterson SN, Ansong C, Hyduke DR, Metz TO, Adkins JN. Salmonella modulates metabolism during growth under conditions that induce expression of virulence genes. *Molecular bioSystems*. 2013;9:1522-1534
 253. Metz TO, Page JS, Baker ES, Tang K, Ding J, Shen Y, Smith RD. High resolution separations and improved ion production and transmission in metabolomics. *Trends in analytical chemistry : TRAC*. 2008;27:205-214
 254. Want EJ, Wilson ID, Gika H, Theodoridis G, Plumb RS, Shockcor J, Holmes E, Nicholson JK. Global metabolic profiling procedures for urine using uplc–ms. *Nature Protocols*. 2010;5:1005-1018
 255. Karpievitch YV, Taverner T, Adkins JN, Callister SJ, Anderson GA, Smith RD, Dabney AR. Normalization of peak intensities in bottom-up ms-based proteomics using singular value decomposition. *Bioinformatics*. 2009

256. Sangster T, Major H, Plumb R, Wilson AJ, D. WI. A pragmatic and readily implemented quality control strategy for hplc-ms and gc-ms-based metabonomic analysis. *The Analyst*. 2006;131:1075-1078
257. Leek JT, Storey JD. Capturing heterogeneity in gene expression studies by surrogate variable analysis. *PLoS Genetics*. 2007;3
258. Lai L, Michopoulos F, Gika H, Theodoridis G, Wilkinson RW, Odedra R, Wingate J, Bonner R, Tate S, Wilson ID. Methodological considerations in the development of hplc-ms methods for the analysis of rodent plasma for metabonomic studies. *Molecular bioSystems*. 2010;6:108-120
259. Benjamini Y, Hochberg Y. Controlling the false discovery rate: A practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society*. 1995;57:289-300

Publications Arising From This Thesis

These articles have been removed for
copyright or proprietary reasons.

Nikolic SB, Sharman JE, Adams MJ, Edwards LM. Metabolomics in hypertension. *J Hypertens*. 2014; 32:1159-1169

Nikolic SB, Wilson R, Hare JL, Adams MJ, Edwards LM, Sharman JE. Spironolactone reduces aortic stiffness via blood pressure-dependent effects of canrenoate. *Metabolomics*. 2014;10:105-113

Nikolic SB, Abhayaratna WP, Leano R, Stowasser M, Sharman JE. Waiting a few extra minutes before measuring blood pressure has potentially important clinical and research ramifications. *Journal of human hypertension*. 2014;28:56-61