An investigation into Gilbert’s syndrome: Understanding the role of unconjugated bilirubin in targeting platelet and haemostatic mechanisms associated with thrombotic risk factors

Avinash Reddy Kundur

(B.Pham, JNTU University, MSc, RMIT University)

School of Medical Science
Heart Foundation Research Centre
Menzies Health Institute
Griffith University

Submitted in fulfilment of the requirements of the degree of

Doctor of Philosophy

August 2017
STATEMENT OF ORIGINALITY

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

Name      Avinash Reddy Kundur

Signature:

Date:     25/08/2017
ABSTRACT ........................................................................................................... 20

1. Review of Literature .................................................................................. 24
   1.1 Introduction ............................................................................................... 25
   1.2 Elevated bilirubin, Gilbert’s syndrome and cardiovascular disease ......... 28
   1.3 Role of Platelet membrane glycoproteins ................................................. 30
      1.3.1 Adhesion ............................................................................................. 30
      1.3.2 Activation ........................................................................................... 31
      1.3.3 Aggregation ......................................................................................... 32
   1.4 Haemostasis ............................................................................................... 32
   1.5 Role of free radicals in platelet function ...................................................... 34
   1.6 Role of Platelets in atherogenesis and atherothrombosis ............................ 37
   1.7 Risk factors for thrombosis ......................................................................... 43
   1.8 Antioxidants in the prevention of atherothrombotic events ......................... 46
   1.9 Bilirubin: structure and metabolism ........................................................... 48
      1.10 Bilirubin as an antioxidant and possible cardio-protectant .................... 52
         1.10.1 Low bilirubin and cardiovascular disease ........................................ 52
         1.10.2 Bilirubin, lipid metabolism and oxidation ....................................... 57
         1.10.3 Bilirubin, inflammation and endothelial function ........................... 58
   1.11 Effect of bilirubin as an antioxidant on platelet function ......................... 61
      1.11.1 The hypothesized antithrombotic potential of bilirubin ....................... 61
      1.11.2 Bilirubin and platelet function ........................................................... 64
      1.11.3 Bilirubin, platelet and hematological characteristics .......................... 66
      1.11.4 Inflammation and platelet activation ............................................... 68
      1.11.5 Free radical induced platelet activation ............................................ 70

2. Aims & Hypothesis ....................................................................................... 73

3. Materials and Methods ............................................................................... 77
   3.1 Research Ethics .......................................................................................... 78
   3.2 Volunteer recruitment ................................................................................ 78
   3.3 Sample Collection and processing ............................................................. 79
      3.3.1 Blood collection .................................................................................... 79
      3.3.2 Sample processing ............................................................................... 80
   3.4 Full Blood Examination ............................................................................ 81
4. Dose dependent inhibition of platelet aggregation by unconjugated bilirubin in vitro

4.1 Introduction

4.2 Methods

4.3 Results

4.4 Discussion

5. Mildly elevated unconjugated bilirubin is associated with reduced platelet activation, P-selectin expression and inflammation in Gilbert’s syndrome
5.2.1 Recruitment and blood collection ............................................................ 116
5.2.2 Platelet aggregation assay ........................................................................ 116
5.2.3 Determination of platelet activity ............................................................. 116
5.2.4 Tests of coagulation ................................................................................. 117
5.2.5 Biochemical analysis ................................................................................ 117
5.2.6 Statistical analysis .................................................................................... 117
5.3 Results ............................................................................................................... 118
5.3.1 Platelet aggregation and activation .......................................................... 120
5.3.2 Coagulation assays ................................................................................... 126
5.3.3 General biochemistry, inflammation and lipid status ............................... 126
5.4 Discussion ......................................................................................................... 128

6. Reduced platelet activity after acute intense exercise in Gilbert’s syndrome ................................................................. 133

6.1 Introduction ....................................................................................................... 134
6.2 Methods ............................................................................................................ 135
6.2.1 Incremental exercise test to exhaustion .................................................... 135
6.2.2 Experimental trial and blood collection ................................................... 136
6.2.3 Determination of Platelet activation and aggregation .............................. 137
6.2.4 Tests of coagulation ................................................................................. 137
6.2.5 Measurement of Cytokines ....................................................................... 137
6.2.6 Biochemical analysis ................................................................................ 137
6.2.7 Statistical analysis .................................................................................... 138

6.3 Results ................................................................................................................. 138
6.3.1 Exercise test .............................................................................................. 141
6.3.2 Platelet aggregation assay ...................................................................... 142
6.3.3 Flow cytometric evaluation of platelet activity ........................................ 144
6.3.4 Coagulation profile .................................................................................. 145
6.3.5 Inflammation and cytokine measurement .............................................. 147

6.4 Discussion ......................................................................................................... 150

7. Comparing the gene expression of SELP and eNOS genes in individuals with Gilbert’s syndrome and normal healthy population ................................................................. 154

7.1 Introduction ....................................................................................................... 155
7.2 Materials and Methods .................................................................................. 156
## LIST OF TABLES

**Table 1.** Therapeutic reference ranges adapted from Royal College of Pathologists Australiasia. ................................................................. 79

**Table 2.** Concentrations of various agonist used for the studies and their respective pathways of platelet activation. ................................................................. 83

**Table 3.** Concentrations of different antibodies used to prepare antibody master mix........................................................................................................ 90

**Table 4.** Concentrations of isotype controls used to prepare isotype control master mix........................................................................................................ 90

**Table 5.** Full blood examination Results........................................................................................................ 102

**Table 6.** Effect of unconjugated bilirubin (UCB) treatment on coagulation and biochemical parameters........................................................................ 105

**Table 7.** General characteristics and full blood examination results of recruited Gilbert’s syndrome and control subjects........................................................................... 119

**Table 8.** Multiple regression analysis for platelet function markers as dependent variables........................................................................................................ 125

**Table 9.** Coagulation assay results of Gilbert’s syndrome and control subjects........................................................................................................ 126

**Table 10.** General biochemistry, inflammation and lipid status in Gilbert’s syndrome and control subjects........................................................................................................ 127

**Table 11.** General and hematological parameters of recruited control and Gilbert’s syndrome subject’s pre and post intense acute exercise trial........................................................................................................ 139

**Table 12.** Mean power, VO$_{2\text{max}}$ and heart rate values attained during the intense acute exercise trials........................................................................................................ 141

**Table 13.** Coagulation profile results pre and post exercise of control and Gilbert’s syndrome subjects........................................................................................................ 146

**Table 14.** Serum cytokine levels of control and Gilbert’s syndrome results at pre and post-acute exercise. ........................................................................................................ 147
Table 15. Biochemical parameters pre and post acute exercise of control and Gilbert’s syndrome and control subjects…………………………………………………………. 149

Table 16. Real time PCR primers………………………………………………………………………………………………………………………………………………………………………………. 159

Table 17. Concentrations of various reagent and cDNA used to make up the final volume for each amplification……………………………………………………………………. 160
LIST OF FIGURES

Figure 1. The extrinsic and intrinsic pathways of the coagulation cascade leading to clot formation................................................................. 34

Figure 2. Pathogenesis of ROS induced atherosclerosis.......................... 42

Figure 3. Synthesis and metabolism of bilirubin and conditions of impaired bilirubin metabolism .......................................................... 50

Figure 4. Cardio-protective mechanisms of mildly elevated bilirubin ......... 56

Figure 5. Possible mechanism of inhibition of platelet activation/aggregation by bilirubin................................................................. 63

Figure 6. Showing an example platelet aggregation report developed by the Helena aggregometer...................................................... 84

Figure 7. Step by step protocol followed in the preparation of compensation controls for BD LSR Fortessa TM Cell analyzer.......................... 88

Figure 8. Step by step protocol followed in the evaluation of activation-dependant platelet surface marker expression using flowcytometer......... 91

Figure 9. Flow cytometric analysis of human platelet activity............... 92

Figure 10. Flow cytometric analysis of human platelet activity.............. 93

Figure 11. Dose responsive and time responsive effect of unconjugated bilirubin on platelet aggregation with 10 min incubation ................. 103

Figure 12. Effect of UCB on collagen and ADP induced platelet aggregation... 104

Figure 13. Mean platelet aggregation (MPA%) in response to the agonists in Gilbert’s syndrome and control................................................. 121

Figure 14. The expression of P-selectin and PAC-1 recorded as mean fluorescence intensity (MFI) on the surface of activated platelets from Gilbert’s syndrome and control subjects .................................. 122
**Figure 15.** The relationship between circulating bilirubin and AA induced platelet aggregation collagen induced platelet aggregation; B) and P-selectin expression in control and GS subjets………………………………………………124

**Figure 16.** Mean platelet aggregation in response to various agonists in Gilbert’s syndrome and control pre and post intense acute exercise......................143

**Figure 17.** Activation dependent surface marker expression on surface of activated platelets in Gilbert’s syndrome and control pre and post-acute exercise........................................................................................................144

**Figure 18.** Showing the various incubation steps involved in the syntheses of cDNA from RNA........................................................................................................159

**Figure 19.** Relative expression of SELP gene in control and Gilbert’s syndrome individuals.................................................................162

**Figure 20.** Relative expression of eNOS gene in control and Gilbert’s syndrome individuals........................................................................162
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AGE</td>
<td>Advance glycation end-products</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AOX</td>
<td>Antioxidant</td>
</tr>
<tr>
<td>Apo-A1</td>
<td>Apolipoprotein-A1</td>
</tr>
<tr>
<td>Apo-B</td>
<td>Apolipoprotein-B</td>
</tr>
<tr>
<td>aPTT</td>
<td>Activated partial thromboplastin time</td>
</tr>
<tr>
<td>BDGs</td>
<td>Bilirubin di-glucunorides</td>
</tr>
<tr>
<td>BMGs</td>
<td>Bilirubin mono-glucunorides</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium</td>
</tr>
<tr>
<td>CB</td>
<td>Conjugated bilirubin</td>
</tr>
<tr>
<td>CAC</td>
<td>Coronary artery calcification</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>CD40L</td>
<td>CD40 ligand</td>
</tr>
<tr>
<td>eGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary heart disease</td>
</tr>
<tr>
<td>CO</td>
<td>Carbon monoxide</td>
</tr>
<tr>
<td>COX-1</td>
<td>Cyclooxygenase-1</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>C&lt;sub&gt;t&lt;/sub&gt;</td>
<td>Cycle threshold</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra-acetic acid</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>FBE</td>
<td>Full blood examination</td>
</tr>
<tr>
<td>Fe&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Iron</td>
</tr>
<tr>
<td>FH</td>
<td>Familial hypercholesterolemia</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GP</td>
<td>Glycoprotein</td>
</tr>
<tr>
<td>GS</td>
<td>Gilbert’s syndrome</td>
</tr>
<tr>
<td>Hb</td>
<td>Haemoglobin</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Hmox</td>
<td>Heme oxygenase</td>
</tr>
<tr>
<td>HO-1</td>
<td>Heme oxygenase-1</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular Adhesion Molecule 1</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>IHD</td>
<td>Ischemic heart disease</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1β</td>
</tr>
<tr>
<td>IL-2</td>
<td>Interleukin-2</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin-8</td>
</tr>
<tr>
<td>IMD</td>
<td>Intima-media thickness</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MAC</td>
<td>Membrane attack complex</td>
</tr>
<tr>
<td>MCH</td>
<td>Mean cell haemoglobin</td>
</tr>
<tr>
<td>MCHC</td>
<td>Mean corpuscular haemoglobin concentration</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MPA</td>
<td>Mean platelet aggregation</td>
</tr>
<tr>
<td>MPV</td>
<td>Mean platelet volume</td>
</tr>
<tr>
<td>MRP&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Multidrug resistance-associated protein 2</td>
</tr>
<tr>
<td>MTB</td>
<td>Modified Tyrode’s buffer</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NF-Kβ</td>
<td>Nuclear transcription factor- Kappa β</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>Nrf2</td>
<td>Nuclear factor eruthroid-2</td>
</tr>
<tr>
<td>O&lt;sub&gt;2&lt;/sub&gt;^-</td>
<td>Superoxide anion</td>
</tr>
<tr>
<td>oxLDL</td>
<td>Oxidised low density lipoprotein</td>
</tr>
<tr>
<td>PAD</td>
<td>Peripheral artery disease</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen activator inhibitor-1</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PerCp</td>
<td>Peridinin chlorophyll protein</td>
</tr>
<tr>
<td>PF3</td>
<td>Platelet factor 3</td>
</tr>
<tr>
<td>PGI&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Prostacyclin</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase-C</td>
</tr>
<tr>
<td>PLA&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Phospholipase A&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>PLT</td>
<td>Platelet count</td>
</tr>
</tbody>
</table>
PPP  Platelet poor plasma
PRP  Platelet rich plasma
PT   Prothrombin time
PWV  Pulse wave velocity
RAGE Receptor for advance glycation end-products
RBC  Red blood cell
RCPA Royal college of pathologists Australasia
RONS Reactive oxygen and nitrogen species
ROS  Reactive oxygen species
RT   Room temperature
SD   Standard deviation
SEL P Selectin-P
SLCO1B1 Solute carrier anion transporter protein 1B1
SST  Serum separator tubes
T2DM Type 2 diabetes mellitus
TC   Total cholesterol
TE   Trolox equivalents
TG   Triacylglycerol
TNF-α Tumour necrosis factor-α
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>t-PA</td>
<td>Tissue plasminogen activator</td>
</tr>
<tr>
<td>TRAP</td>
<td>Thrombin receptor-activating peptide</td>
</tr>
<tr>
<td>TxA₂</td>
<td>Thromboxane A₂</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
</tr>
<tr>
<td>UCB</td>
<td>Unconjugated bilirubin</td>
</tr>
<tr>
<td>UGT1A1</td>
<td>Uridine Glucuronosyl Transferase 1A1</td>
</tr>
<tr>
<td>V-LDL</td>
<td>Very low density lipoprotein</td>
</tr>
<tr>
<td>VO₂PEAK</td>
<td>Peak oxygen uptake</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular smooth muscle cell</td>
</tr>
<tr>
<td>vWF</td>
<td>von-Willebrand factor</td>
</tr>
<tr>
<td>WB</td>
<td>Whole blood</td>
</tr>
<tr>
<td>WBC</td>
<td>White blood cell</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENTS

First, I would like to express my sincere gratitude to my supervisor, Dr. Indu Singh for believing in me and giving me the opportunity to peruse my dream as a researcher. Her constant encouragement and support has always been the source of inspiration for me to grow not only as an experimentalist but also as an independent thinker. After working under my supervisor’s guidance over the past few years, with great confidence I can say that I have become a better human being, and understood several aspects of life, hence “Thank you Ma’am”.

I would like to thank my associate supervisor Dr. Andrew C Bulmer, who has accepted me as his student with open arms after my masters. He has always been an oasis of great intellect and the person who has constantly pushed me to achieve greater heights. It was a great experience to work under a person who has the ability to achieve great things in the field of science. Thank you for being such an amazing supervisor/friend. I am grateful to Dr. Surendran Sabapathy for his patience and guidance throughout the research project. I would also like to thank Dr. Natalie Colson for her patience and guidance throughout my research project.

I will forever be grateful to my best friend, brother, guide and colleague Dr. Abishek Bommannan for his support throughout my masters and PhD. I would also like to thank my colleague Ms. Lada Tucakovic for her assistance throughout the project. My heartfelt thanks to the honours research project student and friend Mr. Roy Robson for rendering a helping hand in my projects.

I wish to thank the Head of School, Prof. Mark Forwood and Deputy Head Research Prof. Nigel McMillan for their help and support throughout my PhD. Also I am grateful
to the Heart Foundation Research Centre and the Menzies Health Institute for providing me with valuable resources for my projects.

My years at Griffith University would have been long and boring without my great friends whom I have made over the past few years. My sincere and heart-felt thanks to my best friend Victoria Ozberk, who has been a constant source of motivation and support throughout my PhD “thank you Vicky”. I would sincerely like to thank my friends Yun-Mi Nguy, Karishma Sachaphibulkij, Veerendra Nethi, Elham Nikbakht, Paulina Janeczek, Brittany McCormack, Brad Patterson, Pramod Sharma, Lokesh Madireddi, Bruno Vieira, Alex R Comm and many more.

Finally, I would like to thank my unique, stereotypical and perfectly imperfect family from India. My mother, who has never stopped praying for me, from the day I was born, My father who inspired me to dream big and cross the oceans and then gave me the resources to turn my dreams into reality. My sweet brother, who never stopped loving me from the day we first met.

*This thesis is dedicated to my family and my supervisor for their constant love and support*

*“Thank you”*
Acknowledgement of Published and Unpublished Papers included in this Thesis

Included in this thesis are papers in Chapters 1, 4 and 5 for which I am the primary author. Appropriate acknowledgements of those who contributed to the research but did not qualify as authors are included in the acknowledgement section.

The bibliographic details (published or submitted for publication) for these papers are:


Chapter 1: Copyright © 2014 Elsevier Ireland Ltd. All rights reserved.
Chapter 2: Copyright © 2014 Informa Plc. All rights reserved.
Chapter 3: Copyright © 2017 Informa Plc. All rights reserved.

(Signed) __________________________________ (Date) __________
Avinash Reddy, Kundur

(Countersigned) ______________________ (Date) __________
Supervisor: Dr. Indu Singh
ABSTRACT

Gilbert’s syndrome (GS) is a common genetic condition associated with mildly elevated unconjugated bilirubin (UCB) concentrations and increased protection against development of cardiovascular disease (CVD). Mutation in the uridine diphosphate glucuronosyltransferase (UGT1A1) gene causing a reduction in UGT1A1 enzyme activity that is responsible for conjugation and elimination of UCB, is considered as the cause for GS. Numerous studies have shown that elevated levels of UCB are negatively associated with risk of developing CVD. While, several in vitro and in vivo trials have shown that UCB at physiological concentrations can improve endothelial function, lipid profile and reduce vascular inflammation, thereby imparting its cardiovascular protection. Cardiovascular disease is a major cause of death globally, an estimated 17.3 million CVD related deaths are reported annually by the World Health Organization (WHO), making it the largest cause of human mortality. Furthermore, several studies have predicted that these numbers would continue rising in the coming years, due to factors such as aging, lifestyle choices and environmental changes.

Platelets are small anuclear cells with the blood that play a key role in regulating haemostasis. Under physiological conditions platelet circulate within the vasculature and only get activated in the presence of trauma to prevent blood loss by mediating primary and secondary hemostasis. However, undesired intravascular platelet activation is known to be associated with thrombosis and subsequent cardiovascular events. Elevated oxidative stress and increased free radical production is considered as one of the major reasons for platelet hyperactivity, especially in individuals with co-morbidities such as Type 2 diabetes mellitus. Diabetic individuals with elevated oxidative stress experience an increased risk developing CVD, due their positive association with several risk factors such as endothelial dysfunction, inflammation and lipid peroxidation.
Antioxidants have always been the topic of great interest, as years of research has shown that elevated exogenous or endogenous antioxidant can hold significant cardiovascular benefits, primarily by constraining the free radical induced oxidative damage. Furthermore, the increased cardiovascular protection of several antioxidants was shown to be associated with its ability to inhibit platelet hyper-activity. Mildly elevated levels of UCB in individuals with Gilbert’s syndrome (GS) was shown to be associated with elevated Antioxidant capacity. Although literature has shown that elevated UCB in GS may be negatively associated with several CVD risk factors, the effect of UCB on thrombotic markers such as platelet activation, aggregation and haemostasis is yet to be investigated. Hence, through a combination of *vitro* and *in vivo* experimental models, the aim of the studies undertaken for this thesis was to investigate the effect of mildly elevated UCB on markers associated with platelet hyperactivity and to unveil the mechanistic pathways that may be responsible for providing GS individuals with improved protection against CVD.

The aim of the first study was to investigate the *in vitro* effect of mildly elevated UCB on platelet aggregation, haemostasis and general biochemical parameters. Unconjugated bilirubin at a concentration of 35μM significantly inhibited collagen induced platelet aggregation. Furthermore, at higher concentrations UCB inhibited adenine diphosphate (ADP) and collagen stimulated platelet aggregation in a dose dependent fashion. The results from this study suggest that mildly elevated UCB at 35μM may impart its cardiovascular protection by reducing collagen induced platelet aggregation (chapter 4). Based on the results from the first study (chapter 4), it was hypothesised that individuals with GS, who have mildly elevated levels of UCB may have a reduced risk of platelet hyperactivity and subsequent thrombosis. Based on the results from the first study GS and normal healthy individuals were recruited to (chapter 5) investigate whether mildly elevated circulating unconjugated bilirubin (UCB) is
negatively associated with multiple thrombotic risk factors including platelet activity, haemostatic function and inflammation in individuals with GS. A statistically significant decrease in the expression of P-selectin on activated platelets was observed in GS subjects. Collagen and AA induced platelet aggregation were significantly reduced in GS versus control group. Elevated UCB and HDL in addition to reduced LDL and hsCRP were also observed in GS when compared to the control group. Reduced P-selectin expression suggests decreased platelet activation-dependent degranulation. While reduced platelet aggregation by AA and collagen, indicate a quantitative decrease in platelet aggregation consequently targeting the COX-1 and GP VI pathways respectively. Intense acute exercise in sedentary but not trained individuals is associated with increased platelet activation and aggregation, which is related to processes underpinning thrombosis and subsequent CVD. To evaluate whether elevated levels of unconjugated bilirubin (UCB) in GS can attenuate thrombotic risk factors in sedentary individuals after intense acute aerobic exercise (chapter 6) platelet activity, haemostatic function, inflammation and lipid profile were performed pre and post intense acute aerobic exercise bout. A significant reduction in the expression of platelet surface marker P-selectin in GS pre and post exercise was observed. Platelet aggregation using exogenous agonists, AA and collagen were significantly inhibited pre and post exercise in GS. Furthermore, serum concentrations of hsCRP and IL10 were significantly reduced in GS when compared to controls. To further evaluate if the observed favourable effects of mildly elevated UCB in GS was due to change in the gene expression of SELP (P-selectin; platelet degranulation) and eNOS3 (endothelial nitric oxide synthase; endothelial function) was performed (chapter 7). A significant increase in the relative expression of SELP and eNOS3 genes was observed in GS when compared with controls.

In conclusion, the novel findings obtained from the studies undertaken for this thesis suggest that elevated UCB in GS is associated with inhibition of platelet activity,
which may be responsible for reduced risk of thrombosis thereby providing increased protection against the development of CVD. Furthermore, by mechanisms such as altering lipid profile and reducing vascular inflammation in normal healthy asymptomatic GS population at rest as well as after acute intense exercise may be responsible for reduced incidence of CVD in GS. However, further studies are needed to elucidate the exact mechanism by which UCB may alter platelet function in individuals with co-morbidities and to confirm UCB’s role elevated antioxidant capacity and improved cardio-protection in GS population.
1. Review of Literature

Adapted from:


**Author Contributions:**

Kundur AR: Conducted the literature search, prepared the manuscript

Singh I, Bulmer AC: Critically reviewed the manuscript
1.1 Introduction

Gilbert’s syndrome (also known as Gilbert-Meulengracht syndrome) is a benign condition affecting 3-10 % of the population and is caused by a genetic polymorphism in the promoter region of the uridine diphosphate glucuronosyltransferase (UGT1A1) gene on chromosome 2. This mutation reduces hepatic UGT1A1 synthesis and therefore, bilirubin conjugation and thus excretion (1-3). Impaired bilirubin excretion is associated with a mild and chronically elevated serum unconjugated bilirubin (UCB) in GS (>17 µM) (2, 3). Several in vitro and in vivo studies have already established an inverse relationship between mildly elevated levels of UCB, as seen in individuals with GS and the incidence of CVD.

Platelets are minute anucleated cells which are known to play key role in preventing blood loss, by aggregating and forming a clot at the site of injury. However, platelet hyperactivity is regularly associated with the development of thrombosis and subsequent cardiovascular diseases (CVD). Increased oxidative stress, inflammation and endothelial dysfunction are considered major risk factors for increased platelet activity. These risk factors act synergistically, can elevate the rate of platelet activation and coagulation, thereby significantly increasing the risk of thrombosis and CVD. Hence, platelet function has evolved as an important marker for predicting future CVD risk, especially in individuals with co-morbidities including obesity and type 2 diabetes mellitus (T2DM) (4).

According to recent World Health Organization (WHO) reports, an estimated 17.3 million deaths annually are attributed to CVD, approximating 30% of all deaths (~7.3 million due to CHD and 6.2 million due to stroke) and is predicted to continue rising in the coming years (5-9). Increased free radical production and oxidative stress is emerging as a key risk factor in the development of atherosclerosis and CVD (10). An imbalance
between free radical production and the body’s endogenous antioxidant defence system to scavenge them leads to oxidative stress and potentially oxidative damage to cellular macromolecules (11). Elevated oxidative stress induced by auto-oxidation of glucose leads to further production of reactive oxygen species, lipid peroxidation, inflammation, endothelial dysfunction, smooth muscle proliferation, matrix metalloproteinase activation and reduced nitric oxide (NO) bioavailability, and therefore could play an important role in the initiation and progression of atherosclerosis (see fig. 1) (10, 12, 13). Reactive oxygen species, including hydrogen peroxide, when generated in large quantities and in the context of platelet function, can mobilize intravascular arachidonic acid (AA) via activation of phospholipase A2 (PLA2). Such activation stimulates calcium (Ca2+) dependent and independent signalling pathways, thus encouraging platelet hyper-activation and subsequent thrombosis, leading to atherothrombosis, embolus and peripheral organ ischemia (4).

Recent developments in our understanding of the factors that trigger the development of atherosclerosis, have led us to consider of that antioxidants may alter CVD risk by inducing anti-atherosclerotic and anti-thrombotic mechanisms within the artery wall. Unconjugated bilirubin is a naturally occurring potent antioxidant and several studies have revealed new biological roles for unconjugated bilirubin (UCB), including anti-mutagenic, immune-modulatory and possibly lipid lowering effects (14-18). This is in contrast to its well-known potentially cytotoxic property, in neurons (19). Hence, Gilbert’s syndrome (GS), a condition of mild and benign hyperbilirubinemia has recently become a topic of great interest due to these individuals experiencing reduced risk of developing CVD (17, 20-22). Evidence from a recent meta-analysis supports the above statement, showing that UCB at mildly elevated concentrations, as seen in GS, is associated with decreased risk of developing CVD, which might be caused by inhibition of free radical lipid and LDL (i.e. oxLDL) oxidation (20, 23). Unconjugated bilirubin
scavenges free radicals in in vitro and in vivo models (17, 20-22). Elevated serum UCB in GS increases circulating antioxidant capacity, which might delay the process of atherosclerosis (17). Large epidemiological studies indicate that individuals with GS have a reduced risk of CVD, cancer and all-cause mortality (24-26). Furthermore, in a longitudinal study, Horsfall and colleagues show a striking 50% reduction in all-cause mortality in GS, when compared to individuals without GS (27). Therefore, GS represents an invaluable and clinically relevant translational model to test the physiological importance of bilirubin in preventing disease. Genetic polymorphism in UGT1A1 is also seen in some other conditions such as Crigler-Najjar syndrome. However in this case complete absence of UGT1A1 function is associated with toxic accumulation of circulating UCB (>345 µM), exceeding the albumin binding capacity of the blood and thus leading to neonatal and adult kernicterus (neuronal toxicity) (19).

Although several studies have shown the cardio-protective effects of mildly elevated UCB, however, the mechanisms by which UCB may reduce the risk of CVD is still unclear. One possible mechanism can be by reducing platelet activity in individuals with GS, thereby reducing the risk of thrombus formation and subsequent CVD. Previously, several antioxidants have been shown to reduce the platelet activity and subsequent thrombus formation through mechanisms such as inhibiting the expression of platelet surface membrane glycoproteins and platelet granule release. Recent studies have shown that mildly elevated UCB at concentrations seen in GS can inhibit collagen induced platelet aggregation and binding. However, further research is needed to understand the effect of UCB on platelet glycoproteins.
1.2 Elevated bilirubin, Gilbert’s syndrome and cardiovascular disease

A negative relationship between total serum bilirubin concentration and the development of cardiovascular diseases in the general population is also observed, with protection being greatest in individuals with Gilbert’s syndrome. Gilbert’s syndrome is a benign condition diagnosed by the presence of mildly elevated concentrations of unconjugated bilirubin (>17.1 µM) in the absence of elevated serum liver enzyme activities or hemolysis (17, 20-22, 28). A significantly reduced incidence of coronary heart disease was observed in GS, when compared with individuals without GS (29-31). Novotny et al. extended upon these findings using a meta-analytical approach showing a reliable inverse and dose responsive relationship between the total serum bilirubin concentrations and development of atherosclerosis in men (20). Hopkins et al. demonstrated that serum bilirubin concentrations were negatively associated with the severity of coronary artery disease (CAD) in both sexes and confirmed that individuals with increased total serum bilirubin concentrations (>17 µM) have an 80% reduced risk of developing CAD (32). Mildly elevated concentrations of UCB are associated with reductions in multiple cardiovascular outcomes including myocardial infarction (MI), peripheral arterial disease (PAD) and ischemic heart disease (IHD) (33-35). Turfan et al. recently reported a significant independent and negative association between total serum bilirubin levels and severity of disease in patients with stable coronary artery disease, suggesting that total serum bilirubin levels may be useful in assessing and reducing the severity of CAD in these patients (36).

Increased total serum bilirubin concentrations are hypothesized to protect individuals with familial hypercholesterolemia (FH) from CVD (37). A 7% increase in circulating total serum bilirubin concentration in FH individuals, due to statin administration, might play a significant role in protection against future CVD (37). Furthermore, individuals undergoing statin treatment and with a median baseline bilirubin
concentration of $\geq 10 \mu M$ had significantly reduced risk of experiencing a CVD event, MI or dying from any cause, when compared with individuals on statin treatment, but with low bilirubin concentration ($\leq 5 \mu M$) (26). Inoguchi et al. were the first to demonstrate that a diabetic population with underlying GS had a reduced risk of vascular complications including CAD and cerebrovascular disease compared to individuals with diabetes only (38). Several other studies have reported a negative relationship between bilirubin concentration/GS and incidence of metabolic disorders (39-42). Diabetes and metabolic syndrome induce a state of oxidative stress, which is believed to contribute to increased CVD risk (38). The mechanism of protection in GS is not yet fully understood, however mildly elevated levels of UCB might increase insulin sensitivity, insulin signalling and glucose tolorence (39, 43, 44). Admistration of bilirubin (20 μmol/kg) improved insulin sensitivity in leptin-receptor defecient mice and, reduced body weight and insulin signalling in hyperglycemic diet-induced obese mouse models (44). Although much evidence suggests that low total serum bilirubin/UCB concentrations are associated with increased risk of cardiovascular disease, these data do not provide a strong indication for a role of bilirubin per se in mediating CVD susceptibility. However, studying individuals with mildly elevated UCB, for example individuals with Gilbert’s syndrome, and their associated risk of CVD could provide a stronger argument for protection.

Initially, Vitek et al. showed that GS patients with mildly elevated UCB levels have markedly reduced risk of CVD. In this three year follow up study only 2% of GS patients developed IHD compared with 12.1% of the general population (34). Bilirubin appears to reduce circulating lipids, in addition to being associated with reduced risk of type 2 diabetes (44, 45). Protection from diabetes also appears to protect from subsequent renal impairment and CVD, arguing that mildly elevated bilirubin may protect broadly from chronic degenerative disease (46).
1.3 Role of Platelet membrane glycoproteins

The process of platelet adhesion, activation and aggregation during vessel wall injury is regulated by highly specialized transmembrane receptors called platelet membrane glycoproteins (GP) (47). These glycoproteins (GP) bind with adhesion molecules and plasma proteins such as collagen, von Willibrand factor and fibronectin to mediate cell-cell and cell-substrate interactions, thus initiating platelet activation (47). On stimulation platelet undergoes a series of rapid alterations within itself leading to shape change, release of cytoplasmic granules and inside-out signalling leading to platelet activation. The GP Ib/IX/V complex located on platelet surface plays a key role in interaction of platelets with vWF on subendothelial matrix of the vessel wall. This initial binding between GP Ib/IX/V and vWF activates GP IIb/IIIa receptor and contributes to the initiation of pre-developmental stages of thrombus formation, the GP IIb/IIIa dependent platelet adhesion, activation and aggregation (48).

1.3.1 Adhesion

Vascular trauma or injury tethers platelets at the site of injury via secretion of various adhesion molecules and plasma proteins that bind with platelet membrane glycoproteins (48). The attachment of GP Ib/IX/V complex with the vessel wall mediated by the vWF stimulates the activation of fibrinogen receptor GP IIb/IIIa (49). With an expression of 50,000-80,000 copies on a resting platelet, the GP IIb/IIIa is the most expressed integrin on a platelet membrane (50). Furthermore, on activation additional pools of GP IIb/IIIa integrin are released from α storage granules and open canalicular system (48). The collagen receptor Integrin IIa/Ib plays an important role in platelet adhesion with sub-endothelial collagen. The interactions between GPIIb and vWF are quick and quite unstable on the cell surface and hence are continuously translocated through “rolling” mechanism (51). During this process the GPVI collagen receptor
belonging to immunoglobulin super family attaches itself to the cell surface. Since GP VI is weakly bound, it triggers an array of intracellular signals causing exocytosis of α and dense granules releasing various agonist such as Adenosine diphosphate (ADP), Adenosine triphosphate (ATP), calcium (Ca\textsuperscript{2+}), fibronectin, β-thromboglobulin, thrombospondin, P-selectin (52). Introduction of these agonists further aggravates the process of platelet activation thus forming an initial layer of platelets at the disrupted site.

**1.3.2 Activation**

The initiation of platelet activation is induced by the activation of collagen receptor GP VI which plays a key role in platelet adhesion, aggregation, degranulation and activation of coagulation (52). The activated GP VI phosphorelates the γ-chain by tyrosine kinase in the Src family, creating a tandem phosphotyrosine motif that is recognized by the Syk, tyrosine kinase and activation of phospholipase Cγ2 (PLC γ2) (52). Hydrolysis of phosphatidylinositol-4 and kinase C leads to thromboxane A2 (TXA2) production and degranulation (53). Exocytosis of platelet α and dense granules release an array of stored agonists which triggers rapid platelet activation in a large scale via “inside out” signalling (54).

Most of the platelet agonists activate platelets by interacting with G coupled receptors on platelet surface. Agonists bind 5-biphosphate (PI-4,5-P\textsubscript{2}) by PLC γ2 and produces 1,4,5,-inositol triphosphate (1,4,5-IP\textsubscript{3}) and diacylglycerol, thus, raising the cytosolic Ca\textsuperscript{2+} concentrations and activating proteins with glycoprotein surface receptors causing conformational shape change and activation the G proteins (53). Activation of Rho family members via G\textsubscript{12} causes shape change and rearrangement of actin cytoskeleton in platelets. The G\textsubscript{4} mediated activation of phospholipase Cβ increases cytosolic Ca2+ and activation of phospholipase A\textsubscript{2} and protein kinase C. Phospholipase A\textsubscript{2} further hydrolyses arachidonic acid (AA) to produce thromboxane A2 (TXA2) causing active recruitment of platelets to the growing thrombus (51, 53).
1.3.3 Aggregation

Thrombin plays a significant role in maintaining the haemostatic and coagulatory functions. High affinity binding of thrombin with GP Ib platelet receptor contributes to platelet activation and induces the release of TXA2 (48). Further release of these agonists by autocrine or paracrine-positive feedback loop amplifies the response of platelets to stimulation. Activation of G\textsubscript{i} family members on platelets suppress PGI\textsubscript{2} mediated adenylyl cyclase stimulation, thus arresting cyclic AMP production, which can inhibit generalized platelet response towards an agonist (51). Tissue factor (TF) produced from the monocytes and endothelial cells initiate further production of thrombin which activates platelets by cleaving protease activated receptors PAR-1 & PAR-4 and converts fibrinogen to fibrin. Fibrin binds with activated GP IIb/IIIa receptors and facilitates subsequent clot retraction by platelet cytoskeleton linkages that assist in clot consolidation (53).

1.4 Haemostasis

The process of platelet plug formation at the site of injury is referred to primary hemostasis. In a vascular breach the endothelial cell lining is broken, releasing TF and factor VIIa, followed by the release of von Willebrand factor (vWF) onto the negatively charged subendothelial structures (55). The platelets circulating in close contact with the vessel wall adhere to the sub-endothelium via vWF, which triggers platelet adhesion and activation followed by the release of platelet granules and interaction between platelets called aggregation (56). Furthermore, platelet activation leads to the activation of various surface phospholipids which accelerate the process of fibrin formation and coagulation (55).

The coagulation cascade can be divided into three different pathways, namely the intrinsic pathway, the extrinsic pathway and the common pathway (57). The extrinsic
pathway is initiated by the release of tissue factor from the monocytes and damaged endothelial walls which activates factor VII to VIIa. The factor VIIa activates factor X to Xa which is the primary activator common pathway (57). Factor Xa in the presence of activated factor Va converts prothrombin to active thrombin. The intrinsic pathway in initiated by the activation of Factor XII leading to the production of thrombin as seen in Figure1.

The extrinsic pathway is quickly inhibited by the production of tissue factor pathway inhibitor, the sole purpose of extrinsic pathway it to initiate the coagulation cascade. The common pathway is known to amplify the cascade through thrombin produced from the extrinsic pathway (58). Once activated, thrombin is known to stimulate the production tenase complex (IXaVIIIa complex; factor X activating factor) and prothrombinase complex (VaXa complex; prothrombin activating factor), thus generating large amounts of thrombin called “thrombin burst”(59). The thrombin produced cleaves fibrinogen to fibrin monomers, which is then polymerized into fibrin fibers followed by the activation of factor XIII to XIIIa which along with calcium, stabilizes the fibrin polymer by cross linking the fibrin in-between platelets and forms the platelet plug (58). Increased thrombin production enhances platelet aggregation and platelet to platelet interaction.
1.5 Role of free radicals in platelet function

Free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) act as signalling molecules in several biological processes such as angiogenesis, cell proliferation, immunity, gene expression and apoptosis (61, 62). Changes in the redox balance by production of intracellular reactive oxygen species (ROS) in platelets are known to play a key role in platelet activation and aggregation (53, 63-66). Several studies have reported that platelet activity and fate are strictly controlled through mechanisms involving the formation of ROS and redox reactions (62, 64). Specific research surrounding the generation of platelet free radicals have defined the role of ROS in several physiological mechanisms and pathological diseases associated with platelets. Platelet free radicals such as superoxide anions (O$_2^•$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radicals (OH) are produced in response to stimulation of platelet by specific agonists such
as collagen and thrombin (67-69). Like many other cell in the body, platelet-derived ROS can have several different enzymatic sources, such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX), phosphatidylinositol 3-kinase (PI3K), cyclooxygenase (COX), protein kinase C (PKC), xanthine oxidase (XO) and cytochromes (Cyts) (70, 71). Generation of platelet-derived ROS is dependent on the type of agonist used to stimulate the receptors on the platelet surface for activation. For example, soluble agonists such as adenine diphosphate (ADP) that stimulate platelet activation via the G protein coupled receptors fail to induce intracellular ROS production, indicating that the production of ROS in platelets is linked to the receptors (72).

Platelet isoform of NADPH oxidase (NOX) is considered as one of the important enzymes associated with ROS generation in platelets stimulated using agonists such as collagen and thrombin (69, 71). Stimulation of GPVI receptors (using agonists such as collagen, convulxin and collagen related peptide (CRP)) and the PAR receptors (using thrombin) were shown to generate intracellular ROS through the NOX pathway (69, 71, 72). NOX is a highly conserved complex of enzymes that exist in multiple isoforms. Till date, seven different isoforms of NOX enzymes have been identified in mammalian cells, with NOX1 and NOX2 confirmed as present in human platelets (71, 73, 74). Both NOX1 and NOX2 are involved in ROS production and platelet activation. The NOX complex is constituted by cytosolic –localised (gp40phox, p47phox, p67phox) and membrane (p22phox, gp91phox) subunits (61, 74). NOX2 mediated free radical generation is triggered by the localization of p47phox to the membrane that brings with the activator subunit p67phox and small gp40phox subunit into contact with NOX2 (74). The subsequent interaction of GTPase RAC with NOX2 and activator subunit p67phox activates the complex and generates O2•− by transferring an electron from NADPH in the cytosol to oxygen in the extracellular space (74). NOX1 mediated generation of superoxide requires the activation of novel cytosolic subunits NOXO1 (NOX organiser 1: a homologue of p47phox subunit)
and NOXA1 (NOX organiser 1: a homologue of p67phox subunit) along with membrane subunit p22phox (74).

In a study done by J. F. Arthur and colleagues it was observed that, intracellular ROS generation in platelets is agonist specific and are generated when platelets are stimulated via the collagen receptor GPVI/FcRγ or immunoglobulin Fc receptor. The generation of ROS in platelets involves two distinct phases, an initial burst that is independent of the spleen tyrosine kinase (Syk) signalling pathway, followed by a second phase that is Syk pathway dependent. Generation of ROS via the collagen receptor GPVI/FcRγ involves linking the receptors to NOX2 by tumour necrosis factor associated factor (TRAF4) (72). Furthermore, an increase in ROS production was observed in procoagulant platelets suggesting a the synergistic effects of G protein-coupled receptors and ITAM receptors of FcRγ and FcγRIIa for ROS generation (72). NOX mediated H₂O₂ generation play key role in collagen induced platelet aggregation and thrombosis. Oxidative inactivation of SHP-2 by H₂O₂ produced from collagen stimulation leads to tyrosine phosphorylation of Syk, Vav1 and Btk in the LAT signalling complex, which promotes the PLCγ2 induced IP3 action of Ca²⁺ channels, which subsequently leads to an increase in cytosolic Ca²⁺, platelet aggregation and thrombosis (67). H₂O₂ is also known to play a significant role in production of TXA₂ from the COX-1 pathway and in the production of F2-isoP by oxidising AA. Both ligands TXA₂ and F2-isoP are known to activate platelets (75).

Inhibition of platelet ROS using various compounds such as antioxidants has shown to alter platelet function. Superoxide dismutase (SOD) and catalase are potent endogenous antioxidants that are known to scavenge free radicals (64, 76). Pre-incubating platelets with SOD and catalase have shown to significantly reduce αIIβ3 activation and P-selectin release, highlighting the role of ROS generation in platelet function (64). Furthermore, NOX1 knockout in mice caused a defect in GPCR mediated platelet
activation, while NOX2 knockout mice experienced defect in both GPCR and GPVI mediated platelet activation (77). In humans, X-linked chronic granulomatous disease (CGD) is associated with NOX2 deficiency. Platelets from patients with CGD showed significantly less ROS production and CD40L expression in response to agonist’s collagen and AA (78). CD40L is released from activated platelet and is known to have proinflammatory and prothrombotic properties. Although lower level of free radicals are known to play an important role as secondary messengers in the signal transduction process, elevated levels of ROS is known to be associated with increased platelet activation, atherogenesis and subsequent atherothrombotic complications.

1.6 Role of Platelets in atherogenesis and atherothrombosis

Platelets are multi-functional anuclear cell fragments that prevent blood loss by mediating primary and secondary hemostasis. Under physiological conditions, circulating platelets are activated in the presence of tissue trauma to arrest hemorrhage, which requires rapid cellular activation and adhesion of platelets to the exposed intravascular surface. Platelet activation occurs in a well synchronized series of events that involves the release of various agonists from intracellular granules and activation glycoproteins on their surface (79). Activation of platelets results in their degranulation and release of multiple cytokines (CD40L, IL-1β, P selectin), chemokines (epithelial neutrophil factor 78, platelet factor 4) and adhesive/coagulatory factors (fibrinogen, vWF, factor V, factor XI, plasminogen and tissue factor) (79, 80). The secretion of platelet agonists, ADP, thromboxane A2 and thrombin from the platelet dense granules allows the thrombus to grow by ensuring the activation and accumulation of nearby platelets and fibrin formation.

Atherogenesis is the process of forming fatty streaks or lipid masses in the arterial wall, through a series of steps such as adhesion and migration of leucocytes to the
endothelial cell surface and into the sub-endothelial space; lipid peroxidation and foam cell formation, which then leads to the formation of atherosclerotic lesions (80, 81). Increased platelet activation is known to play a key role in atherogenisis and accelerating the process of atherosclerosis. Atherogenesis is considered as one of the first steps in the formation of atherosclerosis (81). Growing evidence suggests that activated platelets play a central role in initiation and progression of atherogeneis (80). When activated, platelets are known to interact with circulating leucocytes and the endothelial cells. Once adherent to the endothelial cell surface, platelets provide a sticky surface for circulating leucocytes. Several chemoattractants including CD40L, IL-1β and P-selectin released from degranulated platelets induce inflammation by attracting monocytes to the site of activation (82). Interactions between platelets and leucocytes is initiaally mediated via the engagement of platelet surface P-selectin with leucocyte surface P-selectin glycoprotein ligand-1 (PSGL-1), followed by expression of tissue factor and binding of the Mac-1 (CD11b/CD18, αMβ2) on leucocytes to the GP1bα on the platelet surface. P-selectin expressed on platelet surface promotes the ligation of leucocyte PGSL-1, which then activates leucocyte β2 integrins such as Mac-1 and LFA-1, which are necessary for platelet leucocyte adhesion. Leucocyte β2 is known to engage with several platelet receptors such as GP1bα, JAM-3, ICAM-2 and αIIbβ3. Release of chemokines such as RANTES, platelet factor 4 and matrix degradation enzymes (e.g. MMP 2, 9 from platelets) at the site of atherogenesis facilitate monocyte differentiation into macrophages, which then release 12,15-lipoxygenase, leading to metabolism of arachidonic acid (80, 82). Uptake of oxLDL by macrophages and formation of foam cells is considered an important step in the pathogenesis of atherosclerosis (82). Platelets during their interactions with monocytes were shown to donate cholesterol to monocytes, based on which it was concluded that oxLDL-mediated platelet aggregation induce monocyte migration and foam cell formation (83). Additionally, Daub and colleagues have
identified platelet dense granules containing oxLDL in normal healthy individuals (84). OxLDL and platelets can therefore initiate atherogenesis by inducing foam cell formation, inhibiting endothelial cell regeneration and promoting vascular inflammation within the vascular wall (84). These processes which are regarded as key events in early atherogenesis, play a crucial role by stimulating a cascade of pathological events that can promote the development of atherosclerotic lesion in an otherwise normal artery.

Atherosclerosis can be considered as an inflammatory disease, characterised by the presence of arterial lesions or plaques that have been infiltrated by macrophages, foam cells and lipids. Erosion or rupture of these unstable atherosclerotic lesion can trigger the formation of intravascular platelet rich thrombus that can occlude the blood vessel. In diseased arteries with atherosclerotic lesions or a disrupted endothelial cell lining, exposure of adhesion molecules such as collagen may lead to thrombotic occlusion via platelet hyperactivity (79). In humans, evidence from the atherosclerotic lesions suggest recurrent ulceration or minor fissuring of the plaques, which results in frequent deposition of platelets, accumulation of monocytes and incorporation of microthrombi (85). Resealing of these ulcerations along with the deposited cells further contribute to the development and progression of atherosclerotic lesion (85, 86).

Adhesion of platelets to the exposed subendothelium of a damaged or dysfunctional endothelium, is considered as the first step in the formation atherothrombosis. The extracellular matrix of the subendothelium contains a variety of adhesion proteins such as collagen fibrils, laminin, fibronectin, thrombospindin and Von Willibrand factor (vWF) (48). The exposed collagen fibrils tend to associate with circulating vWF, which play a key role in recruiting platelets via a tethering mechanism. Once bound to the subendothelial collagen, the vWF macromolecule unfolds itself, exposing linear array of A1 domains that binds to the α-chain of GP1b of the GP Ib/IX/V receptor complex (48, 87). However, the platelet adhesion from vWF-dependent
interactions have a fast dissociation rate resulting in platelet rolling and translocation. Adhesion receptors GPVI and $\alpha_2\beta_1$ on these platelets engage with the exposed subendothelial collagen resulting in firm platelet adhesion and aggregation (87). Interactions between the collagen receptor GPVI and subendothelial collagen results in the activation of a cascade of intracellular signalling pathways, that induce calcium mobilization, cytoskeletal reorganization, activation fibrinogen receptor GP IIb/IIIa and release of soluble agonists such as ADP and $\text{TXA}_2$ (51, 87). Soluble agonists such as ADP and $\text{TXA}_2$ released form the dense granules of the activated platelets act in an autocrine and paracrine manner to amplify platelet activation at the site by engaging G protein coupled receptors (P2Y$_1$ and P2Y$_{12}$) and thromboxane receptors TP$\alpha$ and TP$\beta$ (88). Release of tissue factor from the damaged endothelial cells and monocytes accelerates the thrombin generation by forming a complex with coagulation factor FVII(a) of the extrinsic pathway (89). Thrombin is known to stimulate platelet activation through proteolytic cleavage of PAR receptors. Furthermore, thrombin production is central to the generation of fibrin polymers, which are essential in forming a stable clot. This mechanism of platelet activation and release of agonists along with the release and activation of coagulation factors, at the site of damaged vessel wall creates a vicious cycle, which leads to the development of a thrombus with the blood vessel.
Figure 2. Pathogenesis of ROS induced atherosclerosis.

Steps 1-4 Progression of atherosclerotic plaque 1) Increased production of oxidized low density lipoprotein (oxLDL) by ROS induces endothelial dysfunction causing inflammation and recruitment of macrophages; 2) phagocytosis of oxLDL by macrophages; 3) release of inflammatory cytokines and MPO; MPO in the presence of H$_2$O$_2$ and Cl$^-$ forms HOCl, which can induce oxidative modifications to LDL,
induce endothelial dysfunction and further produce ROS; 4) Foam cell apoptosis and cell debris, promote the evolution of atherosclerotic lesion;

**Steps 5-8** Process of platelet activation and thrombus formation 5) Platelet activation due to elevated inflammation, ROS and endothelial dysfunction leads to platelet degranulation and production of other oxidants; O$_2^•$ reacts with NO to produce ONOO$^-$ increasing platelet aggregation and endothelial dysfunction; H$_2$O$_2$ oxidises AA and forms 8-iso-PGF$_{2α}$; 6) increased intravascular inflammation, elevated CRP, AGEs production and ROS increase endothelial dysfunction by scavenging eNOS and NO; 7) Agonists from degranulated activated platelets induce platelet aggregation and activate coagulation cascade, thus forming a solid clot i.e. thrombus; 8) rupture of endothelial cell lining at atherosclerotic lesion exposes collagen and vWF causing the thrombus to bind the site recruit circulating platelets and block the blood vessel.

AA, arachidonic acid; ADP, adenine diphosphate; AGEs, advanced glycation-end products; C$^{2+}$, calcium; CD40L, CD40 ligand; CRP, C-reactive protein; Cl$^-$, chloride; eNOS, endothelial nitric oxide synthase; FIB, fibrinogen; H$_2$O$_2$, hydrogen peroxide; HOCl, hypochlorous acid; IL-1β, interleukin 1β; IL-6, interleukin 6; IL-10, interleukin 10; IL-12, interleukin 12; LDL, low density lipoprotein; LMP, leukocyte microparticles M-CSF, macrophage colony-stimulating factor; MMP, matrix metalloproteins; MPO, myeloperoxidase; NO, nitric oxide; O$_2^•$, superoxide anion; ONOO$^-$, peroxynitrite; oxLDL, oxidised low density lipoprotein; ROS, reactive oxygen species; TF, tissue factor; TGF β, transforming growth factor β; TNF, tumour necrosis factor.
1.7 Risk factors for thrombosis

Elevated body mass index (BMI), glucose concentrations, total cholesterol, hypertension, alcohol and tobacco use are also considered major risk factors of CVD and metabolic disorders (90, 91). Auto-oxidation of glucose is believed to increase oxidative stress status by increasing ROS production (92-94). Elevated oxidative stress in metabolic disorders, especially uncontrolled type 2 diabetes, can significantly increase the risk of cardiovascular complications. Excess ROS production in diabetic conditions further elevates the risk of platelet hyperactivity by various mechanisms including: (i) increasing the production of F2-isoprostane (8-epiprostaglandin F2α) which can amplify platelet response to agonists, (ii) reduced endothelial nitric oxide synthase (eNOS) activity and nitric oxide (NO) production (iii) increasing platelet receptor signalling due to increased oxidative stress (92, 95-97).

Presence of co-morbidities such as metabolic syndrome and T2DM were shown to be positively associated with an increased risk of having cardiovascular complications such as atherothombosis (92, 98-100). Individuals with type 2 diabetes are known have a significantly increased risk (two- to four fold increased risk compared to general population) of suffering from cardiac and cerebrovascular complications (101). This increase is often associated with alterations in the individual’s haemostatic and thrombotic parameters. Individuals with type 2 diabetes are often non-responsive to existing anti-platelet therapies such as aspirin (101, 102). Furthermore, aspirin has failed in the primary prevention of cardiovascular events in individual with low to high risk of CVD (101, 102). Several studies have failed to show the possible beneficial effects of aspirin in patients with type 2 diabetes when compared with general population (103). Initially, elevated oxidative stress along with lower antioxidant defences were believed to be a likely explanation for non-responsiveness to aspirin treatment. However, it is
believed that aspirin non-responsive in individuals with type 2 diabetes was due to a combination of several mechanisms such as individual compliance, reduced platelet sensitivity due to factors such as increased platelet turnover and cyclooxygenase-1 polymorphisms. Platelet COX-2: The inducible COX isoform, COX-2 contributes to the production of TXA2 during times of stress such as inflammation (104). Due to a difference in the sequence homology between COX-1 and COX-2, COX-2 is approximately 170 times less sensitive to the action of aspirin (105).

Endothelial cells can greatly influence platelet activity by activating the L-arginine/nitrergic oxide, arachidonic acid and endothelial ecto-adenosine diphosphate (ecto-ADPase) pathways (79, 106). Hence, the initiation of platelet activation in circulating blood can greatly depend on the function of the endothelial cell lining/vascular surface. Various studies show that ROS are key modulators of platelet reactivity, which initially cause endothelial damage and dysfunction. Excess production and accumulation of intracellular NADH increases in conditions such as type 2 diabetes and metabolic syndrome, via the mitochondrial proton gradient, thus transferring single electrons to oxygen and forming ROS (100). Generation of intracellular ROS such as O2•- and H2O2 can selectively regulate the signalling pathways that are involved in platelet adhesion, activation and aggregation (63-65). Conditions such as obesity, metabolic syndrome and diabetes are associated with increased platelet intracellular ROS production and therefore are associated with an increased risk of thrombosis (76, 107, 108). Platelet adhesion (a primary reversible state) under physiological conditions disaggregates naturally via the actions of NO (109). However, under conditions of oxidative stress, activated platelets aggregate and initiate the formation of intravascular thrombus by promoting inflammation, free radical production and endothelial dysfunction at the site of activation. Superoxide anion (O2•-) and H2O2 interact with NO forming peroxynitrite and mobilize AA respectively, thus creating a positive feedback loop of platelet activation (108, 109).
Furthermore, increased production of peroxynitrite induces lipid peroxidation and production of F2 isomers (isoprostanes) of arachidonic acid (110). These mechanisms are believed to accelerate the rate of atherosclerosis and atherothrombosis development and are clearly activated in individuals with prothrombotic conditions such as diabetes, metabolic syndrome and hypertension. With a population that has been shown to increasingly become non-responsive to current antiplatelet therapies, finding a new alternative that can inhibit platelet hyperactivity via multiple mechanisms is essential.

Antioxidants, which inhibit platelet hyper-activation, are products of renewed interest due to their antithrombotic potential. Individuals with GS, whom possess mildly elevated circulating bilirubin, also have reduced levels of soluble P-selectin, CD 40 ligand (CD40L), inflammation and have improved endothelial function, which together indicate reduced thrombotic risk and hyper-coagulability (111-116). Despite this, the mechanisms to explain protection from CVD in GS are clearly multifactorial, and exist beyond bilirubin’s commonly accepted antioxidant potential. For example, several reports of the relationship between bilirubin on cholesterol/lipid metabolism and immuno-modulatory function indicate new areas of investigation of great significance, in the context of cardiovascular protection (18, 45, 117, 118). Understanding these mechanisms could play a crucial role in explaining the role of UCB in reducing the incidence of CVD in GS (17, 22, 39, 117, 119-121). This review summarises the relationship between UCB, GS and several cardiovascular risk factors that can directly influence platelet function and activation, which can alleviate the risk of intravascular thrombosis. This information will assist in fully appreciating the multifactorial potential of bilirubin as a CVD protectant, providing defence against mortality, by modifying platelet activation in vivo. The antioxidant properties of bilirubin are generally underappreciated, however, these properties could explain its platelet inhibitory effects. Literature over the past few decades
have reported a negative association between consumption of antioxidant rich diet and the risk of cardiovascular events (122-138).

1.8 Antioxidants in the prevention of atherothrombotic events

Many observational and human as well as animal interventional studies have previously shown the ability of natural antioxidants obtained from various plant and animal sources, to reduce the risk of cardiovascular events such as atherothrombosis (11, 122). For example, individuals following a Mediterranean diet had significantly reduced probability of dying from cardiovascular complications (126, 128). Mediterranean diet is rich in fruits, vegetables, legumes, fish and low fat dairy products. Evidence shows that adhering to Mediterranean diet increases antioxidant capacity and decreases oxidised LDL cholesterol, thereby benefitting the cardiovascular system (139). Similarly, a lower incidence of cardiac events and overall mortality rate was reported in myocardial infarction survivors who were on a diet rich in fruit and vegetables (130). A similar trend was also observed in individuals who consumed fish 2-4 times a week, showing up to 38% reduction in CVD risk (140). Mezzano and colleagues have reported longer bleeding times in individuals on a Mediterranean diet compared to individuals on a high fat diet (141). In addition, Dyerberg and Bang observed reduced ADP induced platelet aggregation and longer bleeding times in Eskimos (142). It is believed that the n-3 fatty acids from diet rich in fish is responsible for observed antithrombotic effect (143). The n-3 polyunsaturated fatty acids of the fish oil, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are considered as beneficial antioxidants that hold antiplatelet potential (132, 143). Several studies have reported that EPA and DHA supplementation was associated with reduced platelet activity aggregation in healthy, diabetic and hypertensive individuals (132, 134, 143). Furthermore, 12 month
supplementation of n-3 fatty acids in acute myocardial infarction survivors was shown to be associated with significantly reduced levels of adhesion molecule CD40L (133).

Phytochemicals such as anthocyanins and polyphenols from various plant sources have also shown to have shown to inhibit platelet activity are currently being considered to possibly reduce the risk of cardiac events (122). Several dietary interventional studies have shown that fruits such as grapes, berries, green tea and cocoa that are rich in polyphenols, hold anti-platelet activity (123-125, 129, 131, 144). Carnevale et al., have shown that polyphenol rich dark chocolate reduces platelet derived ROS production and platelet 8-ISO-prostaglandin production by inhibiting NOX2 in smokers (135). Similarly, consumption of flavonal rich chocolate was shown to improve endothelial function and decreased platelet adhesion after 2 hours of ingestion in patients with congestive heart failure (136). In addition, berry consumption inhibited ADP induced platelet activity in individuals with more than one CVD risk factors (hypertension, hyperglycaemia, hyperlipidaemia) (137).

Vitamins are another group of antioxidants that was widely researched for their cardio protective benefits. Vitamin E supplementation for 6 weeks was shown to inhibit thrombin induced platelet aggregation in hypercholesteremic patients and increase platelet NO production in individuals with T2DM (138, 145). Furthermore, vitamin E was shown to exert anticoagulant effect as it was associated with reducing the incidence of venous thromboembolism in women (146). Similarly, oral administration of vitamin C inhibited ADP induced platelet aggregation in healthy subjects and increased the anti-aggregatory effects of glycerol trinitrate and sodium nitroprusside in patients with chronic heart failure (147, 148).

However, there have been some antioxidant based clinical trials showing no beneficial effects of antioxidants on cardiovascular health (149). A meta-analysis of trials involving vitamin E supplementation shows that vitamin E supplementation does not
favourably influence vascular outcomes in low or high risk patients (149, 150). On the other hand, vitamin supplementation was found to significantly increase the risk of haemorrhagic stroke in male smokers (149). It was hypothesised that the alpha-tocopherol and beta carotene supplementation may increase the risk of intracerebral haemorrhage due to their antiplatelet effects (151). These results from clinical trials involving antioxidant supplementation necessitate the need to further examine the effectiveness of other antioxidant that could target different mechanisms of CVD development due to oxidative stress. Unconjugated bilirubin is a yellow coloured pigment produced within the body. Unconjugated bilirubin is believed to have antioxidant properties and mildly elevated levels of UCB have been associated with reduced incidence of CVD, most probably due its antioxidant effect on endothelial function, lipid profile and inflammation.

1.9 Bilirubin: structure and metabolism

Unconjugated bilirubin is derived from cells of the reticulo-endothelial system, as a result of heme catabolism in mammals. Approximately 3.8 mg/kg or 250-300 mg of UCB is produced and excreted daily in adults (152). The formation of bilirubin involves enzymatic oxidative heme ring-opening at the α carbon bridge of heme by heme oxygenase (hmox). Two hmox genes are documented (hmox1 and 2), with hmox1 being inducible and hmox2 constitutively expressed. Heme catabolism results in biliverdin IXα production and the release of iron (Fe^{2+}) and carbon monoxide (CO; fig. 2). In the presence of cytosolic biliverdin reductase, biliverdin is reduced to UCB, which then diffuses into blood and binds to albumin (152, 153). The solute carrier anion transporter protein 1B1 (SLCO1B1, also known as OATP2) aids in the uptake of UCB at the liver, transporting it across the basolateral membrane of hepatocytes (153). Furthermore, Zucker et al. suggested that UCB uptake is dependent on both active and passive processes (154). The functional role of SLCOs is yet to be fully understood, however,
mutations in SLCO1B1 and SLCO1B3 reduce the elimination of UCB, thereby causing idiopathic mild hyperbilirubinemia (155). Indeed, complete deficiency of SLCO1B1 and 1B3 was shown to induce conjugated hyperbilirubinemia in Rotor syndrome (155).
**Figure 3.** Synthesis and metabolism of bilirubin and conditions of impaired bilirubin metabolism. The symbol ⚫ refers to conditions associated with impaired bilirubin excretion, causing mild to marked increase in circulating bilirubin concentrations, depending on the existing condition. BDG, bilirubin diglucuronide; BMG, bilirubin monoglucuronide; BV, biliverdin; BVR, biliverdin reductase; CB, conjugated bilirubin; CO, carbon monoxide; Fe^{2+}, iron; HSA, human serum albumin; Hmox, Heme oxygenase; MRP2, multidrug related resistance protein 2; SLCO1B1, solute carrier anion transporter protein 1B1; UCB, unconjugated bilirubin, UGT1A1, uridine diphosphate glucuronosyltransferase 1A1.
Bilirubin conjugation takes place within hepatocytes and the intestinal epithelium by uridine diphosphate glucuronyltransferase (UGT1A1) (1, 156). Congenital gene mutations (insertion of TA repeats into the promoter region of the human UGT1A1 gene located on chromosome 2q37) can reduce the frequency and accuracy of gene transcription initiation, causing 70% reduction in hepatic UGT1A1 activity (1). Reduced UGT1A1 activity contributes to increased circulating UCB concentrations, which can lead to a diagnosis of GS. The esterification of one or two molecules of glucuronic acid by UGT1A1 to UCB forms a water-soluble form of bilirubin i.e. mono- and diglucuronides (BMGs and BDGs). These molecules are commonly referred to as conjugated bilirubin (CB) and can be excreted into the bile canaliculus by the canalicular membrane transporter multidrug resistance related protein 2 (MRP2), also referred to as ABCC2 (152). Excretion of bilirubin and its conjugates, thereby regulating the bilirubin concentration within the hepatocyte, is a major function of MRP2. Mutations in the cMOAT gene, which is responsible for the transcription of MRP2, can lead to the rare genetic condition of Dubin-Johnson syndrome (fig. 2), inducing a conjugated and unconjugated hyperbilirubinemia (157). Bile is then directed into the gastrointestinal tract where bilirubin glucuronides are first deconjugated by β-glucuronidase containing bacteria including *Escherichia coli* and *Clostridium perfringens* and then further metabolised to urobilinogens and stercobilinogen by enteric bacteria including *C. Perfringens* (158, 159). Unconjugated bilirubin and urobilinogen are reabsorbed via the enterohepatic circulation and are metabolised by the liver or excreted within the urine, with the remainder further metabolised to stercobilinogen and excreted in faeces (45).
1.10 Bilirubin as an antioxidant and possible cardio-protectant

Bilirubin possesses strong reductive potential and this antioxidant capacity is currently believed to represent the main mechanism whereby bilirubin may protect from the development of cardiovascular disease (160). Bilirubin may exert protection from cardiovascular disease by inhibiting lipid peroxidation, which is implicated in initiating and promoting atherosclerosis (160). This antioxidant activity protects from lipid and protein oxidation in numerous in vitro assay systems, however, whether bilirubin can protect from physiologically relevant models of oxidative stress, remains poorly described. Bilirubin’s antioxidant effect may be amplified when bilirubin is oxidised to biliverdin in the presence of RNOS, including peroxynitrite. Biliverdin can be chemically reduced back to bilirubin becoming available for oxidation again (161). This cycle has the potential to amplify the antioxidant potential of bilirubin up to 10000 times. For example, 10 nM of UCB protected HeLa cells from a 10,000 fold excess of H$_2$O$_2$ (162). However, a current debate exists concerning the antioxidant cycle of UCB because the amount of BV formed from UCB depends on the type of radical formed in addition to bilirubin’s physical state in solution (i.e. whether albumin bound or free in solution) (163-165). Despite these arguments, several studies have reported a robust negative association between mildly elevated bilirubin and the prevalence and severity of CVD as discussed below.

1.10.1 Low bilirubin and cardiovascular disease

A low serum bilirubin concentration (i.e. <10 μM) is an emerging predictor of current or future CVD risk with numerous studies indicating that such concentrations are associated with increased CVD risk (22, 29, 32, 166, 167). Schwertner et al. were the first to show that individuals with decreased serum bilirubin concentrations had an increased risk of developing coronary heart disease (CHD), where a 50% reduction in serum
bilirubin levels was associated with a 47% increased risk of coronary artery disease (CAD) (168). These data were further supported by Hopkins et al. who concluded that individuals with CAD had significantly lower concentrations of serum bilirubin when compared to control subjects (32). Decreased serum bilirubin concentrations were also reported in individuals with a familial history of CVD, suggesting a genetic component was implicated in reducing bilirubin concentrations (169). Lower serum bilirubin concentrations are also associated with severity of disease in individuals with CAD (36). In addition, lower baseline levels of serum bilirubin are inversely associated with increased risk of CVD in patients with familial and non-familial hypercholesterolemia (26, 37). Furthermore, a 10 year follow up study in statin treated individuals concluded that individuals with median serum bilirubin levels of 5 µM or less had an 18% increased risk of CVD events, 34% increased risk of myocardial infarction and a 33% increased risk of mortality from any cause, when compared to individuals with median bilirubin levels of 10 µM or more (26). Breimer et al. has reported a U shaped relationship between serum bilirubin concentrations and risk of ischemic heart disease (IHD) among middle aged British men, and concluded that low levels of circulating bilirubin are significantly associated with increased risk of IHD (33). Reduced serum bilirubin concentrations are also inversely associated with the risk of ischemic and all stroke types in men, but not women (166).

Endothelial dysfunction (ED), increased carotid intima-media thickness (IMD) and reduced flow mediated dilation (FMD) are reported in individuals with reduced levels of serum bilirubin (167). These effects contribute to reduced vascular reactivity and therefore are major predictors of atherosclerosis (167). Coronary artery calcification (CAC) score is an established quantitative measure of coronary artery atherosclerosis, and reduced serum bilirubin concentrations are also associated with increased risk of CAC and coronary atherosclerosis (170). A significant association between reduced
serum bilirubin concentrations and CVD risk was further explored in a Chinese population (43). Low bilirubin concentrations were associated with increased concentrations of glycated hemoglobin, Triacylglycerol, V-LDL, apolipoprotein B and reduced concentrations of HDL (43, 169). Additionally, an association between lower serum bilirubin concentrations and abnormal glucose tolerance was documented (43). These findings were further supported by Lin et al. who showed that individuals with reduced serum bilirubin concentrations had a greater prevalence of metabolic syndrome (MS) (40). Metabolic syndrome is diagnosed in the presence of three out of five risk factors i.e. abdominal obesity, elevated blood glucose, elevated blood Triacylglycerol, hypertension and/or reduced HDL (40). The presence of metabolic syndrome can significantly elevate the risk of heart disease and stroke (40). An increased incidence of CVD risk factors, events and deaths in relation to reduced serum bilirubin concentrations shows an important emerging indication for the significance of bilirubin as a physiological antioxidant and potentially as a predictor of future CVD (171).
Figure 4. Cardio-protective mechanisms of Mildly elevated bilirubin. Mildly elevated bilirubin has shown to inhibit several risk factors for CVD, while up regulating various cardio-protective components, thereby reducing inflammation, dyslipidemia and platelet hyperactivity, while upregulating endothelial function thus reducing the risk of thrombosis and CVD(18, 106, 111, 172-176). AGEs, advanced glycation-
end products; CRP, C-reactive protein; eNOS, endothelial nitric oxide synthase; HDL, high density lipoprotein; IL-2, interleukin 2; IL-6, interleukin 6; MPV, mean platelet volume; NADPH, nicotinamide adenine dinucleotide phosphate; NF-kβ, NF-kappaβ; NO, nitric oxide; PKC, protein kinase C; RAGE, receptor for advanced glycation-end products; ROS, reactive oxygen species; sdLDL, small dense low density lipoprotein; TAG, Triacylglycerol; TNFa, tumour necrosis factor α.
1.10.2 Bilirubin, lipid metabolism and oxidation

Increased UCB in GS protects lipids and LDL from copper induced oxidation, suggesting an important role for bilirubin’s antioxidant properties in protecting from atherogenesis (17, 177). Subsequent studies show that elevated UCB concentrations in vivo might have an essential physiological role as an antioxidant, inhibiting the deleterious effects of free radical damage (14-16). Individuals with GS have increased circulating antioxidant status, improving resistance to lipid oxidation in serum (17). Furthermore, previous study reports decreased oxidative stress in men with GS, indicating a dose dependent reduction in urinary excretion of 8-hydroxy-2’-deoxguanosine (oxidative stress biomarker) in relation to UCB concentration (106, 116). However, whether urine collection was conducted acutely or over a period of 24 hours, was not reported (116). Furthermore, the relationship between serum UCB and urinary 8-hydroxy-2’-deoxguanosine are not consistent with no difference in spot urinary 8-hydroxy-2’-deoxguanosine reported in a smaller cross-sectional study including men and women with GS (178). In vitro studies have shown that UCB inhibits photo-oxidation of proteins and DNA damage (179). Furthermore, the antioxidative potential of UCB in scavenging free radicals and inhibiting free radical induced apoptosis in vitro is also documented (160, 162, 176). Effective neutralization of free radicals by elevated antioxidant capacity, thereby reducing oxidative stress may inhibit the production of oxLDL in GS (176). Interestingly, Boon and colleagues reported that individuals with GS are protected against LDL and thiol oxidation, however, discovered that decreased oxLDL concentrations were strongly related to decreased LDL concentrations in GS (176).

These data argue against the importance of UCBs antioxidant effect and implicate a novel role for bilirubin in regulating lipid metabolism and therefore protecting from CVD (176). The importance of altered bilirubin metabolism in influencing sterol
metabolism is strengthened by the finding of dramatically reduced total cholesterol concentrations in the hyperbilirubinemic (UCB) Gunn rat (112, 178). Negative relationships between circulating UCB and serum lipid concentrations exist in multiple cross sectional studies, which are strongly associated with reduced oxLDL (45). Decreased LDL, Triacylglycerol and total cholesterol, in addition to elevated HDL/LDL ratios are reported in individuals with GS (112, 173, 176, 180, 181). Furthermore, Tapan and colleagues reported reduced sd-LDL and oxLDL concentrations in individuals with GS (173). Wallner et al also report a negative association between elevated UCB and protection from age-related dyslipidemia in GS (112). The results from the study suggested that, older individuals (> 30 y.o.) with GS had significantly reduced levels of total cholesterol, LDL-C, TAG and Apolipoprotein-B/Apolipoprotein-A1 (Apo-B/Apo-A1) ratio when compared with matched controls (112). Furthermore, supporting the above cross-sectional studies, a 4 year longitudinal study has concluded that total serum bilirubin is negatively associated with the incidence of future hypertriglyceridemia and MS (182). Reduced lipid concentrations in GS individuals is an interesting finding with important implications for endothelial and platelet function, as discussed in the following sections.

1.10.3 Bilirubin, inflammation and endothelial function

Free radical induced oxidation of LDL can induce inflammation, endothelial dysfunction, and platelet activation (120, 168, 183, 184). Oxidised LDL also induces inflammatory responses in macrophages, which act as a catalyst for the development of CVD (82). Circulating inflammatory mediators also play a critical role in regulating hemostatic pathways that can lead to thrombosis (185). A significant dose dependent reduction in C-reactive protein (CRP; a chronic inflammatory biomarker) is consistently reported in individuals with greater circulating total serum bilirubin, being lowest in individuals with GS (111, 112, 186, 187). Immunomodulatory effects of UCB such as
inhibition of cytokine release (IL-2, INF-γ, TNF-α), T-cell response and disruption of antigen presenting cells are also published (18). Unconjugated bilirubin also exerts cytoprotective properties by intercepting complement proteins namely C3, C1q-IgM and IgG complexes (18). Tapan et al. also reported reduced concentrations of inflammatory mediators CD40L and soluble P-selectin in GS (111). Soluble P-selectin and CD40L, released from activated platelets exhibit potent thrombotic and chemotactic effects (111). CD40L and platelet derived IL-1β induce the expression of E-selectin, ICAM and VCAM on leukocytes and endothelial cells leading to inflammation and endothelial dysfunction (188, 189). Despite a general inhibitory effect of UCB on inflammatory biomarkers it should be noted that mildly elevated UCB, may also increase cytokine release. For example, baseline UCB concentration is positively associated with whole blood IL-1β and IL-8 gene expression, but not with plasma concentrations of these cytokines (190). Interestingly, as UCB levels increase above 17.1 μM, so too does IL-1β in humans, which is supported by elevated IL-1β concentrations in hyperbilirubinemic Gunn rats versus wistar controls (190). Therefore, it is possible that mildly elevated UCB may reduce lipid concentrations, however, may act as an immuno-modulatory agent at concentrations above normal levels (i.e. > 17.1 μM). The importance of these findings in the context of human health, remains to be evaluated.

Endothelial dysfunction is associated with inflammation, increased expression adhesion molecules and platelet reactivity (191). Advanced glycation end-products (AGEs) are produced under oxidative stress conditions, particularly in diabetic individuals as a result of non-enzymatic oxidation of sugars and fatty acids in hyperglycemic conditions (122). When AGEs accumulate on the endothelial cell surface they activate receptors for AGEs (RAGE), which can increase vascular permeability and rigidity. Activation of RAGE also increases the expression of the NF-KappaB transcription factor, which stimulates cellular activation (VCAM), inflammation (release
of IL-6, TNF-α) and procoagulant activity (tissue factor and thrombomodulin) within the vasculature (122). AGEs and activated protein kinase C isoforms (PKC) inhibit endothelial cell eNOS and thus NO synthesis, which is an important regulator of vascular tone, smooth muscle cell proliferation and inhibitor of intravascular platelet aggregation (122, 191). Inhibition of protein kinase C (PKC), nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) activity and vascular smooth muscle cell (VSMC) proliferation by UCB may represent a possible mechanism involved in preventing endothelial/vascular dysfunction in animals and humans with mildly elevated UCB/total serum bilirubin (18, 192). Furthermore, in vitro experiments show that UCB prevents endothelial dysfunction through reducing inflammatory cytokine expression by inhibiting the activation of transcription factors including NF-KappaB, thereby increasing NO bioavailability and reducing expression of adhesion molecules on the endothelial surface (18, 193, 194). Reduced concentrations of AGEs have been reported in GS, suggesting another plausible mechanism for improved endothelial function (172). Unconjugated bilirubin, at physiological concentrations, promotes endothelium dependent vascular relaxation, eNOS expression, and vasodilation (183). Flow mediated dilation (reflecting endothelial cell function and NO release) is positively associated with levels of UCB in GS and normal individuals suggesting a role of UCB in maintaining endothelial function (116, 167). Moreover, a significantly reduced pulse wave velocity (PWV) was reported in individuals with GS when compared with normal control subjects (113). Pulse wave velocity is a measure of arterial stiffness, a significantly reduced PWV is associated with improved endothelial function and reduced risk of CVD (113). Increased UCB concentrations might, therefore, preserve vascular reactivity and inhibit arterial stiffening. Elevated UCB concentrations are also associated with reduced CAC score (an established quantitative measure of coronary artery atherosclerosis) and preservation of coronary artery blood flow reserve and coronary microvascular function (120, 170). Even though
mildly elevated UCB has potent antioxidative effects, in excess it can have significant deleterious, pro-oxidant effects (195). Hence, a thorough understanding of its absorption, metabolism, biodistribution and pathways of activation will be critical to revealing the therapeutic potential of this molecule in future (196).

1.11 Effect of bilirubin as an antioxidant on platelet function

In support of the role that ROS/oxidative stress play in activating platelets, antioxidants can inhibit platelet activity. Small and well controlled studies do suggest that antioxidants hold a significant anti-atherosclerotic and antithrombotic potential (122, 123, 129, 131, 150, 197-199). For example, it is clear that consumption of a mediteranean diet or diets containing fresh fruits and vegetables, rich in antioxidants, reduces the risk of IHD and cardiovascular mortality (200). Mildly hyperbilirubinemic individuals (i.e. GS), with elevated antioxidant capacity also experience significantly reduced risk of CVD (17, 34). It is therefore, possible that the antioxidant potential of bilirubin, contributes to cardiovascular protection in vivo, by influencing platelet function. However, understanding whether bilirubin influences platelet activation and understanding the mechanisms of action is an underexplored area of great interest with particular relevance to CVD prevention.

1.11.1 The hypothesized antithrombotic potential of bilirubin

Although platelets play a key role in inducing endothelial dysfunction, thrombosis and, therefore, myocardial infarction, a dearth of knowledge exists to link bilirubin to platelet function and prevention of CVD. Previously, it has been shown that mildly elevated levels of unconjugated bilirubin have shown to negate several thrombotic risk markers such as endothelial dysfunction, inflammation and oxidative stress. The following paragraphs summarise possible mechanisms by which mildly elevated levels
of bilirubin could minimise platelet hyperactivity, thereby reducing the risk of thrombosis and ischemic event.
**Figure 5.** Possible mechanism of inhibition of platelet activation/aggregation by bilirubin. Figure adapted from Santhakumar et al. (122). Bilirubin can scavenge $\text{H}_2\text{O}_2$ (14, 162, 201); bilirubin could reduce arachidonic acid induced platelet activation by preventing $\text{H}_2\text{O}_2$ mediated arachidonic acid mobilization; bilirubin may block collagen receptor function; bilirubin inhibits the production of platelet derived thromboplastin and PF3, which can catalyse the production of thrombin from prothrombin; bilirubin prevents complement mediated platelet activation by inhibiting several complement proteins; reduced levels of circulating P-selectin was reported in GS, also administration of bilirubin inhibits thrombus formation by inhibiting P-selectin release. $\text{H}_2\text{O}_2$, hydrogen peroxide; PF3, platelet factor 3; vWF, von willebrand factor.
1.11.2 Bilirubin and platelet function

To date no publications have reported whether the improved antioxidant capacity in individuals with mildly elevated bilirubin, is associated with improved platelet function in vivo. Suvansri and colleagues were the first to demonstrate that elevated levels of UCB can affect platelet function and clot formation (202). Increased UCB levels inhibited platelet derived thromboplastin, ADP induced platelet factor 3 (PF3) activation and clot retraction in platelets obtained from hyperbilirubinemic neonates (202). Thromboplastin and PF3 play a key role in the formation of fibrin and with platelets form a stable clot by binding with GPIIb-IIIa receptors on the platelet surface (54, 122, 202). Hence, inhibition of platelet derived thromboplastin and PF3 in hyperbilirubinemic conditions suggests that, UCB at greater concentrations can significantly reduce the quality of clot formation by affecting platelet function. Similarly, elevated serum bilirubin levels were associated with increased clotting time in one month old infants (203). Recent study by Naveen Kumar et al., has demonstrated the pro-apoptotic effect of UCB on platelets. However, they also observed a dose dependent inhibition of collagen induced platelet aggregation by UCB in washed platelets in vitro (204). It was observed that inhibition of platelet adhesion by UCB could by both receptor mediated or agonist mediated. This was shown by performing two separate experiments, firstly stimulating UCB treated washed platelet using collagen and then in second experiment UCB treated collagen was used to stimulate the platelets for adhesion testing. Furthermore, UCB levels greater than 50 µM have shown a dose dependent increase in ROS generation in platelets, with mitochondria being the primary source for ROS generation. It was also observed that elevated levels of UCB were associated with dose dependent increase in Ca\(^{2+}\) levels, reduced G6PDH activity and increased GGT activity in vitro and in vivo in humans (204). However, other studies indicate contradictory results and suggest that platelets can be hyper-activated when treated with UCB (205, 206). The variation between the results of these studies is likely
due to the difference in UCB concentrations tested, UCB solubility limitations and methods used to assess platelet aggregation (205, 206). Some of the techniques applied are no longer used in practice, because they induce platelet aggregation (205, 206). For example, EDTA and anticoagulant citrate dextrose (ACD-A) were used as anticoagulants from which platelets were isolated (205, 206). Platelet aggregation studies require blood to be collected in sodium citrate anti-coagulant tubes, because both EDTA and ACD-A influence platelet aggregation results (207, 208). Furthermore, use of chloride solutions, especially CaCl$_2$, is used to initiate the intrinsic and the extrinsic pathway of the coagulation cascade and also lead to the formation of bilirubin calcium salts (209). Additionally, bilirubin induced platelet hyperactivity according to these studies, is greatly influenced by the presence of albumin. Previous reports show that bilirubin, in the presence of albumin, has no effect on platelet activity (205, 206). Only minimal aggregation was reported by Maurer et al. when UCB at concentrations $\geq$ 10mg/dL (i.e., $\geq$ 172 $\mu$M) was added to PRP, questioning whether UCB, at physiological concentrations, has the potential to induce platelet aggregation within the vascular compartment (206).

Lindenblatt et al. initially discovered the antithrombotic potential of bilirubin in vivo in a mouse model (174). Administration of 10 $\mu$M bilirubin in addition to hemin (a hmxox1 inducer) significantly prolonged ferric chloride induced intravascular thrombus formation and reduced platelet bound P-selectin levels (174). This study indicated that bilirubin may represent a key effector of the antioxidative HO-1 enzyme and that its antithrombotic efficacy was associated with reduced platelet P-selectin expression (174). P-selectin is an adhesion molecule released from $\alpha$ granules of activated platelets and weibel-Palade bodies of activated endothelial cells, mediating the adhesion of platelets and inflammatory cells to the endothelial surface (114, 210). Tapan et al. have also shown decreased serum concentration of circulating CD40 ligand (CD40L) and soluble P-selectin in GS (111). P-selectin is a marker of platelet activation, and when released from
activated platelets, can further attract inflammatory cells, induce endothelial cell activation and platelet aggregation (114, 115). Soluble P-selectin and Platelet bound P-selectin plays an essential role in thrombus formation by facilitating the development of large stable platelet-leukocyte aggregates (211). Although, elevated levels of soluble P-selectin is associated with an increased the risk of deep vein thrombosis and it is often confused as a marker for increased platelet activation, previously studies have shown that increased soluble P-selection is an independent CVD risk marker and is not a marker for platelet hyperactivity (212, 213). Ridker et al. demonstrated that healthy individuals with elevated concentrations of soluble P-selectin may have increased risk of suffering a future cardiovascular event (214). Moreover, inhibition of circulating platelet bound P-selectin is associated with a reduced risk of thrombus formation and is evolving as a therapeutic target for treatment and prevention of future CVD (215). These observations suggest that GS may be less likely to experience thrombosis due to an indirect effect of bilirubin reducing P-selectin expression/concentrations. CD40L is a co-stimulatory molecule expressed in platelets and lymphocytes and is essential for platelet aggregation and thrombus stability (115). Furthermore, activated CD40L on platelets accelerates atherosclerotic development via the induction of adhesion molecules, tissue factor, matrix metalloproteinases and pro-inflammatory cytokines (216, 217). Therefore, reduced CD40L levels in GS further strengthens the possibility of a thrombotic resistant phenotype in individuals with mild hyperbilirubinemia.

1.11.3 Bilirubin, platelet and hematological characteristics

Research studies comparing GS and control subjects have unveiled key differences in hematological parameters in individuals with mild hyperbilirubinemia. Buyukasik et al. initially reported a positive correlation between hemoglobin concentration, hematocrit and red cell count and serum UCB concentrations, the levels of which even though elevated, were within the reference ranges in GS (218). These data suggest that increased
heme catabolism may be partly responsible for increasing UCB concentrations *in vivo* (219, 220). In support of this conclusion, free heme, iron and carboxy hemoglobin concentrations were positively correlated to circulating UCB in another study and were greater in GS individuals (187). These data are further supported by elevated bilirubin concentrations in men compared to women, with men possessing greater total hemoglobin and hematocrit values (221, 222). Similarly, Rodrigues *et al.* reported elevated concentrations of total hemoglobin, mean cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC) in GS (222). Interestingly, reduced platelet counts were reported in GS; a statistically significant and concentration dependent decrease in platelet count was observed in relation to total bilirubin concentrations (222). However, platelet numbers even though reduced, the difference was but not clinically significant (222). Similarly, reduced mean platelet volume (MPV) was reported in individuals with GS (175). Platelets were shown to undergo morphological change and cell lysis, when incubated with exogenous bilirubin, supporting the above observation, that it may be influencing platelet volume and numbers in neonates and adults with elevated levels of bilirubin (202). Further supporting the above findings, Naveen kumar *et al.*, have shown that elevated levels of UCB was associated with platelet apoptosis through p38 mediated mitochondrial dysfunction. Mitogen-activated protein kinase in platelets is known to be associated with mitochondrial dysfunctional mediated apoptosis. UCB at elevated concentrations was shown to induce mitochondrial dysfunction in platelets by increasing the phosphorylation p38 MAP kinase and p53. Furthermore, an upregulation in pro-apoptotic protein Bax (Bcl-2 family) and downregulation of pro-survival proteins phosphor Bad and Bcl-2 (204). However, the observed pro-apoptotic effect of UCB was negated in the presence of albumin, suggesting that UCB at physiological concentrations as seen in normal healthy and GS individuals may not induce thrombocytopenia. This can validated by studies showing very little or no difference in platelet numbers in GS when
compared with normal healthy (175, 204, 222). However, if elevated levels of total serum bilirubin (both UCB and conjugated bilirubin) have similar effect on platelet numbers and volume is yet to be determined. Together, these observations suggest a reduction in the quantitative potential of platelet aggregation and activation. Furthermore, smaller platelets are less responsive to activation and aggregation due to a reduced number of glycoprotein receptors on their surface (223).

1.11.4 Inflammation and platelet activation

Inflammation and activated complement system are known to induce platelet adhesion and activation. Bilirubin has been shown to reduce the activity of complement proteins. Complement mediated platelet activation and granule release can also lead to increased P-selectin expression (185, 224). Several complement proteins could increase the thrombogenicity of blood by activating platelets and coagulation proteins (224). For example, C3a triggers platelet adhesion and activation. C3a formation also contributes to the formation of C3b and C5a and C5b. The C5b-9 complex, also known as Membrane Attack Complex (MAC), induces platelet membrane polarization, shape change and platelet micro particle release (224). In addition, C1q multimers synergistically activate platelets and induce granule release by binding with FCγRIIa receptors and activating GPIIb-IIIa receptors on the platelet surface (fig. 4) (224). Complement proteins C5 and C5a induce tissue factor expression from leukocytes (185, 224). Tissue factor is a potent stimulant of coagulation and is responsible for activation of the external pathway of the coagulation cascade, while C5a induces the expression of plasminogen activator inhibitor-1 (PAI-1) on mast cells and basophils (224). Plasminogen is an important proteolytic enzyme that plays a key role in endogenous fibrinolysis or breakdown of the clot and is activated by tissue plasminogen activator (t-PA) (225). Expression of PAI-1 under pro-coagulant conditions can elevate the risk of thrombosis, due to inhibition of t-PA, thereby preventing the disintegration of thrombus and leading to the formation of
embolus (225). Circulating emboli can then occlude arteries, restricting the blood flow and causing myocardial infarction and ischemic stroke (225).

Unconjugated bilirubin inhibits complement mediated inflammation via interacting with C1q, C1s, and C3 as demonstrated in vitro and in animal models (18). Furthermore, biliverdin, a precursor of bilirubin, significantly reduces C5a receptor gene and protein expression in macrophages, in addition to inhibiting the C-EDTA step which requires the participation of a range of complement proteins i.e., C3, C5, C6, C7, C8, and C9 suggesting a possible mechanism of reducing complement mediated platelet activation (226, 227).

In vitro testing indicates that IL-6 when incubated with platelets can increase agonist induced platelet activation and thromboxane B2 and B thromboglobulin secretion from platelets in a dose dependent manner (228). Furthermore, IL-6 at concentrations representative of chronic inflammation, caused increased hypercoagulability of whole blood and has shown to induce platelet hyperactivation and spreading (229). Significantly reduced plasma IL-6 has been reported in GS, indicating another possible mechanism of reduced platelet activity in vivo (112). Reduced concentrations of CRP, as seen in GS, might also reduce thrombus formation by inhibiting platelet adhesion to the endothelial surface (112). Increased endothelial CRP modulates autocrine prothrombotic activity within the vasculature (230). Increased secretion of endothelial cell derived CRP is associated with elevated platelet adhesion under flow conditions in a “cone and plate” flow system (230). Increased platelet adhesion was modulated by increased expression of P-selection, which symbolises platelet activation and granule release (230). Furthermore, circulating CRP can induce thrombotic complications by stimulating monocytes to express tissue factor (231). These anti-inflammatory and complement inhibitory properties of UCB and its precursors appear to have great potential to reduce the risk of thrombosis by reducing platelet activation, coagulation system activation, whilst
preserving the fibrinolytic system. Activation of platelet receptors such as FCγRIIa is known to be associated with platelet-derived ROS generation and granule release. Production of ROS by activated platelets and granule secretion can create a positive feedback loop of undesirable platelet activation, P-selectin over-expression and thrombus formation. Moreover, complement can also activate the coagulation system and inhibit the fibrinolytic system, increasing the risk of thrombosis (72, 224).

1.11.5 Free radical induced platelet activation

The antioxidative potential of UCB, namely the scavenging of H$_2$O$_2$ and O$_2$•−, has the potential to reduce platelet activation by mechanisms including: i) inhibiting AA mobilization; ii) preventing O$_2$•− mediated NO scavenging; and iii) reduced isoprostane production (4, 14, 79, 162, 201, 232). Furthermore, collagen induced platelet activation induces H$_2$O$_2$ release, which creates a positive feedback loop of agonist mediated platelet aggregation by activation of phopholipase C and arachidonic acid metabolism (122).

Inhibition of free radical induced endothelial dysfunction in GS could importantly reduce the risk of thrombosis (106). For example, inhibition of free radical induced NO scavenging by UCB is considered a possible mechanism in preventing hypertension and improving flow mediated dilitation (46). However, endothelial derived NO also inhibits intravascular platelet aggregation by activation of the cGMP pathway (79). Furthermore, improved vascular reactivity can greatly reduce shear stress induced platelet activation and adhesion, which might be relevant to reducing platelet activation in hyperbilirbinemic individuals (233). Therefore, the antioxidant effects of bilirubin, improving endothelial dependent vasodilatation, may also function to inhibit platelet activation. Furthermore, free radical induced production of oxLDL can significantly increase the risk of thrombosis. CD 36 located on surface of platelets and leukocytes is a receptor for oxLDL (234). Oxidised LDL, even at low concentrations, induces platelet activation by stimulating the phospholipase A$_2$/cyclooxygenase-dependent pathway in platelets
isolated from healthy individuals *in vitro* (235). Furthermore, a study demonstrated that oxLDL can induce rapid platelet aggregation and shape change via the tyrosine kinase-dependent pathway (234). Reduced levels of oxLDL and increased serum resistance to lipid oxidation due to elevated total antioxidant capacity, could conceivably reduce the risk of oxLDL induced platelet activation in GS (17, 24, 176). Therefore, it is possible that elevated UCB with its hypothesized antioxidant properties may directly and indirectly inhibit platelet hyper-activation and initiation of the coagulation cascade. However, further comparative studies of platelet activation testing using traditional aggregometry and advanced flow cytometry, along with coagulation profile studies under normal and oxidative stress conditions between age, sex and BMI matched normal and GS individuals is necessary to further understand the possible role of UCB in preventing platelet activation *in vivo*.

In conclusion, Gilbert’s syndrome has become a topic of great interest due to this syndrome apparently protecting against chronic inflammatory disorders including CVD, cancer and all-cause mortality. The mechanisms inducing protection within GS are clearly multi-factorial and are yet to be fully explored. Revealing these mechanisms will be of great significance because they will improve our understanding of the physiological importance of bilirubin and implicate it in inducing CVD protection. Unconjugated bilirubin is emerging as a physiologically essential endogenous antioxidant with anti-inflammatory, lipid-modifying, cytoprotective and hypothesized anti-thrombotic functions all potentially contributing to disease resistance and reduced all-cause mortality. Platelet hyper-activation and increased hemostatic function are established predictors of CVD. This chapter of thesis links bilirubin to inhibition platelet activation leading to reduced rate of thrombus formation. Therefore, we hope that documenting these platelet inhibitory effects will lead to further exploration of the mechanisms of action and encourage the manipulation of heme catabolism/bilirubin biology as
therapeutic targets for CVD protection in the general population. Manipulating bilirubin synthesis, metabolism, reabsorption and clearance could therefore represent possible therapeutic avenues to reduce the risk of thrombotic complications in normal and at risk populations (196, 236).
2. Aims & Hypothesis
The studies conducted as a part of this thesis were based on the hypothesis that the reduced incidence of CVD related complications in GS individuals could be due to mildly elevated levels of UCB that may cause reduced platelet and haemostatic activity. The role of increased oxidative stress in CVD has been clearly demonstrated by several studies. The oxidant effect of free radical on risk factors such as platelet activity, endothelial dysfunction, vascular inflammation and lipid profile has been well documented in the literature. Research has shown that intravascular platelet activation in the initial stages of atherogenesis promotes the formation of leucocyte-platelet aggregates, lipid peroxidation and foam cell formation, thereby accelerating the development of atherosclerosis. In addition, platelet hyperactivity and hypercoagubility are one of primary causes for late stage CVD related complications such as atherothrombosis. Current anti-platelet therapy such as aspirin, in some cases, has been ineffective in the primary prevention of CVD in individual with low and high risk of CVD. Moreover, individuals with T2DM were often non-responsive or resistant to the current antiplatelet therapy.

Elevated oxidative stress and increased ROS are key modulators of platelet activation, increased inflammation and endothelial dysfunction. By targeting multiple pathways oxidative stress can accelerate the process of atherosclerosis, thereby, increasing the risk of atherothrombosis. Antioxidants have become a topic of great interest due to their hypothesised anti-platelet effects. Several studies have shown the positive effects of antioxidant supplementation. However, there have been some antioxidant based clinical trials showing no beneficial effects of antioxidants on cardiovascular health. Furthermore, several studies have shown the ineffectiveness of aspirin in preventing primary cardiovascular events. Therefore, the need to find an effective antioxidant that can target multiple mechanisms of CVD development is crucial to reduce the incidence of cardiovascular events. Unconjugated bilirubin acts as an
endogenous antioxidant. Researchers have hypothesised numerous mechanisms by which elevated UCB may be associated with reduced CVD risk in GS, however, an exact mechanism is yet to be known. Furthermore, the effect of mildly elevated UCB in GS on platelet activity is still untouched and has to be explored. Therefore, the hypothesis for this thesis is based on the theory that UCB, as an antioxidant, may reduce platelet activity, thereby reducing the risk of late stage atherothrombotic complications, as seen in GS individuals. The anti-atherothrombotic effects of UCB, if observed, could play a key role in developing a therapeutic target to inhibit multiple risk factors of CVD including intravascular thrombus formation. Hence the aim of this research thesis is to understand the mechanisms by which mildly elevated UCB may impart cardiovascular benefits and unveil, if it can negatively influence platelet activity, haemostasis, inflammation and lipid profile, thereby reducing the risk of late stage atherothrombotic complications in GS.

The aim of the first *in vitro* study was to evaluate the effect of UCB on platelet aggregation, coagulation time and lipid profile, at concentrations seen in GS. The hypothesis for this *in vitro* study was that, mildly elevated UCB at concentration 35 μM may impart favourable effects by inhibiting platelet aggregation, increasing coagulation time and improving lipid profile. As studies previously have shown elevated UCB may increase coagulation time and improve lipid profile.

The aim of the second study was to i) investigate if elevated levels of UCB in GS may alter platelet activation, aggregation, haemostatic function, lipid status and inflammation, in GS individuals when compared with normal healthy individuals ii) to validate the previous *in vitro* findings that mildly elevated UCB may inhibit collagen induced platelet aggregation. Based on the results from the first study, the hypothesis of the second study was that mildly elevated UCB in GS may be associated with reduced platelet activation/ de-granulation and platelet aggregation, along with reduced inflammation and improved lipid profile.
Increased oxidative stress is associated with platelet hyperactivity and increased risk of thrombosis. Current anti-platelet therapy and some antioxidants have shown to be ineffective in conditions such as T2DM that are associated with increased oxidative stress due to a multitude of reasons. Acute intense exercise by sedentary population has been associated with an elevated risk of atherothrombotic complications, due to reasons such increased oxidative stress. Hence, the aim of the third study was to evaluate if elevated UCB in sedentary GS individuals may impart similar positive effects as seen previously. Mildly elevated levels of UCB in GS was shown to associated with increased antioxidant capacity along with improved resistance to serum oxidation, based on which the hypothesis of the third study is that mildly elevated UCB may provide these sedentary GS individuals improved resistance against platelet hyper-activation, de-granulation/ P-selectin expression along with improved lipid profile and lower inflammation markers after acute intense exercise.

The aim of the final study was to investigate if the observed favourable effects of UCB influence the genetic expression of P-selectin and eNOS. Based on the results of the first three studies of this thesis and the literature, it can be hypothesised that UCB may down regulate the genetic expression of SELP (P-selectin; adhesion molecule released from platelets and endothelial cells) while upregulating the genetic expression of nitric oxide synthase (eNOS; responsible for the production of nitric oxide, physiological platelet activation inhibitor).
3. Materials and Methods
3.1 Research Ethics

The human *ex-vivo* study protocols chapters 5, 6 and 7 were conducted after obtaining approval from Griffith University Human research Ethics Committee (HREC), Griffith University, Gold Coast, Queensland, Australia (GU Protocol No: MSC/12/12HREC). The blood collection for preliminary *in-vitro* analysis (chapters 4 and 5) were approved by Griffith University HREC (GU protocol No: MSC/13/11/HREC).

Please see Appendix 1 for further details.

3.2 Volunteer recruitment

Normal healthy volunteers between 18-65 years of age, with or without Gilbert’s syndrome (GS) were recruited from the general population using advertisement flyers (see Appendix 2), mass emails and word of mouth from Griffith University and the local community. A plain language statement (see Appendix 3) was provided to all the individuals explaining the study protocol and its requirements in detail in layman’s language. Recruited individuals were carefully screened after obtaining an informed consent (see appendix 4) using a volunteer screening questionnaire (see Appendix 5) based on inclusion criteria being non-smoking individuals with no previous history of hepatitis, liver disease, heart disease and recent acute respiratory inflammation/bacterial infection. The cardiovascular risk assessment and medical history questionnaire (see Appendix 5) were also used to exclude subjects based on other parameters such as alcohol consumption, high blood pressure, BMI, musculoskeletal complications, family history of cardiovascular disease and individuals on any form of medication or antioxidant supplementation (see Appendix 6) during the two weeks prior to participation in the study. A baseline Full blood examination (FBE) and lipid profile along with glucose concentration was performed on all the volunteers to aid in the recruitment process. Volunteers with abnormal FBE results as per Royal College of Pathologists of Australasia
(RCPA) and/or abnormal lipid profile were excluded from the study (237). Allocation of individuals into the GS group was performed either based on a confirmed diagnosis of GS from a general practitioner and/or individuals with circulating UCB concentration >17.1 µM.

Table 1. Therapeutic reference adapted from Royal College of Pathologists Australasia.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Reference range</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC (x10^{12}/L)</td>
<td>3.8 - 6.5</td>
</tr>
<tr>
<td>Hb (g/L)</td>
<td>120 - 180</td>
</tr>
<tr>
<td>WBC (x10^{9}/L)</td>
<td>4.0 – 11.0</td>
</tr>
<tr>
<td>Platelet count (x10^{9}/L)</td>
<td>150 - 400</td>
</tr>
</tbody>
</table>

RBC, red blood cells; Hb, hemoglobin; WBC, white blood cells.

3.3 Sample Collection and processing

3.3.1 Blood collection

Blood collections for all the studies were performed using standard venepuncture techniques after gaining professional phlebotomy competence (Certificate training in Phlebotomy practices, Gold Coast hospital, Queensland, Australia). On each occasion around 20-25 mL of blood was drawn from the median cubital vein using a 21-gauge 1 inch sterile needle (Thermo Medical Corporation, Australia). A light sterile tourniquet was used to palpate the vein, followed by cleaning and disinfecting the area of venepuncture with an alcohol swab (BD biosciences, Australia). The amount of blood collected (22mL) poses no medical risks. A 20 mL disposable syringe (BD biosciences, Australia) or closed blood drawing system was employed for blood collection. The blood sample was dispensed into tri-sodium citrate (3.8%; BD biosciences, Australia) for platelet aggregation, flow cytometry and coagulation studies followed by collection into serum separation tube (SST; BD biosciences, Australia) was collected to perform routine
biochemical analysis (total bilirubin, lipid profile, glucose, uric acid, Triacylglycerol). A tri-potassium ethylene diamine tetraacetic acid (EDTA 1.8 mg/ml; Vacuette Greiner Bio- one, Australia) vacutainers for full blood examination (FBE), while blood sample collected into the PAXgene Blood RNA Tube (PreAnalytiX, BD biosciences, Australia) was used to perform DNA/RNA extraction. The particular sequence of blood collection was followed at all times to prevent the artefactual activation of platelets for platelet flow cytometry and aggregation studies. Instead of discarding the first 2mls of drawn whole blood, it was aliquoted into SST tubes, the following sequence of blood collection was in accordance with order of collection set by RCPA.

3.3.2 Sample processing

The blood samples collected were processed as per the type of sample needed to conduct individual tests. The tri sodium citrate tubes with the blood sample were centrifuged at 180 × g for 10 min at 37°C using a swing rotor centrifuge (Thermo Scientific, Australia). Minimal braking was applied during the centrifugation process to prevent any artifactual activation of platelets. Post centrifugation, a dense pale yellow fluid was obtained which is the platelet rich plasma (PRP). Pasteur pipettes were used to separate the PRP from the whole blood, which was then transferred into a sterile 1.5 mL eppendorf tube and was used for platelet aggregation assay, for detailed description, refer to section 3.5.

Following the isolation of PRP from the tri sodium citrate tubes, the blood tubes were again centrifuged at 2000 × g for 10 min at 37°C, along with the SST tubes. A clear solution called platelet poor plasma (PPP) was obtained from the tri sodium citrate tubes, which was used as blank for the platelet aggregation assay and also to perform coagulation profile analysis. The serum obtained from the SST tubes was aliquoted into 1.5 mL sterile eppendorf tubes and was used to analyse biochemical parameters. All the PAXgene blood tubes were mixed well after blood collection followed by storing them
at room temperature for 2 hours. The RNA was isolated from the PAXgene blood tubes using the PAXgene RNA blood kit (Qiagen, PreAnalytiX, Australia) and following the manual RNA extraction protocol provided from Qiagen (see Appendix 7). The extracted RNA was then converted into cDNA followed by using it to analyse gene expression.

3.4 Full Blood Examination

Full blood examination was performed on the whole blood samples obtained from the volunteers involved in all the study protocols. Full blood examination is an important means to identify individuals with pre-existing disease conditions that can influence the integrity of analysis e.g.: thrombocytopenia. The blood sample from the EDTA tube was used to perform a full blood examination within a period of 10-15 minutes of blood collection. Beckman Coulter AcT™ 5Diff CP hematology analyzer (Coulter Corporation, Miami, Florida, USA) was used for analyzing the whole blood. Quality control (QC) and calibration of the instrument were validated prior to analysis (Beckman Coulter™, Miami, Florida, USA).

3.5 Platelet Aggregometry

Platelets are a multifunctional blood component that plays a crucial role in maintaining haemostasis, clot retraction, inflammation, thrombosis and promotion of atherosclerosis. Platelet aggregometry on platelet rich plasma (PRP) for decades has been the gold standard procedure to assess platelet activity in clinical and research settings. This procedure has proven to be quite useful detecting several platelet related disorders such as Bernard-soulier syndrome and Glanzmann thrombasthenia. Platelet aggregation studies can also be used to analyse several factors in plasma which can regulate platelet activity. In terms of research platelet aggregometry has been of great importance in elucidating the mechanisms of platelet-drug interactions.
In the past, literature has shown that increased platelet activity is associated with the development of occlusive arterial thrombi which could lead to coronary heart disease or myocardial infarction. Platelet aggregometry could play a key role in detecting individuals with elevated pro-thrombotic tendencies. However, this could acquire importance only when the factors influencing the test results could be understood. Platelet aggregation is usually measured by recording the percentage change in the optical density i.e., transmission of light passing through the cuvette with platelet rich plasma (PRP) stimulated by an agonist for aggregation under conditions of constant stirring and temperature. Standardized concentrations of agonists such as ADP, collagen and arachidonic acid (Helena laboratory, Beaumont Texas, USA) were added to PRP to mimic *in vivo* activation of platelets.

Platelet aggregation studies were conducted using 4 channel Helena AggRam platelet aggregometer (Helena laboratory, Beaumont Texas, USA). Platelet aggregation studies were carried out using platelet rich plasma (PRP), obtained by centrifuging the tri-sodium citrate (3.8%) blood tubes. Platelet poor plasma (PPP), 250 μL pipetted into the cuvette and run in all four channels, acts as blank for the instrument. Similar volume of PRP was added to the cuvettes and incubated at 37°C for 1-3 minute. Standardized volumes simulating physiological concentrations of platelet stimulating agents or agonists were added to the respective well. A dose response curve determining the optimal concentration required to stimulate maximal platelet aggregation was performed prior to undertaking any work for this thesis. The agonist concentrations used in this thesis were similar to or lower than the agonist concentrations used by several other antioxidant studies that have been published previously (123, 124, 131, 199, 238-246). The capacity of platelets to aggregate is determined by amount of platelet aggregation induced by a known amount of agonist added to PRP. The absorbance of the un-stimulated PRP with free floating platelets, mixed with the aggregation reagent represents 0% aggregation and
the absorbance of PPP with no floating platelets represents 100% aggregation. As platelets aggregate, the number of floating platelets decreases, causing a reduction in light absorption by PRP. The change in light absorption is recorded as optic’s analog signals which are converted into digital signals and represented as an aggregation curve and maximum aggregation percentage which was recorded for 6 minutes.

Table 2. Concentrations of various agonist used for the studies and their respective pathways of platelet activation.

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Concentration used</th>
<th>Platelet activation pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>5μM</td>
<td>P2Y1 and P2Y12</td>
</tr>
<tr>
<td>Collagen</td>
<td>1μg/mL</td>
<td>GP VI and GP Ia/IIa</td>
</tr>
<tr>
<td>Arachidonic acid (AA)</td>
<td>200μg/mL</td>
<td>COX-1 pathway</td>
</tr>
</tbody>
</table>

COX-1, cyclooxygenase-1; GP, glycoprotein.
Figure 6. Showing an example platelet aggregation report developed by the Helena aggregometer.
3.6 Flow cytometry

Flow cytometry using whole blood is considered as one of the most powerful technique for investigating platelet function. This technique allows rapid detection of platelet surface antigens on a larger number of cells in “real time” in a specific and sensitive manner. Hence analysis of platelet surface receptors using flow cytometry was employed in understanding mechanisms of vascular thrombosis leading to cardio vascular diseases (247). Whole blood flow cytometry was first introduced by Shattil et al and is considered as a major development in evaluating platelet function, since this technique minimises artefactual activation of platelets in vitro (248). Before analysing the sample the whole blood was diluted to reduce the formation of platelet aggregates followed by the addition of antigen specific monoclonal antibodies conjugated with a fluorophore. The hydro-dynamically focused stream of cells passes through a single beam of laser light of a specific wave length at the rate of 1000 – 10,000 cells per minute. The fluorescence detectors aimed at the flow chamber measure the light scattered by the cell and the fluorescence emitted by the excited fluorophore on the cell. The collected data is processed and presented as Forward scatter (FSC) and Side scatter (SSC). The forward represents the cell volume, while the side scatter represents the cytoplasmic complexity i.e. granularity of a cell. Because of several advantages such as volume of sample required (2 µl), increased sensitivity and decreased sample manipulation along with semi quantitative and standardization problems involved with platelet aggregometry, flow cytometry is rapidly replacing the traditional platelet aggregometry techniques.

Development of several activation dependent & independent monoclonal antibodies has allowed researchers to evaluate various platelet glycoproteins such as activation marker P-selectin and fibrinogen receptor GP IIb/IIIa. Antibodies such as CD41/CD61 bind to GP IIb/IIIa on resting platelet, while activation dependent PAC-1 antibody attaches to the fibrinogen binding site of only activated platelets exposed by
conformational shape change in GP IIb/IIIa complex (249). CD62P is also an activation dependent antibody directed towards P-selectin and confirms the release of α granules from activated platelets. These activation dependent antibodies bind specifically to activated platelets only, but not to resting platelets (250). Fluorophore’s such as Allophycocyanin (APC), Peridinin Chlorophyll protein (perCP), Fluorescein isothiocyanate (FITC), Phycoerythrin- Cy5 and phycoerthrin Texas Red (RED- 670) are commonly used to conjugate antibodies for flow cytometric analysis.

BD LSR FortessaTM Cell analyzer (Becton Dickinson and company, San Jose, CA, USA) was used to perform flow cytometric analysis. Prior to testing the flow cytometer was calibrated with BD FACSDiva software using CaliBRITE beads (Becton Dickinson and company, San Jose, CA, USA). Addition of fluorophore conjugated antibody to the CaliBRITE beads provided distinct positive and negative stained populations which allowed us to setup compensation controls automatically for the multicolour flow cytometric analysis.

3.6.1 Panel design and antibody titration

A three colour panel was designed to assess platelet activation using conventional flow cytometry. The panel comprised of fluorophore peridinin chlorophyll protein (PerCp-Cy5.5) conjugated anti-human CD42b, fluorescein isothiocyanate (FITC) conjugated anti-human PAC-1 and allophycocyanin conjugated anti-human CD62P. The following panel was specifically selected to minimise spectral overlap, as each monoclonal antibody was conjugated with a fluorophore that was excited at a different wavelength. The fluorophore FITC was excited by 488nm solid state laser and the emission was collected with a 530/30 bandpass (BP) filter. PerCp-Cy5.5 was excited with by a 532nm laser and the emission was collected using a 695/40 BP filter. APC was excited by a 633nm laser and the emission was collected using a 660/20 BP filter. The volume of antibodies used for investigating platelet activation in this thesis were
determined by performing serial titration of each antibody on unstimulated and stimulated whole blood. Platelets in the whole blood were stimulated using ADP (5µM) for 15 mins at room temperature. Optimal antibody concentration was determined using staining index, which was calculated using median fluorescence intensity of stimulated and unstimulated cells. The following formula was employed to calculate staining index and the antibody volume that with peak staining index was adopted for this thesis.

\[
\text{Staining index} = \frac{\text{(MFI of positive or stimulated cells) – (MFI of negative or unstimulated cells))}}{2 \times \text{SD of negative or unstimulated cells)}
\]

### 3.6.2 Compensation

The antibody panel designed to evaluate platelet activation required the use of multiple fluorophores, therefore signals from one fluorophore can optically interfere with other fluorophores. Compensation was performed to correct the spectral overlap or spillover values. Compensation controls were performed using BD CompBead particles, which contain both positive and negative population of beads. Each tube containing compensation controls had positive and negative beads that were stained using a single colour/antibody. In this case, 4 tubes were labelled, the first tube being unstained beads and the next three tubes specific for each fluorophore as shown in Figure 7. Using the dot plot, the PMT voltages were adjusted on the unstained control tube if the negative population was too high or on the stained controls if the beads are off scale, followed by which the population was gated (For PMT voltages, see Appendix G, Table G2). The PMT voltages for the unstained and stained controls were set before recording the single stained controls. Using positive population for the stained controls was gated using the auto-interval tool, which was further adjusted if needed. Once all the histograms were gated, the compensation was automatically calculated and saved by the BD FACSDiva software.
3.6.3 Sample preparation, staining and gating

Tri-sodium citrate (3.8%) anticoagulated blood was used to analyse platelet function. Blood samples from EDTA blood tube were not used since it could decrease the binding capacity of PAC-1 monoclonal antibody as it is pH and Ca2+ sensitive. The antibody “master mix” with appropriate fluorophore was prepared and 20 μL of antibody cocktail
was added to the diluted blood sample. Isotype controls were run along with the test to ensure that the cells are not binding to the antibodies non-specifically. Agonist ADP (5 μM) was added to induce platelet activation in the tubes labelled as test, followed by the addition of fixative 1% paraformaldehyde (PFA), to prevent secondary platelet activation and aggregation. The amount of agonist used to stimulate platelet activation was similar to the concentration of agonist used to stimulate platelets for platelet aggregation. Furthermore, the concentration of ADP used for platelet activation was 5 μM, which is similar to the concentration used to stimulate platelets for aggregation study and also other studies in literature (124, 125, 251-253).

Instead of ADP, modified tyrode’s buffer (MTB) was added to the unstimulated control tubes. Addition of fixative 1% PFA was always done after the final incubation step as it can CD62P binding to the platelets. The process of data collection using a flow cytometer is called “acquisition”. Acquisition and analysis of data was done by scatter gating. Platelets were identified using forward scatter and scatter, followed by which the platelet population was gated using platelet specific marker CD42b conjugated with PerCp-Cy5.5. The gated population was then used to identify the PAC-1 and P-selectin positive platelets in stimulated and unstimulated whole blood. Aggregated samples and sample with low platelet count can be difficult to gate. Fluorescence of activation dependent platelet makers and light scatter profile of positive population was analysed to develop results.
Table 3. Concentrations of different Antibodies used to prepare antibody master mix.

<table>
<thead>
<tr>
<th>Antibody &amp; conjugated fluorochrome</th>
<th>Reacting Antigen</th>
<th>Ratio of antibody to final volume</th>
<th>Actual volume needed to make a master mix of 1000μL</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD42b-peridinin chlorophyll protein (PerCp-Cy5.5)</td>
<td>GPIb-IX receptor</td>
<td>1:6</td>
<td>166.65μL</td>
</tr>
<tr>
<td>PAC-1-fluorescein isothiocyanate (FITC)</td>
<td>GPIIb-IIIa receptor</td>
<td>1:4</td>
<td>250μL</td>
</tr>
<tr>
<td>CD62P-allophycocyanin (APC)</td>
<td>P-selectin</td>
<td>1:3</td>
<td>330μL</td>
</tr>
<tr>
<td>Modified tyrode’s buffer (MTB)</td>
<td>N/A</td>
<td></td>
<td>253.35μL</td>
</tr>
</tbody>
</table>

Table 4. Concentrations of isotype controls used to prepare isotype control master mix.

<table>
<thead>
<tr>
<th>Isotype control</th>
<th>Ratio of isotype control to final volume</th>
<th>Actual volume needed to make a master mix of 1000μL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse IgG1, CD42b- PerCp-Cy5.5</td>
<td>1:6</td>
<td>166.6μL</td>
</tr>
<tr>
<td>Mouse IgM, PAC-1-FITC</td>
<td>1:4</td>
<td>250μL</td>
</tr>
<tr>
<td>Mouse IgG1, CD62P-APC</td>
<td>1:3</td>
<td>330μL</td>
</tr>
<tr>
<td>Modified tyrode’s buffer (MTB)</td>
<td>N/A</td>
<td>503.4μL</td>
</tr>
</tbody>
</table>
Figure 8. Step by step protocol followed in the evaluation of activation-dependant platelet surface marker expression using flow cytometer.
**Figure 9.** Flow cytometric analysis of human platelet activity. A) Platelets were identified using forward scatter and side scatter characteristics; B) platelet population was gated using platelet specific marker CD42a-perCP-CY5.5-A and characteristic laser scatter; C) unstimulated platelets showing no expression of PAC-1-FITC (negative control); D) showing increased fluorescence intensity indicating the expression of PAC-1-FITC.
Figure 10. Flow cytometric analysis of human platelet activity. A) Platelets were identified using forward scatter and side scatter characteristics; B) platelet population was gated using platelet specific marker CD42a-perCP-CY5.5-A and characteristic laser scatter; C) Platelets showing no expression of CD62P-APC (negative control); D) showing increased fluorescence intensity indicating the expression of CD62-APC.
3.7 Coagulation Studies

The coagulation studies were performed using platelet poor plasma (PPP). A 4 channel C4 coagulation analyser (Helena Laboratory, Beaumont Texas, USA) was used to assess coagulation which was calibrated using reference plasma S.A.R.P (Helena Laboratory, Beaumont Texas, USA). Quality control (QC) samples were run prior to testing using low, medium and high test controls (Helena Laboratory, Beaumont Texas, USA). Test results were recorded as the time when initial clot formation was detected in the microcuvette. All the tests and QC’s were run in duplicate and the sample mean was taken as final result for both the assays.

3.7.1 Prothrombin time (PT)

Prothrombin time (PT) was used to measure the efficacy of extrinsic pathway of the coagulation cascade. 25 µL of PPP sample was pipetted into the C4 microcuvettes and incubated at 37°C for 120 seconds in the incubation wells, followed by the addition of 50 µL of pre warmed PT reagent (thromboplastin; Helena Laboratory, Beaumont Texas, USA) to the sample. Test and the duplicate were run simultaneously.

3.7.2 Activated partial thromboplastin time (APTT)

Activated partial thromboplastin time (APTT) was used to assess the activity of intrinsic coagulation pathway. All reagents and samples were pre warmed to 37°C. Initially 25 µL of PPP sample was pipetted into the C4 microcuvettes followed by addition of 25 µL of APTT reagent (Helena Laboratory, Beaumont Texas, USA). After incubating the sample for 3 minutes at 37°C 25 µL of CaCl2 was added to generate results.
3.7.3 Fibrinogen

Fibrinogen assay is a quantitative assay to determine the concentration of fibrinogen in plasma. Citrated PPP was diluted in 1:10 ratio with Owren’s Veronal buffer (OVB; Helena Laboratory, Beaumont Texas, USA). 50 µL of diluted PPP was added into the C4 microcuvettes and incubated for 2 minutes at 37°C, followed by the addition of 25 µL of pre-equilibertaed thrombin reagent (Helena Laboratory, Beaumont Texas, USA) to generate results.

3.8 Biochemical analysis

Biochemical analysis was performed on automated Integra Cobas 400 Biochemistry Analyser (Roche Diagnostics, Switzerland). Blood sample collected into the serum separating tube (SST) was centrifuged at 2000 × g for 10 minutes at room temperature. Separated serum was collected and stored at -80°C for testing. A batch analysis of all the samples was performed by pipetting 200 µL of test sample into eppendorf tubes to determine serum concentrations of cholesterol, Triacylglycerol, uric acid, unconjugated bilirubin and glucose. A serum level of inflammation marker, C-reactive protein (CRP) was also evaluated using the same analyser.
4. Dose dependent inhibition of platelet aggregation by unconjugated bilirubin \textit{in vitro}

Adapted from:


Author contribution:

Kundur AR: Performed experimental and data analysis, prepared the manuscript

Bulmer AC: Critically reviewed the manuscript

Singh I: Experimental design and critically reviewed the manuscript
4.1 Introduction

Unconjugated bilirubin (UCB) is a yellow coloured endogenous antioxidant produced in the body as result of heme catabolism (152). According to the Royal College of Pathologists Australia (RCPA), the normal reference range for serum total bilirubin concentration in infants is < 115 µM and < 20 µm for adults, whereas unconjugated bilirubin < 16 µM is seen in normal healthy individuals. Unconjugated bilirubin at concentrations > 50 µM is associated with jaundice (254). Numerous studies have shown a negative relationship between UCB concentration and the development of cardiovascular diseases (CVD) (20, 255). This phenomenon was reported in a group of patients experiencing the relatively common disorder of Gilbert’s syndrome (GS) which is characterised by presence of mildly elevated levels of UCB (> 1 mg/dL i.e. > 17.1 µM) (22, 256). Gilbert’s syndrome patients have a decreased prevalence of CVD, including ischemic heart disease (IHD). These observations suggest that mildly elevated UCB levels might delay the process of atherosclerosis by increasing the circulating total antioxidant capacity and reducing the susceptibility of lipids to oxidation and reducing circulating cholesterol concentrations (17, 176, 257).

The antioxidant capacity of UCB is imparted by a mechanism involving donation of hydrogen atoms to scavenge secondary oxidants formed during oxidative biological processes (257). Unconjugated bilirubin is considered to play a vital role in protection against oxidative stress, and prevention of tissue damage during inflammation (24, 165, 258). Various experimental and epidemiological studies have demonstrated that low serum bilirubin levels significantly increase the risk of developing atherosclerosis (20, 255). A meta-analysis showed a reliable negative and dose response relationship between serum bilirubin concentrations and the development of atherosclerosis in men (20). A 50% decrease in serum bilirubin is associated with a 47% increased risk of developing severe CVD (259). Unconjugated bilirubin positively influences endothelial function and
reduces vascular wall thickness, vascular stenosis and coronary artery calcification which are direct markers in the prediction of CVD (170, 183, 260). These studies were successful in proving that decreased bilirubin concentration is an independent risk factor for CVD in men (261, 262). Despite these findings, very little evidence implicating bilirubin in preventing haemostasis is documented, which would provide an additional mechanism to explain reduced mortality rates from ischemic heart disease in people with elevated bilirubin.

Previously, Filiz Tiker et al. observed an negative relationship between levels of bilirubin and activated partial thromboplastin time coagulation results in one month old infants (263). These results provided an indication that increased bilirubin concentrations might perturb haemostasis, which requires activation of platelets and coagulation proteins. Platelets are a multifunctional blood component that play a crucial role in maintaining haemostasis, clot retraction, thrombosis and promotion of atherosclerosis (264). The platelet surface receptors play a key role in the formation and stabilization of the platelet clot (265). Adenosine diphosphate (ADP) and collagen are the two major platelet agonists released during blood vessel injury. These agonists rapidly stimulate platelet aggregation by activating various surface receptors and glycoproteins (265).

Increased platelet activity is clearly implicated in IHD. The causes for increased platelet reactivity are multi-factorial, including oxidative stress, which causes endothelial dysfunction, initiating platelet activation and activation of the haemostatic coagulation cascade (59, 266, 267).

There have been a number of studies showing the anti-oxidative effect of bilirubin in vitro (268). That rationale of this study is that, previously several antioxidants have shown to reduce the risk of thrombosis by inhibiting platelet activity and improving coagulation function (269). However to date no studies have been done to evaluate the effect of UCB, a potent antioxidant, on platelet aggregation and haemostatic function at concentration of
UCB, as seen in GS individuals. Hence, the aim of the following study was to show the effect of mildly elevated concentrations of UCB, equivalent to those seen in GS individuals on platelet activation and haemostatic factors.

**4.2 Methods**

**4.2.1 Participants**

Sixteen normal healthy volunteers from the general population were recruited after giving their informed consent and obtaining clearance from the Human Research Ethics Committee of Griffith University. None of the participants were on medication or taking any antioxidant supplements. Smokers and volunteers with a history of hepatitis, liver disease and recent inflammation were screened using a questionnaire and excluded from the study. Volunteers were further excluded if they possessed abnormal full blood examination (FBE) results showing red cell, leukocyte or platelet characteristics outside normal healthy reference ranges established by RCPA (RBC: 3.8-6.5 × 10^{12}/L, WBC: 4.0-11.0 × 10^9/L, platelets: 150-400 × 10^9/L).

**4.2.2 Blood collection**

Refer to Materials and methods Chapter 3, section 3.3.1 and 3.3.2. Volunteers with abnormal FBE results were excluded. Please refer to Materials and Methods Chapter 3, section 3.4 for a detailed description of FBE analysis.

**4.2.3 Bilirubin working solution**

A bilirubin stock solution was prepared by dissolving crystalline UCB 99% pure (Frontier Scientific. Inc, Logan, UT, USA) in dimethyl sulfoxide (DMSO; Sigma Aldrich, castle Hill, NSW, Australia) to obtain a final concentration of 5 mM. For the dose response experiments, stock solutions with different concentrations were prepared in order to keep the volume of the vehicle DMSO constant. The concentrations of different stock
solutions used for the dose response experiment are provided in Appendix G; Table G1. All bilirubin solutions were covered by silver foil to avoid photo oxidation. A clear yellow coloured solution was obtained, and centrifugation (4000 × g; 5 mins) confirmed the absence of bilirubin aggregation/precipitation. The solution was prepared no more than 10 minutes before addition to test samples and performing experiments.

4.2.4 Sample preparation & platelet aggregation assay

Test sample for platelet aggregation assay was prepared according to the protocol followed in Materials and Methods Chapter 2, Section 3.5. A final UCB concentration of 35 µM was attained by adding 1.25 µL of 5 mM UCB stock solution to 250 µL of PRP (test sample). The control sample was prepared by adding 1.25 µL of DMSO to 250 µL PRP. Both the samples were incubated at 37°C for 10 min in a water bath before performing the test. A third tube with only 250 µL of PRP was incubated under same circumstances to act as a baseline. Aggregation was induced by adding 6.25 µL of ADP to 243.75 µL of PRP to obtain a final concentration of 5 µM of ADP, while 2.5 µL of collagen was added to 247.5 µL of PRP to obtain a final concentration of 1 µg/mL of collagen. Platelet aggregation testing was performed at 37°C at a constant stirring speed of 600 rpm in a 4 channel Helena aggregometer (Helena Corporation, Beaumont, TX, USA).

4.2.5 Tests of coagulation

Coagulation studies were performed using platelet poor plasma (PPP). Test samples (35 µM of UCB) were prepared as above in 250 µL of PPP. All samples were incubated at 37°C for 10 min prior to the test run. A 4 channel C4 coagulation analyser (Helena Corp., USA) was used to assess coagulation which was calibrated using reference plasma S.A.R.P (Helena Corp., USA). Quality control (QC) samples were run prior to testing using low, medium and high test controls (Vital Diagnostics, Bella Vista, NSW, Australia). Test results were recorded as the time when initial clot formation was detected.
All the tests and QC’s were run in duplicate and the sample mean was taken as final result for both the assays.

4.2.5.1 Prothrombin time

Please refer to Materials and Methods chapter 3, section 3.7.1.

4.2.5.2 Activated partial thromboplastin time

Please refer to Materials and Methods chapter 3, section 3.7.2.

4.2.6 Biochemical analysis

Please refer to Materials and Methods chapter 3, section 3.8.

4.2.7 Statistical analysis

GraphPad StatMate 2.00 was used to determine the sample size and power of the study. The sample size and power calculations were made using mean platelet volume (270). A sample size of 14 in each group had 95% power to detect a difference between the means of each group, with a significance level alpha=0.05. The data obtained from the platelet aggregation studies were analysed using the repeated measures ANOVA (Bonferroni post-test) to determine the statistically significant difference between baseline, control and test samples. The coagulation and biochemical were analysed using the paired t-test results (control vs. test solutions). A p-value < 0.05 was considered significant.
4.3 Results

A total of 16 normal healthy volunteers were included in the study based on their FBE results (Table 5).

Table 5. Full blood examination Results.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Mean</th>
<th>Reference ranges</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBC ($\times 10^{12}$/L)</td>
<td>4.7</td>
<td>3.8 – 6.5</td>
</tr>
<tr>
<td>WBC ($\times 10^9$/L)</td>
<td>6.0</td>
<td>4.0 – 11.0</td>
</tr>
<tr>
<td>Hb (g/L)</td>
<td>140</td>
<td>120 – 180</td>
</tr>
<tr>
<td>Platelet Count ($\times 10^9$/L)</td>
<td>260.6</td>
<td>150 – 400</td>
</tr>
<tr>
<td>MPV (fL)</td>
<td>7.7</td>
<td>7 – 10</td>
</tr>
</tbody>
</table>

RBC, Red Blood Cells; Hb, Hemoglobin; MPV, Mean Platelet Volume.

4.3.1 Bilirubin

The mean baseline serum bilirubin concentration for the volunteers was 6.15±2.82 μM. When treated with additional UCB in-vitro plasma concentrations bilirubin increased to 35±3.95 μM (P < 0.01; Table 6). The baseline serum bilirubin-albumin molar ratio was 0.009, while post treatment with UCB the ratio was 0.05.

4.3.2 Platelet aggregation

In vitro addition of UCB indicated dose (Figure 11A) dependent inhibition of platelet aggregation induced by ADP and collagen. A clear time dependent effect on platelet aggregation also existed (Figure 11B). Using these data a final UCB dose of ~35μM and duration of 10 mins was chosen to study the potential effect of Gilbert’s syndrome on platelet aggregation. Subsequently, collagen induced platelet aggregation decreased significantly from 88.9% to 80.9% (P < 0.05; Figure 12 A) in UCB treated samples. While ADP induced platelet aggregation did not change significantly after UCB treatment (P = 0.46; Figure 12 B).
Figure 11. (A) Dose responsive effect of unconjugated bilirubin (UCB) on platelet aggregation with 10 min incubation. (B) Time responsive effect of bilirubin (UCB) on platelet aggregation at 35µM concentration of UCB.
Figure 12. (A) Effect of UCB on collagen-induced platelet aggregation. (*) Bilirubin treated samples show a statistically significant decrease in aggregation vs. both baseline and control (p < 0.05). (B) Effect of UCB on ADP induced platelet aggregation (p ¼ 0.46).
Table 6. Effect of unconjugated bilirubin (UCB) treatment on coagulation and biochemical parameters.

<table>
<thead>
<tr>
<th>Test</th>
<th>Treatment</th>
<th>Mean</th>
<th>SD</th>
<th>P value</th>
<th>RR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Coagulation Assay</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PT(S)</td>
<td>Baseline</td>
<td>13.7</td>
<td>0.52</td>
<td>0.70</td>
<td>11-15</td>
</tr>
<tr>
<td></td>
<td>UCB Treated</td>
<td>13.7</td>
<td>0.59</td>
<td>0.70</td>
<td>11-15</td>
</tr>
<tr>
<td></td>
<td>Baseline</td>
<td>41.5</td>
<td>8.35</td>
<td>0.80</td>
<td>18-45</td>
</tr>
<tr>
<td></td>
<td>UCB Treated</td>
<td>42.2</td>
<td>8.19</td>
<td>0.80</td>
<td>18-45</td>
</tr>
<tr>
<td><strong>Biochemical Assay</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol (mM)</td>
<td>Baseline</td>
<td>3.85</td>
<td>0.34</td>
<td>0.60</td>
<td>2.97-4.99</td>
</tr>
<tr>
<td></td>
<td>UCB Treated</td>
<td>3.96</td>
<td>0.71</td>
<td>0.60</td>
<td>2.97-4.99</td>
</tr>
<tr>
<td>Triacylglycerol (mM)</td>
<td>Baseline</td>
<td>0.81</td>
<td>0.23</td>
<td>0.30</td>
<td>0.59-1.19</td>
</tr>
<tr>
<td></td>
<td>UCB Treated</td>
<td>0.96</td>
<td>0.40</td>
<td>0.30</td>
<td>0.59-1.19</td>
</tr>
<tr>
<td>Total Bilirubin (μM)</td>
<td>Baseline</td>
<td>6.15</td>
<td>2.82</td>
<td>&lt; 0.01</td>
<td>&lt; 20</td>
</tr>
<tr>
<td></td>
<td>UCB Treated</td>
<td>35</td>
<td>3.95</td>
<td>&lt; 0.01</td>
<td>&gt; 17.1*</td>
</tr>
</tbody>
</table>

PT, prothrombin time; SD, standard deviation; RR- reference ranges. (* Reference ranges in GS)

4.3.3 Coagulation studies

No significant difference in coagulation time was observed in PT (P = 0.70) and APTT (P = 0.80) after addition of UCB to samples (Table 6).
4.3.4 Lipids

There were no significant changes in the concentration of cholesterol ($P = 0.60$) or Triacylglycerol ($P = 0.30$) after the addition of UCB to samples (Table 6).

4.4 Discussion

The novel finding of this study is that a mildly elevated bilirubin concentration (~35 µM) in platelet rich plasma reduces platelet activation induced by collagen. However, a similar UCB concentration did not influence ADP induced platelet aggregation. Despite these findings, bilirubin clearly inhibits collagen and ADP induced platelet aggregation in a dose dependent manner. Mildly elevated bilirubin concentrations found in Gilbert’s syndrome are associated with marked protection from cardiovascular diseases, cancer, and all-cause mortality (20, 24, 271). The effect of additional unconjugated bilirubin on the activation of platelets obtained from adult blood has not been investigated previously. These results could provide additional insight into the possible mechanisms providing protection from CVD mortality in GS.

Previous studies suggest that mildly elevated UCB provides Gilbert’s syndrome individuals with protection from CVD possibly by increasing the total antioxidant capacity of the blood (17, 257). The results from this study suggest that mildly elevated bilirubin, similar to that seen in GS, attenuates platelet activation in response to collagen stimulation in vitro. Adenosine diphosphate and collagen are two endogenous agonists that are released at the site of injury to initiate rapid activation and aggregation of platelets in order to form a haemostatic plug and minimise bleeding (272). However these agonists differ in their mechanistic pathways of activation. The current study found that UCB at ~35 µM does not inhibit ADP stimulated platelet aggregation.
Inhibition of collagen induced platelet activation by UCB provides an additional mechanism whereby mildly elevated levels of UCB could protect from mortality associated with CVD. Based on the results of this study we propose unconjugated bilirubin might inhibit the process of collagen stimulated platelet activation *in vivo*, therefore, influencing the process of thrombus formation. Collagen is the most abundant platelet aggregation stimulant present within the vessel wall which plays a key role in stabilization and localisation of a clot and also activating various collagen receptors on platelet surface (273). Glycoprotein VI is a collagen receptor located on the platelet surface that play a crucial role in the activation and aggregation of platelets (274).

Increased intravascular platelet collagen interactions in activated platelets can be induced by various factors such as oxidative or shear stress. Increased interactions between platelets can progressively occlude the blood vessel by forming platelet clumps or aggregates, thus significantly increasing the risk of various cardiovascular and thrombotic events such as myocardial infarction, stroke and IHD (273, 275). Significant inhibition of collagen induced platelet activation indicates that UCB might alter the interaction between the platelet receptors and collagen, similar to other commonly used anti platelet drugs such as revacept, which showed a similar effect on collagen induced platelet aggregation and thus might aid in reducing the risk associated with intravascular thrombosis (276). Aspirin is an anti-platelet drug used to reduce the risk of atherothombosis by inhibiting arachidonic acid and collagen induced platelet aggregation (277). However, research has shown that consumption of low dose aspirin may be ineffective in preventing primary cardiovascular events in diabetes (103). The reported decrease in collagen induced platelet aggregation by UCB does not compare to aspirin induced inhibition of collagen induced platelet aggregation. However, UCB being a potent antioxidant, has shown to inhibit several cardiovascular risk factors such as inflammation (reduced CRP levels reported in individuals with mild hyperbilirubinemia),
improved flow mediated dilation (a marker of improved endothelial function) and scavenging free radicals such as H$_2$O$_2$ (14, 17, 120, 186, 232, 278). By targeting multiple pathways of CVD development, mildly elevated UCB may delay the onset of primary cardiovascular events, as observed in GS individuals (29, 32, 36, 118).

Recent study by Naveen Kumar et al., has demonstrated the pro-apoptotic effect of UCB on platelets. However, they also observed a dose dependent inhibition of collagen induced platelet aggregation by UCB in washed platelets in vitro (180). It was observed that inhibition of platelet adhesion by UCB could by both receptor mediated and agonist mediated. This was shown by performing two separate experiments, firstly stimulating UCB treated washed platelet using collagen and then in second experiment UCB treated collagen was used to stimulate the platelets for adhesion testing. Furthermore, UCB levels greater than 50 µM have shown a dose dependent increase in ROS generation in platelets, with mitochondria being the primary source for ROS generation. It was also observed that elevated levels of UCB were associated with dose dependent increase in Ca2+ levels, reduced G6PDH activity and increased GGT activity in vitro and in vivo in humans. Furthermore, UCB at elevated concentrations was shown to induce mitochondrial dysfunction in platelets by increasing the phosphorylation p38 MAP kinase and p53. Furthermore, an upregulation in pro-apoptotic protein Bax (Bcl-2 family) and downregulation of pro-survival proteins phosphor Bad and Bcl-2 (204). However, the observed pro-apoptotic effect of UCB was negated in the presence of albumin, suggesting that UCB at physiological concentrations as seen in normal healthy and GS individuals may not induce thrombocytopenia. The bilirubin to albumin ratio within this study approximated (normal <0.01; GS<0.1), suggesting that the observed anti-platelet effect of UCB may not be due to platelet apoptosis. This can validated by studies showing very little or no difference in platelet numbers in GS when compared with normal healthy (175, 222). Although platelet viability assay was not performed, platelet numbers were
monitored using ACT5Diff FBE analyser to evaluate the effect of UCB on platelet count at different concentrations, which showed no significant difference.

The coagulation tests prothrombin time (PT) and activated partial thromboplastin time (APTT) were performed to study the effect of UCB on the extrinsic and intrinsic pathways of the coagulation cascade. Hyper-activation of coagulation proteins often lead to arterial or venous thromboembolism (VTE) that are significantly associated with the development of CVD (279). In this study no significant effect of UCB on coagulation was found. However, Filiz Tiker et al. has shown that serum bilirubin at concentrations greater than 100 µM is associated with prolonged APTT in one month old infants. This suggests that bilirubin might have an antithrombotic effect at very high concentrations mediated by the intrinsic pathway of the coagulation cascade in vivo (263). However the author did not discuss the possible mechanism, whereby of unconjugated bilirubin could affect coagulation time and proteins.

A final concentration of approximately 35 µM of UCB was selected for two reasons; 1) to allow comparison of its effect to individuals with Gilbert’s syndrome and 2) explore the possible effects of exogenous administration of bilirubin at physiological concentrations to be assessed (196). Prior to selecting, an initial dose response analysis tested the effect of UCB on platelet aggregation at various concentrations was completed which showed a dose responsive effect of UCB on ADP and collagen induced platelet aggregation (Figure 11A, 11B). A time response analysis was performed at 35 µM revealing 30 min to have greatest inhibition. This particular time period was not selected due to potential confounding influence of bilirubin oxidation. In the system, UCB on 5 min incubation showed insufficient inhibition of aggregation in the presence of both ADP and collagen, hence 10 mins was chosen for the final assay. The incubation temperature at 37°C was decided upon due to it representing physiological body temperature and its use in previously published trials (280).
Interestingly, these results are in opposition to those presented in previous studies, which showed hyper activation of platelets upon exposure to increased levels of UCB (205, 281). This variation can be attributed to the difference in procedures and concentrations of unconjugated bilirubin used. For example, both previously cited papers isolated and washed platelets prior to experimentation, which activates these cells. Such techniques are no longer used in the field due to this important limitation. Furthermore, these articles used sodium hydroxide (NaOH) as the solvent to dissolve UCB. Bilirubin is much more stable in DMSO, as used in this study, whereas bilirubin undergoes auto-oxidation in basic solution (282). Furthermore platelet suspensions were incubated with potassium chloride and calcium chloride prior to aggregation investigation (205, 281). Chloride solutions, particularly CaCl$_2$ initiates the coagulation cascade and the formation of bilirubin calcium salts, which could give false positive results of platelet aggregation. Also platelet suspensions were obtained from blood collected in anticoagulant EDTA tubes, which could have further confounded platelet aggregation results (281). Evidence presented by Moiny et al., suggesting that unconjugated bilirubin results in platelet hyper activity, as discussed by the authors, were influenced by the UCB to serum albumin ratio. These authors clearly stated when unconjugated bilirubin concentration remained below the albumin concentration, no activation was observed. The bilirubin to albumin ratio within this study approximated (normal <0.01; GS<0.1) and therefore, hyperactivation of platelets was never observed. Indeed, the results of this study are entirely novel and evaluated the effect of mildly elevated, but physiological, concentrations of UCB in their natural physiological milieu without altering the albumin concentration.

In conclusion, results obtained from this study are the first to show that mildly elevated unconjugated bilirubin concentration attenuates platelet aggregation induced by collagen. This finding is important, because it may explain an additional mechanism contributing to reduced CVD mortality in individuals with GS. These data suggest the
possible use of mechanisms to mildly elevate UCB levels in vivo, for therapeutic means (196).
5. Mildly elevated unconjugated bilirubin is associated with reduced platelet activation, P-selectin expression and inflammation in Gilbert’s syndrome

Adapted from:


Author contribution:

Kundur AR: Performed experimental and data analysis, volunteer recruitment and prepared the manuscript

Santhakumar AB: Assistance with experimental procedures

Bulmer AC, Singh I: Experimental design and critically reviewed the manuscript
5.1 Introduction

Platelets are key components of blood that restrict blood loss during injury by initiating a sequential process of adhesion, activation and aggregation (79). Agonists released at the site of injury activate platelet surface glycoproteins followed by the release of various stimulants and cytokines from platelet granules, leading to the formation of a stable clot in the presence of fibrinogen (79). However, platelet hyperactivation and aggregation significantly increases the risk of cardiovascular disease (CVD) by accelerating the rate of atherosclerosis and thrombus formation (79, 283). Endothelial dysfunction, oxidised low density lipoprotein (oxLDL) and inflammation also increase platelet activity thereby accelerating the risk of thrombosis and subsequent CVD (79, 84).

Mild unconjugated hyperbilirubinemia in individuals with Gilbert’s syndrome (GS) is associated with protection against the development of cardiovascular diseases (CVD) and associated mortality (20-22). Previous reports indicate an increased antioxidant capacity in individuals with GS, suggesting a mechanism by which mildly elevated bilirubin might protect GS individuals from CVD (17). Gilbert’s syndrome is frequently associated with mutation in the promoter region of the human uridine diphosphate glucuronosyltransferase (UGT1A1) gene causing a ~70% decrease in UGT1A1 enzyme activity decreasing bilirubin conjugation and thus mildly elevating serum unconjugated bilirubin levels (>17 µM; UCB) in GS, when compared with general population (approximately 10 µM) (17). Bilirubin is normally bound to plasma albumin in blood, however, extreme accumulation of UCB above the albumin binding capacity (>335 µM) is considered toxic, because ‘free’ UCB diffuses into tissues causing jaundice and kernicterus (neuronal toxicity) (19). Despite this, UCB concentrations remain well below toxic levels in adults with GS, suggesting it may possess physiological importance, particularly within the vascular compartment (46).
Antioxidant therapies remain a topic of great interest and debate, in the context of preventing free radical damage and potentially disease risk (122). Elevated circulating antioxidant status in individuals with GS is associated with protection against development of CVD and cancer (24, 117). A recent study by Horsfall et al. concluded that individuals with GS/elevated bilirubin levels have a 50% reduced risk of all-cause mortality (27). Furthermore, reduced circulating bilirubin concentrations are associated with increased risk of CVD and death (284). Recent reports demonstrate that GS individuals are also less likely to present with cardiovascular risk factors. For example, GS subjects possess improved endothelial function, reduced C-reactive protein (CRP), low density lipoprotein (LDL), oxLDL and increased high density lipoprotein (HDL) cholesterol and that these parameters are related to increased bilirubin concentrations (45, 176, 284). However, very few investigations have explored the effects of UCB on platelets and the association between mildly elevated UCB and platelet function, which might contribute to protection from CVD and related mortality in GS (284). *In vitro* studies show that UCB at concentrations observed in GS (35 μM) inhibits collagen induced platelet activation (270). Collagen is a platelet stimulant released at the site of damaged endothelium and under pre-thrombotic conditions collagen induced platelet activity can significantly increase the risk of thrombosis (270). The rational of this study is to determine whether existing *in vitro* observations, indicating inhibition of platelet activation, are reflected in a population with mildly elevated UCB such as GS, when compared to normal healthy population. The current study aims to determine the relationship between elevated UCB concentrations and platelet activation, aggregation, hemostatic function, lipid status and inflammation, in GS individuals versus normal healthy individuals.
5.2 Methods

5.2.1 Recruitment and blood collection

Fourteen Gilbert’s syndrome and fourteen normal healthy volunteers (8 male and 6 female in each group) from the general population were recruited after providing informed consent. The process of volunteer recruitment was commenced after obtaining approval from the Griffith University Human Research Ethics Committee. Recruited individuals were carefully screened based on inclusion criteria being non-smoking individuals with no previous history of hepatitis, liver disease and recent acute respiratory inflammation/bacterial infection. Subjects were also excluded if they had consumed any form of medication or antioxidant supplementation during the two weeks prior to participation in the study. Full blood examination (FBE) was performed on all the volunteers using Coulter® Ac.T™ 5diff CP hematology analyser (Beckman Coulter, Inc., Lane Cove, NSW, Australia). Volunteers with abnormal FBE results as per Royal College of Pathologists of Australasia (RCPA) were excluded from the study (237). Body mass index (BMI) of all the volunteers was calculated using standard scales for height and weight measurement. Furthermore, allocation of individuals into the GS group was performed either based on a confirmed diagnosis of GS from a general practitioner and/or individuals with circulating UCB concentration >17.1 μM.

For blood collection protocol, refer to Materials and methods, Chapter 3, section 3.3.1.

5.2.2 Platelet aggregation assay

Refer to Materials and Methods Chapter 3, Section 3.5.

5.2.3 Determination of platelet activity

Refer to Materials and Methods Chapter 3, Section 3.6.
5.2.4 Tests of coagulation

Refer to Materials and Methods Chapter 3, Section 3.7.

5.2.5 Biochemical analysis

Refer to Materials and Methods Chapter 3, Section 3.8. The LDL concentration of the samples was calculated using Friedewald equation (285). The concentrations of UCB were measured using high performance liquid chromatography as previously published (17). All tests were performed in duplicate, with the average of duplicate measures recorded as the final result.

5.2.6 Statistical analysis

GraphPad StatMate 2.00 was used to determine the sample size and power of the study. The sample size and power calculations were made collagen induced platelet aggregation, based upon the effect of exogenously elevated unconjugated bilirubin on collagen induced platelet aggregation (270). A sample size of 14 in each group had 95% power to detect a difference between the means of each group, with a significance level alpha=0.05. Data obtained from platelet aggregation, activation, coagulation and biochemical studies were analysed using unpaired t-tests to determine the statistically significant difference between GS and control groups. Graphpad Prism was used to perform correlation analysis between variables. Furthermore, Sigmaplot was used to perform Forward stepwise regression analysis in order to predict multiple independent variables on dependent variables.
5.3 Results

The general characteristics of subjects showed no significant differences between the groups (Table 7). Individuals with GS had elevated RBC mean cell volume (MCV), mean cell hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) in comparison with control individuals. However, all parameters were within normal reference ranges as established by Royal Collage of Pathologists Australasia (RCPA; Table 7) (237).
Table 7. General characteristics and full blood examination results of recruited Gilbert’s syndrome and control subjects

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control (n = 14)</th>
<th>GS (n = 14)</th>
<th>P value</th>
<th>Reference range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median ± Range</td>
<td>Median ± Range</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>31 ± 26</td>
<td>30 ± 27</td>
<td>0.860</td>
<td>N/A</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24 ± 6</td>
<td>22 ± 9</td>
<td>0.833</td>
<td>22 – 26</td>
</tr>
<tr>
<td>WBC (×10⁹/L)</td>
<td>6.05 ± 5.5</td>
<td>6 ± 5.2</td>
<td>0.843</td>
<td>4.0 – 11.0</td>
</tr>
<tr>
<td>RBC (×10¹²/L)</td>
<td>4.9 ± 1.03</td>
<td>4.7 ± 1.37</td>
<td>0.296</td>
<td>3.8 – 6.5</td>
</tr>
<tr>
<td>Haemoglobin (g/L)</td>
<td>136 ± 27</td>
<td>141 ± 42</td>
<td>0.245</td>
<td>120 – 180</td>
</tr>
<tr>
<td>Haematocrit (%)</td>
<td>0.40 ± 0.05</td>
<td>0.40 ± 0.12</td>
<td>0.577</td>
<td>0.36 – 0.54</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>83 ± 11</td>
<td>84.5 ± 7</td>
<td>0.025*</td>
<td>80 – 100</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>27.7 ± 5.8</td>
<td>29.4 ± 4.8</td>
<td>0.004*</td>
<td>27 – 31</td>
</tr>
<tr>
<td>MCHC (g/L)</td>
<td>341 ± 29</td>
<td>345 ± 16</td>
<td>0.029*</td>
<td>320 – 360</td>
</tr>
<tr>
<td>Platelets (×10⁹/L)</td>
<td>241 ± 143</td>
<td>259 ± 179</td>
<td>0.453</td>
<td>150 – 400</td>
</tr>
<tr>
<td>MPV (fL)</td>
<td>8.35 ± 4.1</td>
<td>8.75 ± 2.8</td>
<td>0.137</td>
<td>6.0 – 10.0</td>
</tr>
</tbody>
</table>

Values are represented as median ± range.

Abbreviations: BMI, body mass index; fL, femtolitre; pg, picogram; GS, Gilbert’s syndrome; MCV, mean cell volume; MCH, mean cell haemoglobin; MCHC, mean corpuscular haemoglobin concentration; MPV, mean platelet volume; RBC, red blood cell; WBC, white blood cell. * = P<0.05.
5.3.1 Platelet aggregation and activation

Reduced platelet aggregation in individuals with GS was observed in response to arachidonic acid and collagen (Figure 13; both $P<0.05$) when compared to controls. Furthermore, the inhibition of AA and collagen induced platelet aggregation was significantly correlated with bilirubin concentrations ($r=0.165$, $P<0.05$, Figure 15A; $r=0.458$, $P<0.0001$, Figure 15B; respectively).

However, no difference in platelet aggregation occurred in response to ADP stimulation. The expression of activation-dependent platelet surface marker P-selectin/CD62P on ADP stimulated platelets was significantly reduced in GS group, compared to controls (Figure 14; $P<0.05$). A significant correlation between bilirubin concentrations and P-selectin expression was observed ($r=0.413$, $P<0.001$; Figure 15C). No difference in the expression of PAC-1 relating to platelet conformational change was observed.

Subsequently, multiple regression analysis was performed to determine the ability of independent variables to explain the observed inhibition of platelet activation and aggregation. Bilirubin, by itself was able to explain the variations in AA (37%), collagen (44%) and ADP (15%) induced platelet aggregation (Table 8). Furthermore, bilirubin estimated 43% of variance in activation-dependent platelet surface marker, P-selectin expression (Table 8).
Figure 13. Mean platelet aggregation (MPA%) in response to arachidonic acid ($P=0.032$), collagen ($P=0.018$) and ADP ($P=0.166$) platelet agonists in Gilbert’s syndrome and control subjects (n=14 per group). Data presented as median ± range; *$P<0.05$; GS versus control. AA, arachidonic acid; ADP, adenosine diphosphate; GS, Gilbert’s syndrome, MPA, mean platelet aggregation.
Figure 14. The expression of P-selectin (A) and PAC-1 (B) activation dependent platelet surface markers in stimulated and unstimulated platelets of Gilbert’s syndrome and control subjects. P-selectin expression was significantly ($P=0.030$) reduced in stimulated platelets of GS versus control subjects. PAC-1 expression between GS and control subjects was not significantly different ($P=0.146$). (n = 14 per group). Data presented as median ± range; * $P<0.05$; GS versus control.
Figure 15. The relationship between circulating bilirubin and AA induced platelet aggregation ($r=0.165; P<0.05; A$), collagen induced platelet aggregation ($r=0.458; P<0.0001; B$) and P-selectin expression ($r=0.413; P<0.001; C$) in control (filled circles) and GS subjects (open circles).
Table 8: Multiple regression analysis for platelet function markers as dependent variables.

<table>
<thead>
<tr>
<th>Dependent Variable</th>
<th>Independent Variable</th>
<th>$R^2$</th>
<th>Delta $R^2$</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA induced platelet aggregation</td>
<td>Bilirubin</td>
<td>0.378</td>
<td>0.378</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>HDL</td>
<td>0.521</td>
<td>0.142</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Collagen induced platelet</td>
<td>Bilirubin</td>
<td>0.444</td>
<td>0.444</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>aggregation</td>
<td>HCT</td>
<td>0.713</td>
<td>0.269</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>MCV</td>
<td>0.778</td>
<td>0.065</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>BMI</td>
<td>0.824</td>
<td>0.046</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>ADP</td>
<td>Bilirubin</td>
<td>0.159</td>
<td>0.159</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>MCV</td>
<td>0.341</td>
<td>0.183</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>P-selectin expression</td>
<td>Bilirubin</td>
<td>0.43</td>
<td>0.43</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>MCH</td>
<td>0.513</td>
<td>0.083</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

$R^2$ adjusted correlation coefficient and cumulative explanation of dependent variable in %. Multiple linear regression.
5.3.2  **Coagulation assays**

No statistically significant difference in clotting times (PT and aPTT), or fibrinogen concentrations were observed between GS and controls (Table 9).

**Table 9.** Coagulation assay results of Gilbert’s syndrome and control subjects.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Control (n=14)</th>
<th>GS (n=14)</th>
<th>P value</th>
<th>Reference range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median ± Range</td>
<td>Median ± Range</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PT (sec)</td>
<td>15 ± 5.2</td>
<td>14.5 ± 5.8</td>
<td>0.41</td>
<td>11 – 15</td>
</tr>
<tr>
<td>aPTT (sec)</td>
<td>37.5 ± 18.4</td>
<td>38.6 ± 26.7</td>
<td>0.41</td>
<td>18 – 45</td>
</tr>
<tr>
<td>Fibrinogen (mg/dL)</td>
<td>370 ± 219</td>
<td>405 ± 379</td>
<td>0.35</td>
<td>200 – 400</td>
</tr>
</tbody>
</table>

Values are represented as median ± range.

Abbreviations: aPTT, activated partial thromboplastin time; GS, Gilbert’s syndrome; PT, prothrombin time; sec, seconds.

5.3.3  **General biochemistry, inflammation and lipid status**

Serum biochemistry profile and inflammatory marker concentrations are shown in Table 10. Individuals with GS had significantly increased concentrations of unconjugated bilirubin (P<0.001; Table 10) and demonstrated improved lipid profile including elevated HDL (P<0.05; Table 10) and significantly reduced LDL (P<0.05; Table 10). High-sensitivity CRP was significantly reduced in GS (P<0.05; Table 10) in comparison to the control group. Concentrations of total cholesterol, uric acid, triglycerides and serum glucose were similar between the groups.
Table 10. General biochemistry, inflammation and lipid status in Gilbert’s syndrome and control subjects.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control (n = 14)</th>
<th>GS (n = 14)</th>
<th>P value</th>
<th>Reference range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median ± Range</td>
<td>Median ± Range</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total bilirubin</td>
<td>7.8 ± 8.2</td>
<td>23 ± 50</td>
<td>0.001**</td>
<td>&lt; 21</td>
</tr>
<tr>
<td>(µM)</td>
<td></td>
<td></td>
<td></td>
<td>GS &gt; 17.1</td>
</tr>
<tr>
<td>TC (mM)</td>
<td>4.54 ± 2.65</td>
<td>4.56 ± 3.07</td>
<td>0.830</td>
<td>&lt; 5.5</td>
</tr>
<tr>
<td>TG (mM)</td>
<td>0.83 ± 1.38</td>
<td>0.89 ± 1.75</td>
<td>0.412</td>
<td>&lt; 2.26</td>
</tr>
<tr>
<td>HDL (mM)</td>
<td>1.21 ± 0.91</td>
<td>1.44 ± 1.33</td>
<td>0.033*</td>
<td>&gt; 1.1</td>
</tr>
<tr>
<td>LDL (mM)</td>
<td>3.13 ± 2.43</td>
<td>2.43 ± 2.61</td>
<td>0.024*</td>
<td>2.0 – 3.4</td>
</tr>
<tr>
<td>hsCRP (mg/L)</td>
<td>1.01 ± 1.89</td>
<td>0.35 ± 0.68</td>
<td>0.043*</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>Uric acid</td>
<td>276 ± 206</td>
<td>310 ± 273</td>
<td>0.482</td>
<td>202 – 416</td>
</tr>
<tr>
<td>Glucose (mM)</td>
<td>4.96 ± 1.8</td>
<td>4.79 ± 1.76</td>
<td>0.142</td>
<td>4.11 – 6.05</td>
</tr>
</tbody>
</table>

Values are represented as median ± range.

Abbreviations: CRP, C-reactive protein; GS, Gilbert’s syndrome; HDL, high density lipoprotein; LDL, low density lipoprotein; TC, total cholesterol; TG, triacylglycerol. * = P<0.05; ** = P<0.001.
5.4 Discussion

The main findings of this study demonstrate novel mechanisms that could contribute to prevention of CVD and subsequent mortality in GS. Reduced collagen and arachidonic acid induced platelet aggregation, suggests a possible mechanism in GS that reduces platelet activity, targeting the GP VI and the COX-1/TXA2 pathways, while decreased platelet surface marker P-selectin expression is suggestive of reduced α-granule release in GS individuals compared to controls. Furthermore, reduced levels of the inflammatory biomarker, hsCRP, an improved lipid status and reduced platelet activity, further support an emerging hypothesis that might contribute to protection against the development of CVD in GS.

Antioxidants and their role in human biology remains an area of great research interest, due to the association between antioxidant consumption, circulating antioxidant capacity and the risk of thrombosis/cardiovascular mortality (17, 122, 124, 125). Several in vitro and in vivo studies suggest that unconjugated bilirubin is a potent antioxidant and possible cardio-protectant (284). Previous reports demonstrate an increased total antioxidant capacity in association with elevated UCB levels in GS, providing a possible explanation for reduced CVD risk in these individuals (17). Although numerous studies report negative relationships between elevated UCB levels in GS and CVD risk, platelet function in these individuals has not been investigated until now.

In this study three different exogenous agonists that are receptor specific and differ in their mechanistic pathways of platelet activation were used (265). Arachidonic acid (AA; cyclooxygenase-1 (COX-1) pathway of platelet activation), ADP (P2Y1/P2Y12 receptor activation) and collagen (interacting with GP VI/integrin α2β1 receptors) are endogenous platelet aggregation and activation stimulants, released at the site of intravascular injury, consequently contributing to thrombus formation (265). Significant
inhibition of AA and collagen-induced platelet aggregation in GS compared to control group, reveals two discrete molecular pathways, which bilirubin might inhibit and thus limit thrombus formation. We believe the inhibition of AA and collagen induced platelet aggregation in relation with UCB in GS is related to the antioxidant properties of UCB, which scavenges hydrogen peroxide (H₂O₂) that initiates platelet activation (68, 162, 286). Furthermore, collagen induced platelet aggregation is associated with a burst of H₂O₂ release from platelets, which acts as a secondary messenger by stimulating AA metabolism and phospholiapse C pathway, suggesting the involvement of these reactive species in platelet activation (4, 68). These findings are further supported by a recent study that has shown a reduced platelet adhesion in response to collagen under the influence of UCB (204). A statistically significant correlation between bilirubin concentration and AA and collagen induced platelet aggregation (Figure 15A; Figure 15B), along with bilirubin explaining the variation in platelet aggregation (Table 8), further strengthens the observed anti-thrombotic effect due to elevated bilirubin in GS.

The results from the current study suggest that increased UCB in GS may reduce platelet aggregation by inhibiting TxA₂ production and interaction between collagen and platelet receptors (277, 287). Previously, in vitro addition of UCB at concentrations of ~35 μM inhibited collagen induced platelet aggregation (270). No significant difference was found between the groups when platelets were stimulated with ADP, suggesting that UCB at concentrations seen in GS may not affect P₂Y₃/P₂Y₁₂ pathway of platelet activation and aggregation. However, a dose dependent inhibition of ADP induced platelet aggregation in vitro was apparent at greater UCB concentrations (270).

P-selectin is a protein released from α-granules of activated platelets and expressed by endothelial cells and leukocytes (288). Compared to control, significantly reduced P-selectin/CD62P mAb expression on platelets was observed in GS individuals, when platelets were stimulated using ADP. Furthermore, reduced P-selectin expression
was significantly correlated with increasing bilirubin concentrations (Figure 15C). Reduced P-selectin expression signifies a reduced activation-dependent de-granulation (α granule) of platelets (114). In support of this finding, Tapan et al. have demonstrated significantly reduced levels of circulating soluble P-selectin in GS (111). Furthermore, some studies have concluded that, inhibition of P-selectin was shown to significantly reduce the risk of thrombosis and future CVD (215, 289, 290). Members of the protein kinase C (PKC) family play a key role in thrombus formation by regulating intra-platelet signalling and platelet granule secretion (291). Inhibition of PKC by UCB can also be a plausible explanation of reduced platelet degranulation and P-selectin expression. Bilirubin dose-dependently inhibits PKC activity in cytosolic and membranous fractions of human skin fibroblasts (292). A 50% reduction in PKC activity was reported in cells at a bilirubin concentration of 45 μM (292). Lindenblatt and colleagues reported that in vivo administration of UCB (10 μM) along with hemin (a heme oxygenase-1 inducer) in animal models significantly inhibits ferric chloride (FeCl₃) induced thrombus formation (174). The authors suggested that the antithrombotic potential of UCB and hemin combined was associated with reduced P-selectin expression, although the authors did not test this hypothesis (174).

GPIIb/IIIa receptors are expressed on the platelet surface as a result of platelet shape change and represent one of initial phases of platelet activation. No significant difference in the expression of PAC-1 on activated platelets was observed between GS and control, suggesting that UCB may have no effect on GPIIb/IIIa receptor function or expression, thereby having no affect the initial stages of platelet activation (Figure 14). However, UCB effectively inhibits the secondary stages platelet activation such as degranulation and α granule secretion. Several antioxidants have demonstrated an inhibition of platelet degranulation and P-selectin expression but not the primary stages of platelet activation i.e, PAC-1 as seen in GS, further supporting the proposed
antioxidative effect of mildly elevated UCB on secondary platelet activation pathway in GS (124, 125).

Coagulation tests prothrombin time (PT) and activated partial thromboplastin time (aPTT) were performed to evaluate the effect of elevated UCB on extrinsic and intrinsic pathways of coagulation cascade. No significant differences in clotting time and fibrinogen concentration were observed between GS and control groups (Table 9). Previously, the coagulation cascade was reported to be unaffected by the addition of exogenous UCB at a concentration of 35 μM, suggesting UCB is unlikely to have any inhibitory effect on the coagulation proteins, at UCB concentrations seen in GS (270). A statistically significant difference in MCV, MCH and MCHC parameters was observed in GS compared to control subjects. A positive correlation was shown between serum bilirubin concentration and hemoglobin, MCH and MCHC levels, suggesting increased erythropoiesis may be partly responsible for increased UCB concentrations (218). Borawski et al., have previously shown a significant inverse logarithmic trend between haemoglobin concentration and ristocetin induced platelet aggregation (293). However similar relationship between haemoglobin concentration and other agonists such as ADP, collagen and AA were not reported in literature. In contrast, cell free haemoglobin released due to hemolysis is known induce platelet aggregation by releasing ADP and lowering nitric oxide bioavailability (294).

Biochemical analysis confirmed improved lipid status (Table 10; reduced LDL, increased HDL) and reduced inflammation in individuals with GS, as documented throughout the literature (45, 112, 176). Furthermore, Tapan et al. have observed decreased levels of small dense LDL and oxidised LDL in individuals with GS (173). The mechanism by which UCB might induce hypocholesterolemia in GS individuals is not yet understood, however, may involve modulation of endocrine function or activation of the aryl hydrocarbon receptor (45). The findings from this study such as lower hsCRP in
GS are also in agreement with several studies which reported a significant reduction in CRP (Table 10) (173, 186, 187).

The results reported in this study, may assist in developing an improved understanding regarding possible mechanisms involved in protection from thrombosis leading to CVD mortality in mildly hyperbilirubinemic individuals (295). However, longitudinal studies investigating the effects of GS on hemostatic function and thrombotic outcomes are clearly necessary to provide stronger causal relationships between GS and reduced mortality.

In conclusion, these data show that elevated levels of circulating bilirubin may be associated with reduced risk of thrombosis by inhibition of platelet aggregation and granule release in GS. These data further support the possible utility of mildly elevating UCB concentrations via physiological/pharmacological means in individuals at increased risk of thrombosis (196).
6. Reduced platelet activity after acute intense exercise in Gilbert’s syndrome
6.1 Introduction

The beneficial effects of a long-term, habitual exercise regime on reducing cardiovascular risk has been clearly established (296). Several studies have previously reported a positive association between long-term exercise participation and reduced platelet activity, however this phenomenon was only observed in trained individuals (297-299). Strenuous acute exercise in sedentary individuals increases the risk of vascular thrombotic events and the incidence of primary cardiovascular complications, especially in individuals with pro-thrombotic conditions such as obesity and type 2 diabetes (299-302). One possible explanation for this observation includes increased oxygen consumption increasing reactive oxygen species (ROS) production, thereby inducing oxidative stress (303, 304). Elevated oxidative stress and its adverse effects on altering the platelet responses to agonists, is one of the primary causes for platelet hyper-activity, causing intravascular thrombus formation and subsequently MI (284). In chronically trained individuals, the undesirable effects of oxidative stress can be negated by mechanisms including upregulation of antioxidant gene expression and enzyme activity (305, 306). Factors such as dehydration, release of platelet activation enhancing hormone (adrenaline) and shear stress can significantly increase the risk of thrombosis and subsequent cardiovascular events in sedentary people performing strenuous exercise (302, 307-309). Therefore, intense exercise serves as a model of oxidative stress induced platelet hyper activation that can be assessed reliably and reproducibly (297, 298, 300-302, 304, 308).

Unconjugated bilirubin (UCB) is considered a potent physiological antioxidant having several cardiovascular protective effects, with many studies showing a negative relationship between elevated UCB and CVD risk (20-22, 45, 46, 76, 284). Gilbert’s syndrome is a relatively common condition characterised by mild unconjugated hyperbilirubinemia in the absence of haemolysis, associated with mutations in human...
uridine diphosphate glucuronosyltransferase (UGT1A1) gene promotor (17, 284). Individuals with GS possess increased total antioxidant capacity and resistance to serum oxidation (17). Furthermore, Horsfall et al. concluded that individuals with GS have 50% reduced risk of all-cause mortality, when compared with normal healthy individuals from the general population (27). The observations are further supported by McCallum et al., who reported a significant negative association between serum bilirubin levels and cardiovascular mortality (310). Acute intense exercise by sedentary population is associated with an increase in platelet activity and P-selectin expression. In the previous study (Chapter 5) it was observed that GS individuals have reduced platelet aggregation and activation compared to normal healthy individuals. Therefore the rationale of this study is to investigate if UCB can impart similar anti-platelet effects even after acute intense exercise, which is usually associated with increased platelet activation and aggregation. The hypothesis for this study was that sedentary individuals with GS, who possess mildly elevated UCB, will experience a reduced risk of platelet activation and aggregation after an intense acute exercise bout.

6.2 Methods

Twenty two healthy and sedentary individuals (11 GS and 11 normal healthy) were recruited in the study after obtaining the approval from Griffith University Human Research Ethics Committee, Queensland, Australia. Prior to the volunteer screening process an informed consent was obtained from volunteer. All the participants included in this study were healthy, non-smoking, sedentary people with no previous history of CVD, bleeding disorders, hepatitis and liver disease. Individuals on any form of medication or antioxidant supplementation were excluded from the study.

6.2.1 Incremental exercise test to exhaustion

The incremental exercise test to exhaustion was performed to evaluate the peak O$_2$ uptake (VO$_2$) of the participants. An electronically braked cycle ergometer (Excalibur Sport
925900, lobe BV, Groningen, The Netherlands) was used to perform the incremental exercise test under standard laboratory conditions. The exercise test was preceded by a warm-up period of 3 minutes at a workload of 30 W, which was then followed by increments in power output of 10-15 W per every 30 seconds until exhaustion. During the exercise test, the participants were required to maintain a constant pedal cadence of 70-80 revolutions per minute (rev/min). The exercise test was terminated once the participants could not maintain a pedal above 65 rev/min despite strong verbal encouragement.

During the exercise test the participants wore a nose clip and breathed through a mouth piece attached to a two-directional differential pressure pneumotachograph (preVent, Medical Graphics Corporation, St Paul, MN, USA). All the gas exchange parameters were acquired breath-by-breath in real-time using a calibrated metabolic measurement device (MedGraphics Ultima CardiO2, Medical Graphics Corporation, St Paul, MN, USA). The gas exchange parameters were subsequently averaged over 30-s intervals and the peak exercise values are the average of the two highest 30 s interval values obtained during the incremental exercise. A CM5 electrocardiograph lead electrode configuration was used to monitor cardiac rhythm and measure heart rate (HR) during exercise.

### 6.2.2 Experimental trial and blood collection

The participants involved in the study were requested to report to the laboratory in the mornings after an overnight fasting of 8-10 hours. Blood sample of 20-22 mL was collected from the median cubital vein after a resting period of 10 mins. The participants then commenced the exercise on a electronically braked cycle ergometer (Excalibur Sport 925900, lobe BV, Groningen, The Netherlands) at a sub maximal cycling load of 70% of their respective VO2max for a period of 1 hour. The participants were provided with water throughout the ride. The cardiac rhythm and HR were constantly monitored during the ride using a CM5-lead electrode configuration.
The blood sample pre and post exercise bout were collected according to the protocol mentioned in Materials and Methods Chapter 3, Section 3.3.1.

6.2.3 Determination of Platelet activation and aggregation

Platelet activation and aggregation assays were performed before and after the incremental exercise. The platelet activation and aggregation assays were performed on fresh blood using Helena Aggram, as described in Materials and Methods Chapter 3, Section 3.5 and Section 3.6.

6.2.4 Tests of coagulation

Prothrombin time, activated partial thromboplastin time and fibrinogen concentration of the samples were performed on platelet poor plasma. Coagulation analysis was performed using C4 coagulation analyser as described in the Materials and Methods Chapter 3, Section 3.7.

6.2.5 Measurement of Cytokines

Human inflammatory cytokine CBA kit (BD Biosciences, San Jose, CA, USA) was used to measure circulating cytokines namely: interleukin-1β (IL-1β), interleukin-8 (IL-8), interleukin-10 (IL-10), interleukin-12 (IL-12), tumour necrosis factor-α (TNF-α). The assay was performed using BD LSRFortessa cell analyzer (BD Biosciences, San Jose, CA, USA) and as per manufacturer’s instructions. The recorded data was analysed using the BD FACSDiva software.

6.2.6 Biochemical analysis

Tests for lipid profile, blood glucose, uric acid and hsCRP were performed using COBAS integra 400 as described in Materials and Methods Chapter 3, Section 3.8. The LDL concentration of the samples was calculated using Friedewald equation. The concentrations of UCB were measured using high performance liquid chromatography. All the tests were performed in duplicate.
6.2.7 Statistical analysis

GraphPad StatMate 2.00 was used to determine the sample size and power of the study. The sample size and power calculations were made collagen induced platelet aggregation, based upon the effect of exogenously elevated unconjugated bilirubin on collagen induced platelet aggregation (270). A sample size of 11 in each group had 80% power to detect a difference between the means of each group, with a significance level alpha=0.05. The data obtained from platelet aggregation, activation, coagulation and biochemical studies were analysed using the non repeated-measures analysis of variance (ANOVA) along with Bonferroni’s post-test, to determine the statistically significant difference between GS and control groups. Data obtained from the exercise test (Table 12) and other parameters such as age and BMI (Table 11) were analysed using unpaired t-tests to determine the statistically significant difference between GS and control groups. Group data are presented as mean ± SD and a $P$-value < 0.05 was considered significant.

6.3 Results

The general characteristics of subjects showed no significant differences between the groups (Table 11). However, all parameters were within normal reference ranges as established by Royal Collage of Pathologists Australasia (RCPA; Table 11) (237).
Table 11. General and hematological parameters of recruited control and Gilbert’s syndrome subject’s pre and post intense acute exercise trial.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control (n = 11)</th>
<th>GS (n = 11)</th>
<th>P value</th>
<th>Reference ranges</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>31.3 ± 2.23</td>
<td>30.7 ± 2.32</td>
<td>0.860</td>
<td>N/A</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.2 ± 1.59</td>
<td>24.0 ± 0.53</td>
<td>0.179</td>
<td>22 – 26</td>
</tr>
<tr>
<td>WBC (×10⁹/L)</td>
<td>6 ± 1.01</td>
<td>7.01 ± 1.56</td>
<td>6.11 ± 1.47</td>
<td>6.48 ± 1.42</td>
</tr>
<tr>
<td>RBC (×10¹²/L)</td>
<td>4.86 ± 0.11</td>
<td>5.05 ± 0.48</td>
<td>4.81 ± 0.41</td>
<td>4.99 ± 0.39</td>
</tr>
<tr>
<td>Hemoglobin (g/L)</td>
<td>138 ± 9.71</td>
<td>143 ± 11.2</td>
<td>142 ± 10.5</td>
<td>148 ± 10.2</td>
</tr>
<tr>
<td>Haematocrit (%)</td>
<td>0.40 ± 0.02</td>
<td>0.42 ± 0.03</td>
<td>0.40 ± 0.03</td>
<td>0.42 ± 0.02</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>83.2 ± 4.36</td>
<td>83.5 ± 4.39</td>
<td>85.95 ± 3.75</td>
<td>86.6 ± 3.57</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>28.4 ± 1.79</td>
<td>28.4 ± 1.71</td>
<td>29.7 ± 1.40</td>
<td>29.8 ± 1.19</td>
</tr>
<tr>
<td>MCHC (g/L)</td>
<td>341 ± 4.70</td>
<td>340 ± 4.17</td>
<td>346 ±4.39</td>
<td>343 ± 2.81</td>
</tr>
<tr>
<td>Platelets (×10⁹/L)</td>
<td>214 ± 49.8</td>
<td>230 ± 54.6</td>
<td>252 ± 51.9</td>
<td>267 ± 63.1</td>
</tr>
<tr>
<td>MPV (fL)</td>
<td>9.27 ± 1.08</td>
<td>9.95 ± 1.03</td>
<td>9.07 ± 0.80</td>
<td>8.9 ± 0.88</td>
</tr>
</tbody>
</table>

Values represented as mean ± SD.
Abbreviations: BMI, body mass index; fL, femtolitre; pg, pictogram; GS, Gilbert’s syndrome; MCV, mean cell volume; MCH, mean cell hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MPV, mean platelet volume; RBC, red blood cell; WBC, white blood cell.
6.3.1 Exercise test

The mean VO2max values attained during the incremental exercise test were not significantly different between groups (GS: 70 ± 1.0 Vs Control: 70.2 ± 0.36 mL/kg/min). The average power, VO2 and heart rates attained during the constant-load exercise tests are shown in Table 12. All the participants were able to complete 60 minutes of exercise at 70% of their respective VO2MAX. No significant differences were observed between GS and controls for the various parameters measured during the constant-load exercise tests.

Table 12. Mean power, VO2max and heart rate values attained during the intense acute exercise trials

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>GS</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Power (W)</td>
<td>105 ± 34</td>
<td>126 ± 29</td>
<td>0.15</td>
</tr>
<tr>
<td>Power (% peak)</td>
<td>49.1 ± 4</td>
<td>50.2 ± 5.1</td>
<td>0.54</td>
</tr>
<tr>
<td>VO2 (L/min⁻¹)</td>
<td>2 ± 0.01</td>
<td>1.77 ± 0.36</td>
<td>0.44</td>
</tr>
<tr>
<td>VO2 (% peak)</td>
<td>70.0 ± 1.0</td>
<td>70.2 ± 0.36</td>
<td>0.92</td>
</tr>
<tr>
<td>Heart rate (beats.min⁻¹)</td>
<td>144 ± 14</td>
<td>147 ± 23</td>
<td>0.73</td>
</tr>
<tr>
<td>Heart rate (%HRMAX)</td>
<td>84 ± 7</td>
<td>83 ± 9.2</td>
<td>0.63</td>
</tr>
</tbody>
</table>

Values represented as mean ± SD.
Abbreviations: GS, Gilbert’s syndrome; VO2, maximal oxygen uptake; W, watts, HR, heart rate.
6.3.2 Platelet aggregation assay

A statistically significant reduction in arachidonic acid (*P=0.04; Figure 16A) induced platelet aggregation was observed in GS at both resting and exercise induced oxidative stress state. A significantly reduced collagen induced platelet aggregation (*P=0.03; Figure 16B) was also seen in GS but only under induced oxidative stress conditions. However, no significant change was observed when platelets were stimulated using ADP (data shown; Figure 16C).
Figure 16. Mean platelet aggregation in response to various agonists in Gilbert’s syndrome and control pre and intense acute exercise. Significantly reduced AA induced platelet aggregation results were observed in GS at resting ($P=0.04$) and under induced oxidative stress conditions ($P=0.04$). (B) Significantly reduced collagen induced platelet aggregation was observed in GS under oxidative stress conditions ($P=0.03$). * signifies statistically significant result ($P<0.05$). No difference was observed pre and post exercise within the groups. AA, arachidonic acid; GS, Gilbert’s syndrome; MPA, mean platelet aggregation.
6.3.3 Flow cytometric evaluation of platelet activity

A significantly reduced activation-dependent platelet surface marker expression, P-selectin, was recorded in GS individuals at resting and after the acute intense exercise bout in comparison with controls (Figure 17). No significant change in the expression of PAC-1 on ADP stimulated platelets was observed between GS and controls at resting or after acute exercise bout.

Figure 17. Activation dependent surface marker expression in Gilbert’s syndrome and control pre and post-acute exercise. Significantly reduced P-selectin expression was observed in GS at resting (P=0.02) and under induced oxidative stress conditions (P=0.003). * signifies statistically significant result (P<0.05). No difference was observed pre and post exercise within the groups. GS, Gilbert’s syndrome; MFI, Mean fluorescence intensity.
6.3.4 Coagulation profile

Elevated levels of UCB did not have any significant influence on the clotting time, as no significant difference was observed with coagulation assays, PT and aPTT, between GS and control (Table 13) at resting and after intense acute exercise bout. Furthermore, no significant difference was observed in fibrinogen concentrations between GS and controls.
Table 13. Coagulation profile results pre and post exercise of control and Gilbert’s syndrome subjects.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Control (n = 11)</th>
<th>GS (n = 11)</th>
<th>P value</th>
<th>Reference ranges</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-ex</td>
<td>Post-ex</td>
<td>Pre-ex</td>
<td>Post-ex</td>
</tr>
<tr>
<td>PT (sec)</td>
<td>15.7 ± 0.84</td>
<td>15.4 ± 0.90</td>
<td>14.9 ± 1.83</td>
<td>14.4 ± 1.32</td>
</tr>
<tr>
<td>aPTT (sec)</td>
<td>36.2 ± 3.98</td>
<td>37.7 ± 4.06</td>
<td>39.5 ± 6.34</td>
<td>36.3 ± 5.22</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>395 ± 97.4</td>
<td>379 ± 95.8</td>
<td>375 ± 101</td>
<td>386 ± 156</td>
</tr>
</tbody>
</table>

Values represented as mean ± SD.
Abbreviations: aPTT, activated partial thromboplastin time; GS, Gilbert’s syndrome; PT, prothrombin time; sec, seconds.
6.3.5 Inflammation and cytokine measurement

Inflammation marker hsCRP and serum cytokine levels of GS and controls were measured pre and post-acute intense exercise (Table 14). Significantly reduced levels of hs-CRP were detected in GS in comparison with controls, however the results were within the normal reference ranges (hsCRP: low risk - <1 mg/L; moderate risk - 1-3 mg/L; high risk - > 3 mg/L). Significantly reduced levels of IL-10 were found in individuals with GS at resting state when compared with normal individuals, however similar effect was not noticed post exercise (Table 14). No further variation in the cytokine levels of IL-1β, IL-8, IL-12 and TNF-α were observed.

Table 14. Serum cytokine levels of control and Gilbert’s syndrome results at pre and post-acute exercise.

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 11)</th>
<th>GS (n = 11)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-ex</td>
<td>Post-ex</td>
<td>Pre-ex</td>
</tr>
<tr>
<td>hsCRP</td>
<td>1.08 ± 0.9</td>
<td>1.06 ± 0.9</td>
<td>0.43 ± 0.2*</td>
</tr>
<tr>
<td>Cytokine (pg/mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>4.25 ± 2.53</td>
<td>4.87 ± 3.23</td>
<td>5.72 ± 3.77</td>
</tr>
<tr>
<td>IL-8</td>
<td>3.43 ± 1.19</td>
<td>1.92 ± 0.86</td>
<td>3.41 ± 1.37</td>
</tr>
<tr>
<td>IL-12</td>
<td>6.11 ± 1.89</td>
<td>4.40 ± 2.89</td>
<td>6.05 ± 3.05</td>
</tr>
<tr>
<td>IL-10</td>
<td>3.97 ± 1.23</td>
<td>2.08 ± 1.6</td>
<td>2.01 ± 0.74*</td>
</tr>
<tr>
<td>TNF-α</td>
<td>4.12 ± 2.48</td>
<td>3.91 ± 1.44</td>
<td>3.76 ± 0.74</td>
</tr>
</tbody>
</table>

Values represented as mean ± SD.
* signifies statistically significant result (P<0.05)
Abbreviations: hsCRP, high sensitive C-reactive protein; IL-1β, interleukin-1β; IL-8, interleukin-8; IL-12, interleukin-12; TNF-α, tumour necrosis factor-α.
6.3.6 Biochemical analysis

Table 15 indicates that individuals with GS had significantly elevated levels of UCB when compared with controls, pre and post exercise. Furthermore, UCB concentration was mildly elevated post exercise. No significant differences were observed between GS and controls for several other biochemical parameters such as blood glucose, TC, HDL, LDL, uric acid and TG.
**Table 15.** Biochemical parameters pre and post acute exercise of control and Gilbert’s syndrome subjects.

Values represented as mean ± SD.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Control (n = 11)</th>
<th>GS (n = 11)</th>
<th>P value</th>
<th>Reference ranges</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-ex</td>
<td>Post-ex</td>
<td>Pre-ex</td>
<td>Post-ex</td>
</tr>
<tr>
<td>Bilirubin (µM)</td>
<td>7.83 ± 2.35</td>
<td>8.51 ± 2.26</td>
<td>30.3 ± 6.20</td>
<td>34.4 ± 6.14</td>
</tr>
<tr>
<td>TC (mM)</td>
<td>4.26 ± 0.59</td>
<td>4.11 ± 0.85</td>
<td>4.53 ± 0.86</td>
<td>4.64 ± 0.95</td>
</tr>
<tr>
<td>TG (mM)</td>
<td>0.82 ± 0.22</td>
<td>0.71 ± 0.25</td>
<td>0.95 ± 0.48</td>
<td>1.03 ± 0.46</td>
</tr>
<tr>
<td>HDL (mM)</td>
<td>1.50 ± 0.43</td>
<td>1.53 ± 0.60</td>
<td>1.69 ± 0.49</td>
<td>1.76 ± 0.55</td>
</tr>
<tr>
<td>LDL (mM)</td>
<td>2.39 ± 0.63</td>
<td>2.26 ± 0.69</td>
<td>2.19 ± 0.75</td>
<td>2.23 ± 0.72</td>
</tr>
<tr>
<td>Uric acid</td>
<td>315 ± 82.2</td>
<td>285 ± 117.2</td>
<td>293 ± 71.3</td>
<td>306 ± 82.8</td>
</tr>
<tr>
<td>Glucose (mM)</td>
<td>5.02 ± 0.42</td>
<td>4.16 ± 1.10</td>
<td>4.77 ± 0.53</td>
<td>4.60 ± 0.38</td>
</tr>
</tbody>
</table>

Abbreviations: CRP, C-reactive protein; GS, Gilbert’s syndrome; HDL, high density lipoprotein; LDL, low density lipoprotein; TC, total cholesterol; TG, Triacylglycerol
6.4 Discussion

The results from this study suggest that individuals with GS may have increased protection from developing acute exercise associated platelet activation and aggregation. Reduced arachidonic acid induced platelet aggregation (COX-1 pathway) at rest and after exercise was observed in GS individuals when compared to the non-GS healthy population. Reduced collagen induced platelet aggregation (GP VI) were observed in GS group, only after exercise. Several studies have previously demonstrated that acute intense exercise by sedentary individuals can increase ROS production, thereby elevating oxidative stress in these individuals (311, 312). Inhibition of these platelet aggregation pathways could be related to the additive antioxidant potential of elevated UCB in GS. Reduction in the expression of activation-dependent platelet surface marker, P-selectin at rest and after exercise, signifies reduced release of P-selectin from α-granules of activated platelets, independent of exercise related stress. Lower levels of the circulating inflammatory biomarker hsCRP at rest and after exercise and decreased IL-10 at rest in GS supports a hypothesis that, reduced platelet activation and aggregation along with lower levels of inflammation may represent mechanisms by which sedentary GS individuals may have reduced prevalence of CVD, which is usually associated with increased oxidative stress.

Intense and unaccustomed exercise by sedentary individuals can result in elevated free radical production and oxidative stress, as result of elevated oxygen consumption (303). In the current study, participants with a sedentary lifestyle were required to perform exercise at 70% of their VO\textsubscript{2PEAK}, thus leading to an acute state of metabolic, oxidative stress (125, 198, 298, 300, 308). This protocol induces a robust and reliable oxidative stress as published by us and other groups (198, 297, 298, 300, 301, 308). Conditions such as obesity, metabolic syndrome and diabetes are associated with increased free radical production and oxidative stress, which is considered as one of the many possible
causes for the development of a pro-thrombotic state in these individuals (76, 144, 198). The platelet aggregation results from this study suggest that elevated levels of UCB may target the COX-1 pathway of platelet activation, thereby inhibiting the production of thromboxane A$_2$ (TxA$_2$) and subsequent platelet aggregation (162). Hydrogen peroxide (H$_2$O$_2$) is an oxidant that plays a significant role in the activation of the COX-1 pathway (313). Scavenging H$_2$O$_2$ required for the production of TxA$_2$ by UCB could be considered as a possible explanation for reduced AA induced platelet aggregation in GS at rest and after exercise (14, 162, 232, 313). Inhibition of collagen receptor activity namely, GP VI/integrin α2β1, could also represent one of several possible reasons for reduced collagen induced platelet aggregation (270, 314, 315). The results of this study are in agreement with the previous in vitro trial (Chapter 4) where 35μM UCB concentration significantly reduced collagen induced platelet aggregation. Equivalent concentrations of UCB (35μM) were also seen in GS individuals recruited in the current study. Other studies have also demonstrated that GS individuals have circulating UCB levels close to 35μM (17, 22, 175). Interestingly there was no effect on ADP stimulation at 35μM UCB (270). Similarly, in the current study, no significant reduction in ADP-induced platelet aggregation was observed in GS at rest or after exercise suggesting that physiological UCB concentrations has no influence on P$_2$Y$_{12}$/P$_2$Y$_1$ ADP receptors. These data also support the findings of Naveen Kumar et al. who showed that UCB significantly inhibits collagen induced aggregation and adhesion of washed platelets (204).

Inoguchi et al, have reported that diabetic GS individuals have reduced prevalence of vascular complications and CVD in comparison with normal diabetic individuals (38). Improved endothelial function, nitric oxide (NO; natural platelet aggregation inhibitor) production and reduced oxidative damage might represent possible mechanisms by which UCB may negate the pro-thrombotic effect of elevated oxidative stress in GS individuals with diabetes and other co-morbidities (284).
Significant inhibition in P-selectin expression was observed in GS at rest and after exercise. Several other studies have reported decreased P-selection expression in response to exogenous antioxidant supplementation. We speculate that the anti-platelet effect of UCB in GS may be related to enhanced circulating antioxidant capacity (17). P-selectin expression on platelets increases after exercise and could be associated increased oxidative and shear stress post exercise (307, 309). However, no increase in P-selectin expression in GS individuals, post exercise was observed, suggesting that GS are resistant to acute exercise induced platelet hyper-activity. Although, not statistically significant, an increase in the P-selectin expression post exercise was observed in control subjects, which is in agreement with the previous studies, showing increased P-selectin expression post exercise. A statistically significant result could have been obtained by increasing the numbers in the study. The results from the flow cytometric evaluation of P-selectin expression suggests that in GS individuals, acute exercise activates platelets less intensely when compared normal healthy individuals. Several studies have previously shown that intense acute aerobic exercise in sedentary population is associated with increased platelet activity and subsequent P-selectin expression (299-302, 307-309). In agreement with the current findings, Tapan and colleagues reported a negative association between elevated UCB levels and plasma P-selectin concentration with significantly reduced soluble P-selectin in GS individuals (111). With platelets being one of the primary sources for circulating P-selectin, elevated plasma P-selectin is directly associated with increased platelet activity and the risk of future CVD (215, 316). Furthermore, UCB (10 μM) in the presence of hemin (Heme-oxygenase substrate and enzyme inducer) significantly inhibited ferric chloride (FeCl$_3$) induced thrombus formation (174). The authors hypothesised that UCB may inhibit FeCl$_3$ induced thrombus formation by restraining P-selectin expression, which was confirmed, using a physiological model of oxidative stress (174). However, the exact mechanism by which UCB inhibits P-selectin is yet to be
confirmed. Elevated UCB in GS had no significant effect on PAC-1 expression on activated platelets both at rest and after exercise, suggesting that UCB does not affect the GP IIb-IIIa, fibrinogen receptor mediated platelet activation.

The results from this study add importantly to understanding the importance of mildly elevated levels of UCB in GS, in alleviating the risk of platelet hyperactivity after acute exercise in sedentary individuals. Constant–load exercise at 70% of VO\textsubscript{2PEAK} was used to induce oxidative stress in participants. Previously several studies have successfully shown that intense and unaccustomed exercise by sedentary individuals can significantly induce oxidative stress (125, 144, 198). Several other studies have reported an increase in pro-thrombotic activity after a bout of intense exercise (297, 298, 308). Increased platelet sensitivity to agonists, glycoprotein activation, and expression of adhesion molecules were some of the mechanism associated with increased platelet activity due to intense exercise induced oxidative stress (297, 298, 308, 309). Furthermore intense exercise increases P-selectin expression on platelets, as seen in the current study (309).

In conclusion, this study suggests that GS may be protective against platelet hyperactivity at rest and after metabolic stress. Inhibiting platelet aggregation by two discrete agonists ex vivo may represent a possible mechanisms for protection in GS individuals.
7. Comparing the gene expression of *SELP* and eNOS genes in individuals with Gilbert’s syndrome and normal healthy population
7.1 Introduction

Gilbert’s syndrome (GS) is a common genetic condition seen in about 3-17% of the general population and is inherited in an autosomal recessive fashion (17). Uridine diphosphate-glucuronosyltransferase (UGT1A1) is an enzyme responsible for conjugating and clearing bilirubin. In GS individuals the mutation in UGT1A1 gene responsible for the UGT1A1 enzyme, results in reduced conjugation and excretion of unconjugated bilirubin (UCB), thus leading to mild unconjugated hyperbilirubinemia (284). The negative association between GS and the risk of CVD has already been well established (284). Mutations in several other genes controlling bilirubin metabolism (both UCB and conjugated bilirubin) have also been associated with reduced CVD risk, however, mutation in UGT1A1 is considered as the most significant of all (28). Changes in the relative expression of a gene can greatly influence the production of their respective proteins that play a crucial role in the pathogenesis of chronic disease. Several genes have been identified, that are known to play a key role in regulating platelet and haemostatic activity (317-323). Increased expression of these genes and their associated polymorphisms were shown to have a significant impact on the risk of thrombosis and subsequent CVD (323-326).

P-selectin is a cellular adhesion molecule, released from activated platelets and endothelial cells (327). Increased levels of soluble and platelet bound P-selectin have shown to be associated with increased risk of thrombosis and future risk of developing CVD (327-329). The SELP gene at 1q21, contains 17 exons and is >50 kb in length. SELP is responsible for P-selectin expression (327). P-selectin in humans is produced as a result of the alternative splicing of exons containing transmembrane domain (327). Tregouet DA et al., have shown that increased expression of specific haplotypes of the SELP genes are associated with increased risk of myocardial infarction (329, 330).
Endothelial nitric oxide synthase (eNOS) is an enzyme responsible for the production of nitric oxide (NO) within the endothelial cells (284). Nitric oxide plays a central role in regulating endothelial function, as a vasodilator and more importantly an intravascular platelet aggregation inhibitor (109, 284). Reduced levels of NO were associated with increased risk of thrombosis (109, 284, 331). The production of NO is associated with the availability of eNOS enzyme, which is encoded by the *eNOS3* gene located at 7q36 (332, 333). An increased expression of *eNOS3* has been associated with increased NO availability, which can reduce the risk of thrombosis (334, 335). Thrombin is a serine protease formed as a result of proteolytic cleavage of prothrombin and is known to convert fibrinogen to fibrin, thus enabling clot formation (336). Thrombin was previously shown to inhibit *eNOS3* expression and NO production (336).

The aim of this study was to evaluate if individuals with GS may have different expression of *SELP* and *eNOS3* in comparison to normal healthy individuals. From the previous studies that were conducted as a part of this thesis, it was seen that individuals with GS have reduced platelet activity and P-selectin expression. However, it is unclear if the observed reduction in platelet activity and P-selectin expression is due to downregulation of *SELP* gene, and upregulation of *eNOS* gene. The hypothesis for this study is that individuals with GS may have a favourable expression of *SELP* and *eNOS3* genes that play a key role in regulating thrombus formation. **Materials and Methods**

### 7.2.1 Recruitment and sample collection

Fourteen normal healthy and fourteen individuals with Gilbert’s syndrome were recruited from the general population via advertisements in and around Griffith University, Gold Coast, QLD, Australia. A written consent was obtained from all the volunteers prior to their participation in the study. Please refer to Chapter 2 Materials and Methods section 3.2 for a detailed description on volunteer recruitment and screening. Three ml of fasting blood sample was collected from the median cubital vein into the
PAXgene® blood RNA tubes. After blood collection the blood tubes were gently mixed for couple of minutes, and incubated at room temperature for 2 hours, followed by storing at -80°C for future analysis.

7.2.2 Isolation of RNA and cDNA synthesis

The PAXgene® blood RNA kit was used to extract total RNA from the PAXgene® blood RNA tubes, according to manufacturer’s protocol (Qiagen, VIC, Australia; refer to Chapter 10, Appendices Section 10.7, Appendix 7 for a manufacturer’s protocol). Prior to commencing the RNA extraction process the PAXgene® blood RNA tubes were briefly thawed, followed by using various stabilization buffers, digestion of proteinase K and spin column clean up, for extraction and isolation of RNA. The quality of isolated RNA was measured by calculating the total absorbance of the sample using a Nanodrop at 260 nm (Thermo Scientific, Australia). The isolated total RNA was used to synthesise cDNA using iScript cDNA Synthesis kit (BioRad Laboratories, Inc., CA). After adding the iScript reverse transcriptase along with the total RNA into the 0.2 ml PCR tube, the mixture was incubated as per manufacturer’s instructions to facilitate cDNA reactions as seen in Figure 18.

Figure 18. Showing the various incubation steps involved in the syntheses of cDNA from RNA.
7.2.3 Primer selection

The primer sequences used in this study were selected from literature (337). The Nucleotide Basic Local Alignment Search Tool from the GenBank non-redundant nucleotide sequence database (National Centre for Biotechnology Information) was used to further verify the specificity of the primers. The selected primers were then ordered for synthesis by the Gene-Works Pty Ltd. (Thebarton, SA, Australia).

Table 16. Real time PCR primers

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SELP</td>
<td>Forward-TGCACCACCAACTGCTTAGC</td>
</tr>
<tr>
<td></td>
<td>Reverse-GGCATGGACTGTGGTCATGAG</td>
</tr>
<tr>
<td>eNOS</td>
<td>Forward- GGGACCACATAGGTGTCTGC</td>
</tr>
<tr>
<td></td>
<td>Reverse- CCAGCACAGCTACAGTGAGG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward-GGATTGTTCATGACACTCGTGG</td>
</tr>
<tr>
<td>(Reference gene)</td>
<td>Reverse-GAGGTTGAGCAGTTCATCG</td>
</tr>
</tbody>
</table>

7.2.4 Evaluating gene expression using Real-Time PCR

The Corbett RotorGene 3000 (Corbett life Science, Qiagen, VIC, Australia) was used to perform real-time PCR reactions, with SYBR Green as the detection dye. The optimal amplification of the target gene at minimum primer concentration, along with minimising non-specific gene amplification was ensured by optimising the primer concentrations prior to evaluating the gene expression. The reaction mix for each RT-PCR reaction consisted of a measured volume of various reagents, which were calculated using primer optimization, as shown in table 17 below.

Table 17. Volume of various reagents and cDNA used to make up the final volume for each amplification.
<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>iQ SYBR Green</td>
<td>10 μL</td>
</tr>
<tr>
<td>cDNA (1/50 dilution)</td>
<td>2 μL</td>
</tr>
<tr>
<td>Oligonucleotide primers</td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>4 μL</td>
</tr>
<tr>
<td>Reverse</td>
<td>4 μL</td>
</tr>
<tr>
<td>Final Volume</td>
<td>20 μL</td>
</tr>
</tbody>
</table>

A pool of cDNA samples were used alongside each primer set to act as a positive control to confirm primer amplification, while no-template samples were used as negative control. The PCR reaction was performed at 60°C as the annealing temperature and the cycling parameters are as follows: 95°C for 10 min, 60 cycles of 95°C for 15 sec, annealing (60°C) for 30 s, 72°C for 30 s and ramping from 72°C to 95°C rising by 1°C each step. The endogenous control gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to calculate the ΔCₜ values. The amplification of all the test and control samples was performed in triplicates. The results obtained from determining the cycling parameters were analysed using the comparative Cₜ method as the means of relative quantification, and normalised to the endogenous control gene (housekeeping gene; GAPDH) and expressed as $2^{(ΔCₜ)}$.

### 7.2.5 Statistical analysis

BestKeeper© software was used to analyse the results and to further ensure the accurate selection of reference gene. The difference in cycle threshold values against the housekeeping genes was calculated using Microsoft Excel (Microsoft office 2007). An unpaired *t*-test was performed using GraphPad Prism version 6.0 (GraphPad Software,
La Jolla, California, USA) to statistically analyse the data. A $p$ value of less than 0.05 was considered as statistically significant difference between the test and the control

7.3 Results

A statistically significant difference in the genetic expression of $SELP$ and eNOS genes was observed between GS and controls. Individuals with GS expressed significantly increased expression of $SELP$ gene when compared with the control subjects. Similarly a significant increase in the relative expression of eNOS gene was also observed in individuals with GS when compared with control subjects.

![Relative expression of P-selectin gene expression](image)

**Figure 19.** Relative expression of $SELP$ gene in control and Gilbert’s syndrome individuals. A statistically significant decrease in the relative expression of $SELP$ gene was seen in control compared to GS individuals ($p<0.001$).
Relative expression of eNOS gene in control and Gilbert’s syndrome individuals. The relative expression of eNOS gene was significantly upregulated in GS individuals compared to control ($p<0.01$).

**Figure 20.** Relative expression of eNOS gene in control and Gilbert’s syndrome individuals. The relative expression of eNOS gene was significantly upregulated in GS individuals compared to control ($p<0.01$).

7.4 Discussion

In this study, the difference in the relative expression of thrombus regulating SELP and eNOS genes between GS and control individuals was evaluated. The genes of interest SELP and eNOS were selected based on the previous results (chapter 5 and 6) which showed that individuals with GS have reduced P-selectin expression and platelet activity. The SELP gene regulates the protein expression of P-selectin, while eNOS gene was responsible for the production of endothelial nitric oxide (327, 334). Both SELP and eNOS genes were significantly upregulated in GS individuals when compared with the control subjects. The PAXgene® blood RNA kit is a system that specializes in stabilization, purification and collection of intracellular RNA. The cDNA required for evaluating the relative gene expression of SELP and eNOS were extracted from whole blood using PAXgene blood tubes. Previously, studies have used PAX gene tubes to extract mRNA to evaluate the expression of SELP and eNOS genes, as the expression of
certain genes in whole blood is representative of gene expression in tissues (338-340). White blood cells circulate throughout the body and constantly interact with several other cells such as endothelial cells in the blood vessels, thus making them the most important cells to study the changes in the gene expression of several markers that are known to play a key role in the development of CVD (341). Circulating white cells such as peripheral blood mononuclear cells (PBMNCs) have been considered as an effective model in unveiling the various pathways leading to CVD. Previously, whole blood was used to study the changes in gene expression of several markers associated with blood pressure, inflammation and antioxidants enzymes within the body (341-344). Therefore, gene expression profiling of circulating white blood cells could potentially act as a diagnostic tool in detecting early stages of CVD development.

P-selectin is an important pro-coagulant cell adhesion molecule, and is known to play an important role in the process of clot formation by stimulating tissue factor release, leukocyte adhesion and inflammation within the vasculature (284, 345). Several studies have shown a direct relationship between the relative expression of SELP, soluble/platelet bound P-selectin concentration, and increased risk of vascular complications (327, 329). Herrmann SM et al., have reported that the SELP gene is highly polymorphic and several polymorphisms were associated with an increased risk of myocardial infarction (MI) (328). Interestingly, the Pro715 allele polymorphism in the SELP gene was associated with reduced risk of MI (328). Previously studies have shown that a 1.8 fold increase in the relative expression of the SELP gene was linked to an increased leukocyte accumulation and monocyte adhesion (337). Results from the previous studies (study 3 & 4) have shown reduced P-selectin expression on activated platelets in GS individuals at rest and after acute intense exercise bout, suggesting reduced P-selectin gene expression in GS individuals. Furthermore, Tapan et al., have reported a
significantly reduced levels soluble P-selectin levels in GS (111). Interestingly, in the current study a statistically significant increase in the relative expression of SELP was reported in GS. Even though the observed results were statistically significant, the clinical significance of this result may be questionable, as the fold change in gene expression between GS and control is too small. Elevated SELP gene expression is associated with increased levels of soluble P-selectin, a decreased soluble P-selectin levels and platelet bound P-selectin expression suggests the presence of an alternate mechanism by which GS individuals may inhibit P-selectin protein expression. Previous in vitro have shown that bilirubin can reduce the genetic expression of several adhesion molecules including P-selectin suggesting that bilirubin might be partially responsible for inhibiting expression of adhesion molecules (174, 193). However further studies are strongly needed to support the above mentioned hypothesis.

The relative expression of eNOS gene was significantly upregulated in GS, when compared with control subjects. Nitric oxide synthase (NOS) exists in 3 different forms endothelial NOS (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS) within the body and is expressed in several types of cells such as endothelial cells, leukocytes and platelets (346). Most leukocytes and platelets express eNOS gene, making them an ideal model to study the changes in the gene expression of eNOS in pathological conditions such as CVD (342, 344). Furthermore, Sase K and Michel T initially observed eNOS mRNA in human platelets and have hypothesised that platelet eNOS expression may play a role in the regulation of platelet function by an endogenous NO pathway (347). Changes in the activity of platelet eNOS has been suggested to be responsible for abnormal platelet activity as reported in pro-thrombotic conditions such as diabetes (346, 347). Previous studies have reported improved flow mediated dilation in individuals with GS (106, 113, 116, 191). Improved flow mediated dilation is a direct marker for improved endothelial function and NO bioavailability. A decrease in NO bioavailability has been associated
with reduced eNOS expression and endothelial dysfunction. Based on the current results, an increase in the relative expression of eNOS gene can increase the production of eNOS enzyme, that can subsequently help in the production of increased vascular NO concentrations. Increased eNOS genetic expression was shown to be associated with activation of PI3 kinase/Akt signalling pathway, which are known to play a significant role in the production of eNOS enzyme and NO (348, 349). Furthermore, increased eNOS genetic expression was previously shown to be associated with reduced risk of thrombosis and vascular disease (332, 334, 349).

The results from this study are the first to shown a difference in the relative expression of SELP and eNOS genes in GS individuals when compared with control subjects. It was observed that individuals with GS had an elevated SELP and eNOS gene expression when compared with control subjects. Although increased SELP gene expression is associated with increased risk of platelet activation and inflammation, the change in SELP gene expression in GS was relatively small (Figure 19) (284). Furthermore, evaluating the gene expression in a larger cohort could have given a different outcome. The finding of increased eNOS gene expression can be considered as one of many possible explanations for a reduced CVD risk in GS. An increased eNOS genetic expression is negatively associated with several cardiovascular risk factors such as increased platelet activity, endothelial dysfunction and reduced vascular elasticity (332). However further studies comparing the relative gene expression of genes such as SELP and tissue factor are required to establish a strong correlation between GS and reduced risk of CVD.
8. Discussion and Future directions
8.1 Discussion

The overall aim of the experiments undertaken as a part of this thesis was to evaluate and unveil the effect of mildly elevated UCB in GS in reducing various markers that are associated with CVD. The results from the *in vitro* and *in vivo* studies completed for this thesis indicate that mildly elevated levels of UCB as seen in GS individuals are associated with inhibiting AA and collagen induced platelet aggregation, ADP induced platelet degranulation and P-selectin expression, reduced inflammation and improved lipid profile. After decades of research, investigators have concluded that elevated oxidative stress and free radical production are one of the primary perpetrators for increased risk of cardiovascular events (92, 100). Elevated oxidative stress was shown to positively influence several risk factors such as platelet hyper-activity along with increased vascular inflammation and lipid peroxidation (76, 96). Antioxidants in recent years have become a topic of great interest due to their hypothesised ability to reduce the risk of cardiovascular risk factors by scavenging free radicals and reducing oxidative stress (11, 122, 161). Unconjugated bilirubin is a potent endogenous antioxidant, which is physiologically produced as a result of heme-catabolism. Several studies have shown that elevated levels of UCB are associated with attenuating several cardiovascular risk factors such as, inflammation and improving lipid profile (also observed in the results of studies undertaken for this thesis) as well as endothelial dysfunction (14, 22, 171, 232). The above mentioned phenomenon was reported in individuals with GS, who experience mildly elevated levels of UCB. Gilbert’s syndrome is a common genetic condition seen in about 3-17% of the general population and is inherited in an autosomal recessive fashion. In GS individuals the mutation in *UGT1A1* gene responsible for the UGT1A1 enzyme, results in reduced conjugation and excretion of UCB, thus leading to mild unconjugated hyperbilirubinemia (22). Mildly elevated UCB in GS allows these individuals to have several cardiovascular benefits such as reduced oxidative stress,
vascular inflammation and improved lipid profile (112). However, a dearth of knowledge exists on the effect of elevated UCB in GS on pro-thrombotic markers such as platelet activation, aggregation and coagulation markers under at rest as well as after intense strenuous exercise. We hypothesised that mildly elevated UCB in GS may impart its cardio-protective effects by reducing platelet activity.

In order to support the above mentioned hypothesis, a total of four different studies were conducted. The first study evaluated the \textit{in vitro} effect of UCB at concentrations seen in GS on platelet aggregation, coagulation profile and biochemical parameters. A final concentration of approximately 35 μM of UCB was selected to explore the possible effects of exogenous administration of bilirubin at physiological concentrations seen in GS individuals. The results from this study demonstrated mildly elevated UCB at concentration 35 μM significantly inhibited collagen induced platelet aggregation. Furthermore, \textit{in vitro} UCB at higher concentrations also inhibited collagen and ADP induced platelet aggregation in a dose dependent manner. Previously, study done by Naveen Kumar and colleagues observed a dose dependent inhibition of collagen induced platelet aggregation by UCB, similar to the results obtained in the first \textit{in vitro} study of this thesis (204). However, Naveen \textit{et al.}, used washed platelets, while the \textit{in vitro} study conducted for this thesis used whole blood. It was also reported that inhibition of platelet adhesion by UCB could be both receptor mediated and agonist mediated. On the other hand, Maurer \textit{et al.} reported that UCB at concentrations \( \geq 10\text{mg/dL} \) (i.e., \( \geq 172 \mu\text{M} \)) when added to PRP, induced minimal aggregation. This observed variation might be to the difference in techniques and the high concentrations of UCB employed to perform the study (206). No significant effect of UCB at 35 μM was observed when ADP was used to stimulate platelets. ADP is a soluble agonist released from platelet dense granules, and is known to stimulate platelet activation by interacting with purinergic receptors \( \text{P}_2\text{Y}_{12} \) and \( \text{P}_2\text{Y}_1 \) (79). A significantly reduced collagen induced platelet aggregation by \textit{in vitro}
addition of UCB suggested that, mildly elevated UCB inhibits the platelet aggregation, thereby showing the potential to reduce the risk of intravascular thrombosis at concentrations that are physiologically seen in GS individuals. This could be one the many mechanisms by which mildly elevated UCB in GS may impart its cardio-protective effects.

Based on the results from the first study, the next study was designed to evaluate if mildly elevated UCB in GS may hold similar anti-platelet effect in vivo. A statistically significant reduction in AA and collagen induced platelet aggregation was observed in GS when compared with normal healthy individuals. These results suggest UCB in GS significantly reduced platelet aggregation by targeting two discrete pathways of platelet activation namely the arachidonic acid COX-1 pathway and the GP VI collagen receptor function. A statistically significant correlation between bilirubin concentration and AA and collagen induced platelet aggregation, along with bilirubin explaining the variation in platelet aggregation, further strengthens the observed anti-thrombotic effect due to elevated bilirubin in GS. Significantly reduced P-selectin/CD62P mAb expression on platelet surface was observed in GS individuals when compared to control. Furthermore, reduced P-selectin expression was significantly correlated with increasing bilirubin concentrations. Reduced P-selectin expression signifies a reduced activation-dependent de-granulation (α granule) of platelets. P-selectin is an adhesion molecule released from the α granules of activated platelets. P-selectin is known to play a key role in increasing the size of thrombus by recruiting circulating leukocytes at the site (79). The novelty of this study is that no previous work has been conducted to evaluate the platelet function in GS. Upon literature review, it was found that this was the first study to report reduced platelet activity in GS individuals. The only other study examining platelets in GS observed a reduced mean platelet volume (MPV) in GS individuals when compared normal healthy individuals (175). Although no platelet function assay was performed
similar to those in this thesis, Tapan et al., observed reduced plasma levels of soluble P-selectin in GS (111).

Reduced levels of the inflammatory biomarker hsCRP and an improved lipid status were also reported in GS individuals when compared with normal healthy individuals. These findings, further support an emerging hypothesis that might contribute to protection against the development of CVD in GS. The overall results of this study demonstrate that reduced platelet activity in GS could be responsible for hypothesized cardio-protective benefits in these individuals.

In the light of these findings, the following study investigated if individuals with GS, who are hypothesised to have an increased total antioxidant capacity, exhibit platelet hyper-activity after acute intense exercise bout. Reduced AA induced platelet aggregation at rest and after exercise was observed in GS individuals when compared to the non-GS healthy population. An inhibition of collagen induced platelet aggregation was observed in GS individuals after exercise. However, in normal healthy no such decrease was observed in collagen induced platelet aggregation post exercise. Several studies have previously shown that, unaccustomed exercise at 70% of individuals VO2PEAK was associated with increased platelet activation, de-granulation and P-selectin expression (297, 306). The results of this study suggest that GS individuals may have improved protection from platelet hyperactivity after acute intense exercise, when compared to normal healthy individuals. Furthermore, significantly reduced platelet adhesion molecule P-selectin along with lower levels of inflammation marker hsCRP were also observed pre and post-acute exercise bout in GS individuals. Reduced P-selectin expression is associated with a reduction in thrombus size, as P-selectin is known to actively recruit circulating leucocytes, thereby increasing the size of thrombus. Acute and intense exercise by sedentary individuals was associated with an increased risk of developing thrombosis and subsequent ischemic attack (299, 300, 311). Furthermore,
intense and unaccustomed exercise by sedentary individuals can result in elevated free radical production and oxidative stress, as result of elevated oxygen consumption (311). The results from this study demonstrate that individuals with GS may have greater protection from the risk of having increased platelet activation and subsequent CVD even under conditions such as exercise induced oxidative stress. Conditions such as type 2 diabetes are known to be associated with increased oxidative stress. The results for the study have shown that diabetic GS individuals may have a reduced risk of developing vascular complications when compared with the diabetic population without GS (38). These findings of Inoguchi et al., agree with the results of this thesis which showed decreased platelet activity after exercise in GS may be due to increased antioxidant capacity of GS neutralising the effects of oxidative stress. Improved endothelial function, nitric oxide (NO; natural platelet aggregation inhibitor) production and reduced oxidative damage might represent possible mechanisms by which UCB may negate the pro-thrombotic effect of elevated oxidative stress in GS individuals with diabetes and other co-morbidities.

To further evaluate, if the observed reduction in thrombotic markers was due to mildly elevated UCB in GS influencing the genetic expression of thrombotic markers, P-selectin (SELP) and endothelial nitric oxide synthase (eNOS), the next study compared the relative gene expression of these genes in the whole blood of GS and control. Interestingly, a significant increase in the relative expression of SELP gene and eNOS gene were observed in GS when compared with control subjects. Whole blood collected in PAXgene tubes was used to evaluate gene expression, as the relative expression of certain genes in whole blood are representative of gene expression in tissues. Furthermore, it is more invasive to obtain vascular endothelial cells from GS volunteers for eNOS gene expression. Although SELP is expressed in platelets, the whole blood was collected in PAXgene blood tubes because it has an additive that reduces mRNA
degradation and preserve intracellular mRNA (350). A statistically significant increase in the relative expression of SELP gene in GS was observed. A decreased platelet bound P-selectin expression as observed in the previous studies completed in this thesis and decreased levels of soluble P-selectin as reported in the literature (111), suggests the presence of an alternate mechanism by which GS individuals may inhibit P-selectin protein expression on platelets. A significant increase in the genetic expression of eNOS in GS was observed. Although no researchers have investigated the gene expression of SELP and eNOS in GS, some previous studies have reported improved flow mediated dilation in individuals with GS (106, 113, 116, 191). Improved flow mediated dilation is a direct marker for improved endothelial function and NO bioavailability. A decrease in NO bioavailability has been associated with reduced eNOS expression and endothelial dysfunction. Based on the current results, an increase in the relative expression of eNOS gene can increase the production of eNOS enzyme, that can subsequently help in the production of increased vascular NO concentrations. The results from the final study have shown that improved endothelial function in GS may be due to the upregulation in the relative expression of eNOS gene. However, further studies are required to prove this hypothesis.

In conclusion, the results obtained from the studies undertaken for this thesis, were the first to show a positive association between mildly elevated levels of UCB in GS and attenuation of platelet activity, which may be responsible for reduced risk of thrombosis, thereby possibly providing an increased protection against the development of CVD in GS individuals. It is still unclear if the hypothesised elevated total antioxidant capacity in GS may be responsible for improved platelet activity. However, inhibition of platelet aggregation by two discrete pathways may suggest a novel mechanism by which mildly elevated levels of UCB may reduce the risk of cardiovascular events, even in individuals who experience elevated oxidative stress as a result of co-morbidities such as T2DM and
obesity. Furthermore, the observed improved lipid profile and reduced inflammation marker suggest that elevated UCB in GS may play a key role in delaying the atherosclerotic processes. However, the current research projects do have some important limitations. The platelet surface marker expression, in particular P-selectin was not analysed in the first *in vitro* study, which would have further complemented the findings in chapter five and six. Secondly, total antioxidants and oxidative stress markers were not measured for the studies conducted for this thesis. However several studies have successfully demonstrated that GS population have higher antioxidant capacity and lower levels of oxidative stress markers.

The results from this research have provided novel insights into one of the many possible mechanisms associated with increased protection against CVD in GS. This thesis was the first to demonstrate the mechanistic pathways involving platelet activity that may be responsible for reduced incidence of CVD in GS. However, there are several questions that are yet to be addressed, such as understanding of the exact mechanism by which UCB inhibits platelet activation and de-granulation, improve lipid profile and reduce inflammation. Furthermore, future research should be focused on evaluating the effect of UCB on thrombotic markers in diabetic individuals with and without GS. The outcome of the future studies addressing these issues may play a key role unveiling the mechanistic pathways by which UCB may be associated with increased protection against the development of CVD.


9. References


167. Erdogan D, Gullu H, Yildirim E, Tok D, Kirbas I, Ciftci O, Baycan ST, Muderrisoglu H. Low serum bilirubin levels are independently and inversely related to impaired flow-mediated vasodilation and increased carotid intima-media thickness in both men and women. Atherosclerosis. 2006;184(2):431-7.


253. Kamruzzaman SM, Endale M, Oh WJ, Park SC, Kim KS, Hong JH, Kwak YS, Yun BS, Rhee MH. Inhibitory effects of Bulnesia sarmienti aqueous extract on agonist-


326. McManus DD, Beaulieu LM, Mick E, Tanriverdi K, Larson MG, Keaney JF, Benjamin EJ, Freedman JE. Relationship among circulating inflammatory proteins,


10. Appendices
This certificate generated on 15-07-2015.

This certificate confirms that protocol 'Approved procedure: Venepuncture, umbrella ethical cover for venous blood collection from human volunteers to maintain, develop and standardise the techniques and procedures in Hematology research laboratory.' (GU Protocol Number MSC/13/11/HREC) has ethical clearance from the Griffith University Human Research Ethics Committee (HREC) and has been issued with authorisation to be commenced.

The ethical clearance for this protocol runs from 05-09-2011 to 26-09-2016.

The named members of the research team for this protocol are:

Dr Andrew Bulmer
Miss Connie Boon
Dr Indu Singh
Mr Abishek Santha Kumar
Mr Avinash Kundur

The research team has been sent correspondence that lists the standard conditions of ethical clearance that apply to Griffith University protocols.

The HREC is established in accordance with the National Statement on Ethical Conduct on Research Involving Humans. The operation of this Committee is outlined in the HREC Standard Operating Procedure, which is available from www.gu.edu.au/or/ethics.

Please do not hesitate to contact me if you have any further queries about this matter.

Rick Williams
Manager, Research Ethics
Office for Research
Bray Centre, N54 Room 0.15 Nathan Campus
Griffith University
Phone: 07 3735 4375
Facsimile: 07 373 57994
Email: rick.williams@griffith.edu.au
HUMAN RESEARCH ETHICS COMMITTEE

ETHICAL CLEARANCE CERTIFICATE

This certificate generated on 15-07-2015.

This certificate confirms that protocol 'Comparative evaluation of thrombotic risk factors in Gilbert's syndrome and healthy population upon induced oxidative stress.' (GU Protocol Number MSC/12/12/HREC) has ethical clearance from the Griffith University Human Research Ethics Committee (HREC) and has been issued with authorisation to be commenced.

The ethical clearance for this protocol runs from 19-09-2012 to 24-12-2013.

The named members of the research team for this protocol are:

Dr Andrew Bulmer
Dr Indu Singh
Mr Avinash Kundur

The research team has been sent correspondence that lists the standard conditions of ethical clearance that apply to Griffith University protocols.

The HREC is established in accordance with the National Statement on Ethical Conduct on Research Involving Humans. The operation of this Committee is outlined in the HREC Standard Operating Procedure, which is available from www.gu.edu.au/or/ethics.

Please do not hesitate to contact me if you have any further queries about this matter.

Rick Williams
Manager, Research Ethics

Office for Research
Bray Centre, N54 Room 0.15 Nathan Campus

Griffith University
Phone: 07 3735 4375
Facsimile: 07 373 57994
Email: rick.williams@griffith.edu.au
VOLUNTEERS REQUIRED

Do you have Gilbert’s syndrome?

With no history of heart disease, bleeding disorders and currently not on any form of special diet

Researchers from the School of Medical Science are currently seeking participants for a study on individuals with Gilbert’s Syndrome. We would like to hear from you if you fit the above criteria

- Gilbert’s syndrome is a benign condition seen in 10% of population and is characterised by mildly elevated levels of unconjugated bilirubin
- Many studies have shown an inverse relationship between the prevalence of cardiovascular diseases and increased serum concentrations of unconjugated bilirubin in Gilbert’s syndrome individuals
- We hypothesize that increased antioxidant status of individuals with Gilbert’s syndrome may lead to decreased platelet and clotting activity, reducing inflammation and improving lipid profile thus providing protection from cardiovascular diseases under induced oxidative stress conditions including acute exercise

What would you be required to do?

- You will be required to complete 2 questionnaires
- You will be required to exercise for about 45 minutes under the supervision of an accredited exercise physiologist and a physician
- Pre and post exercise blood sample will be collected

TOTAL TIME OF INVOLVEMENT IN STUDY: NO MORE THAN 3 HOURS IN TWO SEPERATE VISITS
A full explanation of the study and all your blood test results will be provided to you and all your information will be handled in strict confidence.

Your participation will be greatly appreciated.

If you are interested and require further information:

- Mr. Avinash Kundur: a.kundur@griffith.edu.au or (07) 5552830; Mob: 0424740027
- Indu Singh: i.singh@griffith.edu.au or (07) 55529821
- Andrew C Bulmer: a.bulmer@griffith.edu.au or (07) 55528215
INVITATION TO PARTICIPATE IN A RESEARCH PROJECT

PLAIN LANGUAGE STATEMENT

Project Title: Effect of Unconjugated bilirubin on thrombotic risk factors in patients with Gilbert’s Syndrome under induced oxidative stress conditions

Investigators: Mr. Avinash Reddy Kundur, Dr. Indu Singh and Dr. Andrew C Bulmer

Dear Participant

You are invited to participate in a research project being conducted by GRIFFITH UNIVERSITY. This information sheet describes the project in straightforward language or ‘plain English’. Please read this sheet carefully and be confident that you understand its contents before deciding whether to participate. If you have any questions about the project please ask one of the investigators.

Who is involved in this project? Why is it being conducted?

- The above mentioned investigators want to evaluate the protective antioxidative properties of unconjugated bilirubin and its effect on blood clotting and stickiness in blood vessels, one of the major causes of cardiovascular diseases. Many studies have shown an inverse relationship between the prevalence of cardiovascular diseases and the serum concentrations of unconjugated bilirubin. This research project is one of the early phases of a number of possible trials to examine the possible protective effects of unconjugated bilirubin against development of cardiovascular diseases by reducing the process of blood clotting and stickiness. The ultimate aim of our research is to demonstrate if unconjugated bilirubin could be effective in reducing thrombotic risk factors.
- The project needs your consent to be part of the research ever after the Human Research Ethics Committee approval

Why have you been approached?

- You have been selected for this research because you volunteered in response to our advertisement.

What is the project about? What are the questions being addressed?

- The project is about understanding the protective antioxidant effects of unconjugated bilirubin on platelet and clotting activity of patients with Gilbert’s Syndrome under increased oxidative stress conditions
- We hypothesize that increased antioxidant status of patients with Gilbert’s disease may lead to decreased platelet and clotting activity, reducing inflammation and
improving lipid profile thus providing protection from cardiovascular diseases under induced oxidative stress conditions including acute exercise

If I agree to participate, What will I be required to do?

- You will be required to complete 2 questionnaires and give a small amount of blood from your arm vein on day one
- Following the first blood collection procedure you will be required to exercise for about 45 minutes under the supervision of a accredited exercise physiologist and a physician
- A second blood sample will be taken post exercise

What are the risks or disadvantages associated with participation?

- Blood will be collected from your arm vein using the same method as for routine medical blood tests by an experienced and qualified person. A sterile needle is passed into the vein and blood is drawn. The needle is then withdrawn; a cotton swab is gently pressed over the puncture site for approximately two minutes. Adverse effects of taking blood are the minor discomfort associated with the needle passing through the skin and the possibility of minor bruising near the puncture site. The risk of infection is only minima when venepuncture is performed under sterile conditions as described above. Some people may feel briefly dizzy after blood collection; please let us know if this occurs.
- You can feel lethargic or tried during the exercise, however you will be monitored during the whole process by an accredited exercise physiologist and a physician. If you feel dizzy during or after the exercise please notify us.

What are the benefits associated with the participation?

- There are no direct benefits to you form your participation; however it will help the research in this area and may benefit the patients and doctors in future by making guided therapy in cardiovascular disease population.

What are my rights as a participant?

- We want to draw your attention to your rights, which include
  - The right to withdraw their participation at any time, without prejudice.
  - The right to have any unprocessed data withdrawn and destroyed, provided it can be reliably identified, and provided that so doing does not increase the risk of the participant.
  - The right to have any questions answered at any time.

Whom should I contact if I have any questions?

- Avinash Reddy Kundur (07 55528320) or Indu Singh (07 55529821)

What other issues should I be aware of before deciding whether to participate?

- There are no other issues we can suggest at this time which you should also consider before you decide whether to participate or not

The ethical conduct of this research

Griffith University conducts research in accordance with the National Statement on Ethical Conduct in Human Research. If you have any concerns or complaints about the
ethical conduct of the research project, you should contact the Manager, Research Ethics on 3735 5585 or research-ethics@griffith.edu.au

Privacy Statement

The conduct of this research involves the collection, access and/or use of your identified personal information. The information collected is confidential and will not be disclosed to third parties without your consent, except to meet government legal or other regulatory authority requirements. A deidentified copy of this data may be for other research purposes. However, your anonymity will at all times be safeguarded.

Yours sincerely,

Indu Singh (i.singh@griffith.edu.au)
Avinash Reddy Kundur (a.kundur@griffith.edu.au)
Andrew C Bulmer (a.bulmer@griffith.edu.au)
Comparative evaluation of thrombotic risk factors in Gilbert’s Syndrome and healthy population upon induced oxidative stress.

INFORMED CONSENT FORM

Research Team: Avinash Kundur, Indu Singh & Andrew C Bulmer

School of Medical Science

61(7) 55529821

a.kundur@griffith.edu.au

By signing below, I confirm that I have read and understood the information package and in particular have noted that:

- I understand that my involvement in this research study will include giving some blood & urine sample at two different occasions, performing 1 hour of exercise and completing a questionnaire;
- I have had any questions answered to my satisfaction;
- I understand risks involved and how they will be managed;
- I understand that there will be no direct benefit to me from my participation in the research;
- I understand that my participation in the research is voluntary;
- I understand that if I have any additional questions I can contact the research team;
- I understand that I am free to withdraw any time, without comment or penalty;
- I agree to participate in the project.

Volunteers Name: ________________________________

Volunteers Signature: ________________________________

Date: _______ _______ _______

Witness name: ________________________________

Witness Signature: ________________________________
Volunteer Screening

Title of project: **Comparative evaluation of thrombotic risk factors in Gilbert's Syndrome and healthy population upon induced oxidative stress.**

**Confidentiality:** The information given on this form will be treated as confidential. The information will not be copied and will be destroyed if a subject is not selected for the study. Only the researchers involved will have access to this data.

**Name:** ____________________________  **SUBJECT CODE** [ ]  **M/F**

**Date:** ____________________________

**Address:** ____________________________

**Telephone:**  
- Home: ____________________________  
- Work: ____________________________

**Date of Birth:** ____________________________  
- Age: ____________________________  
- Weight: ____________________________  
- Height: ____________________________

What is the earliest time you would be available in the mornings?

**VOLUNTEER INCLUSION CRITERIA:**
1. Between 20-60 years of age.
2. Non-smoker
3. Healthy
4. No known problems with venepuncture

**VOLUNTEER EXCLUSION CRITERIA:**
1. Excessive bleeding tendency
2. Anti-Coagulant therapy
3. Recent GI bleed
4. Liver Disease
5. Anti-inflammatory Drugs affecting platelet
6. Platelet count of <125 & >450
7. History of Hepatitis

**PLEASE COMPLETE THE FOLLOWING DETAILS:**

<table>
<thead>
<tr>
<th>Have you had, or do you have:</th>
<th>Yes</th>
<th>No</th>
<th>Not sure</th>
</tr>
</thead>
<tbody>
<tr>
<td>High blood pressure</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Angina</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart attack</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Do you wish to volunteer for any further studies done by our group in future:

Thank you for your co-operation: We will be selecting 20 normal and 20 Gilbert’s Syndrome patients and only two blood and urine sample will be collected pre and post exercise. Please do not be offended if you are not included in the study, since the selection is based on first come first choice basis and inclusion criteria, we would however like to keep your name for involvement with further studies if you agree.
CVD RISK FACTOR ASSESSMENT PROFORMA

Date: ___________  Name: ____________________________

DOB: ___________  Age: ___________  □ Male  □ Female

POSITIVE RISK FACTORS

1. Age: Men ≥ 45 yr; Women ≥ 55 yr
   □ Yes  □ No

2. Family history: MI, coronary revascularisation, or sudden death before 55 yr in father or male 1° relative (i.e., brother or son), or before 65 yr in mother or female 1° relative (i.e., sister or daughter).
   Notes:

3. Cigarette smoking: Current cigarette smoker or those who quit within the previous 6 months.
   Notes:

4. Hypertension: Systolic blood pressure ≥ 140 mm Hg or diastolic ≥ 90 mm Hg, confirmed by at least 2 measurements on separate occasions, or on antihypertensive medication.
   Notes:

5. Dyslipidaemia: LDL ≥ 3.37 mmol L⁻¹ or HDL < 1.04 mmol L⁻¹, or total serum cholesterol ≥ 5.18 mmol L⁻¹, or on lipid-lowering medication.
   Notes:

6. Prediabetes: Impaired fasting glucose (≥ 5.50 mmol L⁻¹) or impaired glucose tolerance (≥ 7.7 mmol L⁻¹, 2-h value). Confirmed by at least 2 measurements on separate occasions.
   Notes:

7. Obesity: BMI ≥ 30 kg m⁻² or waist girth >102 cm (men) and >88 cm (women).
   Notes:

8. Sedentary lifestyle: Not participating in ≥ 30 min of moderate intensity physical activity (40-60% VO₂R) on ≥ 3 d wk⁻¹ for at least 3 months.
   Notes:

NEGATIVE RISK FACTOR

1. HDL ≥ 60 mg dl⁻¹ (1.55 mmol L⁻¹)
   □ Yes  □ No

RISK STRATIFICATION

□ LOW – < 45 y (M) or < 55 y (F) and asymptomatic with ≤ 1 CVD Risk Factor.
□ MODERATE – ≥ 45 y (M) or ≥ 55 y (F) or asymptomatic with ≥ 2 CVD Risk Factors.
□ HIGH – ≥ 1 signs symptoms or known CVD, pulmonary or metabolic disease.

Notes:
MEDICAL HISTORY QUESTIONNAIRE

Name: ___________________________ Date: ___________

Date of Birth: ___________ Age: ___________ Male □ Female □

Address: ____________________________________________________________

Phone: ( ) ___________ (Home) Phone: ( ) ___________ (Work/Mob)

1. Family history. Indicate if any of your immediate family (parents, brothers, sisters, grandparents) has experienced any of the following, the age at which diagnosis occurred, and the person’s relationship to you.

   Relationship & Age
   High Blood Pressure ___________
   High Cholesterol ___________
   Heart Disease ___________
   Stroke ___________
   Diabetes ___________
   Cancer ___________

2. Personal medical history. Indicate symptoms that apply to you.
   □ Pain or discomfort in chest following exercise, eating or exposure to cold.
   □ Frequent heart palpitations or flutter.
   □ Very poor exercise tolerance.
   □ Frequent dizziness.
   □ Chronic cough.
   □ Other current symptoms that exercise may affect.
   Details ____________________________________________________________

3. Are you presently experiencing, or have you ever been treated by a doctor for any of the following?
   Allergies: Hay fever, eczema, other rashes.
   □ Yes □ No
   Details ____________________________________________________________

4. Lung problems.
   (Asthma/Emphysema/Bronchitis/Shortness of Breath/Other)
   □ Yes □ No
   Details ____________________________________________________________

5. Heart problems. (Rheumatic fever/Chest pain/Palpitations/Ankle swelling/Other)
   □ Yes □ No
   Details ____________________________________________________________

   □ Yes □ No
   Details ____________________________________________________________

7. Cholesterol problems.
   □ Yes □ No
   Details ____________________________________________________________

8. Gut problems. (Ulcer/Abdominal pain/Diarrhoea/Constipation/Hernia/Other)
   □ Yes □ No
   Details ____________________________________________________________

   □ Yes □ No
   Details ____________________________________________________________

10. Urinary problems. (Burning/Difficulty with control of urine)
    □ Yes □ No
    Details ____________________________________________________________

    □ Yes □ No
    Details ____________________________________________________________
MEDICAL HISTORY QUESTIONNAIRE

12. Easy bruising.
   □ Yes
   □ No
   Details

13. Endocrine problems. (Diabetes/Thyroid/Other)
   □ Yes
   □ No
   Details

14. Fitting, fainting, blackouts, loss of consciousness, muscle weakness, loss of sensation.
   □ Yes
   □ No
   Details

15. Headaches.
   □ Yes
   □ No
   Details

16. Sight or hearing problems.
   □ Yes
   □ No
   Details

17. Nervous conditions.
   □ Yes
   □ No
   Details

18. Bone or joint injury.
    (Back/Knee/Ankle/Hip/Shoulde)
   □ Yes
   □ No
   Details

19. Other joint problems. (Aches or pains/Arthritis)
   □ Yes
   □ No
   Details

20. Work related injuries.
   □ Yes
   □ No
   Details

21. Sleeping patterns. How many hours do you sleep on average per night?
   ________ hours

22. Do you ever have trouble falling asleep?
   □ Yes
   □ No
   □ Occasionally

23. Smoking status.
   □ Never smoked.
   □ Quit smoking ________ years/months ago.
   □ Currently smoke ________ years/months

24. How many cigarettes did/do you currently smoke per day? ________

25. Physical activity. How many times per week do you perform exercise such as walking/running, cycling, swimming or organised sporting activities for at least 20-30 minutes continuously?
   □ Do not have a regular program.
   □ Once per week.
   □ 2-3 times per week.
   □ 4-5 times per week.
   □ more than 5 times per week.

26. On average, how would you rate the intensity of the exercise that you perform?
   □ Light – Slight or minimal increase in perceived effort and breathing intensity (able to comfortably hold a conversation while exercising).
   □ Moderate – Noticeable increase in perceived effort and breathing intensity (but still able to hold a conversation while exercising).
   □ Heavy – High level of effort and heavy breathing (unable to comfortably hold a conversation while exercising).

27. Alcohol consumption. List how many days you consumed an alcoholic beverage in the past two weeks.
   □ Did not drink in the past 6 months.
   □ Did not drink in the past 2 weeks.
   □ 1-2 days.
   □ 3-4 days.
   □ 5-7 days.
   □ 8-10 days.
   □ 11-14 days.

28. List how many alcoholic beverages on average you had per day in the past two weeks.
   □ Did not drink in the past 6 months.
   □ Did not drink in the past 2 weeks.
   □ 1 drink.
   □ 2-3 drinks.
   □ 4-6 drinks.
   □ 7 or more drinks.

Official Use - Notes:
**MEDICAL HISTORY QUESTIONNAIRE**

**Medications.** Please list all medications you take in the table below.

<table>
<thead>
<tr>
<th>Name of Drug</th>
<th>What is it for?</th>
<th>Dose</th>
<th>Frequency</th>
<th>Started Year/date</th>
</tr>
</thead>
<tbody>
<tr>
<td>e.g. Accupril</td>
<td>Hypertension</td>
<td>20 mg</td>
<td>Once a day</td>
<td>April 2003</td>
</tr>
</tbody>
</table>

**Prescription Drugs**

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
</table>

**Over-the-counter medication**

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
</table>

**Vitamins and other medications**

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
</table>

**Medications discontinued in past 6 months**

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
</table>

**Operations.** Please list all surgeries you have undergone including the approximate date or year.

<table>
<thead>
<tr>
<th>Surgery</th>
<th>Diagnosis</th>
<th>Date/year</th>
</tr>
</thead>
</table>

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
</table>

**Official Use - Notes**
Emergency Contact Form

Name: ____________________________

In case of emergency I authorise the research team, or a representative thereof, to contact the following persons.

Person One:
Name: ____________________________

Relationship: ______________________

Phone Number(s): __________________

Person Two:
Name: ____________________________

Relationship: ______________________

Phone Number(s): __________________

Official Use - Notes
### Dietary Antioxidant Questionnaire

**GENERAL INSTRUCTIONS**

- Answer each question as best you can. Estimate if you are not sure. A guess is better than leaving a blank.
- Use only a black or blue ball-point pen. Do not use a pencil or felt-tip pen. Do not fold, staple, or tear the pages.
- Put an X in the box next to your answer.
- If you make any changes, cross out the incorrect answer and put an X in the box next to the correct answer. Also draw a circle around the correct answer.
- If you mark NEVER, NO, or DON’T KNOW for a question, please follow any arrows or instructions that direct you to the next question.

BEFORE TURNING THE PAGE, PLEASE COMPLETE THE FOLLOWING QUESTIONS.

**Today's date:**

<table>
<thead>
<tr>
<th>MONTH</th>
<th>DAY</th>
<th>YEAR</th>
<th>In what month were you born?</th>
<th>In what year were you born?</th>
<th>Are you male or female?</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>Jan</td>
<td>1</td>
<td>2012</td>
<td>2013</td>
<td>2014</td>
</tr>
<tr>
<td>02</td>
<td>Feb</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>03</td>
<td>Mar</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>04</td>
<td>Apr</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>05</td>
<td>May</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>06</td>
<td>Jun</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>07</td>
<td>Jul</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>08</td>
<td>Aug</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>09</td>
<td>Sep</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Oct</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Nov</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Dec</td>
<td>9</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Are you taking oral contraceptive pills? Oral contraceptive may act as an antioxidant hence we need to ask this question.

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>Yes</td>
</tr>
<tr>
<td>02</td>
<td>No</td>
</tr>
<tr>
<td>03</td>
<td>Not applicable (Male)</td>
</tr>
</tbody>
</table>

What is your current weight?

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>&lt;50 kg</td>
</tr>
<tr>
<td>02</td>
<td>51-60 kg</td>
</tr>
<tr>
<td>03</td>
<td>61-70 kg</td>
</tr>
<tr>
<td>04</td>
<td>71-80 kg</td>
</tr>
<tr>
<td>05</td>
<td>81-90 kg</td>
</tr>
<tr>
<td>06</td>
<td>91-100 kg</td>
</tr>
<tr>
<td>07</td>
<td>101-110 kg</td>
</tr>
<tr>
<td>08</td>
<td>&gt;110 kg</td>
</tr>
</tbody>
</table>

How long have you been at your current weight?

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>&lt;1 month</td>
</tr>
<tr>
<td>02</td>
<td>1-6 months</td>
</tr>
<tr>
<td>03</td>
<td>7-12 months</td>
</tr>
<tr>
<td>04</td>
<td>12 months or longer</td>
</tr>
</tbody>
</table>

What is your lightest adult weight?

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>&lt;50 kg</td>
</tr>
<tr>
<td>02</td>
<td>51-60 kg</td>
</tr>
<tr>
<td>03</td>
<td>61-70 kg</td>
</tr>
<tr>
<td>04</td>
<td>71-80 kg</td>
</tr>
<tr>
<td>05</td>
<td>81-90 kg</td>
</tr>
<tr>
<td>06</td>
<td>91-100 kg</td>
</tr>
<tr>
<td>07</td>
<td>101-110 kg</td>
</tr>
<tr>
<td>08</td>
<td>&gt;110 kg</td>
</tr>
</tbody>
</table>

How many years have you been involved in the sport?

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>&lt;2 years</td>
</tr>
<tr>
<td>02</td>
<td>2-3 years</td>
</tr>
<tr>
<td>03</td>
<td>&gt;3-5 years</td>
</tr>
<tr>
<td>04</td>
<td>&gt;5-7 years</td>
</tr>
<tr>
<td>05</td>
<td>&gt; 7 years</td>
</tr>
</tbody>
</table>

How many hours do you spend doing water training a week?

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>&lt;3 hours a week</td>
</tr>
<tr>
<td>02</td>
<td>3-4.9 hours a week</td>
</tr>
<tr>
<td>03</td>
<td>5-6.9 hours a week</td>
</tr>
<tr>
<td>04</td>
<td>7-9 hours a week</td>
</tr>
<tr>
<td>05</td>
<td>&gt;9 hours a week</td>
</tr>
</tbody>
</table>

How many hours do you spend doing additional aerobic training a week?

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>&lt;3 hours a week</td>
</tr>
<tr>
<td>02</td>
<td>3-4.9 hours a week</td>
</tr>
<tr>
<td>03</td>
<td>5-6.9 hours a week</td>
</tr>
<tr>
<td>04</td>
<td>7-9 hours a week</td>
</tr>
<tr>
<td>05</td>
<td>&gt;9 hours a week</td>
</tr>
</tbody>
</table>

How many hours do you spend doing resistance training (weights) a week?

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>&lt;3 hours a week</td>
</tr>
<tr>
<td>02</td>
<td>3-4.9 hours a week</td>
</tr>
<tr>
<td>03</td>
<td>5-6.9 hours a week</td>
</tr>
<tr>
<td>04</td>
<td>7-9 hours a week</td>
</tr>
<tr>
<td>05</td>
<td>&gt;9 hours a week</td>
</tr>
</tbody>
</table>

Do you typically eat breakfast before training?

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>Yes</td>
</tr>
<tr>
<td>b</td>
<td>No</td>
</tr>
</tbody>
</table>

Have you been ill in the last week, to a point it has hindered your training?

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>Yes</td>
</tr>
<tr>
<td>b</td>
<td>No</td>
</tr>
</tbody>
</table>

Have you required on going consumption of anti-inflammatory medication?

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>Yes</td>
</tr>
<tr>
<td>b</td>
<td>No</td>
</tr>
</tbody>
</table>
1. Over the past 1 month, how often did you drink tomato juice or vegetable juice?

☐ NEVER (GO TO QUESTION 2)

☐ 1 time per month or less
☐ 2–3 times per month
☐ 1–2 times per week
☐ 3–4 times per week
☐ 5–6 times per week

1a. Each time you drank tomato juice or vegetable juice, how much did you usually drink?

☐ Less than ¾ cup (200mL)
☐ ¾ to 1½ cups (200 to 300mL)
☐ More than 1¼ cups (300mL)

2. Over the past 1 month, how often did you drink orange juice, apple, pineapple, cranberry or grape juice?

☐ NEVER (GO TO QUESTION 3)

☐ 1 time per month or less
☐ 2–3 times per month
☐ 1–2 times per week
☐ 3–4 times per week
☐ 5–6 times per week

2a. Each time you drank orange juice, pineapple, apple, cranberry or grape juice, how much did you usually drink?

☐ Less than ¾ cup (200mL)
☐ ¾ to 1½ cups (200 to 300mL)
☐ More than 1¼ cups (300mL)

3. Over the past 1 month, how often did you drink fruit drinks containing blackberry, strawberry, cranberry, raspberry, blackcurrent or blueberry?

☐ NEVER (GO TO QUESTION 4)

☐ 1 time per month or less
☐ 2–3 times per month
☐ 1–2 times per week
☐ 3–4 times per week
☐ 5–6 times per week

3a. Each time you drank fruit drinks containing blackberry, strawberry, cranberry, raspberry, blackcurrent or blueberry, how much did you usually drink?

☐ Less than ¾ cup (200mL)
☐ ¾ to 1½ cups (200-300mL)
☐ More than 1½ cups (300mL)

4. How often did you drink other fruit drinks (such as thriftee, Vitafresh, Kool-Aid, sports drink, diet or regular)?

☐ NEVER (GO TO QUESTION 5)

☐ 1 time per month or less
☐ 2–3 times per month
☐ 1–2 times per week
☐ 3–4 times per week
☐ 5–6 times per week

4a. Each time you drank other fruit drinks, how much did you usually drink?

☐ Less than ¾ cup (200mL)
☐ ¾ to 1½ cups (200 to 300mL)
☐ More than 1¼ cups (300mL)

4b. How often were your fruit drinks enriched (added) with vitamin-C?

☐ Almost never or never
☐ About ¼ of the time
☐ About ½ of the time
☐ About ¾ of the time
☐ Almost always or always
☐ Don’t know

5. How often did you drink hot drinks such as coffee, black, green or oolong tea?

☐ NEVER (GO TO QUESTION 6)

☐ 1 time per month or less
☐ 2–3 times per month
☐ 1–2 times per week
☐ 3–4 times per week
☐ 5–6 times per week

5a. Each time you drank coffee, black, green or oolong tea, how much did you usually drink?

☐ Less than 1 cup (250mL)
☐ 1 to 2 cups (250-500mL)
☐ More than 2 cups (500mL)

6. How many glasses of ICED tea, caffeinated or decaffeinated, did you drink?

☐ NEVER (GO TO QUESTION 7)

☐ 1–3 cups per month
☐ 1 cup per week
☐ 2–4 cups per week

7. How many glasses of ICED tea, caffeinated or decaffeinated, did you drink?

☐ Less than 1 cup
☐ 1 cup
☐ 2–4 cups per week

8. How many glasses of ICED tea, caffeinated or decaffeinated, did you drink?

☐ Less than 1 cup
☐ 1 cup
☐ 2–4 cups per week

9. How many glasses of ICED tea, caffeinated or decaffeinated, did you drink?

☐ Less than 1 cup
☐ 1 cup
☐ 2–4 cups per week

10. How many glasses of ICED tea, caffeinated or decaffeinated, did you drink?

☐ Less than 1 cup
☐ 1 cup
☐ 2–4 cups per week

11. How many glasses of ICED tea, caffeinated or decaffeinated, did you drink?

☐ Less than 1 cup
☐ 1 cup
☐ 2–4 cups per week
7. **Over the past 1 month,** did you drink red wine?

- □ **NO** (GO TO QUESTION 8)
- □ **YES**

7a. How often did you drink **red wine**?

- □ 1 time per month or less
- □ 2–3 times per month
- □ 1–2 times per week
- □ 3–4 times per week
- □ 5–6 times per week
- □ More than 7 times per week

7b. Each time you drank **red wine,** how much did you usually drink?

- □ 1-2 glasses (110-220mL)
- □ 3-4 glasses (330-440mL)
- □ 5-6 glasses (550-660mL)
- □ More than 7 glasses (770mL)

8. How often did you drink **beer**?

- □ **NEVER** (GO TO QUESTION 9)

8a. Each time you drank **beer,** how much did you usually drink?

- □ 1 glass to 1 can (110-375mL)
- □ More than 1 to 2 cans (400mL-750mL)
- □ More than 2 to 3 cans (800-1125mL)
- □ More than 3 cans (1125mL)

9. How often did you eat **blackberries** or **blackcurrents** (fresh, canned, or frozen)?

- □ **NEVER** (GO TO QUESTION 10)

9a. Each time you ate **blackberries** or **blackcurrents,** how many did you usually eat?

- □ Less than 10 berries
- □ Between 10 and 20 berries
- □ More than 20 berries

10. How often did you eat **dried fruit,** such as prunes, raisins or dates?

- □ **NEVER** (GO TO QUESTION 11)

10a. Each time you ate **dried fruit,** how much did you usually eat?

- □ Less than 2 tablespoons
- □ 2 to 5 tablespoons
- □ More than 5 tablespoons

11. **Over the past 1 month,** did you eat **strawberries,** **boysenberries,** **blueberries,** or **raspberries**?

- □ **NO** (GO TO QUESTION 12)
- □ **YES**

11a. How often did you eat **strawberries,** **blueberries,** **boysenberries,** or **raspberries**?

- □ 1–6 times per year
- □ 7–11 times per year
- □ 1 time per month
- □ 2–3 times per month
- □ 1 time per week
- □ 2 or more times per week

11b. Each time you ate **strawberries,** **blueberries,** **boysenberries** or **raspberries,** how much did you usually eat?

- □ Less than ½ cup
- □ ½ to ¾ cup
- □ More than ¾ cup
12. How often did you eat cranberries, or cherries?

   a. NEVER (GO TO QUESTION 13)
   b. 1–6 times per year
   c. 7–11 times per year
   d. 1 time per month
   e. 2–3 times per month
   f. 1 time per week

12a. Each time you ate cranberries or cherries, how much did you usually eat?

   a. Less than ½ cup or less than 10 fruit
   b. ½ to 1 cup or 10 to 30 fruit
   c. More than 1 cup or more than 30 fruit

13. Over the past 1 month, did you eat plums or pineapple?

   a. NO (GO TO QUESTION 14)
   b. YES

13a. How often did you eat plums or pineapple (canned or fresh)?

   b. 1–6 times per year
   c. 7–11 times per year
   d. 1 time per month
   e. 2–3 times per month
   f. 1 time per week

13b. Each time you ate either plums or pineapple, how much did you usually eat?

   a. Less than 1 plum or less than ½ cup
   b. Between 2 – 4 plums or 1-2 cups
   c. More than 4 plums or more than 2 cups

14. How often did you eat pears?

   a. NEVER (GO TO QUESTION 15)
   b. 1–6 times per year
   c. 7–11 times per year
   d. 1 time per month
   e. 2–3 times per month
   f. 1 time per week

14a. Each time you ate pears, how much did you usually eat?

   a. Less than 1
   b. 1 to 2 pears
   c. More than 2 pears

15. How often did you eat oranges or kiwifruit?

   a. NEVER (GO TO QUESTION 16)
   b. 1–6 times per year
   c. 7–11 times per year
   d. 1 time per month
   e. 2–3 times per month
   f. 1 time per week

15a. Each time you ate oranges or kiwifruit, how much did you usually eat?

   a. Less than 1 orange or 2 kiwifruit
   b. 1-3 oranges or 2-4 kiwifruit
   c. More than 3 oranges or 4 kiwifruit

16. How often did you eat bran flakes or whole grain (Weet-bix, VitaBrits) breakfast cereal?

   a. NEVER (GO TO QUESTION 17)
   b. 1–6 times per year
   c. 7–11 times per year
   d. 1 time per month
   e. 2–3 times per month
   f. 1 time per week

16a. Each time you ate bran flakes or whole grain breakfast cereal, how many did you usually eat?

   a. Less than 1 cup
   b. 1-2 cups
   c. More than 3 cups
17. Over the past 1 month, did you eat All Bran, Sultana Bran or Cocoa Krispies (or other chocolate cereal) breakfast cereal?

- □ NO (GO TO QUESTION 18)
- □ YES

17a. How often did you eat All Bran, Sultana Bran or Cocoa Krispies?

- □ NEVER
- □ 1–6 times per year
- □ 7–11 times per year
- □ 1 time per month
- □ 2–3 times per month
- □ 1 time per week

17b. Each time you ate All Bran, Sultana Bran or Cocoa Krispies, how much did you usually eat?

- □ Less than 1 cup
- □ 1 to 2 cups
- □ More than 2 cups

18. How often did you eat Cornflakes, Rice crispies or Grinners breakfast cereal?

- □ NEVER (GO TO QUESTION 19)
- □ 1–6 times per year
- □ 7–11 times per year
- □ 1 time per month
- □ 2–3 times per month
- □ 1 time per week

18a. Each time you ate Cornflakes, Rice crispies or Grinners, how much did you usually eat?

- □ Less than 1 cup
- □ 1 to 2 cups
- □ More than 2 cups

19. How often did you eat artichokes or artichoke hearts?

- □ NEVER (GO TO QUESTION 20)
- □ 1–6 times per year
- □ 7–11 times per year
- □ 1 time per month
- □ 2–3 times per month
- □ 1 time per week

19a. Each time you ate artichokes, how much did you usually eat?

- □ Less than ½ cup
- □ ½ to 1 cup
- □ More than 1 cup

20. How often did you eat cabbage (red or white)?

- □ NEVER (GO TO QUESTION 21)
- □ 1–6 times per year
- □ 7–11 times per year
- □ 1 time per month
- □ 2–3 times per month
- □ 1 time per week

20a. Each time you ate cabbage (red or white), how much did you usually eat?

- □ Less than ½ cup
- □ ½ to 1 cup
- □ More than 1 cup

21. How often did you eat potatoes (such as red (Maori) or orange kumera), regardless of cooking method?

- □ NEVER (GO TO QUESTION 22)
- □ 1–6 times per year
- □ 7–11 times per year
- □ 1 time per month
- □ 2–3 times per month
- □ 1 time per week

21a. Each time you ate potatoes, how much did you usually eat?

- □ Less than 1 medium potato
- □ 1 to 2 medium potatoes
- □ More than 2 medium potatoes
22. How often did you eat spinach?

- a. NEVER (GO TO QUESTION 23)
- b. 1–6 times per year
- c. 7–11 times per year
- d. 1 time per month
- e. 2–3 times per month
- f. 1 time per week

22a. Each time you ate spinach, how much did you usually eat?

- a. Less than ¼ cup
- b. ¼ to 1 cup
- c. More than 1 cup

23. How often did you eat capsicums (red, green or yellow)?

- a. NEVER (GO TO QUESTION 24)
- b. 1–6 times per year
- c. 7–11 times per year
- d. 1 time per month
- e. 2–3 times per month
- f. 1 time per week

23a. Each time you ate capsicum, how much did you usually eat?

- a. Less than ½ cup
- b. ½ cup to 1 cup
- c. More than 1 cup

24. How often did you eat broccoli (fresh or frozen)?

- a. NEVER (GO TO QUESTION 25)
- b. 1–6 times per year
- c. 7–11 times per year
- d. 1 time per month
- e. 2–3 times per month
- f. 1 time per week

24a. Each time you ate broccoli, how much did you usually eat?

- a. Less than ½ cup
- b. Less than ½ cup to 1 cup
- c. More than 1 cup

25. How often did you eat pecans or walnuts?

- a. NEVER (GO TO QUESTION 26)
- b. 1–6 times per year
- c. 7–11 times per year
- d. 1 time per month
- e. 2–3 times per month
- f. 1 time per week

25a. Each time you ate pecans or walnuts, how much did you usually eat?

- a. Less than ¼ cup
- b. ¼ to ½ cup
- c. More than ½ cup

26. Did you eat canned spaghetti, baked beans (navy beans) or kidney beans?

- a. NO (GO TO QUESTION 27)
- b. YES

26a. How often did you eat canned spaghetti, baked beans or kidney beans?

- b. 1–6 times per year
- c. 7–11 times per year
- d. 1 time per month
- e. 2–3 times per month
- f. 1 time per week

26b. Each time you ate canned spaghetti, baked beans or kidney beans, how much did you usually eat?

- a. Less than ¼ cup
- b. ¼ to ½ cup
- c. More than ½ cup

27. Over the past month, how often did you eat unsweetened cooking chocolate or sugar free dark chocolate?

- a. NEVER (GO TO QUESTION 28)
27a. Each time you ate unsweetened cooking chocolate or sugar free dark chocolate, how much did you usually eat?

- □ Less than ¼ cup (fun size bar)
- □ ¼ to 1 cup (standard size bar)
- □ More than 1 cup (1/2 block of chocolate)

28. Over the past 1 month, how often did you eat milk chocolate or dark chocolate (not already mentioned above)?

- □ NEVER (GO TO QUESTION 29)
- □ 1–6 times per year
- □ 7–11 times per year
- □ 1 time per month
- □ 2–3 times per month
- □ 1 time per week
- □ 2 or more times per day

28a. Each time you ate milk or dark chocolate, how much did you usually eat?

- □ Less than ¼ cup (fun size bar)
- □ ¼ to 1 cup (standard size bar)
- □ More than 1 cup (1/2 block of chocolate)

29. How often did you eat chocolate cake or chocolate chip cookies?

- □ NEVER (GO TO QUESTION 30)
- □ 1–6 times per year
- □ 7–11 times per year
- □ 1 time per month
- □ 2–3 times per month
- □ 1 time per week
- □ 2 or more times per day

29a. Each time you ate chocolate cake or chocolate chip cookies, how much did you usually eat?

- □ Less than 1 slice or 3 cookies
- □ 1 slice or 3 cookies
- □ More than 1 slice or 3 cookies

30. How often did you eat lasagna with meat (fresh or frozen and cooked)?

- □ NEVER (GO TO QUESTION 32)
- □ 1–6 times per year
- □ 7–11 times per year
- □ 1 time per month
- □ 2–3 times per month
- □ 1 time per week
- □ 2 or more times per day

30a. Each time you ate lasagna with meat, how much did you usually eat?

- □ Less than 1 cup
- □ 1 to 2 cups
- □ More than 2 cups

Over the past 1 month...

31. How often did you eat condensed (tinned) tomato soup?

- □ NEVER (GO TO QUESTION 33)
- □ 1–6 times per year
- □ 7–11 times per year
- □ 1 time per month
- □ 2–3 times per month
- □ 1 time per week
- □ 2 or more times per day

31a. Each time you ate condensed (tinned) tomato soup, how much did you usually eat?

- □ Less than 1 cup
- □ 1 to 2 cups
- □ More than 2 cups

32. How often did you eat chocolate ice-cream (full, reduced or low fat)?

- □ NEVER (GO TO QUESTION 33)
- □ 1–6 times per year
- □ 7–11 times per year
- □ 1 time per month
- □ 2–3 times per month
- □ 1 time per week
- □ 2 or more times per day

32a. Each time you ate chocolate ice-cream, how much did you usually eat?

- □ Less than 1 cup
- □ 1 to 2 cups
- □ More than 2 cups

33. How often did you consume milk (full, reduced or low fat)?

- □ NEVER (GO TO QUESTION 34)
- □ 1–6 times per year
- □ 7–11 times per year
- □ 1 time per month
- □ 2–3 times per month
- □ 1 time per week
- □ 2 or more times per day
33a. Each time you consumed milk, how much did you usually eat?

- Less than 1 cup
- 1 to 2 cups
- More than 2 cups

34. How often did you drink flavoured milk or eat yoghurt (full or reduced fat)?

- NEVER (GO TO QUESTION 35)
- 1–6 times per year
- 7–11 times per year
- 1 time per month
- 2–3 times per month
- 1 time per week
- 2 or more times per day

34a. Each time you drank flavoured milk or ate yoghurt how much did you usually consume?

- Less than 1 cup
- 1 to 2 cups
- More than 2 cups

35. Please mark any of the following single herbs and spices you consumed more than once per week either fresh or dried:

- Cinnamon
- Cloves
- Oregano leaf
- Ginger
- Mustard seeds
- Tumeric

36. How many servings of fruit (not including juices) did you eat per week or per day?

- Less than 1 per week
- 1–2 per week
- 3–4 per week
- 5–6 per week
- 1 per day

38. How often did you take One-a-day-, Theragran-, or Centrum-type multivitamins?

- Less than 1 day per month
- 1–3 days per month
- 1–3 days per week
- 4–6 days per week
- Every day

38a. Does your multivitamin usually contain antioxidants (such as vitamin C, vitamin E or selenium.)?

- NO
- YES
- Don't know

38b. For how many years have you taken multivitamins?

- Less than 1 year
- 1–4 years
- 5–9 years
- 10 or more years

38c. Did you take any vitamins, minerals, or other herbal supplements other than your multivitamin?

- NO (GO TO QUESTION 44)
- YES (GO TO INTRODUCTION TO QUESTION 39.)

These last questions are about the vitamins, minerals, or herbal supplements you took that are NOT part of a One-a-day-, Theragran-, or Centrum-type of multivitamin.

Please include vitamins taken as part of an antioxidant supplement.
39. How often did you take Beta-carotene (NOT as part of a multivitamin in Question 38)?

- □ NEVER (GO TO QUESTION 40)
- □ Less than 1 day per month
- □ 1–3 days per month
- □ 1–3 days per week
- □ 4–6 days per week
- □ Every day

39a. When you took Beta-carotene, about how much did you take in one day?

- □ Less than 10,000 IU
- □ 10,000–14,999 IU
- □ 15,000–19,999 IU
- □ 20,000–24,999 IU
- □ 25,000 IU or more
- □ Don’t know

39b. For how many years have you taken Beta-carotene?

- □ Less than 1 year
- □ 1–4 years
- □ 5–9 years
- □ 10 or more years

40. How often did you take Vitamin A (NOT as part of a multivitamin in Question 38)?

- □ NEVER (GO TO QUESTION 41)
- □ Less than 1 day per month
- □ 1–3 days per month
- □ 1–3 days per week
- □ 4–6 days per week
- □ Every day

40a. When you took Vitamin A, about how much did you take in one day?

- □ Less than 8,000 IU
- □ 8,000–9,999 IU
- □ 10,000–14,999 IU
- □ 15,000–24,999 IU
- □ 25,000 IU or more
- □ Don’t know

40b. For how many years have you taken Vitamin A?

- □ Less than 1 year
- □ 1–4 years
- □ 5–9 years
- □ 10 or more years

41. How often did you take Vitamin C (NOT as part of a multivitamin in Question 38)?

- □ NEVER (GO TO QUESTION 42)
- □ Less than 1 day per month
- □ 1–3 days per month
- □ 1–3 days per week
- □ 4–6 days per week
- □ Every day

41a. When you took Vitamin C, about how much did you take in one day?

- □ Less than 500 mg
- □ 500–999 mg
- □ 1,000–1,499 mg
- □ 1,500–1,999 mg
- □ 2,000 mg or more
- □ Don’t know

41b. For how many years have you taken Vitamin C?

- □ Less than 1 year
- □ 1–4 years
- □ 5–9 years
- □ 10 or more years

Over the past 1 month...

42. How often did you take Vitamin E (NOT as part of a multivitamin in Question 38)?

- □ NEVER (GO TO QUESTION 43)
- □ Less than 1 day per month
- □ 1–3 days per month
- □ 1–3 days per week
- □ 4–6 days per week
- □ Every day

42a. When you took Vitamin E, about how much did you take in one day?

- □ Less than 400 IU
- □ 400–799 IU
- □ 800–999 IU
- □ 1,000 IU or more
- □ Don’t know

42b. For how many years have you taken Vitamin E?

- □ Less than 1 year
- □ 1–4 years
- □ 5–9 years
- □ 10 or more years

43. How often did you take selenium (NOT as part of a multivitamin in Question 38)?

- □ NEVER (GO TO QUESTION 44)
- □ Less than 1 day per month
- □ 1–3 days per month
- □ 1–3 days per week
- □ 4–6 days per week
- □ Every day
43a. When you took selenium, about how much did you take in one day?
   a. Less than 20 μg
   b. 21–100 μg
   c. 101–200 μg
   d. 201 μg or more
   e. Don't know

43b. For how many years have you taken selenium?
   a. Less than 1 year
   b. 1–4 years
   c. 5–9 years
   d. 10 or more years

The last two questions ask about usual eating patterns and meal quantity.

44. Please mark which of the following best describes your usual eating patterns:
   1. 3 meals and 3 snacks
   2. 3 meals and 2 snacks
   3. 2 meals and 3 snacks
   4. 2 meals and 2 snacks
   5. 5 snacks
   6. No set meal pattern

45. Please mark which of the following best describes your usual meal size.
   1. < 1 cup
   2. >1 to 2 cups
   3. >2 to 3 cups
   4. >3 to 4 cups
   5. >5 cups

Thank you very much for completing this questionnaire! Because we want to be able to use all the information you have provided, we would greatly appreciate it if you would please take a moment to review each page making sure that you:
   - Did not skip any pages and
   - Crossed out the incorrect answer and circled the correct answer if you made any change
## 10.7 Appendix G

### Table G1.

<table>
<thead>
<tr>
<th>Final concentration of UCB in 250 µL of PRP</th>
<th>Unconjugated bilirubin (UCB) stock solution used</th>
<th>Volume of Vehicle DMSO used to spike UCB concentration in PRP</th>
</tr>
</thead>
<tbody>
<tr>
<td>35 µM</td>
<td>5 mM</td>
<td>1.25 µL</td>
</tr>
<tr>
<td>50 µM</td>
<td>10 mM</td>
<td>1.25 µL</td>
</tr>
<tr>
<td>100 µM</td>
<td>20 mM</td>
<td>1.25 µL</td>
</tr>
<tr>
<td>150 µM</td>
<td>30 mM</td>
<td>1.25 µL</td>
</tr>
<tr>
<td>200 µM</td>
<td>40 mM</td>
<td>1.25 µL</td>
</tr>
<tr>
<td>300 µM</td>
<td>60 mM</td>
<td>1.25 µL</td>
</tr>
</tbody>
</table>

### Table G2. BD LSR Fortessa settings – PMT voltages

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Voltage</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSC</td>
<td>490</td>
</tr>
<tr>
<td>SSC</td>
<td>203</td>
</tr>
<tr>
<td>APC</td>
<td>425</td>
</tr>
<tr>
<td>FITC</td>
<td>400</td>
</tr>
<tr>
<td>PerCP-Cy5.5</td>
<td>526</td>
</tr>
</tbody>
</table>
Table G3. Raw data for collagen induced platelet aggregation. GS Vs control

<table>
<thead>
<tr>
<th></th>
<th>Maximal platelet aggregation percentage</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gilbert’s syndrome</td>
<td>Control (normal healthy)</td>
</tr>
<tr>
<td>1</td>
<td>89.8</td>
<td>96.50</td>
</tr>
<tr>
<td>2</td>
<td>92.7</td>
<td>95.00</td>
</tr>
<tr>
<td>3</td>
<td>88.1</td>
<td>94.20</td>
</tr>
<tr>
<td>4</td>
<td>90.4</td>
<td>93.75</td>
</tr>
<tr>
<td>5</td>
<td>92.3</td>
<td>91.50</td>
</tr>
<tr>
<td>6</td>
<td>76.7</td>
<td>95.10</td>
</tr>
<tr>
<td>7</td>
<td>90.7</td>
<td>94.65</td>
</tr>
<tr>
<td>8</td>
<td>90.9</td>
<td>93.60</td>
</tr>
<tr>
<td>9</td>
<td>86.1</td>
<td>89.55</td>
</tr>
<tr>
<td>10</td>
<td>94.1</td>
<td>91.80</td>
</tr>
<tr>
<td>11</td>
<td>85.7</td>
<td>86.60</td>
</tr>
<tr>
<td>12</td>
<td>88.9</td>
<td>93.00</td>
</tr>
<tr>
<td>13</td>
<td>91.7</td>
<td>87.10</td>
</tr>
<tr>
<td>14</td>
<td>87.2</td>
<td>92.00</td>
</tr>
<tr>
<td>Mean</td>
<td>88.9</td>
<td>92.4</td>
</tr>
<tr>
<td>SD</td>
<td>4.1</td>
<td>2.8</td>
</tr>
</tbody>
</table>
10.8 Appendix D


Important points before starting

• Make sure that the kit box is intact and undamaged, and that buffers have not leaked. Do not use a kit that has been damaged.

• When using a pipet, ensure that it is set to the correct volume, and that liquid is carefully and completely aspirated and dispensed.

• To avoid transferring samples to the wrong tube or spin column, ensure that all tubes and spin columns are properly labeled using a permanent pen. Label the lid and the body of each tube. For spin columns, label the body of its processing tube. Close each tube or spin column after liquid is transferred to it.

• Spillages of samples and buffers during the procedure may reduce the yield and purity of RNA.

• Unless otherwise indicated, all steps of this protocol, including centrifugation steps, should be carried out at room temperature (15–25°C).

• Because of the sensitivity of nucleic acid amplification technologies, the following precautions are necessary when handling samples to avoid cross-contamination:

   • Carefully pipet the sample into the spin column without moistening the rim of the column.

   • Always change pipet tips between liquid transfers. Use aerosol-barrier pipet tips
• Avoid touching the spin column membrane with the pipet tip. • After vortexing or heating a microcentrifuge tube, briefly centrifuge it to remove drops from the inside of the lid.

• Wear gloves throughout the entire procedure. In case of contact between gloves and sample, change gloves immediately.

• Close the spin column before placing it in the microcentrifuge. Centrifuge as described in the procedure.

• Open only one spin column at a time, and take care to avoid generating aerosols.

• For efficient parallel processing of multiple samples, fill a rack with processing tubes to which the spin columns can be transferred after centrifugation. Discard the used processing tubes containing flow-through, and place the new processing tubes containing spin columns directly in the microcentrifuge.

**Things to do before starting**

• Blood must be collected in PAXgene Blood RNA Tubes according to the instructions in the PAXgene Blood RNA Tube Product Circular.

• Ensure that the PAXgene Blood RNA Tubes are incubated for at least 2 hours at room temperature after blood collection to ensure complete lysis of blood cells. Incubation of the PAXgene Blood RNA Tube overnight may increase yields. If the PAXgene Blood RNA Tube was stored at 2–8°C or −20°C or −70°C after blood collection, first equilibrate it to room temperature, and then store it at room temperature for 2 hours before starting the procedure.

• Ensure that instruments, such as pipets and the shaker–incubator have been checked and calibrated regularly according to the manufacturer’s recommendations.
• A shaker–incubator is required in steps 5 and 20. Set the temperature of the shaker–incubator to 55°C.

• Buffer BR2 may form a precipitate upon storage. If necessary, warm to 37°C to dissolve.

• Buffer BR4 is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%) as indicated on the bottle to obtain a working solution.

• If using the RNase-Free DNase Set for the first time, prepare DNase I stock solution. Dissolve the solid DNase I (1500 Kunitz units)* in 550 µl of the RNase-free water provided with the set. Take care that no DNase I is lost when opening the vial. Do not vortex the reconstituted DNase I. DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube.

• Current data shows that reconstituted DNase I can be stored at 2–8°C for up to 6 weeks. For long-term storage of DNase I, remove the stock solution from the glass vial, divide it into single-use aliquots (use the 1.5 ml microcentrifuge tubes supplied with the kit; there are enough for 5 aliquots), and store at –20°C for up to 9 months. Thawed aliquots can be stored at 2–8°C for up to 6 weeks. Do not refreeze the aliquots after thawing.

• When reconstituting and aliquoting DNase I, ensure that you follow the guidelines for handling RNA

Procedure

1. Centrifuge the PAXgene Blood RNA Tube for 10 minutes at 3000–5000 x g using a swing-out rotor. Note: Ensure that the blood sample has been incubated in the PAXgene Blood RNA Tube for a minimum of 2 hours at room temperature (15–25°C), in order to achieve complete lysis of blood cells. Note: The rotor must contain tube adapters for round-bottom tubes. If other types of tube adapter are used, the tubes may break during centrifugation.
2. Remove the supernatant by decanting or pipetting. Add 4 ml RNase-free water to the pellet, and close the tube using a fresh secondary BD Hemogard closure (supplied with the kit). If the supernatant is decanted, take care not to disturb the pellet, and dry the rim of the tube with a clean paper towel.

3. Vortex until the pellet is visibly dissolved, and centrifuge for 10 minutes at 3000–5000 x g using a swing-out rotor. Remove and discard the entire supernatant. Small debris remaining in the supernatant after vortexing but before centrifugation will not affect the procedure. Note: Incomplete removal of the supernatant will inhibit lysis and dilute the lysate, and therefore affect the conditions for binding RNA to the PAXgene membrane.

4. Add 350 µl Buffer BR1, and vortex until the pellet is visibly dissolved.

5. Pipet the sample into a 1.5 ml microcentrifuge tube. Add 300 µl Buffer BR2 and 40 µl proteinase K. Mix by vortexing for 5 seconds, and incubate for 10 minutes at 55°C using a shaker–incubator at 400–1400 rpm. After incubation, set the temperature of the shaker–incubator to 65°C (for step 20). Note: Do not mix Buffer BR2 and proteinase K together before adding them to the sample.

6. Pipet the lysate directly into a PAXgene Shredder spin column (lilac) placed in a 2 ml processing tube, and centrifuge for 3 minutes at maximum speed (but not to exceed 20,000 x g). Note: Carefully pipet the lysate into the spin column and visually check that the lysate is completely transferred to the spin column. To prevent damage to columns and tubes, do not exceed 20,000 x g. Note: Some samples may flow through the PAXgene Shredder spin column without centrifugation. This is due to low viscosity of some samples and should not be taken as an indication of product failure.

7. Carefully transfer the entire supernatant of the flow-through fraction to a fresh 1.5 ml microcentrifuge tube without disturbing the pellet in the processing tube.
8. Add 350 µl ethanol (96–100%). Mix by vortexing, and centrifuge briefly (1–2 seconds at 500–1000 x g) to remove drops from the inside of the tube lid. Note: The length of the centrifugation must not exceed 1–2 seconds, as this may result in pelleting of nucleic acids and reduced yields of total RNA.

9. Pipet 700 µl sample into the PAXgene RNA spin column (red) placed in a 2 ml processing tube, and centrifuge for 1 minute at 8000–20,000 x g. Place the spin column in a new 2 ml processing tube, and discard the old processing tube containing flow-through.

10. Pipet the remaining sample into the PAXgene RNA spin column, and centrifuge for 1 minute at 8000–20,000 x g. Place the spin column in a new 2 ml processing tube, and discard the old processing tube containing flow-through. Note: Carefully pipet the sample into the spin column and visually check that the sample is completely transferred to the spin column.

11. Pipet 350 µl Buffer BR3 into the PAXgene RNA spin column. Centrifuge for 1 minute at 8000–20,000 x g. Place the spin column in a new 2 ml processing tube, and discard the old processing tube containing flow-through.

12. Add 10 µl DNase I stock solution to 70 µl Buffer RDD in a 1.5 ml microcentrifuge tube. Mix by gently flicking the tube, and centrifuge briefly to collect residual liquid from the sides of the tube. If processing, for example, 10 samples, add 100 µl DNase I stock solution to 700 µl Buffer RDD. Use the 1.5 ml microcentrifuge tubes supplied with the kit. Note: DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently flicking the tube. Do not vortex.

13. Pipet the DNase I incubation mix (80 µl) directly onto the PAXgene RNA spin column membrane, and place on the benchtop (20–30°C) for 15 minutes. Note: Ensure that the DNase I incubation mix is placed directly onto the membrane. DNase digestion will be
incomplete if part of the mix is applied to and remains on the walls or the O-ring of the spin column.

14. Pipet 350 µl Buffer BR3 into the PAXgene RNA spin column, and centrifuge for 1 minute at 8000–20,000 x g. Place the spin column in a new 2 ml processing tube, and discard the old processing tube containing flow-through.

15. Pipet 500 µl Buffer BR4 to the PAXgene RNA spin column, and centrifuge for 1 minute at 8000–20,000 x g. Place the spin column in a new 2 ml processing tube, and discard the old processing tube containing flow-through. Note: Buffer BR4 is supplied as a concentrate. Ensure that ethanol is added to Buffer BR4 before use (see “Things to do before starting”, page 36).

16. Add another 500 µl Buffer BR4 to the PAXgene RNA spin column. Centrifuge for 3 minutes at 8000–20,000 x g.

17. Discard the tube containing the flow-through, and place the PAXgene RNA spin column in a new 2 ml processing tube. Centrifuge for 1 minute at 8000–20,000 x g.

18. Discard the tube containing the flow-through. Place the PAXgene RNA spin column in a 1.5 ml microcentrifuge tube, and pipet 40 µl Buffer BR5 directly onto the PAXgene RNA spin column membrane. Centrifuge for 1 minute at 8000–20,000 x g to elute the RNA. It is important to wet the entire membrane with Buffer BR5 in order to achieve maximum elution efficiency.

19. Repeat the elution step (step 18) as described, using 40 µl Buffer BR5 and the same microcentrifuge tube.

20. Incubate the eluate for 5 minutes at 65°C in the shaker–incubator (from step 5) without shaking. After incubation, chill immediately on ice. Note: This incubation at 65°C
denatures the RNA for downstream applications. Do not exceed the incubation time or temperature.

21. If the RNA samples will not be used immediately, store at –20°C or –70°C. Since the RNA remains denatured after repeated freezing and thawing, it is not necessary to repeat the incubation at 65°C. If using the RNA samples in a diagnostic assay, follow the instructions supplied by the manufacturer. Note: For quantification in Tris buffer, use the relationship $A_{260} = 1 \times 44 \mu g/ml$