# The impact of metformin on cardiovascular markers in type 1 diabetes: MERIT Study


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## Abstract

Type 1 diabetes mellitus (T1DM) is characterised by an increased risk of cardiovascular disease (CVD). Metformin has been shown to have cardio-protective properties in type 2 diabetes. We aimed to determine if the cardio-protective effect of metformin is mediated by modulating circulatory biomarkers in T1DM whilst maintaining unchanged glycemic control.

Twenty-three T1DM patients without overt CVD were treated with metformin for eight weeks (treatment group-TG). They were matched with nine T1DM patients on standard treatment (SG) and twenty-three age- and sex-matched healthy volunteers (HC). Insulin dose was adjusted to keep unchanged glycaemic control in TG. We evaluated endothelial progenitor cells (cEPCs-$CD45^{dim}CD34^{+}VEGFR2^{+}$), circulatory endothelial cells (cECs-$CD45^{dim}CD133^{-}CD34^{+}CD144^{+}$), , microRNAs (miR), cytokines (all groups), microparticles (MP) and peripheral mononuclear cells (PBMC) (except SG).

At baseline, TG had lower cEPCs, Pro-angiogenic cells (PACs), (Colony forming Unit) CFU-Hills' colonies, and PACs adhesion and higher cECs, Endothelial-MPs(EMP-$AnnexinV^{+}CD31^{+}CD41^{-}$) and Platelet-MPs (PMP-$AnnexinV^{+}CD31^{+}CD41^{+}$) versus HC. Metformin improved cEPCs, PACs, CFU-Hills' colonies, cECs number and PACs adhesion in TG to levels seen in HC whilst EMPs and PMPs levels did not change.

miR-21, miR-222, miR-195, miR-210, miR-223 and miR-320 levels were higher in TG when compared to HC. In TG, miR-21, miR-222, miR-195 and miR-210 levels reduced significantly after metformin treatment. Inflammatory cytokines and tissue inhibitors of metalloproteinase-1 (TIMP-1) were higher in T1DM when compared to HC. Vascular injury markers were not raised in T1DM when compared to HC. After metformin treatment, TIMP-1, IL-6 and thrombomodulin levels reduced significantly.

In TG, metformin treatment significantly modulated eleven genes in PBMC. These genes were involved in MAP signalling kinase pathway, inflammatory response, cell movement, death, signalling and survival.

We have demonstrated that metformin can potentially shift the balance towards vascular repair. This can be mediated via improvement in EPC mobilisation, survival, inflammatory status and proangiogenic miRs. independent of metformin's glucose lowering effect. However further long-term cardiovascular outcome studies in T1DM are still required.

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# Dedication

This work is dedicated to

My parents,

Dr Wali Ahmed and Dr Nusrat Wali

for making me what I am

My wife,

Uzma

for supporting me all the way

My son, Yousuf and daughter, Farah

My brothers, Dr Faisal Wali Ahmed and Dr Fauwad Wali Ahmed

Thank you for supporting and always believing in me.

## Publication

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## Abbreviation

| | |
|---|---|
| ACD | Acid-Citrate-Dextrose |
| ACEi | Angiotensin-converting enzyme inhibitors |
| ac-LDL | acetylated LDL |
| ACS | Acute coronary syndromes |
| AGE | Advanced-glycated end-product |
| AMI | Acute myocardial infarction |
| AMPK | AMP-activated protein kinase |
| ARB | Angiotensin receptor blockers |
| AUC | Area under curve |
| bFGF | basic fibroblast growth factor |
| BMI | Body mass index |
| BP | Blood pressure |
| CAD | Coronary artery disease |
| cEC | circulatory endothelial cells |
| cEPC | $CD45^{dim}CD34^{+}VEGFR2^{+}$ |
| CFU-Hills | Colony forming unit-Hills |
| CGM | Continuous glucose monitoring |
| CHD | Coronary heart disease |
| CIMT | Carotid intima media thickness |
| CK | Creatine kinase |
| CKD | Chronic kidney disease |
| CKD | Chronic kidney disease |
| CPC | Circulatory proginator cells |
| CRP | C-reactive protein |
| CVA | Cerebro-vascular disease |
| CVD | Cardiovascular disease |
| DAFNE | Dose Adjustment for Normal Eating |
| DCCT | The Diabetes Control and Complications Trial |
| DiLDL | 1,19–dioctadecyl–3,3,39,39–tetramethylindocarbocyanine–labeled acetylated low-density lipoprotein |
| DM | Diabetes mellitus |
| DOD | Duration of diabetes |

| | |
|---|---|
| EC | Endothelial cells |
| ECFCs | Endothelial colony forming cells |
| EDIC | Epidemiology of Diabetes Interventions and Complications |
| EDTA | Ethylenediaminetetraacetic acid |
| EF | Endothelial function |
| EMP | Endothelial microparticles |
| eNOS | Endothelial nitric oxide synthase |
| EPC | Endothelial progenitor cells |
| FACS | Fluorescence-activated cell sorting |
| FDA | Food and Drug Administration |
| FITC | Fluorescein isothiocyanate |
| G-CSF | Granulocyte colony-stimulating factor |
| HC | Healthy control |
| HGF | Hepatocyte growth factor |
| HIF-1α | Hypoxia-inducible factor 1α |
| hmga2 | High-mobility group A2 |
| hpf | High power field |
| IDF | International Diabetes Federation |
| IGF-1 | Insulin-like growth factor-1 |
| IHD | Ischaemic heart disease |
| IL | Interleukin |
| INF-γ | Interferon-gamma |
| IP-10 | Interferon gamma-induced protein 10 |
| IQ | Interquartile |
| ISEV | International Society for Extracellular Vesicles |
| LMP | Leukocyte microparticle |
| LOX-1 | Lectin-like oxidized low-density lipoprotein (LDL) receptor-1 |
| MAP | Mitogen-activated protein |
| MCP-1 | Monocyte chemoattractant protein-1 |
| MI | Myocardial ischemia |
| MIP-1β | Macrophage inflammatory protein-1β |
| miRNA | microRNA |
| MMP-9 | Matrix matelloproteinase 9 |

| | |
|---|---|
| MP | Microparticles |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NICE | National Institute for Health and Clinical Excellence |
| PAC | Pro-angiogenic cells |
| PAF | Platelet activating factor |
| PBMC | Peripheral blood mononuclear cells |
| PBS | Phosphate-buffered saline |
| PFP | Platelet-free plasma |
| PMP | Platelet microparticles |
| PRP | Platelet rich plasma |
| PS | Phosphatidylserine |
| PVD | Peripheral vascular disease |
| PVD | Peripheral vascular disease |
| RAGE | Receptor of AGE |
| RBC | Red blood cells |
| RBC-MP | Red blood cells-derived microparticle |
| RCT | Randomised control trial |
| ROS | Reactive oxygen species |
| SD | Standard deviation |
| SDF-1 | Stromal cell-derived factor-1 |
| SE | Standard error |
| SG V1 | Standard group pre observation |
| SG V2 | Standard group post observation |
| sICAM-1 | Soluble intercellular adhesion molecule-1 |
| SIRT-1 | Silent information regulator 1 |
| SIRT-1 | Silent information regulator |
| STAT5A | Signal transducer and activator of transcription 5A |
| sVCAM-1 | Vascular cell adhesion molecules |
| sVCAM-1 | Soluble vascular cell adhesion molecules-1 |
| T1DM | Type 1 diabetes mellitus |
| T2DM | Type 2 diabetes mellitus |
| TG V1 | Treatment group pre-metformin |
| TG V1.5 | Treatment group 4 weeks metformin |
| TG V2 | Treatment group post metformin |
| TIA | Transient ischemic attack |

| | |
|---|---|
| TIMP-1 | Tissue inhibitors of metalloproteinase-1 |
| TNF-α | Tumour necrosis factor alpha |
| VEGF | Vascular endothelial growth factor |
| WCC | White cell count |
| WHO | World Health Organisation |

# Chapter 1: Diabetes and metformin

## 1.1 Diabetes mellitus

Diabetes mellitus (DM) is one of the most common metabolic syndromes characterised by hyperglycaemia (Nathan *et al.*, 1997). In addition to disturbance in glucose metabolism, protein and fat metabolism is affected. The main pathological abnormality is either reduced insulin secretion or decreased insulin sensitivity. The metabolic disturbance observed in diabetes results in the development of long term complications (Alberti *et al.*, 2004). Of major concern is that the burden of disease is increasing. It was estimated that, in 2015, approximately 415 million people of the world population have diabetes mellitus. This is estimated to increase to 642 million by the year 2030 (DiabetesUK, 2015). The prevalence of diabetes among adults in England in 2011 was 6 % and the cost to the National Health Service (NHS) in 2015 was £10 billion (DiabetesUK, 2016).

Common symptoms at presentation are related to hyperglycaemia. Patients usually present with classical symptoms, including polyuria, polydipsia and weight loss despite increased appetite and tiredness (Kasper *et al.* 2008). Current diagnostic criteria for diabetes mellitus are based on World Health Organisation (WHO)/ International Diabetes Federation (IDF) (2006) criteria: “fasting plasma glucose ≥ 7.0mmol/l and/or 2–h plasma glucose ≥ 11.1mmol/l”.

Depending on the pathogenesis DM can be classified into different categories. Two major classifications are type 1 DM (T1DM) and type 2 DM (T2DM) (AmericanDiabetesAssociation, 2010). T2DM is characterised by insulin resistance and abnormal glucose metabolism. T2DM is more prevalent, and the current epidemic is putting the NHS under huge pressure. By contrast, T1DM by recent estimates accounts for 10% and 15% of all cases of diabetes mellitus. However, it presents earlier than T2DM. This results in patients having a metabolic abnormality for a longer duration of time. This results in serious consequence in both short and long terms (Daneman, 2006).

### *1.1.1 Type 1 diabetes mellitus*

T1DM is characterised by the absolute lack of insulin due to pancreatic β-cell destruction. There are two types of T1DM: Type 1A, which is associated with autoimmune destruction of pancreatic β-cells and Type 1B, which is idiopathic in origin. However, both have a similar problem of insulin deficiency. The pathogenesis of T1DM is multifactorial. In addition to genetic susceptibility, exposure to environmental factors, triggers an immune response in the at risk individual, resulting in the destruction of pancreatic β-cells (Kasper DL, 2008). There is a gradual decline in β-cell numbers. However, most patients only become symptomatic when there is depletion of pancreatic β-cells by 80% (Kasper DL, 2008).

#### *1.1.1.1 Epidemiology of type 1 diabetes*

T1DM usually occurs in young individuals. Around 50-60% of the patients are younger than 16-18 years old at the time of the onset of disease (Devendra *et al.*, 2004). It appears that there are two peaks of presentation of T1DM: one between the ages of 5 to 7 years old and a second at or near puberty (Harjutsalo *et al.*, 2008). It is one of most the common chronic diseases in children (Karvonen *et al.*, 2000). Incidence of T1DM is much lower in adults. Approximately 25% of diagnosis of T1DM is made in adults (Haller *et al.*, 2005). Around 5 to 10% of adults with T2DM diagnosis are found to have T1DM (Palmer *et al.*, 2005). Patients present with short duration of symptoms between 3 to 4 weeks and 7 to 8 weeks for children and adults respectively. Most of the patients usually present with the classical symptoms described above. However, few patients have diabetic ketoacidosis at the first presentation (Devendra *et al.*, 2004).

The incidence of T1DM is higher during autumn and winters (Moltchanova *et al.*, 2009). Both genders are affected equally by T1DM (Soltesz *et al.*, 2007). However, there are reports suggesting that more males are affected as adults than females, especially in European populations (Gale, 2001; Kyvik *et al.*, 2004). In areas of high incidence of T1DM, more males appear to be diagnosed with T1DM. Whereas, in areas of lower incidence more females are affected by T1DM (Karvonen *et al.*, 1998).

The worldwide incidence of T1DM is roughly 2-5% (Onkamo *et al.*, 2000; DIAMOND Project Group, 2006; Patterson *et al.*, 2009). In the United Kingdom, approximately 20

in 100000 people present with T1DM per year (Karvonen *et al.*, 2000). The UK has the fifth highest incidence of type 1 diabetes under the age of 14 years (28.2 per 100,0000) (IDF Atlas, 2015). The incidence of T1DM varies considerably depending on geographical location (Figure 1). The incidence increases as we move north of the equator (Karvonen *et al.*, 2000). Scandinavian countries having the highest incidence of T1DM. India, China and Venezuela has the lowest incidence in the world (0.1 per 100000) (Maahs *et al.*, 2010). It is particularly of concern that the age of presentation of T1DM is decreasing (Dahlquist and Mustonen, 2000). Finland has an annual rise in the incidence of T1DM of 2.4%, whereas Sweden and Norway have risen in the incidence of 3.3% (Patterson *et al.*, 2009).

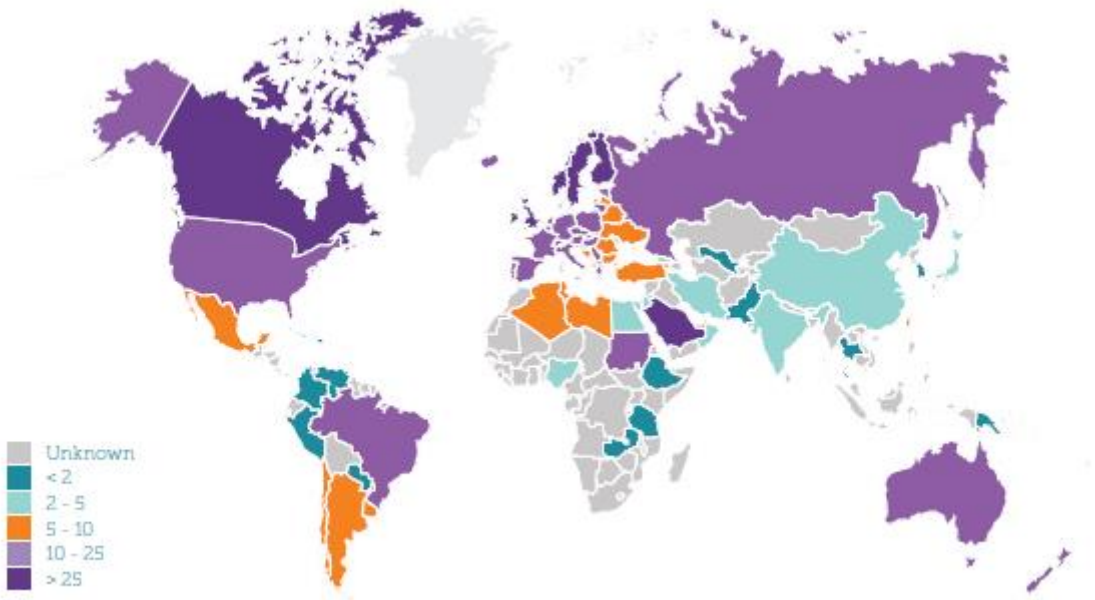


Figure 1: Incidence of T1DM under 15 years old per 100000 children per year (2015).

(IDF Atlas, 2015)

### *1.1.1.2 Natural history of type 1 diabetes*

The pathophysiology of T1DM includes a combination of genetic, environmental and immunological factors acting synergistically leading to destruction of pancreatic β cells. At birth, individuals at risk of developing T1DM have normal pancreatic β cell function and mass. Exposure to environmental trigger in a genetically susceptible individual will lead to the development of autoimmune response against pancreatic β cells. Immunological markers are present in individuals before clinical development in T1DM. There is a loss of pancreatic β cells. The rate of loss is variable depending on many factors. Clinical symptoms of diabetes develop when there is a loss of ~80% of pancreatic β cells. Recent studies have suggested that in some individual symptoms may develop with loss of 40 to 50% of β cells (Akirav *et al.*, 2008). In some individuals, pancreatic β cell function persists for a prolonged period. (Figure 2).

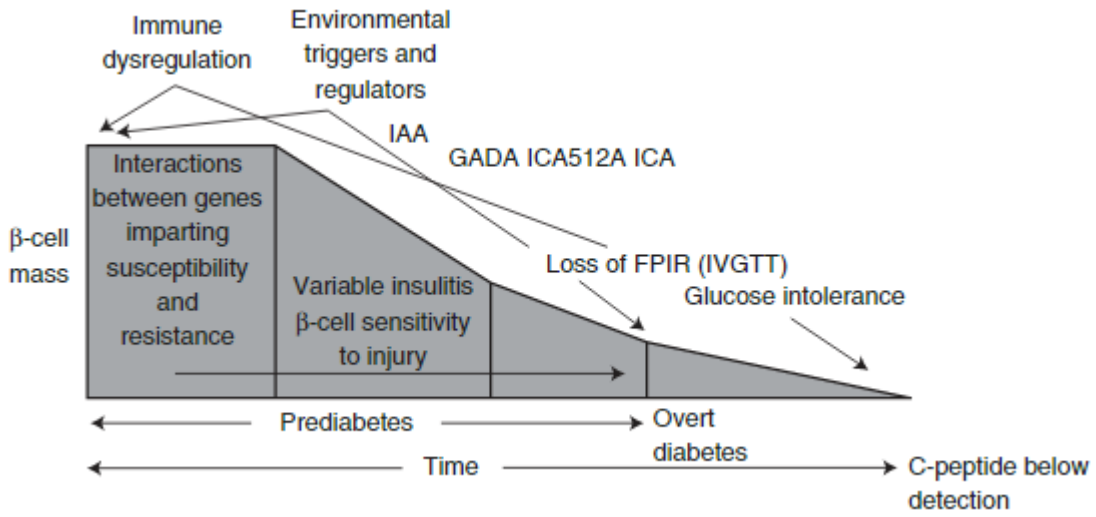


Figure 2: Pathogenesis of T1DM. Adapted from (Atkinson, 2012)

T1DM does not follow any specific inheritance pattern. There is a 2% risk of developing T1DM in children with T1DM mothers, whereas children of T1DM fathers have a risk of 7% (Redondo *et al.*, 2001). 83% of newly diagnosed T1DM do not have any family history of T1DM. The background risk of becoming T1DM in the general population is around 1 in 300. This is increased to one in twenty for individuals with first degree relatives of T1DM (Redondo et al 2001). The concordance rate for monozygotic twins is around 30-50%. For diazygotic twins, this is reduced to around 6 to 10% (Atkinson, 2012).

#### *1.1.1.3 Cardiovascular complication in diabetes*

Cardiovascular disease (CVD) remains the main cause of mortality and morbidity in patients with diabetes despite controlling for traditional factors such as hypertension, smoking, and hypercholesterolemia (Kannel and McGee, 1979; Stamler *et al.*, 1993). DM is linked with 24000 more deaths each year, and up to half of these are due to CVD (The Health and Social Care Information Centre 2011). The rates of CVD are 3-5 fold higher with T1DM patients than in the general population. In addition, poorer outcomes in diabetes have been observed for treatment of acute coronary syndromes (ACS) and coronary revascularisation (Malmberg *et al.*, 2000; Lloyd-Jones *et al.*, 2003; Gurm *et al.*, 2004; Pell *et al.*, 2004). Furthermore, data has shown that patients with diabetes on standard treatment for secondary prevention have significantly worse outcome when compared to non-diabetic individuals ('Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients,' 2002).

There is ample evidence from population based studies that there is an increased risk of CVD and mortality in T1DM. Roy *et al*. (2012) showed CVD rate of 15.5% over 6 year follow up in T1DM (Roy *et al.*, 2012). Waden *et al.* (2009) showed CVD rate of 8.6% over 5.7 years in T1DM (Wadén *et al.*, 2009). The reason for high CVD rate in Roy *et al.* (2012) could be due to a very high HbA1c of 13.5% (vs 8% in Waden *et al.* 2009). In a large UK population based trial, it was demonstrated that patients with T1DM were more likely to develop acute coronary heart disease (CHD) when compared to healthy controls (HR in males and female was 3 and 7.6 respectively).. Overall, it is estimated that CVD events will occur 10 to 15 years earlier in T1DM individual when compared to non-diabetic individuals (Soedamah-Muthu *et al.*, 2006). Incidence rate of CHD in T1DM varies between 2.1% to 19% (Schram *et al.*, 2003a; Conway *et al.*, 2009). On average, the incidence of CHD is around 15% over 15 years period (de Ferranti *et al.*, 2014). The mortality rate from CHD has been reported to be high as well. Conway *et al*. (2009) demonstrated a fatal CHD rate of 8.39 over 15 year follow up. Shankar *et al.* (2007) and Stettler *et al.* (2006) showed a CHD mortality rate of 15% and 17% respectively after more than 10 years follow up (Stettler *et al.*, 2006; Shankar *et al.*, 2007). Mortality from CHD is higher in males than females and in patients of age more than 40 years (Liang et al 2013).

Incidence of CVA is lower than IHD in T1DM. The rate of CVA in T1DM varies between 0.3% to 0.74% (background population rate of 0.2 to 0.3%-(Schram *et al.*, 2003a; Klein *et al.*, 2004; Roger *et al.*, 2012). Peripheral vascular disease is a very common complication in T1DM. Moss *et al.* (1999) demonstrated that the incidence of lower limb amputation was 7.2% (Moss *et al.*, 1999). In Swedish population based study, it was observed that the probability of lower limb amputation was 20% in males and 11% in females in patients with T1DM who areolder than 65 years. Patients with T1DM are 86 times more likely to undergo amputations when compared to the background population (Jonasson *et al.*, 2008).

The patients with T1DM have been shown to develop CVD much earlier than non-diabetic population (Figure 3). CVD usually develops within around two decades of diagnosis of T1DM. This could then occur potentially when the patients are in their 30s if they were diagnosed with T1DM in early teens (de Ferranti *et al.*, 2014). CVD has been shown to be a major cause of mortality in T1DM after 20 years of diagnosis. This occurs at a rate of 3% per year (Secrest *et al.*, 2010). The life expectancy of adults at the age of 20 with type 1 diabetes is reduced by up to 13 years with CVD being the leading cause of premature death (Livingstone *et al.*, 2015).

It has been shown that young patients (mean age of 25 years) with T1DM without any co-morbidities including macrovascular disease or microalbuminuria, show features of early cardiovascular disease, such as increased carotid intima media thickness (CIMT) and endothelial dysfunction as compared with age matched healthy controls (Sibal *et al.*, 2009a). Other studies have also demonstrated increased CIMT in T1DM when compared to healthy volunteers (Yamasaki *et al.*, 1994; Järvisalo *et al.*, 2002; Dalla Pozza *et al.*, 2007; Margeirsdottir *et al.*, 2010). Similarly, T1DM is associated with coronary artery calcifications (Dabelea *et al.*, 2003). All these are makers of early atherosclerosis. Angiographic evidence suggests that T1DM patients have increased coronary artery burden than non-diabetic population (Valsania *et al.*, 1991). This could be 4-fold higher (Pajunen *et al.*, 2000).

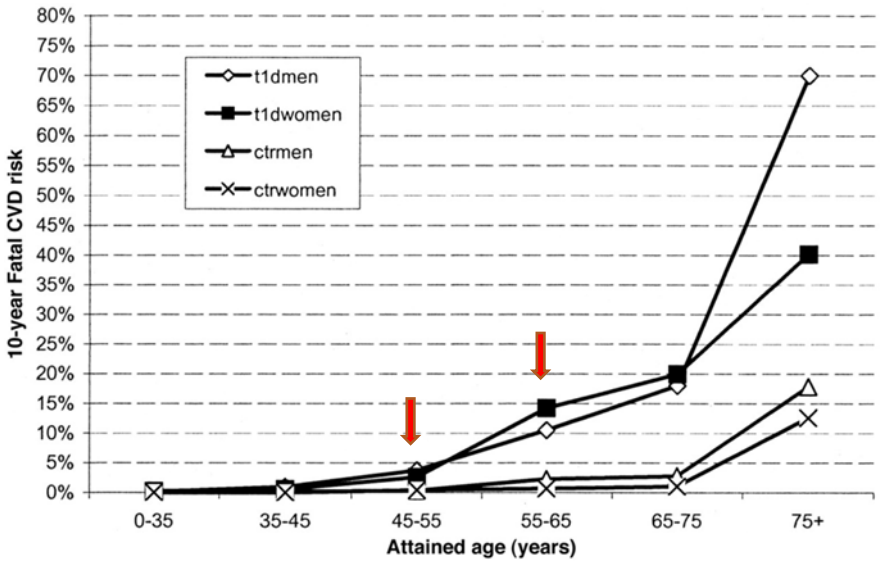


Figure 3:10-year CVD risk in T1DM according to age and gender compared to non-diabetic individuals. Adapted from (Soedamah-Muthu *et al.*, 2006)

### *1.1.1.4 Management of T1DM*

The primary aim of the management of T1DM is to improve glycaemic control (HbA1c <7). This is achieved by insulin replacement and structured education of the patient e.g. DAFNE (Dose Adjustment for Normal Eating). The availability of structured education programme is still limited to the small number of patients with T1DM. However, there is a drive to increase the availability of education to all T1DM. Insulin pump therapy, which delivers insulin as a constant infusion, is also now available, but its use in the United Kingdom is below the recommended level.

The benefits of good glycaemic control are well documented. The Diabetes Control and Complications Trial (DCCT) (between 1982–1993) and Epidemiology of Diabetes Interventions and Complications (EDIC) (between 1994-2006) have both shown benefits of intensive diabetes control. This (DCCT, 1986) was a landmark, multi-centre randomised control trial (RCT) which compared intensive diabetes control with conventional control in T1DM patients. It showed that diabetes-related complications are reduced by as much as 76% (DCCT, 1993). EDIC is a post randomisation study of DCCT (EDIC, 1999a). It showed that good glycaemic control has long term benefits. There was 42% risk reduction in all cause cardiovascular outcomes (myocardial infarction, cardiac catheterisation, etc.) (EDIC, 1999b). It also demonstrated that the intensively treated group had slower progression of atherosclerosis markers (Nathan

*et al.*, 2003) (carotid intima-medial wall thickness progressed slowly and less coronary calcification). A 1% increment in HbA1c has been shown to be associated with an increase (6.4%) in coronary artery stenosis (Larsen *et al.*, 2002). In a large Swedish observation study, it has been shown that a 1% increase in HbA1c was associated with an increase in the risk of CVD (approximately 30% increase) (Eeg-Olofsson *et al.*, 2010).

Based on these studies, good glycaemic control is defined as HbA1c of ≤ 7% (53 mmol/mol). However, in clinical practice this has been difficult to achieve. The national paediatric audit (2012) showed that only 16% of patients under the age of 25 year achieved HbA1c of 7.5% (58 mmol/mol). Median reported HbA1c in Europe and Australia is around 8.5% (Pambianco *et al.*, 2007; Saunders *et al.*, 2008; Donovan and McIntyre, 2010). In EDIC, mean HbA1c was around 8% (63 mmol/mol) (EDIC, 2003; Nathan DM, 2005). If glucose levels are tightly controlled than there is an increased incidence of hypoglycaemia (DCCT: 2.50 – 3 times more common in the intensive group) (DCCT, 1991). A major barrier against achieving good glycaemic control is fear of hypoglycaemia. The recent work by the EURODIAB Prospective Complications Study has demonstrated a U-shaped association between the all-cause mortality and HbA1c (Figure 4). That is, the all-cause mortality is highest at low (5.6%; 37.7mmol/L) and high (11.8%; 105.5mmol/mol) HbA1c (Schoenaker *et al.*, 2014). Even with good glycaemic control the CVD risk remains more than twice that of non-diabetic individuals (Figure 5)(Lind *et al.*, 2014; Snell-Bergeon and Maahs, 2015).

Based on all the available evidence, additional strategies need to be developed to reduce the CV risk in T1DM without causing significant hypoglycaemia.

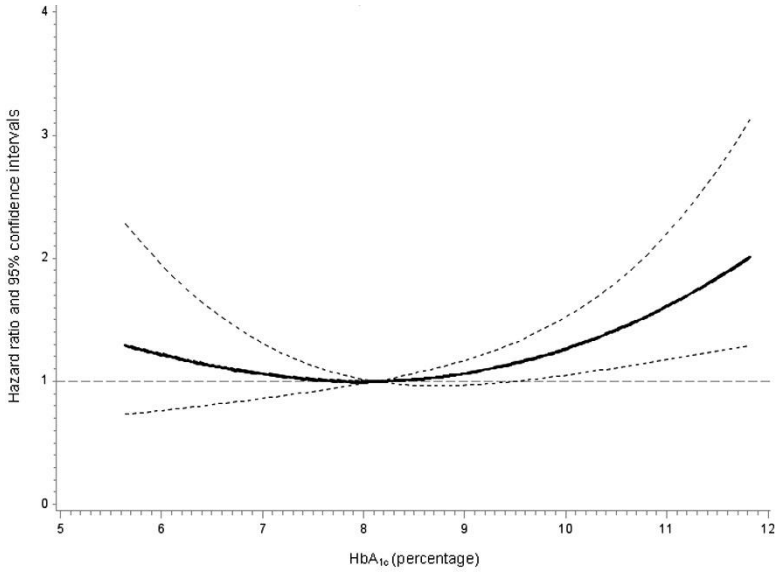


Figure 4: Association between HbA1c and all-cause mortality risk in T1DM patients.

'Restricted cubic spline regression with three knots located at the fifth, 50th, and 95th percentiles (HbA1c 5.6%, 8.1%, and 11.8%). The y-axis represents adjusted HRs for all-cause mortality for any value of HbA1c compared with a reference HbA1c of 8.1%. HRs are adjusted for age and sex (P for overall effect of HbA1c = .008; P for nonlinearity = .03). Dashed lines indicate 95% CIs.'

(Schoenaker *et al.*, 2014)

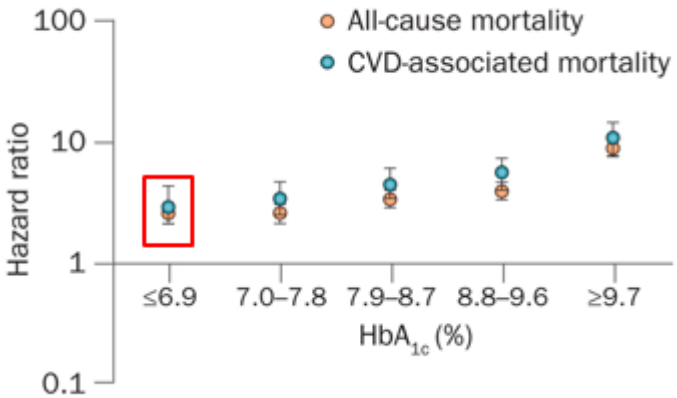


Figure 5: Elevated risk of mortality in type 1 diabetes mellitus

(Snell-Bergeon and Maahs, 2015)

## 1.2 Circulatory markers of vascular repair and damage in diabetes mellitus

The endothelium and its functions play a major role in the maintenance of vascular homeostasis. Damage to the endothelium and subsequent impairment of endothelial function is key to the early development of vascular disease and atherosclerosis. Vascular homeostasis is maintained by a fine balance between vascular repair and damage. Advancement in research has identified circulatory biomarkers which play a critical role in the maintenance of a balance of repair and damage. The circulatory biomarkers of vascular damage and repair are endothelial progenitor cells (EPCs), circulatory endothelial cells (cECs), microparticles (MPs), microRNA (miRNA) and cytokines.

These markers are discussed briefly in this section and in more details in Chapter 4, Chapter 5, Chapter 6 and Chapter 7.

### *1.2.1 Endothelial progenitor cells*

In response to vascular damage, vascular repair is promoted by local endothelial cells and bone-marrow derived cells, called endothelial progenitor cells (EPCs). EPCs were first described in 1997 (Asahara *et al.*, 1997). These cells have the ability to home in on the site of vascular injury, proliferate and contribute to endothelial repair (Urbich and Dimmeler, 2004a), thereby maintaining endothelial health.

Circulatory endothelial progenitor cells (cEPCs) are a heterogeneous population of cells characterised by the expression of surface antigen CD34+, VEGFR-2+ and/or CD133+ identified by flow cytometry. CD34+ and CD133+ are haematopoietic stem cell markers (Timmermans *et al.*, 2009; Yoder, 2012). VEGFR-2 is a surface marker of endothelial lineage. Progenitor cells undergo various stages of maturation. The CD133 marker is lost as cEPCs mature. Thus, more mature cEPCs are positive for CD34 and VEGFR-2 (Hristov *et al.*, 2003). VEGFR-2 plays an important role in angiogenesis by promoting endothelial cell growth and cell permeability (Collet *et al.*, 2014). cEPC levels are reduced in T1DM.

Proangiogenic cells (PACs), previously known as early EPCs are the cultured peripheral blood mononuclear cells (PBMNC) whereas colonies derived from replated PBMNC are known as colony-forming units (CFU-Hill's colonies) (Hill *et al.*, 2003a). PACs and CFU-Hill's colonies are reduced in both type 1 and type 2 diabetes.

### *1.2.2 Circulatory Endothelial Cells*

Vascular damage results in the release of circulatory endothelial cells (cECs) from the vascular intima. cECs are mature endothelial cells characterised by presence of endothelial cell surface markers like CD144 and absence of haematopoietic (e.g. CD45) and progenitor cell markers (e.g. CD133). CD144 is important for maintaining endothelium integrity through cell-to-cell adhesion (Goon *et al.*, 2006).

cECs are formed through detachment from vascular intima due to irreversible loss of integrity as a response to endothelial activation by mechanical stress, inflammatory cytokines, growth factors, infectious agents, lipoprotein, and oxidative stress (Goon *et al.*, 2006; Burger and Touyz, 2012). cECs are elevated in type 1 and type 2 diabetes (McClung *et al.*, 2005; Asicioglu *et al.*, 2010). The cEC count (a marker of vascular damage) is directly related to HbA1c in type 1 diabetes (Asicioglu *et al.*, 2010).

### *1.2.3 Microparticle*

Microparticles (MPs) are submicron (0.1-1 micron) fragments which are released by cells (endothelial cells, platelets, leukocytes etc.), either through activation or apoptosis in response to various stimuli (Jimenez *et al.*, 2003). MPs are identified by surface antigens on their bilayer membrane which they inherit from the cells they originate from (for example; endothelial microparticles (EMPs) are characterised by the presence of CD144, CD62e, CD146, CD51 and CD31). In addition, they also have phosphatidylserine on the outer leaflet of the bilayer membrane (Piccin *et al.*, 2006).

MPs are present in small numbers in healthy controls (Berckmans *et al.*, 2001). However, levels of circulatory MPs increase in patients with CVD risk factors including diabetes mellitus. Levels of MP phenotypes have been shown to be altered in both, type 1 and type 2 diabetes (Dignat-George *et al.*, 2002; Salem *et al.*, 2015). This will be discussed further in Chapter 4.

It is hypothesised that altered glucose levels in diabetes leads to generation of reactive oxygen species and lipid peroxidation. This mechanism plays a role in the generation of MPs (Curtis *et al.*, 2010). MPs are not only a biomarker of vascular damage but also plays a vital role in cell-to-cell communication (Trummer *et al.*, 2013). Cell-to-cell communication can be carried by MPs via membrane or cytoplasmic protein or nuclear material like miRNA (Lozito and Tuan, 2014).

### *1.2.4 MicroRNA*

MicroRNA (miRNA) is a family of small highly conserved endogenous non-coding single-stranded RNA that is produced in every animal and plant cells (Ambros, 2004; David *et al.*, 2004). They are approximately 22 (19-24) nucleotides in length (David *et al.*, 2004; Bhaskaran and Mohan, 2014). They play a significant role in the regulating post-transcriptional gene expression. miRNA binds the complementary target on messenger RNA leading to their degradation on inhibition of transcription. This results in dysregulation of subsequent protein production. However, the exact mechanism is unknown (Bhaskaran and Mohan, 2014). miRNAs are thought to regulate approximately 30% of mRNA (Filipowicz *et al.*, 2008).

With advances in the miRNA field, we are now able to detect miRNA in plasma (Mitchell *et al.*, 2008). The role of miRNA in diabetes has been acknowledged recently. miRNA has been recognised as a potential marker to predict the future development of diabetes mellitus (Zampetaki *et al*., 2010). Various studies have shown that miRNA levels are altered in patients with diabetes. Therefore, there is immense potential in exploring the role of miRNA in developing complications related to diabetes mellitus. Furthermore, new treatments could be developed which can target specific miRNA to reduce the development of diabetes related complications. The MERIT Study is novel as we explored the effect of metformin on selected miRNAs linked with the development of CVD.

### *1.2.5 Cytokines and peripheral blood mononuclear cell*

Cytokines are small glycoproteins that are released by endothelial cells and circulatory cells like peripheral blood mononuclear cells (PBMCs) (Kofler *et al*., 2004). PBMCs play an important role in the maintenance of inflammatory signals and the development of vascular damage and atherosclerosis (Moore and Tabas, 2011; Hedrick, 2015). Cytokines are involved in many physiological and pathological functions including immunity (innate and/or acquired) (Dinarello). They also play an important role in generating an inflammatory response leading to vascular damage or repair (Ait-Oufella *et al*., 2011). Each cytokine has an important role in the process of vascular homeostasis. For example, IL-6, IL-8 and TNF-α are potent inflammatory cytokines, whereas IL-10 is a potent anti-inflammatory cytokine (Han and Boisvert, 2014). Chronic inflammatory response in diabetes plays an important role in the development of diabetes related complication. Inflammatory cytokines act on the endothelium, leading to the activation and generation of adhesion molecules, E-Selectin, ICAM-1 and VCAM-1 (Wang and Huo, 2010). Inflammatory cytokines and adhesion molecules initiate an interaction between leukocytes and the endothelium, leading to development of vascular dysfunction and atherosclerosis in diabetes (Weber *et al*., 2007). bFGF and VEGF plays an important role in vascular repairs (Lindner *et al*., 1991). However, an exaggerated response in diabetes leads to the development of proliferative retinopathy (Alon *et al.*, 1995).

Circulatory cytokines, growth factors and adhesion molecule levels have been studied in both, type 1 and type 2 diabetes. Levels of these markers have been shown to predict the onset and progression of diabetes related complication. This is further discussed in chapter 6.

## 1.3 Metformin

Metformin is the most commonly used oral drug for the treatment of T2DM. It has become the first line oral therapy as per major guidelines for treatment of T2DM (American Diabetes Association (ADA), European Association for the Study of Diabetes (EASD) and National Institute for Health and Clinical Excellence (NICE) 2008). Metformin is currently the only biguanide used in clinical practice (Kaztung *et al* 2007 p-741).

The active component of metformin is guanidine, a compound that has been in use for centuries to treat “intense urination” (Witters, 2001). Guanidine is extracted from a plant called *Galega officinalis* (French Lilac) (Figure 6). Metformin was created by Sterne and colleague in mid 1950s and found to have glucose lowering effect, but needs a pancreas for its action (Sterne, 1957). At the same time, (Ungar *et al.*, 1957) discovered a more potent biguanide called phenformin. The usual effective dose of phenformin is 20 times lower than metformin (100mg vs 2000mg). However, phenformin was reported to cause lactic acidosis and increased cardiovascular mortality in type 2 diabetes patients (Walker and Linton, 1959). This resulted in biguanide falling out of favour. However, the less potent biguanide, metformin gained acceptance among clinicians in Europe. With time, it was clear that metformin-induced acidosis occurred at a very low frequency. In 1995, metformin was approved by the Food and Drug Administration (FDA) for use in the United States. In 1998, metformin was shown to have cardio-protective effect in individuals with type 2 diabetes (UKPDS, 1998). Since then metformin has become an integral part of the management of T2DM.

### *1.3.1 Structure of biguanide*

Three biguanides have been discovered so far; metformin, phenformin and Buformin (Bridges *et al.*, 2014) (Figure 7). Biguanide contains two linked guanidine (Pernicova and Korbonits, 2014). Metformin has two methyl groups attached to the guanidine side chain. Phenformin has a loss of an ammonia group from the two guanidine linked groups. In addition, phenformin contains a phenyl-ethyl ring attached to the guanidine side chain (Pernicova and Korbonits, 2014). In Buformin, butyl ring is attached to the

guanidine side chain (Bailey and Day, 2004). Buformin and phenformin are not used in clinical practice due to an increased risk of lactic acidosis (Schafer, 1983).

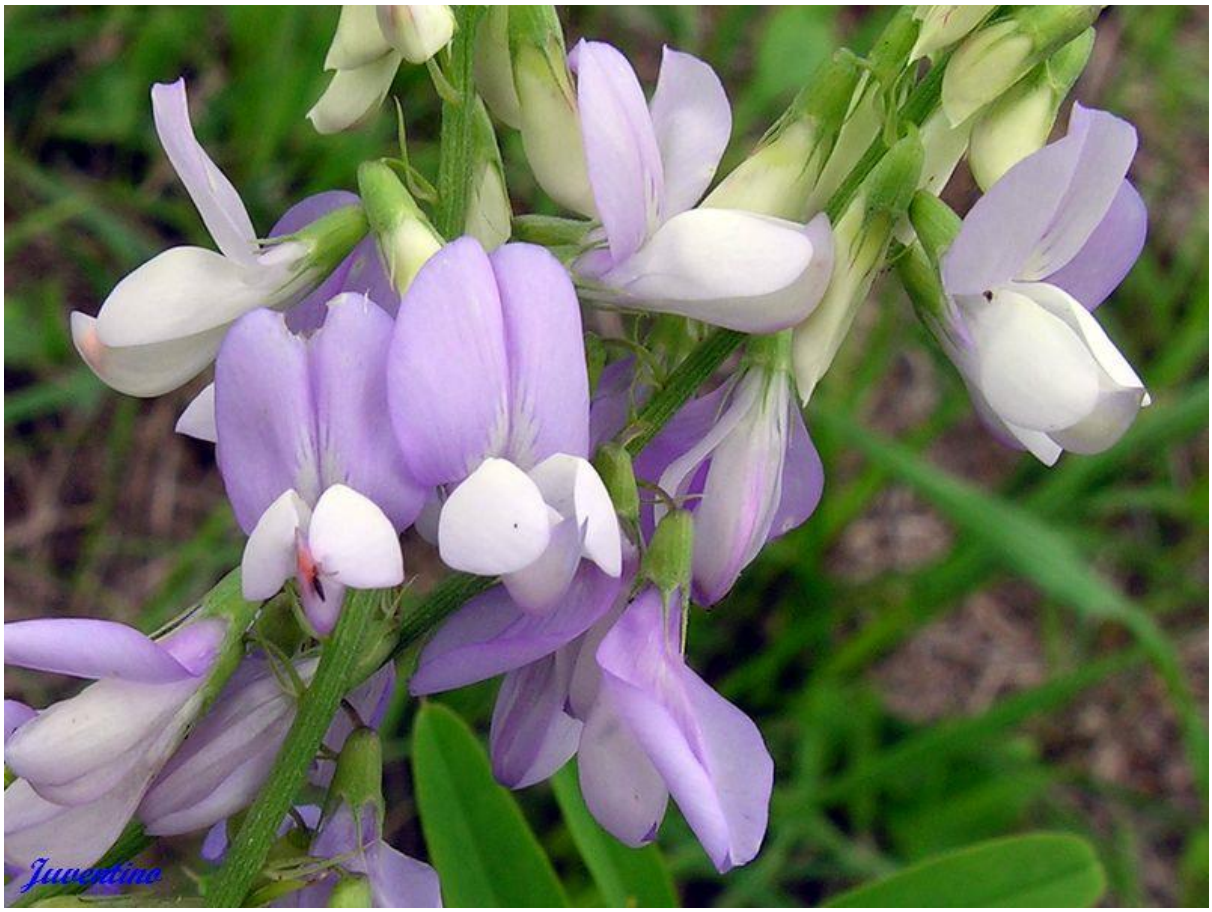


Figure 6: Galega officinalis (French Lilac).

Picture taken from Flore-bis.

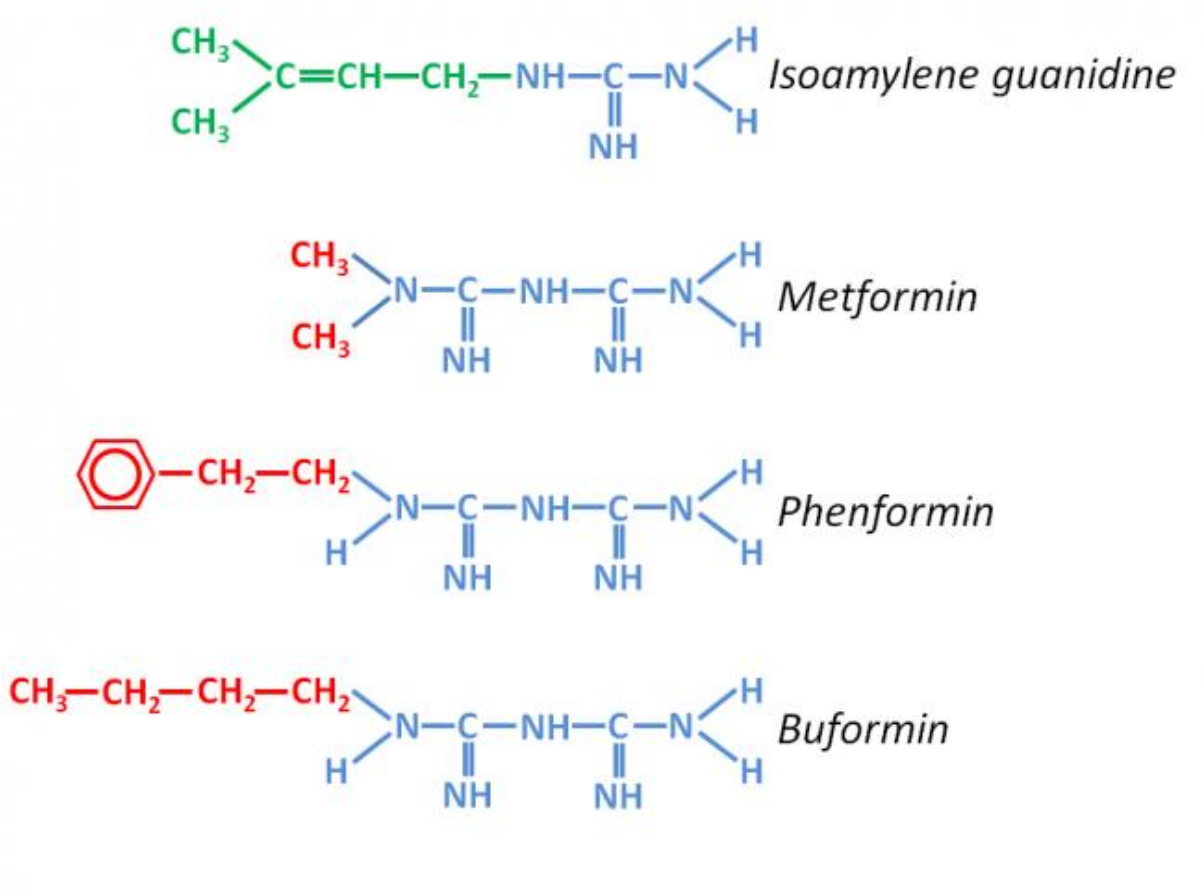


Figure 7: Structure of metformin, phenformin and Buformin.

Adapted from (Holleman, 2014)

### *1.3.2 Pharmacokinetic profile of metformin*

Metformin is entirely absorbed via the small intestine. The stomach and large intestine are not involved in metformin absorption. Bioavailability of metformin is approximately 40-60%. Absorption of metformin finishes at about 6-10 hours after oral administration (Scheen, 1996). The rate of absorption or bioavailability does not increase with an increasing dose of metformin. There is an inverse correlation between metformin dose and absorption (Scheen, 1996). In circulation, metformin does not bind to plasma protein (Papanas *et al.*, 2009). Metformin is not metabolised by liver. It is excreted by the kidney. Half-life is approximately 1.5 to 6.5 hours (Tucker *et al.*, 1981)(Tucker *et al.* 1981). It can be prolonged in case of renal impairment (Papanas *et al.*, 2009). Metformin concentration in plasma is below 5ug/ml or 10-30 umol/L (FDA, 2002; Diamanti-Kandarakis *et al.*, 2009)(Figure 8).

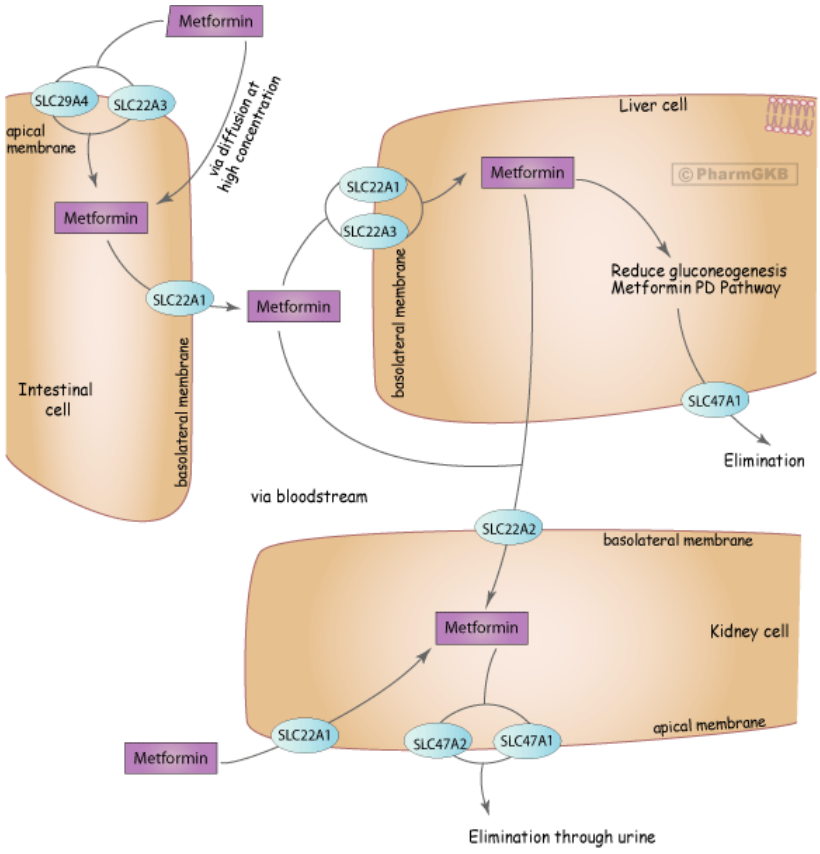


Figure 8: Metformin pharmacokinetic.

Adapted from (Gong Li, 2012)

### *1.3.3 General action of metformin*

Metformin is mainly used to lower blood glucose levels in type 2 diabetes individuals. Metformin reduces hepatic gluconeogenesis and lipogenesis. This effect is speculated to be mediated by several mechanisms. Activation of adenosine monophosphate kinase-activated protein Kinase (AMPK) pathway plays a vital role in action of metformin in liver (Diamanti-Kandarakis *et al.*, 2009). Metformin reduces insulin resistance. This is mediated via its actions in liver, skeletal muscles and the small intestine (Hundal *et al.*, 2000; Viollet *et al.*, 2012; McCreight *et al.*, 2016). This effect is mediated via AMPK pathway activation and recruitment of GLUT4. It is important to note that metformin has been shown to accentuate the insulin mediated glycogen synthesis at physiological relevant doses (Al-Khalili *et al.*, 2005). In addition, metformin inhibits lipogenesis and increases fatty acid oxidation in skeletal muscles (Diamanti-Kandarakis *et al.*, 2009).

Concentration of metformin was 30 to 300 time higher in mucosa of small intestine compared to plasma (Bailey *et al.*, 2008). Metformin may diminish glucose absorption from intestine (Bailey and Turner 1996). This has been demonstrated by Fineman and colleagues, where gut released metformin has been demonstrated to lower glucose absorption. This effect was mediated mainly in the lower part of the small intestine (Buse *et al.*, 2016). Metformin may increase the secretion of GLP-1 and also cause a reduction in appetite (Bailey and Turner 1996; Rotella *et al.*, 2001).

### *1.3.4 Efficacy of metformin in type 2 diabetes mellitus*

Metformin is an anti-hyperglycaemic agent which has been shown to improve glycaemic control, both in monotherapy and in combination with oral hypoglycaemic and injectable agents. In a meta-analysis, metformin has been shown to improve HbA1c by 12 mmol/mol (1.12%) reduction when compared with placebo. As an add-on with oral hypoglycaemic agent and insulin, metformin reduced HbA1c by 11mmol/mol (0.95%) and 9 mmol/mol (0.83%). The reduction has been shown to be maintained in trials with duration of more than 24 weeks (Hirst *et al.*, 2012). Metformin has shown to have dose dependent glucose lowering effect (Garber *et al.*, 1998;

Fujioka *et al.*, 2005). Improvement in glycaemic control has been observed in both obese and non-obese (normal BMI) individuals (Ito *et al.*, 2010).

The effect of metformin on body weight is of great interest as the majority of T2DM individuals are either overweight or obese (Daousi *et al.*, 2006). Unfortunately, data on the effect of metformin on weight is not consistent. The (UKPDS, 1998), one of the longest running trials showed that type 2 diabetes patients on metformin as monotherapy gained 1.5kg in weight. The reviews of other studies does demonstrate a modest effect on weight loss (up to a maximum of 3.8kg) (DeFronzo *et al.*, 1995; Golay, 2007).

Metformin has shown to reduce total cholesterol, LDL cholesterol and triglyceride significantly (-0.26mmol/L, -0.22mmol/L and -0.13 mmol/L respectively). However, metformin has no effect on blood pressure (Wulffele *et al.*, 2004).

### *1.3.5 Efficacy of metformin in type 1 diabetes mellitus*

Metformin has now been recommended in overweight type 1 diabetes patients as an addition to insulin therapy (NICE, 2015). However, the addition of metformin has shown to have no effect on glycaemic control (Vella *et al.*, 2010). It was speculated that the dose of insulin was adjusted toward baseline HbA1c. Indeed, insulin dose has been demonstrated to be reduced by 20%. Metformin has shown a modest reduction of weight in type 1 diabetes (Moon *et al.*, 2007; Vella *et al.*, 2010; Pitocco *et al.*, 2013a; Libman *et al.*, 2015). Furthermore, the reduction of lipid profile has been shown to be of similar magnitude as in type 2 diabetes (Vella *et al.*, 2010).

### *1.3.6 Action on AMPK pathway*

The mechanism of action of metformin is unclear. It has been proposed that metformin primarily acts on an enzyme (AMPK) (Figure 9) (Katzung *et al.,* 2007 page 741).

Metformin is a powerful activator of AMPK. At supra-pharmacological concentrations (1-10 mM), metformin inhibits complex I of the respiratory chain, leading to a reduction in intracellular ATP, which in turn triggers the activation of AMPK (Owen *et al.*, 2000). More importantly, at pharmacologically relevant concentrations (10-20 µM), it has been proposed that metformin phosphorylates and thereby activates AMPK in human endothelial cells (Its upstream kinase, LKB1 is not a direct target of metformin but required in the process of AMPK activation) (Hardie, 2006) Upon activation, AMPK directly promotes the expression of peroxisome proliferator- activated receptor-γ coactivator-1α (PGC-1α) and increases mitochondrial function (Iwabu *et al.*, 2010). Its expression has been reported to decline during ageing, (Petersen *et al.*, 2003; Ling *et al.*, 2004) and is down-regulated in the failing heart (Arany *et al.*, 2005) thus correlating with impairment of mitochondrial function. Both gain and loss-of-function studies indicate that PGC-1α plays a key role in regulating mitochondrial biogenesis (Lin *et al.*, 2002) and is essential for mitochondrial function in all cells, including EPCs. PGC-1α also acts as a transcriptional co-activator. It positively regulates expression of the pro-angiogenic factor like vascular endothelial growth factor (VEGF) leading to angiogenesis *in vivo* (Arany *et al.*, 2008a; Jung *et al.*, 2010). PGC-1α knockout mice show a striking failure to reconstitute normal blood flow to the ischaemic limb. Increased PGC-1α expression in skeletal muscle is protective (Arany *et al.*, 2008b). Since direct activation of PGC-1α might not be amenable to pharmacological intervention, an attractive approach would be the activation of its upstream regulators, such as AMPK.

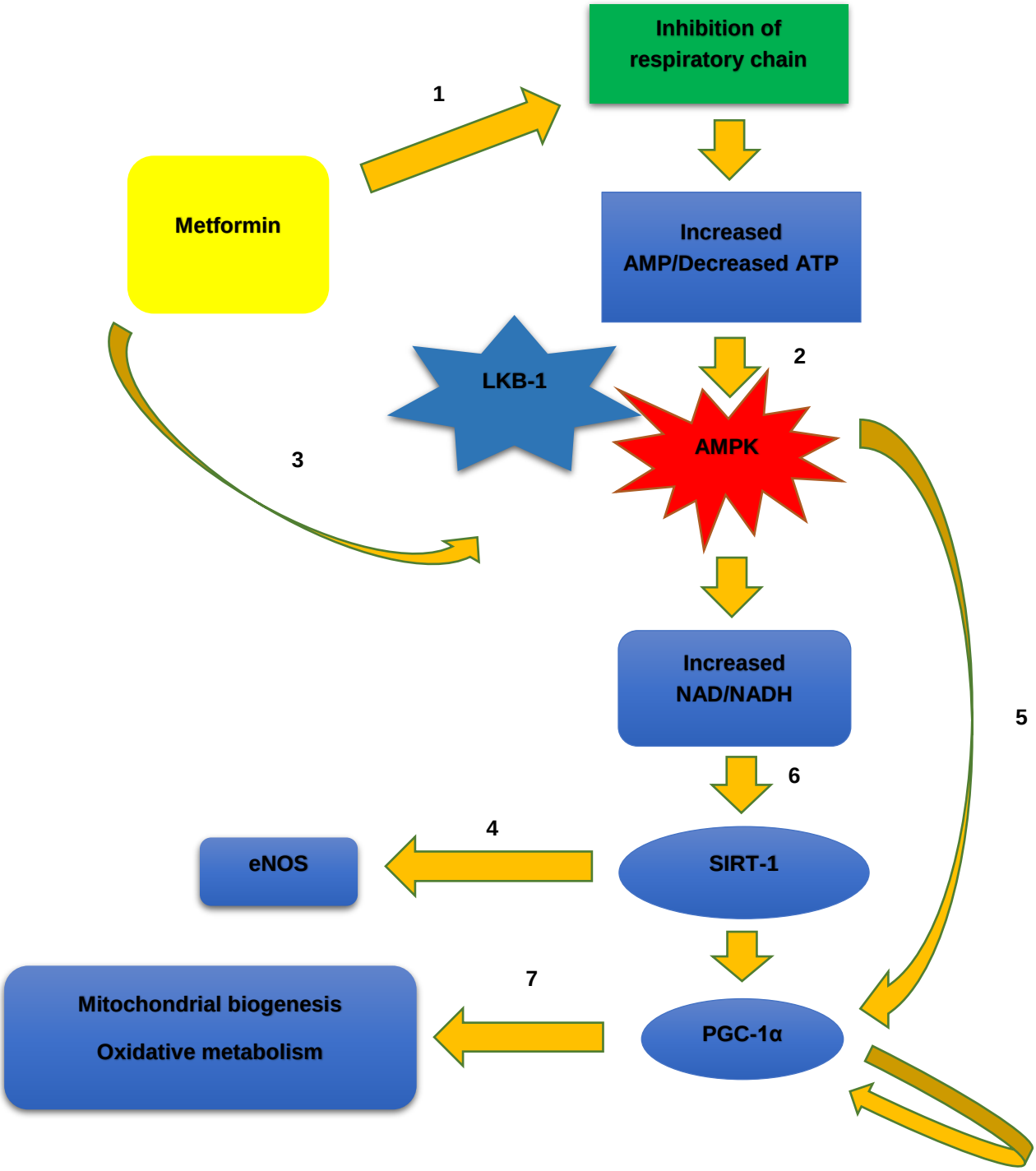


Figure 9: Proposed mechanism of metformin action through AMPK/PGC-1α pathway.

1**.** Metformin in supraphysiological doses inhibits complex I of the respiratory chain, leading to the reduction of intracellular ATP and subsequent increase in AMP concentrations, key monitor of the cellular energy status. **2.** Rising levels of AMP stimulate AMPK. **3.** Metformin directly activates AMPK (via phosphorylation of AMPK kinases (i.e. LKB1). 4. Activation of AMPK increases eNOS production. 5. AMPK directly phosphorylates PGC-1α and this in turn mediates an increase in PGC-1α protein action on the PGC-1α its own promoter. 6. Fasting increases NAD+, substrate and an activator of sirtulin 1, NAD-dependent protein deacetylase (SIRT1) which activates PGC-1α through deacetylation and thus facilitates regulation of target gene expression. 7. Activated PGC-1α regulates target genes i.e. increased translocation of GLUT4.

### *1.3.7 Cardiovascular effect of Metformin*

Metformin has been shown to have vasculo-protective benefits irrespective of its antihyperglycaemic and lipid lowering effects (UK Prospective Diabetes Study 1988). The UK Prospective Diabetes Study (UKPDS) (1998) demonstrated that metformin significantly reduced all-cause mortality and diabetes-related mortality. This reduction was much more than other groups treated with sulfonylurea and insulin. Furthermore, there was no significant difference in the glycaemic control of metformin and insulin or sulphonylurea treated group. In animal models, metformin prevented the development of pulmonary hypertension, luminal occlusion and severe transplant coronary heart disease (CHD) (Verma *et al.*, 2000; Cantin *et al.*, 2002; Romualdi *et al.*, 2008; Teede *et al.*, 2008; Agard *et al.*, 2009). Metformin also improved heart failure and survival in animals via activation of AMPK and its downstream mediators, endothelial nitric oxide synthase (eNOS) and PGC-1α (Gundewar *et al.*, 2009). More recently, it has been demonstrated that metformin (at physiological concentration) and 5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide (AICAR) (another AMPK activator) normalised mitochondrial reactive oxygen species (mtROS) and promoted mitochondrial biogenesis in mature endothelial cells, also partly mediated by the AMPK and PGC-1α pathway (Kukidome *et al.*, 2006; Kim *et al.*, 2007b). Metformin has been demonstrated to lower mortality and morbidity in heart failure patients with type 2 diabetes (Eurich *et al.*, 2005). This effect was most likely independent of glycaemic control.

In chronic ischaemic post MI model, metformin has been shown to have cardio-protective effect (Yin *et al.*, 2011). Chronic pre-treatment of metformin in T2DM individuals was associated with improved outcomes after primary angioplasty for acute myocardial infarction (AMI) (Zhao *et al.*, 2013b). Lexis *et al.* (2014) demonstrated in a retrospective cohort study that the use of metformin is associated with smaller infarct size in patients with MI (Lexis *et al.*, 2014b). In contrast, Basnet and colleagues showed that in AMI patients who have undergone primary angioplasty, infarct size was not associated with metformin use (Basnet *et al.*, 2015). Similarly, metformin has not shown any improvement in the left ventricular ejection fraction despite the improvement in the all-cause mortality (Lexis *et al.*, 2014a; Abualsuod *et al.*, 2015).

Metformin has been shown to improve EF in T2DM (Mather *et al.*, 2001). Similarly, metformin has shown to improve EF in T1DM (Pitocco *et al.*, 2013b). Even in non-diabetic first degree relatives of the patient with diabetes mellitus, metformin has shown to improve EF (de Aguiar *et al.*, 2006). Furthermore, metformin has demonstrated a reduction in CIMT in patients with type 2 diabetes (Matsumoto *et al.*, 2004). However, evidence in non-diabetic individuals does not compliment trials in type 2 diabetes. Metformin did not show any effect on CIMT in high CV risk cardiovascular individuals (Preiss *et al.*, 2013). However, this trial had below mentioned limitation which can explain the results. All participants in this trial were already taking statins. Statin use in previous trials in individual with diabetes was much lower (UKPDS and HOMA). Furthermore, compliance of metformin was less then 80%. Similarly, around 20% of the subjects took lower doses of metformin. The initial design of the trial stipulated 24 months follow-up period. However, this was reduced to 18 months. In addition, fewer participants completed the trial than the calculated sample size. All these factors could have potentially limited the effect of metformin on CIMT. In contrast, metformin reduced CIMT in PCOS (Orio *et al.*, 2005) and improved circulation in PVD patients (Sirtori *et al.*, 1984).

## 1.4 Conclusion

From the above-mentioned discussion, we can identify that type 1 diabetes patients are at increased risk of developing CVD. Even with good glycaemic control, the risk of CVD remains higher. There is a need to develop novel treatment modalities to prevent CVD.

Metformin has been shown to have a cardio-protective effect in type 2 diabetes mellitus. This effect may be mediated independent of glycaemic control. It is likely the cardioprotective mechanism would operate in T1DM in addition to T2DM.

# Chapter 2: Study aims and hypothesis

## 2.1 Study aims

Metformin was the first medication to demonstrate cardiovascular benefit in type 2 diabetes (UK Prospective Diabetes Study 1988). Its cardio-protective effect is likely to be glucose independent and mediated via activation of the AMPK pathway (Katzung *et al.,* 2007 page 741, UK Prospective Diabetes Study 1988). Metformin has been shown to reduce formation of advanced glycation product and improve endothelial function (Pitocco *et al.*, 2013a). Metformin is usually added in type 1 diabetes to improve glycaemic control and reduce insulin requirement (Vella *et al.*, 2010). There is limited information available on the cardiovascular effect of metformin in type 1 diabetes. There was no guidance regarding the use of metformin in type 1 diabetes at the time of setting up of our study. In addition, at the start of our study, there was no randomised controlled trial published on the effect of metformin on CVD in type 1 diabetes.

Type 1 diabetes pathophysiology provided an important reason to study the effect of metformin on circulatory biomarkers in patients with type 1 diabetes. We could adjust the insulin dosage to maintain an unchanged glycaemic control. Changes in insulin resistance are less likely to play a major role in type 1 diabetes. This would not have been possible in patients with type 2 diabetes. Metformin treatment in type 2 diabetes would have resulted in improvement in insulin resistance, glycaemic control and weight. Thus, type 1 diabetes is a useful model to explore the effect of metformin.

We aimed to determine if the cardio-protective effect of metformin is mediated by modulating circulatory biomarkers in type 1 diabetes whilst maintaining unchanged glycaemic control. We aimed to establish the concept of enhancing endogenous vascular stem cell function and a relationship between markers of vascular repair and damage.

### *2.1.1 Primary aim*

The primary aim of our study was to evaluate the effect of metformin on cEPCs in type 1 diabetes.

## 2.2 Hypotheses

In type 1 diabetes, when changes in glycaemia are accounted for, metformin will:

1. Improve the number and function of EPCs in T1DM
2. Improve cEC in T1DM
3. Improve circulatory MPs in T1DM
4. Increase angiogenic miRNA and decreases anti-angiogenic miRNA
5. Increase cytokines related to EPC mobilisation and angiogenesis
6. Decrease cytokines representative of vascular injury markers and inflammatory cytokines
7. Shift the gene expression profile of PBMC in T1DM towards decreased inflammation.

Aims and objectives are again presented in each chapter.

# Chapter 3: Clinical study

## 3.1 Clinical study design

### *3.1.1 Type of study*

To test above-mentioned hypothesis, we desgined an observation study named MERIT Study. The Medicines and Healthcare Products Regulatory Agency (MHRA) judged our study as a Non-Clinical Trial of an Investigational Medicinal Product (non-CITMP).

### *3.1.2 Clinical study method*

We recruited 23 patients with type 1 diabetes with inclusion criteria of HbA1c <8.5% (69 mmol/mmol), absence of macrovascular disease or stage 3b or worse renal impairment (eGFR <45ml/min/1.73 $m^2$) or active proliferative retinopathy, as the 'treatment group' (TG). Nine age-, gender and duration of diabetes matched type 1 diabetes patients were recruited as a standard group (SG). Randomisation was not undertaken. Both, TG and SG did not have any new intervention during the trial except for metformin in TG. Patients with suspected hypoglycemia unawareness were excluded. The study protocol (Figure 10) included a *run-in* phase of 6 weeks to ensure stable glucose control. Following this period metformin was given for 8 weeks to TG with a dose titrated up to a maximum of 1 gram twice a day over 2 to 3 weeks or to the highest tolerated dose. The SG underwent similar follow-up except for metformin treatment. Participants were encouraged not to change their medication other than the dose of insulin therapy during the study. Patients were closely followed up and will attend the clinic at a regular interval. Telephone contact was also made to review the patient's glycaemic control. After the end of the study, patients were given the option to continue Metformin therapy at the discretion of their GPs and followed up in their routine diabetes clinic.

Furthermore, the treatment group was compared with 23 age- and gender-matched non-diabetic healthy controls (HC).

Inclusion and exclusion criteria for TG and SG are given Table 1.

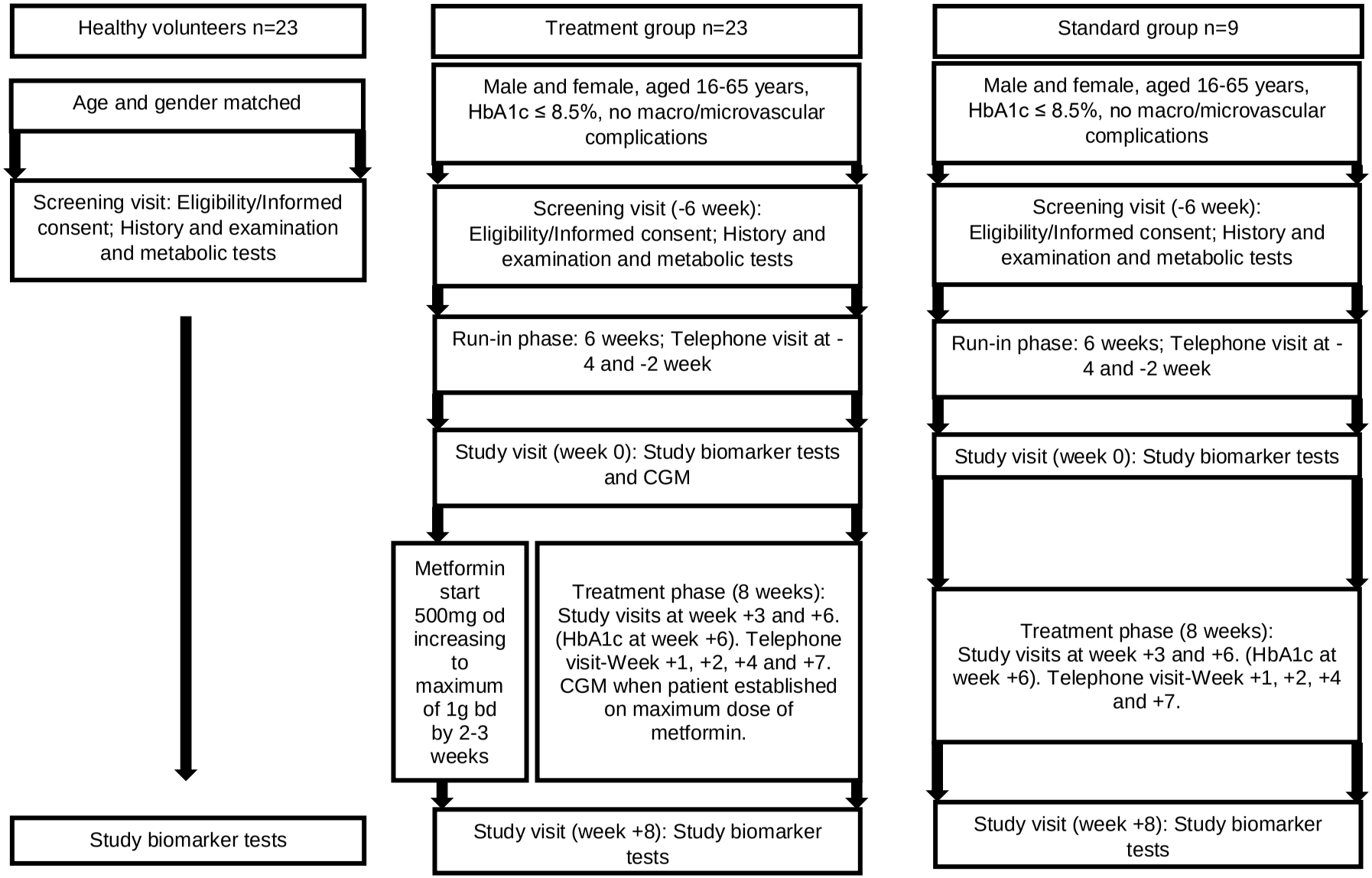


Figure 10: Schematic diagram illustrating MERIT Study design. CGM: Continuous glucose monitoring.

| Inclusion Criteria | Exclusion Criteria |
|---|---|
| • Type 1 diabetes mellitus<br>• Age ≥ 16<br>• HbA1c ≤ 8.5% (69 mmol/mol)<br>• Duration of diabetes ≥ 5 years<br>• Stable diabetes control for ≥ 2 months | • GFR < 45 ml/ min/ 1.73m$^2$<br>• Woman of childbearing age planning pregnancy<br>• Pregnancy and/or lactation<br>• Proliferative diabetic retinopathy<br>• History of CVD/IHD<br>• Peripheral vascular disease<br>• CVA or TIA<br>• Suspected hypoglycaemia unawareness<br>• Impaired cognitive function/ unable to give informed consent<br>• History of lactic acidosis<br>• Contraindications to metformin<br>• History of alcohol problem or drug abuse |

Table 1: Inclusion and exclusion criteria

All subjects gave their written informed consent and the Local Ethics Committee approved the study. Patients with type 1 diabetes were recruited either from, Queen Elizabeth Hospital, Gateshead or Royal Victoria Infirmary, Newcastle, UK. Healthy controls were recruited from the staff from the above or students from Newcastle University, UK.

All participants were advised to attend early morning in the fasting state. The advice was given to avoid caffeinated drinks (tea, coffee, etc.), smoking and exercise since the preceding evening (previously described by Sibal *et al.* 2009). Women will be studied in the follicular phase of their menstrual cycle.

Medical examination included general physical examination, blood pressure, and body weight and height measurement. 12-lead ECG and the Rose angina questionnaire

were performed. Clarke hypoglycaemic awareness questionnaire was performed to assess hypoglycaemia unawareness status before the start of the study.

**Retinopathy status** was confirmed before the start of the study. If retinopathy screening had been done within the last six months, then this was accepted.

### *3.1.3 Blood and other routine care*

A total of 40-mls of blood (Ethylenediaminetetraacetic acid-EDTA and citrate) was collected for the study of potential biomarkers at the start and end of the study for TG and SG. In HC, blood was collected once.

In addition, blood was taken to measure HbA1c, full blood count, thyroid function test, U&E, and LFT as per routine clinical care. In TG, HbA1c was measured four times: at recruitment, the start of treatment, six weeks after the start of treatment and end of the study. In SG, HbA1c was evaluated at the same time points. The aim that no significant change of glycaemic control was observed as judged by HbA1c measurements. HbA1c was measured once in HC at the time of recruitment.

**C-peptide levels** were measured if needed, before the start of the study. Subjects were excluded if C-peptide levels were higher than 200pmol/L (0.6ng/ml).

Urine was collected to document microalbuminuria status (positive or negative). However, if the urine albumin-creatinine ratio had been done in the last six months than it was accepted.

### 3.1.4 *Glycaemic control*

We aimed for unchanged glycaemic control during the study in the treatment group, which was assessed by HbA1c (four times points over 14 weeks) and continuous glucose monitoring (CGM) (Ipro2- Medtronic) (minimum of 48 hours) was performed in those receiving metformin to ensure unchanged glycaemic control. EasyGV Version 8.8.2. R2 was used to calculate the glucose variability index (Hill *et al.*, 2011). CGM was not undertaken in standard group. The CBG target for the duration of the study was 5mmol/l to 11mmol/l.

## 3.2 Statistical analysis

The results are expressed as mean+/-SD (standard deviation) or mean+/-SE (Standard error) unless stated otherwise. Within the group (treatment or standard) comparison was evaluated by paired Student t-test or Wilcoxon Signed Rank test depending on the distribution. Between-groups, the comparison was evaluated by unpaired Student t test or the Mann-Whitney test. Log transformation was carried out for miRNAs before performing a comparison between or within groups. All the data were log transformed before we performed correlation. Correlation between different parameters were calculated by Pearson correlation. One-way ANOVA was used to analyse the difference between HbA1c values. Statistical significance was accepted at $p < 0.05$ (two-tailed significance) unless stated otherwise.

We took advice from the statistician regarding using ANCOVA to assess the change in the treated group (TG) after adjusting for the change in the control group (SG). We were advised that it is better to use Student t-test or Wilcoxon Signed Rank test. This was due to the design of the study. The treatment group (TG) had more subject than the standard group (SG). This will make intrepretation of the result difficult.

As the aim of the study was to assess the effect of metformin on cEPCs in type 1 diabetes, therefore, a statistical power calculation was undertaken only for the TG. Based on our pilot work and in order to reduce CVD risk in patients with type 1 diabetes, minimum of 20 patients were required in treatment group to show a difference of 0.0021 in cEPCs (% leukocytes) with $\alpha=0.05$ and a power of 90%. SPSS v21.0 (SPSS Inc, Ill) was used to perform statistical analysis.

## 3.3 Results

### *3.3.1 Clinical characteristics*

Baseline characteristics of three groups are shown in the Table 2. All groups were well matched for age, gender and blood pressure. TG and SG had a similar duration of diabetes (DOD), HbA1c, baseline insulin dose, lipid profile and creatinine. BMI was lower in SG in comparison to TG.

In the TG, at recruitment, twelve patients took aspirin and/or ACE inhibitor and/or statins in addition to insulin. No new medication other than metformin was started during the trial (except for metformin in the TG). No medication dosage was changed other than the dose of insulin and metformin. The HC group took no aspirin, ACE inhibitors and/or statins. Five patients in the SG took aspirin or/and ACE inhibitor and/or statins in addition to insulin. There was no difference in medication between TG and SG.

After treatment with metformin in the TG, BMI, total cholesterol, triglyceride, blood pressure and HbA1c remained unchanged. Over fourteen weeks HbA1c values were as follows (-6 week (56.4+/-8.3 mmol/mol, 7.3+/-3%), 0 week (56.85+/-10.5 mmol/mol, 7.3+/-0.9%), +6 week (56.8+/-8.5 mmol/mol, 7.3+/-0.8%) and +8 week (56+/-0.8mmol/mol, 7.3+/-0.8%); one-way ANOVA, $p$=0.78). The coefficient of variation of HbA1c over 14 weeks was 4.8%. Furthermore, continuous glucose monitoring confirmed unchanged glucose control and variability (Table 3). Insulin dose, HDL cholesterol and creatinine were significantly reduced in the TG treatment group after metformin treatment. There were no changes in any variables in SG.

| | TG (n=23) | | p value | HC (n=23) | p value | SG (n=9) | | p value | p-value SG V1 vs TG V1 |
|---|---|---|---|---|---|---|---|---|---|
| | **TG V1** | **TG V2*** | TG V1 vs V2 | | HC vs TG V1 | **SG V1** | **SG V2*** | SG V1 vs. V2 | |
| Age year | 46+/-13 | - | - | 46+/-12.6 | 1 | 47.4+/-13.6 | - | - | 0.8 |
| Sex M/F n | 11/12 | - | - | 11/12 | - | 5/4 | - | - | - |
| DOD years | 23+/-13.6 | - | - | - | - | 23.7+/-14.1 | - | - | 0.9 |
| BMI kg/m$^2$ | 28.7 (24-32) | 29 (23-32) | >0.05 | 26.2+/-4.7 | 0.1 | 23.8 (22-27) | 23.7 (21.3-27.1) | 0.3 | <0.05 |
| Systolic BP mmHg | 125+/-10.8 | 121+/-14 | 0.2 | 119.4+/-9 | 0.2 | 132.8 +/-6.2 | 130.8+/-12.1 | 0.7 | 0.05 |
| Diastolic BP mmHg | 76.2+/-9.2 | 74+/-7 | 0.1 | 75.7+/-9 | 0.9 | 77+/-8.2 | 72.9 +/-3.6 | 0.4 | 0.8 |
| HbA1c mmol/mol | 56.9+/-10.5 | 55.9+/-8.5 | 0.5 | 34.8+/-2.9 | <0.0001 | 58.6+/-7.4 | 59 +/-9 | 0.7 | 0.6 |
| HbA1c % | 7.3+/-0.9 | 7.3+/-0.8 | 0.6 | 5.3+/-0.3 | <0.0001 | 7.5+/-0.70 | 7.5+/-0.8 | 0.6 | 0.6 |
| Insulin dose Units | 44 (20-69) | 39 (18-66) | <0.001 | - | - | 52.3+/-11 | 52.9 +/-11 | 0.5 | 0.4 |
| Smoking Y/E/N | 4/2/17 | - | - | 0/0/23 | | 2/1/6 | - | - | - |
| Total Cholesterol mmol/l | 4.5+/-0.8 | 4.4+/-1 | 0.2 | 4.96+/-0.8 | 0.1 | 4.8+/-1.3 | 4.9 +/-1.4 | 0.8 | 0.7 |
| Triglyceride mmol/l | 0.9+/-0.4 | 0.9+/-0.4 | 0.9 | 1.5+/-0.9 | 0.008 | 0.7+/-0.32 | 0.7 +/- 0.3 | 0.6 | 0.2 |
| HDL-cholesterol mmol/l | 1.8+/-0.5 | 1.6+/-0.4 | <0.05 | 1.6+/-0.4 | 0.1 | 1.9+/-0.6 | 2.1 +/- 0.6 | 0.4 | 0.5 |
| Creatinine umol/l | 73 (68-94) | 70 (63-77) | 0.01 | 78 (70-87) | 0.3 | 75 (65-87) | 77 (62.8-83.5) | 0.7 | 0.7 |
| WCC | 6.4 +/- 2.4 | 6.3 +/- 2 | 0.7 | 6.3 +/- 1.6 | 0.9 | 5.8 +/- 1.5 | 5.6 +/- 1.7 | 0.9 | 0.5 |

Table 2: Subject's clinical and metabolic characteristics. Values are given as mean+/-SD or * median (Interquartile range (IQ)). Kg-Kilogram, BMI-body mass index, BP-Blood pressure, M-Male, F-Female, DOD-Duration of diabetes, Y-Yes, E-Ex-smoker, N-No. TG V1: Pre-treatment; TG V2: Post-treatment; SG V1: Pre-observation; SG V2: Post observation; WCC: White cell count. * HbA1c TG V2 vs SGV2 p=0.6.

| | TGV1 | TGV2 | p value |
|---|---|---|---|
| **Average glucose CGM mmo/L** | 9+/-3 | 8+/-2.3 | 0.17 |
| **Blood glucose standard deviation** | 3.3+/-1.1 | 3+/-1.2 | 0.3 |
| **Mean amplitude of glycaemic excursion** | 7+/-2.7 | 6+/-3 | 0.3 |
| **Continuous overall net glycaemic action** | 7.7+/-2 | 7.3+/-2.2 | 0.4 |
| **Total area under curve (AUC) (calculated):** | 12341+/-2900 | 11500+/-3182 | 0.3 |
| **AUC above limit-7.8 (CGM)** | 1.86 | 1.97 | 0.7 |

Table 3: Glucose variability before and after metformin treatment. TG V1: Pre-treatment; TG V2: Post-treatment; CGM: Continous glucose monitor

### *3.3.2 Side effects*

None of the volunteers in the study suffered any side effects requiring discontinuation of metformin. Eighteen patients took the full recommended dose of metformin (1000mg BD or 500mg BD-due to low eGFR-45 to 60 ml/min/1.73 $m^2$). Five patients had gastrointestinal side effects that required dose reduction (two patients took 500mg TDS; two took 500mg BD, and one took 500mg OD). No patient suffered any major episode of hypoglycaemia. A major episode of hypoglycaemia was defined as any episode of low blood glucose requiring the intervention of the third party due to impaired conciousness to resolve the event. There was no significant effect of metformin on minor hypoglycaemic events (% ≤ 3.9 mmol/L and area under curve 3.9 mmol/L on CGMS: 8.6% vs 13.3%; p=0.2 and 0.08 vs 0.1; p=0.5 respectively).

## 3.4 Discussion

We designed the MERIT study to explore the effects of metformin in type 1 diabetes on surrogate CVD circulatory biomarkers. Almost all the studies in type 1 diabetes so far have only explored the effect of metformin on metabolic features rather than cardiovascular markers or outcomes. There is only one study which explored the effect of metformin on endothelial function and oxidative markers (Pitocco *et al.*, 2013b).

We recruited T1DM subject who have well-established diagnosis based on clinical and biochemical features. It was ensured that all patients were diagnosed for more than five years. This gave enough time for patients to have developed subclinical vascular damage without overt disease. The aim of the study was to explore the effect of metformin on early cardiovascular markers in type 1 diabetes. We excluded patients with the overt macrovascular disease. Metformin is contraindicated in patients with eGFR below 30 ml/min. Furthermore, dose reduction is needed when eGFR goes below 45 ml/min. Therefore, we excluded patients with stage 3b CKD. Patients with proliferative retinopathy were excluded. Proliferative retinopathy is characterised by ischaemic induced vessel formation. Proliferative retinopathy will result in increased cEPC and angiogenic circulatory markers driven by ischemia-induced VEGF response. Patients with hypoglycaemic unawareness were excluded to ensure that a recruited cohort can be safely follow up due to the inherent risk of hypoglycaemia.

Subjects in the TG had good overall diabetes control (HbA1c < 57 mmol/mol or 7.3%). The average duration of diabetes was more than 20 years. This made our group fascinating. Our group based on the duration of diagnosis would have developed a subclinical vascular injury. In our centre, we have shown that the young individual with a 10-year diagnosis of type 1 diabetes has features of vascular damage (Sibal *et al.* 2009). Metabolic features were well controlled except for BMI. The cohort in TG were overweight when compared to SG. Increased BMI has been reported in recent studies in type 1 diabetes, including a previous study conducted at our institute in young type 1 diabetes (Age 25.4 years) (Sibal *et al.* 2009). In this study, BMI was 25.3 kg/m$^2$. Similarly, subjects in Pitacco *et al.* (2013) were overweight (27.3 kg/m$^2$). BMI can

affect levels of circulatory markers evaluated. Metformin treatment may influence insulin resistance in TG as they were overweight.

TG cohort were compared with matched SG. SG underwent similar follow up and clinical care during the study period except for treatment with metformin. We only recruited nine subjects in SG. The main aim for SG was to establish that clinical care and follow up during the study will not result in any changes in the measured parameters. TG and SG were well matched for age and gender except for BMI. BMI was significantly lower in SG when compared to TG. This could introduce a bias which can influence the results. Future studies should be planned to explore the effect of metformin on insulin resistance and circulatory biomarkers.

Twenty-three volunteers were recruited in the healthy control group. They were well matched with TG. HbA1c was as expected lower in HC compared to TG. Interestingly, triglyceride was higher in HC when compared with TG. This could be due to the good lifestyle in our TG. Indeed, TG group participant were motivated to improve their health. Our type 1 diabetes individuals had been educated by healthcare professional as a part of education for type 1 diabetes. Furthermore, patients in TG were on lipid lowering agents.

TG was given metformin for eight weeks. The dose of metformin was titrated gradually to reduce the chance of developing of side effects. As expected, a few patients developed metformin-related side effects and were unable to reach the maximum recommended dose. This is in contrast with Pitocco *et al.* (2013), who reported that all subjects managed to take the full recommended dose of 850mg TDS. This could be due to the shorter duration of treatment in our study (8 weeks). By contrast the cohort in Pitocco *et al.* (2013) had more time to gradually increase the dose of metformin.

We ensured adequate follow up to prevent the development of major hypoglycaemia. Insulin dose was reduced to maintain constant glycaemic control. CGMS was performed before the start of metformin and at a maximum dose of metformin. Insulin dose was reduced by five units per day. This was in line with previous studies (Meyer

*et al.*, 2002; Lund *et al.*, 2008a). A meta-analysis estimated a reduction of insulin dose of 6.6 units per day with metformin (Vella *et al.* 2010).

We were able to achieve the aim of our study to keep the glycaemic control unchanged. This is evidence by stable HbA1c and CGMS reading. Furthermore, glucose variability also remained unchanged. It is interesting to note from previous work on metformin in type 1 diabetes that overall HbA1c remained unchanged after metformin treatment (Vella *et al.* 2010 and Pitocco *et al.* 2013).

We performed CGMS in TG. CGMS was not performed in SG. This was due to limitation in funding. Furthermore, performing CGMS provided information which was used to make sure that overall glycaemic control did not change and patients' blood glucose were in safe range. Therefore, we did not have data to compare glucose variability between TG and SG. We believe that this will not lead to significant bias as HbA1c remained similar between TG and SG.

Eight weeks of metformin treatment did not result in any significant change in BMI. This is in contrast with a recent study which showed that six months of metformin in people with type 1 diabetes resulted in the loss of nearly 2.5 kg weight when compared to placebo (Pitocco *et al.*, 2013b). However, we requested that the patients would not aim to improve their diabetic control whilst in the study, so it is a possible explanation for the lack of weight loss. Surprisingly, HDL cholesterol levels were reduced after metformin therapy, though, were similar to the control group and remained well within the normal range. Total cholesterol and triglyceride did not change with metformin treatment.

In summary, types 1 diabetes cohorts (TG) in our study have good overall diabetes control. Overall metabolic parameters were in line with the previously published evidence. They are well matched with HC and SG. Furthermore, metformin treatment did not change glycaemic control and BMI.

# Chapter 4: Effect of metformin on endothelial progenitor cells, circulatory endothelial cells and microparticles

## 4.1 Endothelial Progenitor Cells

Endothelial progenitor cells (EPCs) contribute to the development of new blood vessels in adult (Urbich and Dimmeler, 2004b). EPCs play a pivotal role in vascular repair. Impairment of EPC function is an important factor in the pathogenesis of cardiovascular disease. This has made EPCs remarkably attractive as a biomarker of cardiovascular disease (Silvestre and Lévy, 2009).

EPCs are defined as a group of cells that are mainly derived from bone marrow with an ability to multiply (clonal expansion), proliferate and have a capacity for resistance to stress (stemness characteristics) and can differentiate into mature endothelial cells (Urbich and Dimmeler, 2004b).

### *4.1.1 Historical Perspective*

Before 1997, our general understanding was limited to the fact that the new blood vessel formation in adult (postnatal) life is limited to angiogenesis. Angiogenesis is a process of development of the blood vessels through proliferation, sprouting and migration of fully differentiated cells (endothelial) from the pre-existing vasculature (Risau and Flamme, 1995). Vasculogenesis, the process by which mesodermal cells differentiate into angioblast precursor cells and then subsequently develop into a new vessel like structure, was thought to occur only during embryonic development (Risau and Flamme, 1995; Sabin, 2002).

In 1997, Asahara *et al.* challenged the above theory and postulated that postnatal vessel formation could occur not only by angiogenesis but also by vasculogenesis. Asahara et al. (1997) isolated cells from the adult peripheral blood that differentiated into endothelial cells *in vitro* and *in vivo*. These cells express cell surface markers CD34 and VEGFR2 (KDR). In culture, these cells formed clusters consisting of round cells in the centre and sprouts of spindle shaped cells in the periphery. This is similar to blood island like clusters during embryonic life. This seminal study suggested that there are circulatory cells “progenitor endothelial cells”, which can contribute to new vessel

formation in adult life. These cells also play an important in repair of damaged endothelium (Asahara *et al.*, 1997).

Since 1997, more than 8000 papers have been published (Pubmed as of 12th October 2016, using the search term endothelial progenitor cells). Even with a large number of literature, defining EPCs remains a matter of debate (Yoder, 2012). Isolating true EPCs remains difficult since they lack a specific cell surface marker and their maturation cycle remains unclear (Yoder, 2009). Furthermore, many circulatory cells may contribute to blood vessel repair and formation (Ingram *et al.*, 2005; Yoder, 2012). Two main methods have been used to isolate EPCs in research (Ingram *et al.*, 2005).

1 Culture method
2 Flow cytometry

### *4.1.2 Endothelial progenitor cells isolation: Culture Characterization*

Endothelial progenitor cells can be cultured from peripheral blood mononuclear cells (PBMC) using ficoll density centrifugation or lymphoprep to separate PBMCs from peripheral blood. Two main methods have been extensively used in literature (Prater *et al.*, 2007):

A. Adhesion based method
B. Colony forming assay

#### *4.1.2.1 Adhesion based method*

In method 1 (Figure 11), once PBMCs are separated, they are plated with culture medium supplemented with endothelial growth factors on a fibronectin coated tissue culture plate. After 4 to 7 days in culture, non-adherent cells are removed leaving behind adherent cells (Vasa *et al.*, 2001b; Rehman *et al.*, 2003). These adherent cells are called pro-angiogenic cells (PACs).

PACs have the distinct property of binding plant lectin (*Ulex europaeus*; Ulex) and ingest acetylated LDL (ac-LDL). PACs are identified and quantified by labelling with both ac-LDL and ulex lectin and then are counted. PACs have been used in many studies to define EPCs (Vasa *et al.*, 2001b; Rehman *et al.*, 2003).

Even though ac-LDL bind's cell surface receptors of EPCs and endothelial cells (Voyta *et al.*, 1984), it is not a completely reliable marker of EPCs. ac-LDL receptors are widely distributed on myeloid lineage subset of hematopoietic cells (Rohde *et al.*, 2006). In addition, ulex lectin binds not only vascular endothelial cells but also many circulatory blood cells and epithelial cells (Holthöfer H, 1982; Schwechheimer *et al.*, 1984; Liu Sm, 1996; Graziano *et al.*, 2001). Thus, both LDL and lectin are not specific markers to identify EPCs.

Another confounding factor is contamination of PBMCs with platelets (Prokopi *et al.*, 2009). These platelets can get fragmented *in vitro* culture. On occasion, platelet fragments can get incorporated in the cell membrane of cells in culture plates. This can result in cells with platelet fragment showing some features and function of platelets (Prokopi *et al.*, 2009).

Adhesion based method is fraught with above-mentioned problems. But it is interesting to note that these adherent cells (EPCs or PACs) are known to promote angiogenesis in animal ischaemic models like limb ischemia and myocardial ischemia (MI) (Kalka *et al.*, 2000; Kawamoto *et al.*, 2001) These cells have been shown to be directly associated with CVD (Vasa *et al.*, 2001b).

Vasa *et al*., (2001) demonstrated that PAC numbers were 40% lower in coronary artery disease patients and also demonstrated impaired functional capacity (PAC migration) as compared to healthy volunteers. In addition, there was an inverse correlation between numbers of risk factors and PAC numbers. Smoking was found to be the most important factor which lowered PAC numbers. Whereas, hypertension had the greatest effect on PAC function: reduced PAC migration (Vasa *et al.*, 2001b) and endothelial repair capacity (Giannotti *et al.*, 2010).

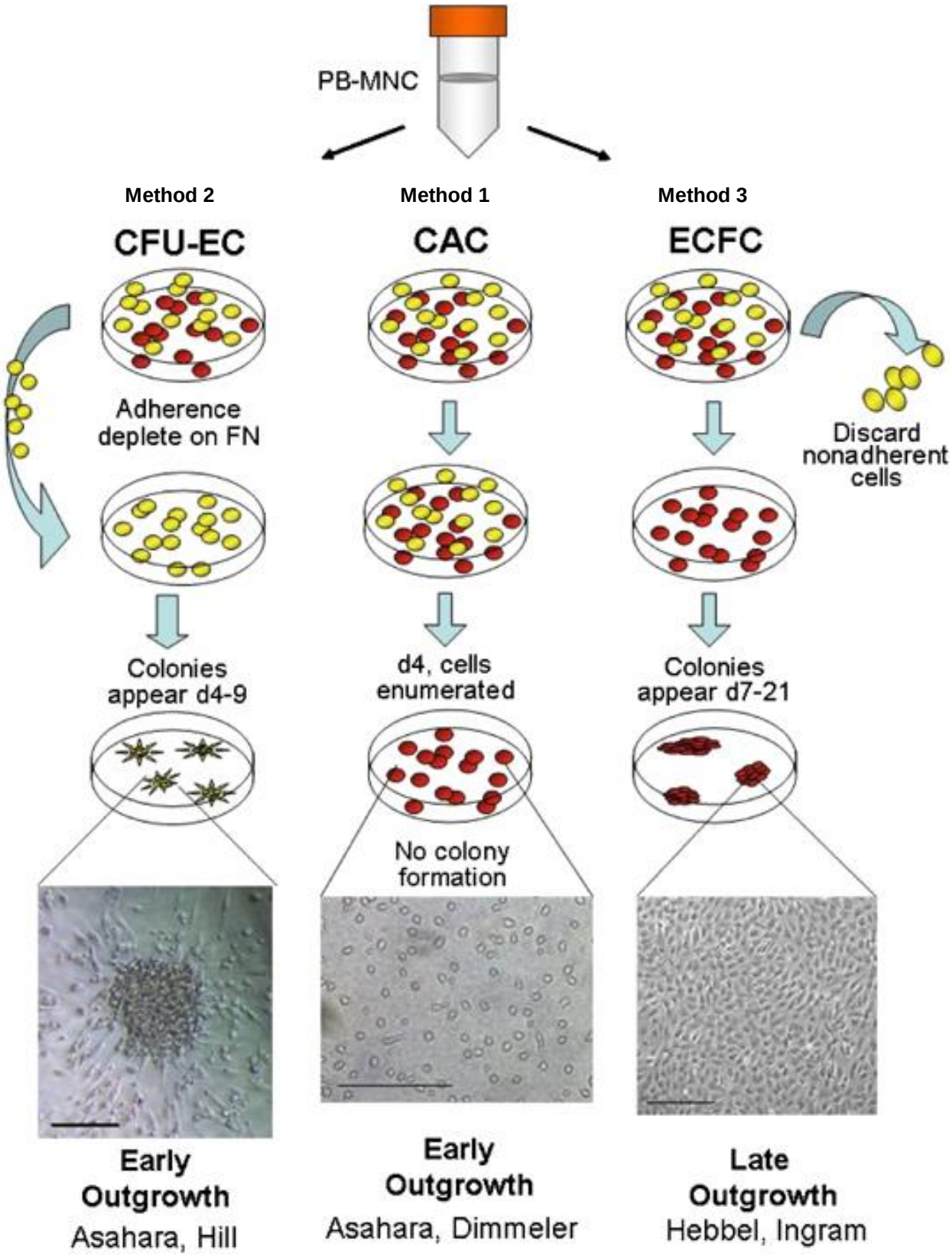


Figure 11: Common methods of EPC culture. Adapted from (Prater *et al.*, 2007).

#### *4.1.2.2 Colony forming assay*

The second method for defining EPCs uses isolated PBMCs in a colony forming assay (Yoder, 2012). Colony forming assay can be performed in two ways (Figure 11).

In method 2, PBMCs are separated from the peripheral blood through ficoll density centrifugation or lymphoprep. PBMCs are plated on a fibronectin coated tissue culture plate. After 48 hours, non-adherent cells are collected. This is to avoid any contamination with other cells like monocyte, endothelial cells, etc. Collected non-adherent cells are plated fibronectin coated tissue culture plate. The culture medium is changed after every 72 hours. After 5 to 7 days discrete colonies are formed. These colonies are characterized by a central collection of round cells surrounded by elongated spindle-shaped cells sprouting at the periphery (Hill *et al.* 2003). These colonies are called colony forming unit-Hills (CFU-Hills) or colony forming unit-EC (CFU-EC) or early EPCs.

Asahara *et al.,* (1997) initially defined the method. It was shown that these clusters of colonies have an endothelial phenotype as they express CD31, CD34, FLK-1 (fetal liver kinase; also called KDR-kinase insert domain receptor or VEGFR2), Tie-2 (CD202B) and E-Selectin. It was thought that these colonies are likely responsible for vasculogenesis.

The Hill colony isolation method has been modified, and the additional step of removing contaminating cells like monocytes or endothelial is now used (Hill *et al.*, 2003a). An important feature of these colonies is the fact that they disappear within 10-14 days (Ingram *et al.*, 2005). CFU-Hills express endothelial, hematopoietic and macrophage antigens. They show some macrophage function of expressing non-specific esterase and bacteria activity (phagocytosis). They have minimal proliferative potential and do not form vessels *in vivo* (Yoder *et al.*, 2007).

Even though CFU-Hills do not have a direct role in vessel formation, it has been shown that CFU-Hills numbers are an important predictor of cardiovascular risk. CFU-Hill

numbers are inversely correlated with the Framingham risk score (Hill *et al.*, 2003a). Patients with CAD (Hill *et al.*, 2003a), diabetes mellitus (Hill *et al.*, 2003a; DiMeglio *et al.*, 2010), hypertension (Hill *et al.*, 2003a) and subclinical atherosclerosis (Cheng *et al.*, 2010a) have significantly lower CFU-Hills (significantly lower in multi vessel coronary disease; (Kunz *et al.*, 2006). Acute vascular injury (angioplasty, unstable angina, etc.) results in an increase in the number of CFU-Hills (George *et al.*, 2004; Bonello *et al.*, 2006).

In method 3, isolated PBMCs are plated on type 1 collagen, and non-adherent cells are discarded. This is done every day until the seventh day and then every other day. Colonies start to form between 5 to 22 days. These colonies are well-circumscribed monolayer of cobblestone endothelial cells (Ingram *et al.*, 2005). These colonies are called endothelial colony forming cells (ECFCs) or late EPCs. These colonies are distinct from those described by Hill et al. (2003) (CFU-Hill). ECFCs do not express haematopoietic markers like CD45 or monocytic markers like CD14. Also, ECFCs do not express CD133, but they do express CD34 and KDR. If $CD34^{+}CD45^{-}$ enriched PBMCs are plated, there is a significant improvement in numbers of ECFCs (Timmermans *et al.*, 2007). These colonies have strong proliferative potential and form vessels in vivo (Ingram *et al.*, 2005; Yoder *et al.*, 2007).

To date our understanding for CFU-Hill and ECFCs is limited. We are still trying to understand the relevance of these colonies. Evidence has shown that these colonies are likely to play an important part in neo-vasculogenesis, either directly or indirectly (Padfield Gj, 2010). In humans, both CFU-Hills and ECFCs are mobilised due to vascular stress (Bonello *et al.*, 2006; Marboeuf *et al.*, 2008; Massa *et al.*, 2009). They both are believed to play an essential role in vascular repair. In a mouse model when CFU-Hill and ECFCs are infused together in an ischaemic limb, there is a synergistic improvement in the blood flow to the limb (Yoon *et al.*, 2005).

### *4.1.3 Circulatory endothelial progenitor cells enumeration: Flow cytometry (FACS)*

An increasingly common method for identifying circulatory EPCs is through flow cytometry of peripheral blood. The technique is based on the fact that each cell will express a specific surface antigen. These surface antigens can be labelled with monoclonal antibodies. This means that through fluorescence-activated cell sorting (FACS), the specific cell population can be enumerated. A small amount of peripheral blood (50-150 microliter) or mononuclear cells after separation in labelled with a fluorescent monoclonal antibody (Vasa *et al.*, 2001b; Xu *et al.*, 2008; Sibal *et al.*, 2009a). After incubation, the cells are identified using FACS analysis.

Identification of cEPCs by flow cytometry is hampered by the fact that there is a lack of specific markers that clearly discriminate EPCs from circulatory endothelial cells (cEC). Circulatory EPCs are thought to include antigens for hematopoietic and endothelial lineage cells (Yoder *et al.* 2007). In the first report (Asahara *et al.*, 1997), CD34+ and VEGFR2+ (KDR) cells were identified as markers of identification. When cells with $CD34^+$ or $KDR^+$ cells were infused in an ischaemic limb, these cells promoted vasculogenesis and were localised in the ischaemic area (Asahara *et al.*, 1997).

However, CD34 and KDR are also expressed on endothelial cells. This makes delineation of cEPCs from cECs difficult. Peichev *et al.* (2000) added that, in addition to CD34 and KDR, cEPCs also express CD133, which is not expressed by endothelial cells (Peichev *et al.*, 2000). Reye *et al.* (2002) extended this and showed that bone marrow derived $CD133^+VEGFR2^+CD34^-$ cells give rise to cells that express endothelial cell surface markers. They function as mature endothelial cells and promote angiogenesis and wound healing *in vitro* and *in vivo* (Reyes *et al.*, 2002). However, no studies have been conducted on $CD34^+VEGFR2^+CD133^+$ cells to see if they promote vasculogenesis or if they localise to new vessel sites (Yoder, 2009).

Cells positive for all three markers, $CD34^+$, $VEGFR2^+$ and $CD133^+$ do not form CFU-Hill or ECFC *in vitro*. However, only $CD34^+$ cell fractions which are $CD45^-$ demonstrated ECFC activity. Endothelial cells do not express the CD45 antigen (Case

*et al.*, 2007; Timmermans *et al.*, 2007). In addition, Timmerman *et al.* (2007) demonstrated that VEGFR2, but not CD133 was expressed by $CD34^+/CD45^-$ cells. Similarly, $CD34^+/CD45^+$ cells expressed CD133 but not VEGFR2.

From the available evidence, $CD34^+$ cells have been used extensively to describe and define the EPC phenotype. In order to improve the identification of more specific populations of EPCs, various combinations of surface antigens like $CD34^+VEGFR2^+$ (Pelosi *et al.*, 2002), $CD34^+CD133^+$ (Allanore *et al.*, 2007), $CD34^+VEGFR2^+CD133^+$ (Peichev *et al.*, 2000), $CD34^+CD133^+CD45^+$ (Martin *et al.*, 2008), $CD34^+CD133^+CD45$, $CD45^-CD34^+VEGFR2^+$ etc. have been used. Other than combinations mentioned above, $CD144^+$, $CD31^+$, $CD146^+$ and $CXCR\text{-}4^+$ have been used in various combinations to identify cEPCs. However, none of these have been studied *in vitro* or *in vivo* to confirm the formation of the endothelium.

$CD34/VEGFR2^+$, $CD34^+CD133^+$ and $CD133^+VEGFR2^+$ have been used in much clinical studied to determine cEPC role in CVD (Schmidt-Lucke *et al.*, 2005; Chironi *et al.*, 2007; Sibal *et al.*, 2009a). CD133 is expressed more in immature cells when compared to CD34. Furthermore, in contrast to CD34, CD33 in not expressed on mature EC. Therefore, $CD34^+CD133^+$ may not be a specific marker of EPCs. Ingram et al. (2005) suggested using $CD45^-$ cells to define cEPCs (Ingram *et al.*, 2005). Making a distinction between CD45 negative, dim and bright events have been possible with improvement in flow cytometry technology and methods resulting in improved sensitivity of rare event. $CD34^+VEGFR2^+$ cells have been shown to express CD45 dimly (Schmidt-Lucke *et al.*, 2010).

Schmidth-Lucke et al. 2010 clarified that the $CD45^{dim}$ population defines the $CD34^+VEGFR2^+$ cells as cEPCs in relation to CVD. Current evidence defines cEPCs as cells which are $CD45^{dim}CD34^+VEGFR2^+$ (Schmidt-Lucke *et al.*, 2010). Using other markers like $CD133^+VEGFR2^+$ and $CD34^+CD133^+VEGFR2^+$ in $CD45^{dim}$ fraction will increase variability as these events are much rarer than $CD45^{dim}CD34^+VEGFR2^+$. $CD34^+VEGFR2^+$ has been shown to provide the best data with regards to cell count and coefficient of variation (Fadini *et al.*, 2008) (Figure 12).

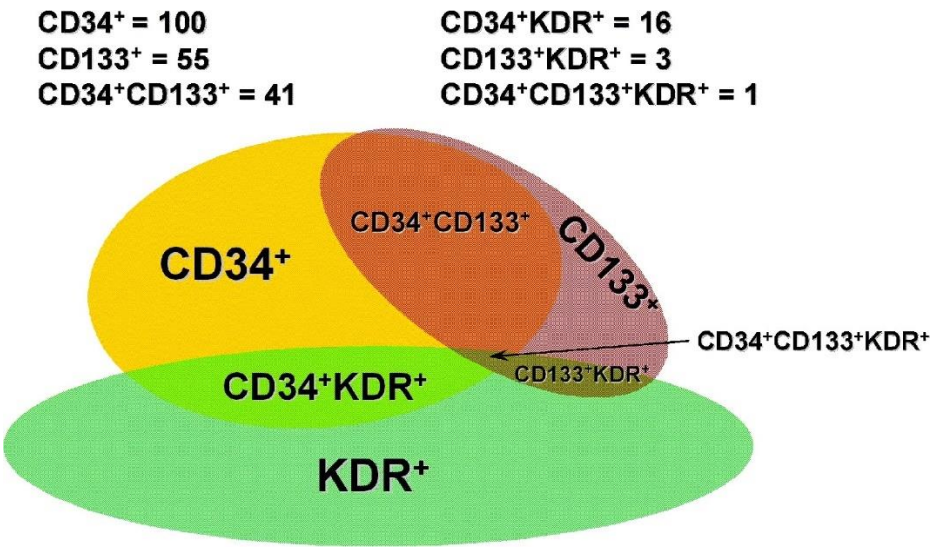


Figure 12: A Venn diagram demonstrating different subgroups of progenitor cells calculated relative to CD34+ cell count. Adapted by (Fadini *et al.*, 2008).

Vasa *et al.* (2001) demonstrated that CD34+KDR+ cells were reduced by 48% in coronary artery patients as compared to healthy individuals. This reduction was strongly correlated with cardiovascular risk factors (r=-0.394; P=0.02). Interestingly, $CD34^+$ or $CD133^+$ alone or $CD34^+CD133^+$ combined cells did not show a correlation with the number of risk factors (Vasa *et al.*, 2001b). Schmidt-Lucke *et al.* (2005) showed similar results and added that patients with lower $CD34^+/VEGFR2^+$ cell count are more likely to have a cardiovascular event over 10+/-12 months follow-up (Schmidt-Lucke *et al.*, 2005).

Circulatory EPCs ($CD34^+VEGFR2^+$) numbers were adversely affected even in the pre-clinical stage of atherosclerosis (Chironi *et al.*, 2007). Furthermore, cEPC levels had been shown to have predictive potential in post cardiac events (Werner *et al.*, 2005). In this large (519 IHD subjects) study, higher cEPC ($CD34^+VEGFR2^+$) levels at baseline results in good prognosis over a period of 12 months. However, no association was found between cEPC levels and acute MI and death from any cause. This may be due to the patients not being classified on the history of acute or recent MI (Kim, 2005).

Acute MI leads to increase in cEPC numbers: $CD34^+$ (Shintani *et al.*, 2001), $CD34^+CXCR4^+$ and $CD34^+CD177^+$ (Wojakowski *et al.*, 2004) and $CD34^+$, $CD34^+KDR^+$, $CD34^+CD133^+$ and $CD34^+CD117^+$ (Grundmann *et al.*, 2007) when compared to stable coronary artery disease (CAD) patients. This is likely due to mobilisation of cEPCs in response to growth factors and cytokine release from ischaemic tissue (Ghadge *et al.*, 2011).

### *4.1.4 Mobilisation and homing*

In response to insult like ischaemia or endothelial damage, progenitor cells mobilise from bone marrow and home to the site of the stress. This is a complex but co-ordinated multistep process (Papayannopoulou *et al.*, 2001) which is regulated by factors such as:

1. Proteases (elastase, cathepsins, and matrix metalloprotein-9)
2. Cytokines (interleukin 8 (IL-8), IL-1, etc)
3. Chemokines and growth factors (VEGF, stromal cell-derived factor-1 (SDF-1), Granulocyte colony-stimulating factor (G-CSF), erythropoietin, hypoxia-inducible factor 1α (HIF-1α).

VEGF and SDF-1 play a very important role in the mobilisation and homing of cEPCs (Asahara *et al.*, 1999; De Falco *et al.*, 2004). Pathological and physiological stimuli like hypoxia and exercise lead to induction of HIF-1α (Jiang *et al.*, 2008; Cheng *et al.*, 2010b). HIF-1α is a transcription factor that promotes VEGF and SDF-1 expression (Ceradini *et al.*, 2004; Jiang *et al.*, 2008; Oladipupo *et al.*, 2011). SDF-1 binds to the CXCR-4 chemokine receptor which is highly expressed in EPCs (Shiba *et al.*, 2007) (Figure 13). HIF-1α increase expression of erythropoietin, a powerful mobiliser of EPCs (Heeschen *et al.*, 2003a).

eNOS has been shown to increase EPC mobilisation. Stimuli such as exercise and VEGF activate eNOS, which is needed to maintain adequate EPC mobilisation. eNOS is thought to act via MMP-9 (Aicher *et al.*, 2003).

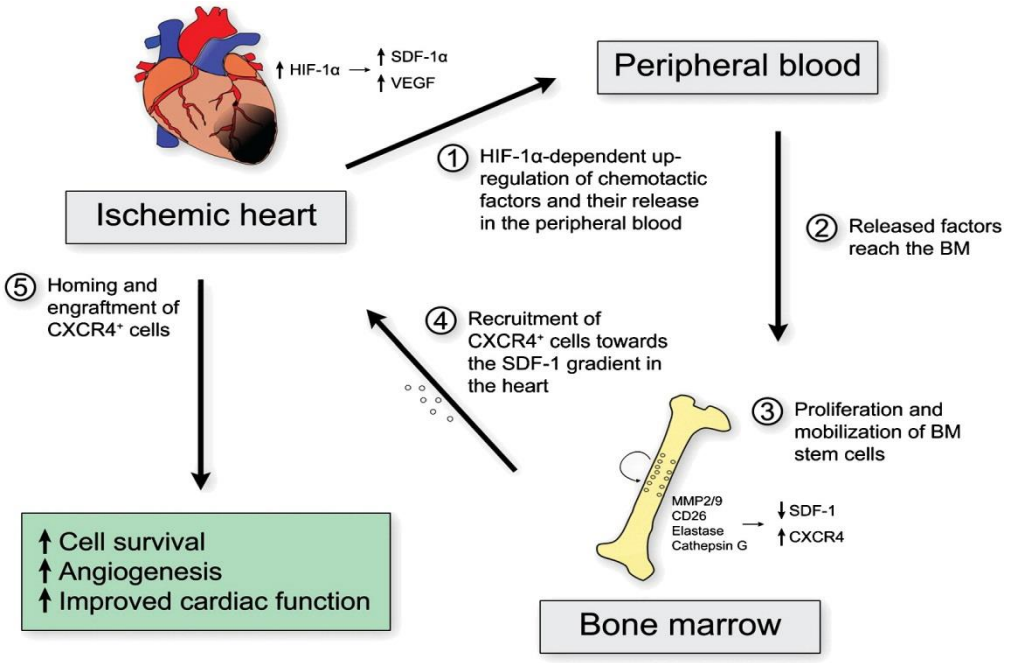


Figure 13: HIF-1α, VEGF and SDF-1 interaction in vascular repair.

1.) Ischemia increases up-regulation of the HIF-1α, which then increases SDF-1α and VEGF. 2.)" These signals reach the bone marrow (BM). 3.) This leads to alterations in the BM microenvironment (e.g. induction of MMP2/9, CD26, elastase and cathepsin G) resulting in proliferation and increased CXCR4 receptor expression of stem and progenitor cells as well as SDF-1 degradation. 4.) The changes in the BM SDF-1 gradient lead to the translocation of stem and progenitor cells into the PB and their homing towards the local SDF-1 gradient initiated in the myocardium by ischemia. 5.) BM stem and progenitor cells selectively home to the ischaemic myocardium where they are involved in myocardial repair through cardio-protective functions and induction of angiogenesis". Adapted from (Ghadge *et al.*, 2011).

The initial step in mobilisation is thought to depend on proteinases like MMP-9, which acts on a membrane bound kit ligand and converts it to the soluble kit ligand. This is needed by progenitor cells to move to the vascular zone of bone marrow (Heissig *et al.*, 2002). VEGF and SDF-1 activate bone marrow MMP-9 (Figure 3) (Heissig *et al.*, 2002; Hao *et al.*, 2011).

Powerful chemokines and cytokines like VEGF, MCP-1 and SDF-1; all play an important role in homing EPCs to the injury site (Chavakis *et al.*, 2005). For cEPCs to adhere to damaged endothelium, the integrin receptor plays a critical role. These proteins are involved in the adhesion and transmigration of progenitor cells. β2-integrin and α4β1-integrin are two important integrins. In experimental models, it has been shown that blocking β2integrin prevents homing of EPCs to the site of ischaemia

(Chavakis *et al.*, 2005). Furthermore, blocking of α4β1-integrin results in the reversal of adhesion of EPCs from vascular cell adhesion molecules-1 (VCAM-1) (Qin *et al.*, 2006). Once the EPCs adhere to the damaged endothelium, they need to transmigrate through the monolayer. This is mediated by endothelial protease like MMP-9 and cathepsin (Urbich *et al.*, 2005; Iwakura *et al.*, 2006).

Above mentioned activity plays a vital role in neovascularisation. However, our understanding of mobilisation and homing of EPCs is limited and continues to be investigated (Mayr *et al.*, 2011).

### *4.1.5 Function of endothelial progenitor cells*

From extensive literature, EPCs have a positive effect on vascular function. EPCs were thought to play a direct role in neovascularisation (incorporation into neo-endothelium). It is now understood that there are different types of circulating progenitor cells in peripheral blood. These different types of cells act directly and indirectly (paracrine and autocrine) during the process of vascular repair.

Rehman et al. (2003) showed that early EPCs have limited proliferation potential, but secrete high levels of pro-angiogenic growth factors like VEGF, G-CSF and hepatocyte growth factor (HGF) (Rehman *et al.*, 2003). He et al. (2005) showed that early EPCs release IL-8, a powerful angiogenic cytokine. Cultured medium from early EPC increases proliferation of HUVEC cells. This effect is attenuated by inhibiting IL-8 (He *et al.*, 2005). CD34+ cells in culture release IL-8, IL-6, macrophage inflammatory protein-1β (MIP-1β), MIP-1α and monocyte chemoattractant protein-1 (MCP-1) (Scheubel *et al.*, 2010). Furthermore, endothelial sprout preferentially grows towards CD34 cells. In addition, CD34+ cells augment the growth of endothelial sprout in culture.

Yoon *et al.* (2005) studied the effect of early and late EPCs *in vitro* and *in vivo*. They found that IL-8 and VEGF were secreted from early EPCs. Late EPCs had high levels of receptors for VEGF and IL-8. When late EPCs were cultured with conditioned

medium from early EPCs, there was significantly improved proliferation, migration, invasion of collagen gel and tubulisation. This effect was neutralized when VEGF and IL-8 were inhibited. Yoon *et al.* (2005) performed in vivo experiment by injecting early and late EPCs together in the ischaemic hind limb. This resulted in significant improvement in perfusion when compared to either early or late type of EPC alone (Yoon *et al.*, 2005).

Cytokines released from EPCs have cytoprotective effects on endothelial cells. This is due to the increased expression of Catalase, Cu/ZnSoD and MnSOD in endothelial cells. These cytokines released from cEPCs have an anti-apoptotic effect. This reduction in endothelial cell apoptosis is through increased Bcl-2/Bax expression ratio (Bcl-2; anti-apoptotic factor; Bax; pro-apoptotic factor). This effect is not reversed with an inhibitor of cytokines like VEGF, HGF, MM-9, IL-8 alone or in combination. Therefore, it is likely that there are some unidentified cytokines or factors responsible. (Yang *et al.*, 2010).

Recently, it has been postulated that the paracrine effect that release of exosomes or microparticles from EPCs may be partly responsible for the paracrine effect. These exosomes/microparticles are a conduit of transport, moving an important message from one cell to another. This could be cytokines or mRNA or microRNA (Lässer *et al.*, 2013). Sahoo *et al.* (2011) showed that $CD34^+$ cells secrete exosomes. These exosomes independently increase angiogenesis in vitro and in vivo (Sahoo *et al.*, 2011). These exosomes are rich in pro-angiogenic miRNA (Lässer *et al.*, 2013). The role of miRNA will be discussed in chapter 5.

### *4.1.6 Effect of environmental and physiological factors on cEPCs*

#### *4.1.6.1 Smoking*

Smoking is one of the major risk factors for CVD. Vasa *et al.* (2001) identified smoking as a major predictor of EPC numbers (PAC). Smokers have significantly fewer EPCs ($CD34^+KDR^+$ and $KDR^+CD133^+$) levels (Yue *et al.*, 2010). In addition to lower cEPC numbers, their functional capacity is adversely affected. Smokers have poorer proliferation, migration, adhesion and tubulisation when compared to non-smokers

(Michaud *et al.*, 2006). Interestingly, brief exposure (30minutes) to second-hand smoking results in increased cEPCs. This is likely secondary to vascular injury leading to the mobilisation of EPCs. Nevertheless, the functional capacity of EPCs is adversely affected (Heiss *et al.*, 2008). Smoking cessation results in recovery of EPC numbers, but this effect is reversible. That is, when smoking is resumed, EPC numbers reduce (Kondo *et al.*, 2004).

#### *4.1.6.2 Obesity*

Obesity is a common problem and associated with cardiovascular risk. Circulatory EPC ($CD34^+KDR^+$) numbers are reduced in healthy but obese subjects (Westerweel *et al.*, 2008). EPC numbers are improved with weight loss (Müller-Ehmsen *et al.*, 2008). Muller-Ehmsen *et al.* (2008) inducted volunteers into diet and six months exercise program group. On average, participants lost 5.8 kgs. In addition, a decrease in BMI correlated with an increase in $CD34^+$ and $CD34^+CD117^+$ cells. Heida *et al.* (2010) demonstrated that obesity had deleterious effects on EPC function (Heida *et al.*, 2010). This could be reversed with weight loss. More recently, Ciuceis *et al.* (2012) showed that EPC numbers were improved after weight loss achieved by bariatric surgery (De Ciuceis and et al., 2012).

#### *4.1.6.3 Exercise*

Physical exercise results in an increase in cEPCs. This even occurs with a single bout of exercise in healthy individuals (Rehman *et al.*, 2004). Different researchers have tried different exercise regimens to assess the effects of exercise on cEPCs. Cesari *et al.* (2012) demonstrated that cEPC numbers improve significantly after 3 months of exercise (Cesari *et al.*, 2012). This improvement was also seen in children and adolescents. Walther *et al.* (2008) compared different durations of exercise regimens (2 hours/week, 5 hours/week and 12 hours/week). Maximal benefit was seen in the group undertaking exercise for 12 hours/week. The migratory capacity of EPCs was also better in this group (12 hours/week) as compared to other groups (Walther *et al.*, 2008).

Even in ischaemic heart disease (IHD) patients, exercise has a positive effect on cEPCs. Again, different research groups have tried different regimen. Laufs *et al.*

(2004) showed that four weeks of controlled exercise program resulted in 78%+/-34% improvement in cEPC levels (Laufs *et al.*, 2004). Steiner et al. (2005) showed similar results after stable CAD patients undertook 12 weeks of endurance training (Steiner *et al.*, 2005).

### *4.1.7 Effect of diabetes mellitus on endothelial progenitor cells*

There is an extensive body of evidence regarding alteration in EPC numbers and function in DM. Both culture and cytometric methods have consistently demonstrated the findings of decreased EPCs number and impaired function (example; adhesion, migration, vessel formation and proliferation) in both T1DM and T2DM compared to HC.

PAC numbers are negatively affected in T2DM (Tepper *et al.*, 2002) and T1DM (Loomans *et al.*, 2004). Furthermore, PAC numbers are inversely correlated with HbA1c (Tepper *et al.*, 2002).

Circulatory EPC levels are lower in T2DM (Egan *et al.*, 2008; Liao *et al.*, 2010) and T1DM (Sibal *et al.*, 2009a) compared to HC. In T1DM patients without any overt CVD complication, cEPCs ($CD34^+CD144^+$, $CD133^+CD144^+$ and $CD133^+CD144^+$) are lower by 46-69% as compared to healthy controls. This correlated with markers of early vascular disease (endothelial function) (Sibal *et al.*, 2009a). EPC ($CD34^+VEGFR2^+$) and $CD34^+$ cells are reduced early during the natural history of T2DM. $CD34^+$ cells are reduced in patients with impaired glucose tolerance compared to HC. (Fadini *et al.*, 2007).

Patients with DM-related complications have lower cEPC as compared to patients without diabetes-related complication. Patients with microalbuminuria (Dessapt *et al.*, 2010), peripheral vascular disease (Fadini *et al.*, 2005) and non-proliferative retinopathy (Brunner *et al.*, 2009) have lower cEPCs ($CD34^+$ and $CD34^+CD133^+$, $CD34^+VEGFR2^+$, $CD34^+CD133^+CD309^+$ and $CD34^+CD133^+CD309^+CD31^+$ respectively). Makino and co-workers (2009) showed that numbers of $CD34^+$ cells

predictor development of microalbuminuria in T2DM patients (Makino *et al.*, 2009). In proliferative retinopathy, a condition with increased vascularisation, there is an increase in cEPC numbers. This result in a phenomenon called diabetic paradox (which is characterized by increased neovascularisation in the retina but limited vascularisation peripherally). This is due to the diabetic related limitation of cEPC to function peripherally and a consequence of growth factor and cytokines released from ischaemic insults resulting in mobilization and homing of cEPC (Fadini *et al.*, 2006a).

The role of hyperglycaemia in reduced EPC numbers is supported by studies in T1DM. EPC numbers have been shown to improve significantly with improving glycaemic control in children with T1DM (Hörtenhuber *et al.*, 2013). Higher glucose variability has been shown to reduce EPC numbers ($CD34^+$ and $CD34^+VEGFR2^+CD133^+$) in T1DM (Maiorino *et al.*, 2015). Furthermore, improvement in glucose variability has been shown to improve EPC numbers (Maiorino *et al.*, 2016). In vitro studies have demonstrated impaired EPC function under very high glucose condition (Chen *et al.*, 2007; Kim *et al.*, 2014). High glucose exposure resulted in increased cellular oxidative stress and mitochondrial function resulting in autophagy of EPC (Kim *et al.*, 2014). Chen et al. (2007) showed that high glucose increased senescence and reduced the function of EPC which is not ameliorated by anti-oxidant treatment. Balestrieri *et al.* (2013) showed that levels of silent information regulator 1 (SIRT-1) are reduced whereas platelet activating factor (PAF) is increased in EPCs of T2DM. This is much more exacerbated in patients with poor control (Balestrieri *et al.*, 2013). Human studies in both T1DM and T2DM have confirmed that EPC functions are in patients with DM. Loomans *et al.* (2005) observed impaired tube formation in patients with T1DM. Similarly, adhesion, proliferation, migratory capacity is reduced in T2DM (Tepper *et al.* 2004). Table 4 present studies published on T1DM and EPC.

Reduced numbers of EPC in patients with DM can occur due to three broad mechanisms. Firstly, the release of EPC from bone marrow of DM patients in response to stimuli may be impaired. Secondly, differentiation of progenitor cells in bone marrow can be impaired. Thirdly, decreased survival of endothelial progenitor cells.

| Reference | Study subject | Study method | Findings |
|---|---|---|---|
| (Reinhard *et al.*, 2011) | T1DM with and without nephropathy | Cell culture | Similar numbers |
| (Hörtenhuber *et al.*, 2013) | T1DM vs. HC | Flow cytometry | EPC lower in T1DM. Increase is related to HbA1c improvement |
| (Palombo *et al.*, 2011) | T1DM vs. HC | Flow cytometry | Reduced EPC numbers and independently and inversely related to CIMT |
| (Hernandez *et al.*, 2014) | T1DM for 50 years or more | Flow cytometry | Normal EPC levels despite the prolonged duration of T1DM. EPC numbers are inversely correlated with CVD and nephropathy |
| (Maiorino *et al.*, 2015) | T1DM and effect of glucose variability | Flow cytometry | EPC is related to glucose variability |
| (Maiorino *et al.*, 2016) | T1DM and effect of improved glucose variability | Flow cytometry | EPC numbers increased with improved glucose variability |
| (Maiorino *et al.*, 2014) | T1DM with and without erectile dysfunction vs. HC | Flow cytometry | EPC numbers reduced in erectile dysfunctional patients |
| (Waclawovsky *et al.*, 2015) | T1DM vs. HC and effect of exercise (aerobic and resistance) | Flow cytometry | EPC levels are similar at baseline between T1DM and HC. EPC numbers did not change with exercise in T1DM in contrast to HC |
| (West *et al.*, 2015) | T1DM vs. HC and exercise | Flow cytometry | EPC number similar in T1DM and HC at baseline. Post-exercise EPC number did not change in T1DM in contrast to HC |
| (Sibal *et al.*, 2009a) | T1DM vs HC | Flow cytometry | EPC count significantly lower in T1DM. EPC levels were directly related to endothelial function. |
| (Loomans *et al.*, 2004) | T1DM vs HC | Cell culture | EPC numbers and function were lower in T1DM when compared to HC. |

Table 4: Available published studies on EPC and T1DM.

### *4.1.8 Effect of medication on endothelial progenitor cells*

Since the discovery of EPCs and our understanding of their clinical significance, many intervention studies have been conducted to see if numbers and function of EPCs can be improved. As discussed above, making lifestyles changes such as smoking cessation and exercise has been shown to improve the numbers and function of EPCs. In addition, medications such as atorvastatin (Vasa *et al.*, 2001a), ramipril (Min *et al.*, 2004), enalapril (Wang *et al.*, 2006a), telmisartan (Pelliccia *et al.*, 2010), pioglitazone (Werner *et al.*, 2007), etc. have shown positive (numbers and/or function) effects on EPCs in uncontrolled studies.

We will briefly discuss the effect of commonly used medication in diabetes on EPCs. The discussion is limited to statins, antihypertensive agents, oral and injectable hypoglycaemic agents.

#### *4.1.8.1 Statins*

Vasa *et al.* (2001) demonstrated a 3-fold increase in EPCs numbers after four weeks of treatment with atorvastatin. Functional capacity as measured by the Boyden chamber showed significant improvement. This seems to be due to increased expression of telomere repeat-binding factor2 (Spyridopoulos *et al.*, 2004). Statin also prevents EPC telomere shortening (Satoh *et al.*, 2008). Statins have been shown to increase eNOS activity. These have been shown to increase EPC mobilisation from bone marrow (Walter *et al.*, 2002b). Mice myocardial infarction models have demonstrated increased mobilisation of EPCs after treatment with a statin when compared to a vehicle treated mice group (Landmesser *et al.*, 2004). Similar results were observed in T1DM animal model (Mohler *et al.*, 2009). Statin had demonstrated an increase in EPC numbers and function in human studies. Statin treatment during the acute phase of ischaemic stroke improved CFU-Hill colonies number when compared to a non-treated group (Sobrino *et al.*, 2012). Similar results were noted in post-menopausal women after eight weeks of pravastatin (Paradisi *et al.*, 2012). Atorvastatin has been shown to increase cEPC numbers in patients with CAD and subjects undergoing coronary artery bypass compared to a non-statin treated group

(Baran *et al.*, 2011) and (Schmidt-Lucke *et al.*, 2010). Statin treatment in patients with DM has been shown to increase EPC numbers (Jaumdally *et al.*, 2010).

Questions have been raised over the long-term effect of statins on EPC. It has been shown that the effect of statin on EPC seems to be lost after three months. Use of statins over long-term decreased EPC numbers in CVD patients (Hristov *et al.*, 2007). Brief discontinuation of statins in patients with T2DM has shown to increase EPC numbers and their function (Fadini *et al.*, 2015b). This was speculated to be caused by reverse activation of statin affected pathways. However, the effect of statin in lowering cholesterol far out weights the risk of CVD in patients with T2DM.

#### *4.1.8.2 Angiotensin-converting enzyme inhibitors (ACEi) and angiotensin receptor blockers (ARB)*

Angiotensin-converting enzyme inhibitors (ACEi) and angiotensin receptor blockers (ARB) are used extensively in patients with DM, especially with hypertension and microalbuminuria. Both ACEi and ARB have shown to increase EPC mobilisation and function in patients with CVD (Pelliccia *et al.*, 2010; Cangiano *et al.*, 2011). Min *et al.* (2004) demonstrated increased EPC numbers and function in patients with CAD treated with Ramipril (Min *et al.*, 2004). Similarly, Bahlman *et al.* (2005) observed an increase in EPC numbers after olmesartan or irbesartan in T2DM. Both of these studies enumerated EPC by culture method. Bahlmann *et al.* (2005) also used flow cytometry to enumerate $CD34^+$ cells but did not show any effect of treatments on these cells (Bahlmann *et al.*, 2005). Sun *et al.* (2013) demonstrated increased cEPC numbers after perindopril in patients with type 2 diabetes after myocardial ischemia (Sun *et al.*, 2013). ACEi has demonstrated an inverse correlation between the increase in EPC numbers and vascular injury as assessed by CIMT in patients with hypertension (Cacciatore *et al.*, 2011).

#### *4.1.8.3 Oral and injectable hypoglycaemic medications*

##### *1.1.1.1 Metformin*

Metformin has been shown to increase the EPCs number in patients with T2DM (Chen *et al.*, 2010; Liao *et al.*, 2010; Esposito *et al.*, 2011). However, in these studies patients had lost weight and their glycaemic control had also improved. Therefore, the drug-only effect on the improvement of EPC numbers cannot be confirmed due to the potential effects of improved glycaemic control and/or weight loss. No studies have been conducted in patients with T1DM. Metformin may exert a positive effect on EPC via multiple mechanisms. Briefly, the mechanism is likely to be centred around AMPK activation, SIRT-1, increased eNOS activity and reduced inflammation/oxidative stress. All these pathways can lead to increased EPC mobilisation and improved survival and function.

##### *1.1.1.2 Sulfonylureas*

Sulfonylureas are commonly used in T2DM. There is very limited literature published on the effect of sulfonylurea on EPCs. Chen *et al.* (2010) demonstrated that the EPC numbers increased after gliclazide treatment. However, it was not clear if this effect was mediated via improved glycaemic control or a direct consequence of the gliclazide.

##### *1.1.1.3 Thiazolidinedione*

Only pioglitazone is now used in T2DM from thiazolidinediones. Multiple studies have demonstrated that pioglitazone increased EPC numbers in T2DM when compared to the non-treated group (Wang *et al.*, 2006b). However, this correlated with the decrease in fasting glucose levels in treated group. Furthermore, pioglitazone improved migration, adhesion, tube formation and viability (Wang *et al.*, 2006b; Spigoni *et al.*, 2012). These studies have been replicated in mouse models of type 2 diabetes. Gensch and co-workers showed increased EPC numbers and function and reduced apoptosis after pioglitazone treatment (Gensch *et al.*, 2007). Werner *et al.* (2007) showed that patients with CAD (normoglycaemic) treated with pioglitazone results in improvement in number and function of EPCs. This was due to the positive effect of

pioglitazone on serum adiponectin concentration (an increase of 322%) (Werner *et al.*, 2007).

#### *1.1.1.4 DPP4 inhibitors*

Incretin-based therapy are now increasing being used in T2DM and may have cardio-protective effects. Animal studies have demonstrated that sitagliptin increased EPC numbers and function (Goncalves *et al.*, 2011; Huang *et al.*, 2012). Fadini and co-worker (2010) replicated findings of animal studies in patients with T2DM. EPC numbers increased after four weeks of sitagliptin treatment. SDF-1α levels also increased significantly. It was speculated that the increase in SDF-1α could be due to inhibition of DDP4 in the bone marrow, leading to increased mobilisation of EPCs (Fadini *et al.*, 2010).

#### *1.1.1.5 Insulin*

Insulin is the mainstay of treatment in T1DM. There is limited evidence on the effect of insulin on EPC in patients with T1DM. Insulin treatment in mice type 1 diabetic model have shown to improve EPC numbers and function (Dong et al. 2011). Most of the evidence regarding insulin and EPCs are in T2DM. Insulin has been shown to improve the cEPCs number and function in type 2 diabetes (Humpert *et al.*, 2005; Humpert *et al.*, 2008; Fadini *et al.*, 2011; Oikonomou *et al.*, 2014). However, the improvement in cEPCs number in Fadini et al. (2011) could be attributed to improvement in HbA1c. This was in contrast with Humpert et al. (2005), who showed that the effect of insulin on the cEPCs number was independent of HbA1c. The effect of insulin can be mediated via insulin-mediated IGF-1 and eNOS activation (Humpert *et al.*, 2008). Insulin also has been shown to reduce oxidative stress (Siegelaar *et al.*, 2009).

### *4.1.9 Methods*

#### *4.1.9.1 Flow cytometry protocol for enumeration of circulatory endothelial progenitor cells (cEPCs) and circulatory endothelial cells (cECs)*

Peripheral blood sample collected in the EDTA bottle was used to flow cytometry analysis of cEPCs and cECs. First 4 millilitres of collected blood was not used for cEPCs analysis to avoid spurious result. Blood samples were processed within 4 hours of collection.100 µl volume whole blood was added to a TruCount tube.

All five antibodies mentioned in Table 5 was added to the whole blood in the Trucount tube. The sample was incubated for 30 minutes at room temperature in the dark. After incubation, 2 millilitres of Pharm Lyse (1x strength, diluted with DI water) was used to lyse RBCs. It was further incubated for 10 minutes in the dark to ensure complete lysis of RBCs. The sample was then analysed using the BD FACS Canto™ II system.

Following antibodies were added to the whole blood in the TruCount tube (Table 5):

| Antibody | Fluorochrome | Volume to add |
|---|---|---|
| **CD34** | PerCP-Cy5.5 | 20µl |
| **CD133** | APC | 5µl |
| **VEGFR2 (KDR)** | PE | 5µl |
| **CD144** | FITC | 10µl |
| **CD45** | V500 | 5ul |

Table 5: Antibodies used for EPC and cEC enumeration.

Before analysing the study samples, optimisation was carried out. Compensation and gating strategy were defined with support from flow cytometry department. This is discussed below.

#### *4.1.9.2 Optimising compensation strategy for enumeration of circulatory endothelial progenitor cells and circulatory endothelial cells*

First, each fluorochrome was analysed separately. Then, V500 CD45 was used to stain the cells. After that, each fluorochrome was added with V500 CD45 one at a time to enumerate the fluorescence in each channel. Compensation was done to make sure there is no overlap of the fluorescence channels (e.g., FITC can spill over into the PE channel). Once the compensation was achieved using each fluorescence antibody with V500 CD45 than all the fluorochromes were added and analysed together. Adequate compensation was deemed adequate once each fluorochrome fluorescence (run together) was similar to fluorochrome fluorescence run separately.

#### *4.1.9.3 Optimisation of gating strategy for flow cytometric analysis of circulatory endothelial progenitor cells*

BD FACSDiva™ software was used for flow cytometry data analysis. Forward and side scatter was plotted. Gate for leukocytes was drawn to exclude debris and beads. Subsequent analysis was done in the lymphocytic gate. Quantification of different cEPCs was performed by gating at the region containing lymphocyte sub-population of the cell. A further gate was set on CD45 vs. side scatter. This gate defined all $CD45^+$ positive events. The events in $CD45^+$ were then plotted on CD34 versus CD45. Gate was configured to define all events which were $CD34^+$ and $CD45^+$.

The lymphocytic gate was set on CD45 vs. side scatter. The gate was set to define all $CD45^{dim}$ positive events. $CD45^{dim}$ events were then plotted on CD34 versus CD45. The gate was set to define all events which were $CD34^+$ and $CD45^{dim}$ positive. Then we plotted all CD34 and $CD45^{dim}$ events on CD34 versus VEGFR2. Upper right quadrant was defined as $CD45^{dim}CD34^+VEGFR2^+$. Furthermore, all $CD34^+$ and $CD45^{dim}$ events were plotted as CD34 versus CD133. Upper right quadrant was defined as $CD45^{dim}CD34^+CD133^+$ (Figure 14).

cEPCs are defined as **$CD45^{dim}CD34^+VEGFR2^+$**. Circulating progenitor cells (CPCs) are defined as **$CD45^+CD34^+$** and **$CD45^{dim}CD34^+CD133^+$**.

### *4.1.9.4 Determination of cEPCs and CPC per 100 leukocytes*

Number of cEPCs and CPCs per 100 leukocytes was calculation by the following formula:

$$\text{cEPCs or CPC per 100 leukocytes} = \frac{number\ of\ events\ in\ cEPC\ or\ CPC\ gate}{total\ number\ of\ events\ in\ leukocyte\ gate} \times 100$$

### *4.1.9.5 Inter-assay and intra-assay variation of cEPCs*

As the primary outcome of our study is the change of cEPCs, it is paramount that the coefficient of variation (CV%) is as low as possible (approximately 5-10%). Four volunteers sample were run on two separate occasions to enumerate inter-assay variation. Nine volunteers samples were analysed twice at the same time to assess intra-assay variation. Inter-assay and intra-assay variation for $CD45^{dim}CD34^{+}VEGFR2^{+}$ events are < 7% and < 8% respectively. This is in concordance with the best available evidence (Table 6).

Table 6: Evidence about intra-assay variations of cEPC.

| **Published evidence** | **Coefficient of variation %** |
|---|---|
| Jialil et al. 2010 | 11% |
| Shmilovich et al. 2007 | <10% |
| Dessapt et al. 2010 | <10 |
| Briguori et al. 2010 | 15.5% |

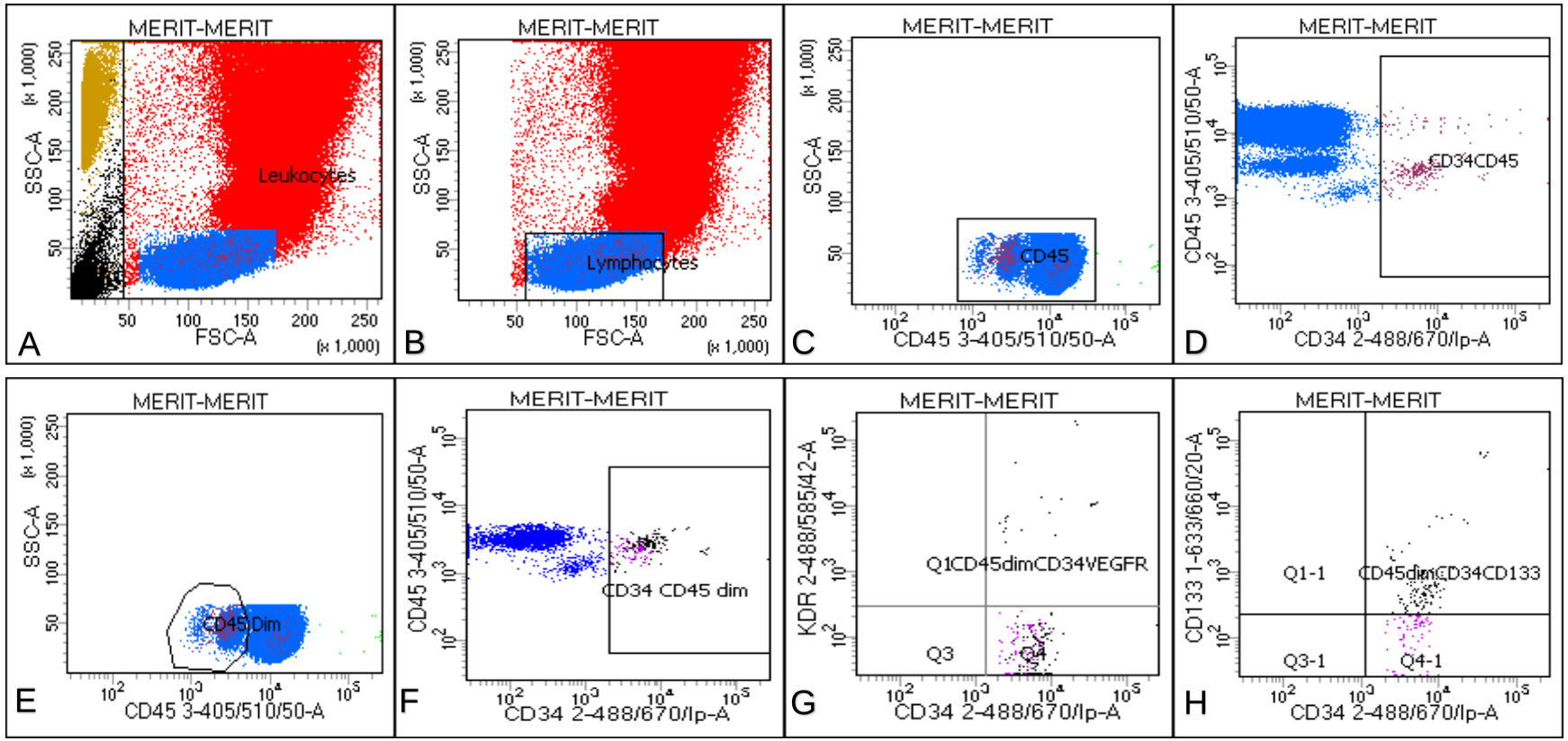


Figure 14: Gating strategy to identify cEPC and different CPC population.

A. Debris (black) and Trucount beads (yellow) were excluded in side vs. forward-scatter graph; B. Gating selecting lymphocyte population; C. Gating selecting CD45 with all bright and dim population; D: Gating selecting $CD45^{+(bright\ and\ dim)}CD34^{+}$; E. Gating selecting $CD45^{dim}$; F. Gating selecting $CD45^{dim}CD34^{+}$; G. Gating selecting $CD45^{dim}CD34^{+}VEGFR2^{+}$; H. Gating selecting $CD45^{dim}CD34^{+}CD133^{+}$.

#### *4.1.9.6 Laboratory Protocol*

Blood volume of 40 mls was collected at the start and end (8 weeks) of the study and was processed within 4 hours of blood collection.

#### 4.1.9.6.1 Isolation of EPCs

#### 4.1.9.6.2 Culture Method

##### *4.1.9.6.2.1 Choice of medium*

EBM2 (EBM-2; Promo cell supplemented with 20% fetal calf serum and growth factors-hEGF, HC, VEGF, hbFGF, R3IGF, AA and Hep-11) was used as a medium for our culture.

##### *4.1.9.6.2.2 Proangiogenic cells*

PACs were cultured according to the method discussed by Vasa *et al.* (2001) (Figure 15). It was modified to suit our laboratory. Approximately 20 mls of venous blood was transferred from vacutainer tubes to a 50mL Falcon tube. Venous blood was diluted with $Ca^{2+}$/$Mg^{2+}$-free phosphate-buffered saline (PBS) (2:1) and mixed by gentle inversion. 13mL of Lymphoprep was added to a fresh 50mL Falcon tube. This was tilted approximately 45° and the dilute blood was carefully overlaid. Sample was then centrifuged at 800*g* for 15 minutes at room temperature (acceleration = 5, deceleration = 3). The buffy coat layer was carefully taken at the Lymphoprep/plasma interface using a plastic pasteur pipette, avoiding any lymphoprep, and transferred to a fresh 50mL Falcon tube. The sample was then centrifuged at 500*g* for 5 minutes at room temperature (deceleration=3). The supernatant was discarded and washed by resuspending in 10mL $Ca^{2+}$/$Mg^{2+}$-free PBS. This was further centrifuge at 500*g* for 5 minutes at room temperature (deceleration=3). The supernatant was discarded and the cells resuspended in 1mL of culture medium. Culture medium consisted of EBM2 (including growth factors) with 20% fetal calf serum (FCS) and 1% penicillin/streptomycin.

The cell density was determined by diluting 10μL cell suspension in 90μL PBS. An aliquot of dilute cell suspension was added to Trypan Blue at a ratio of 1:1. 10μL of diluted/stained cell suspension was pipetted to one chamber of the haemocytometer and the cells were counted in five squares. The cell count per mL was determined by:

$$Number\ of\ cells\ per\ ml = \frac{total\ cells\ counted\ \times 20}{2 \times 10^{-5}}$$

The volume of cells was determined from the stock suspension to give 1 X $10^6$ and 5 X $10^6$ cells as follows:

$$V = \frac{Z \times 1000}{C}$$

*V* = volume of stock suspension containing 1 X $10^6$ and 5 X $10^6$ cells, *Z* = required cell number (1 X $10^6$ and 5 X $10^6$), *C* = cell density of stock suspension.

Culture plates were coated with fibronectin and allowed to dry for 30 minutes. For each sample, at least 3-4 wells of a 24-well culture plate were seeded with 1 X $10^6$ cells from the stock suspension. In addition 5 X $10^6$ cells were seeded into six wells of 6 well culture plate. The plates were incubated at 37°C in 5% $CO_2$/air for 48 hours.

After 48 hours, the supernatant containing non-adherent cells was collected and transferred to a fresh 50mL Falcon tube. Each well was washed twice with sterile PBS and retained with the supernatant. The supernatant was used for plating Hill colonies.

After removing and washing culture plates, the medium was placed, and cells were incubated 37°C in 5% $CO_2$/air for 48 hours. After which PACs were stained with Ac-LDL and Ulex lectin. At least three wells of 24 well plates were stained.

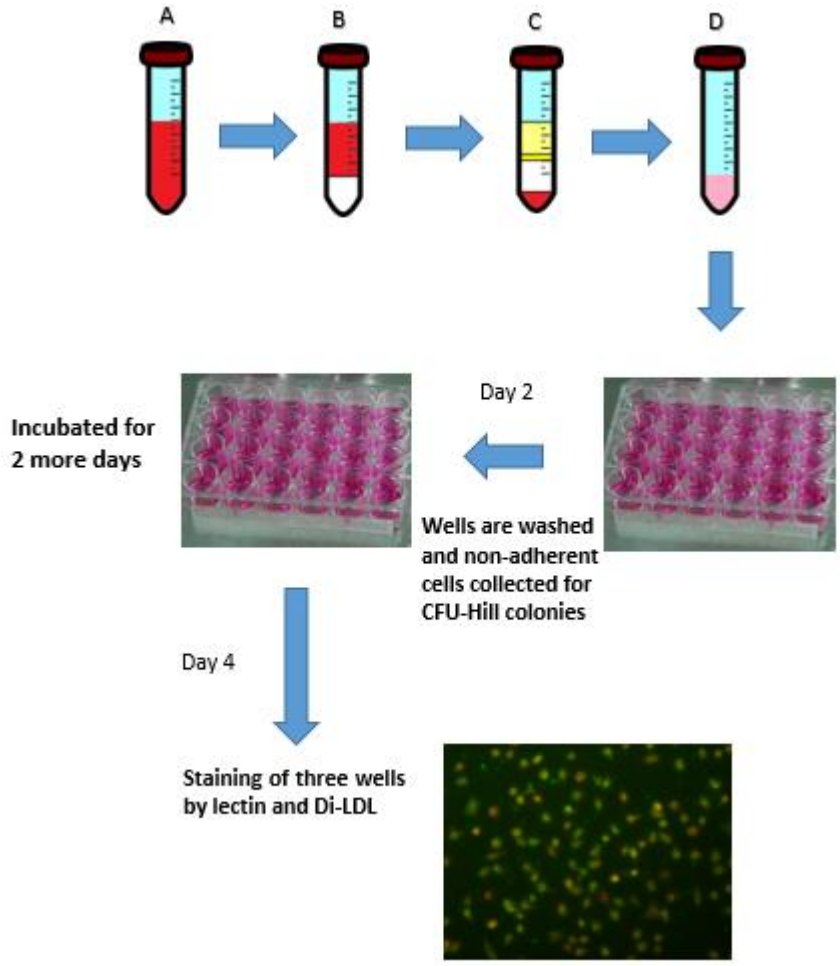


Figure 15: PAC enumeration method.

Staining EPCs were done according to the method discussed by Vasa *et al.* (2001). Cells were incubated with DiLDL (1,19–dioctadecyl–3,3,39,39–tetramethylindocarbocyanine–labeled acetylated low-density lipoprotein) (2.4 ug/mL) for 1 hour at 37°C. 2% paraformaldehyde was used for 10minutes to fix the cells, and then lectin staining was performed by incubating the cells for 1 hour with fluorescein isothiocyanate (FITC)–labelled Ulex europaeus agglutinin I (lectin, ten ug/mL; Sigma). At least 15 randomly selected high-power fields were captured by an inverted microscope. Cells staining for both lectin and Di-LDL (Figure 16) were classed as PACs, and they were counted per high power fields.

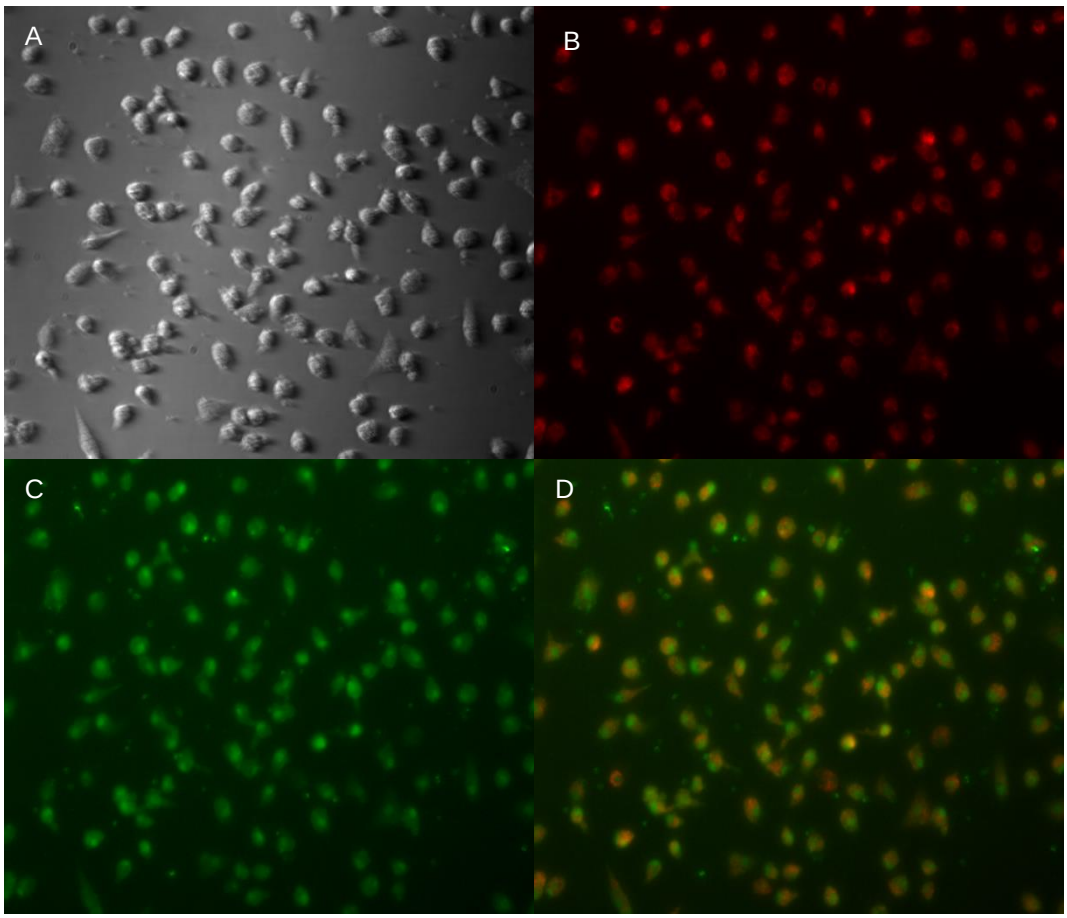


Figure 16: **A**. Pro-angiogenic cells (20X; Brightfield). **B**. Dil-LDL staining of EPCs (pro-angiogenic cells): Red. **C**. Ulex-Lectin staining of EPCs (pro-angiogenic cells): Green. **D**. Double staining of EPCs (superimposed image of EPCs labelled with dil-LDL and ulex-lectin).

#### *4.1.9.6.2.3 Hill colonies*

Characterisation of the Hill colonies was done as per protocol discussed by Hill *et al*. (2003) (Figure 17). Initial steps were similar to section 4.1.9.6.2.3 (discussed above). However, after 48 hours, the culture supernatant was collected. The cultured plate was washed twice with PBS and supernatant collected. The cell suspension collected was centrifuged at 500*g* for 5 minutes at room temperature (deceleration=3). The supernatant was discarded, and the cell pellet was re-suspended in 1mL of medium and counted. $1X10^6$ cells were re-plated in fresh fibronectin-coated 24-well plates. At least three wells were re-plated. The plates were incubated at 37°C in 5% $CO_2$/air, and the medium was changed every three days. After seven days, Hill colonies and clusters were counted.

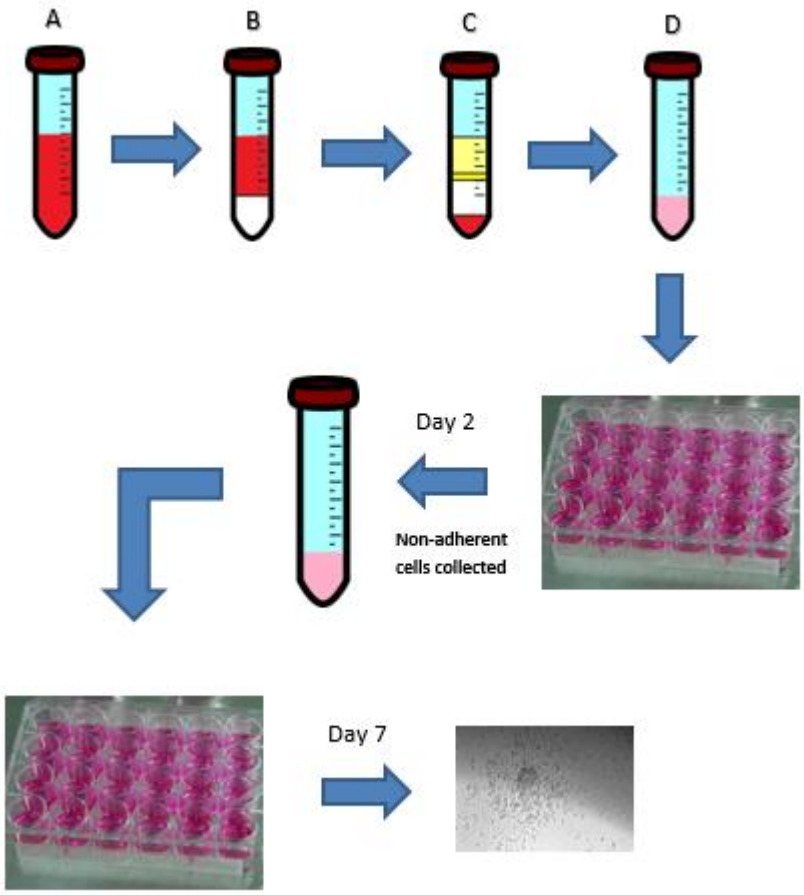


Figure 17: CFU-Hill Colonies enumeration method.

Only those colonies were counted that exhibit multiple thin flat cells emanating from a central cluster of rounded cells as illustrated in the Figure 18. These colonies were called Hill colonies.

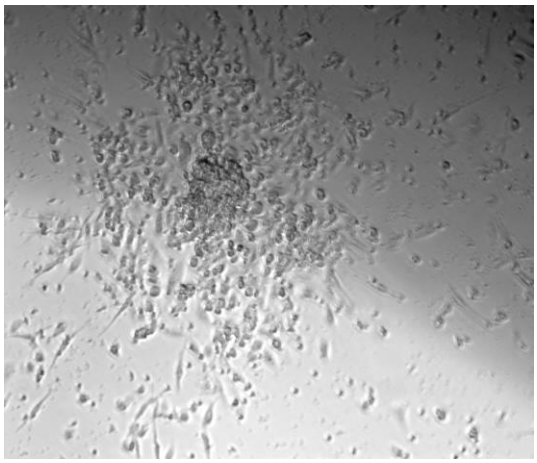

Figure 18: Hill Colony (multiple thin flat cells radiating from a central cluster of rounded cells).

#### *4.1.9.6.2.4Functional Assay*

The fibronectin adhesion assay was performed as a part of the functional assay. This has been described by Huang *et al.* (2007) (Figure 20). On day 4, at least three wells of 6 well culture plates were washed with PBS and gently detached with 5 mmol/l EDTA.

Detached cells were collected in a 50 ml falcon tube. The culture plates were washed twice with PBS and once with EBM2 containing 5% FCS. This was to make sure all detached cells were collected. The suspension was centrifuged at 500g for 5 min. The supernatant was discarded. The pellet was re-suspended in 500 ul of EBM2 containing 5% FCS.

At least four wells of the 48 well culture plate were coated with fibronectin. After drying for 30 minutes, identical cell numbers were plated and incubated at 37°C in 5% $CO_2$/air for 30 minutes. After 30 minutes, each well was washed at least twice to remove all non-adherent cells. Adherent cells are stained with ac-LDL and ulex-lectin, as described above. At least ten randomly selected high-power fields were captured. Cells staining positive for both lectin and Di-LDL were judged to be EPCs, and they were counted per high power fields (Figure 19).

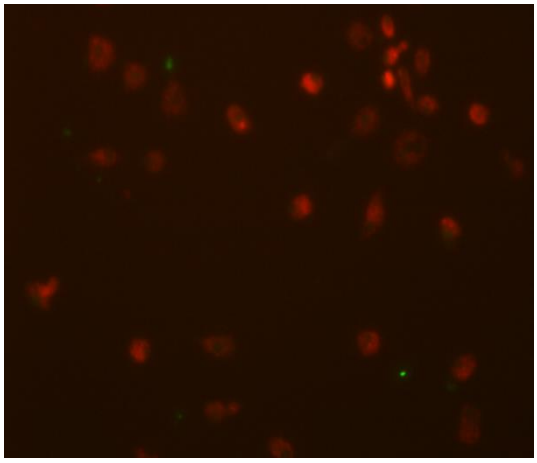

Figure 19: Dual-stained cells positive for both lectin and Di-LDL (Adhesion assay).

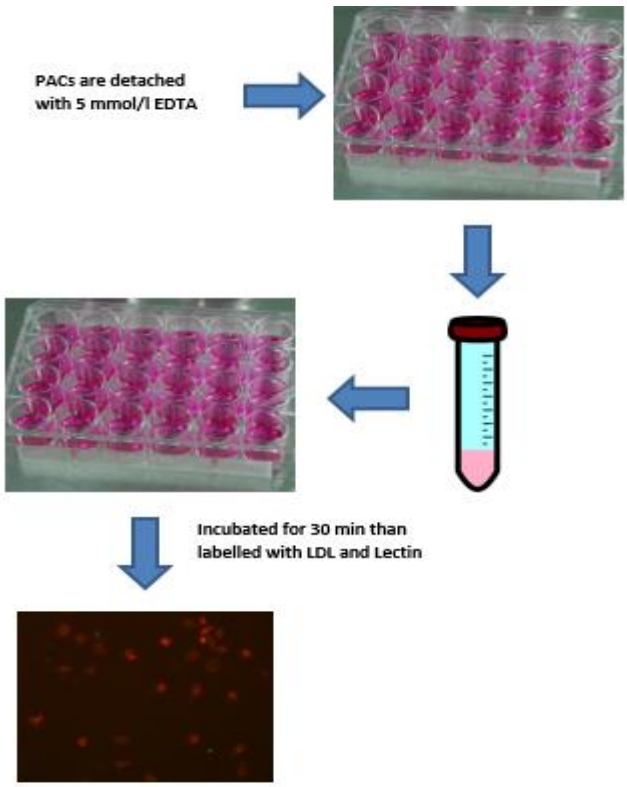


Figure 20: PAC adhesion function method.

### *4.1.10 Specific aims*

In this section, we aimed to evaluate if metformin improved the following in type 1 diabetes when controlled for glycaemic changes:

1. Number of cEPCs,
2. Number of PACs,
3. Adhesion function of PACs, and
4. Number of CFU-Hill colonies.

### *4.1.11 Results*

#### *4.1.11.1 CD34+*

Result is presented in Figure 21.

##### 4.1.11.1.1 Treatment group (Pre metformin) versus Healthy control

$CD34^+$ cells were significantly lower (57%) in TG compared with HC (Treatment group pre-metformin (TG V1) vs HC; median (intraquartile range (IQ): 0.047 (0.038-0.078) vs 0.11 (0.09-0.13) % leukocytes; $p<0.0001$).

##### 4.1.11.1.2 Treatment group (Pre metformin) versus Treatment group (Post metformin)

Eight weeks of metformin treatment did not result in any change in $CD34^+$ cells; TG V1 vs TG V2; median (IQ): 0.047 (0.038-0.078) vs 0.058 (0.04-0.074) % leukocytes; $p=0.1$.

##### 4.1.11.1.3 Treatment group (Post metformin) versus Healthy control

After metformin treatment, $CD34^+$ cells remained significantly lower in TG V2 when compared with HC (TG V2 vs HC); median (IQ): 0.058 (0.04-0.074) vs 0.11 (0.09-0.13) % leukocytes; $p<0.0001$.

##### 4.1.11.1.4 Treatment group (Pre metformin) versus Standard group (Pre observation)

At baseline, $CD34^+$ cell count was similar in TG and the SG (TG V1 vs standard group pre observation (SG V1); median (IQ): 0.047 (0.038-0.078) vs 0.047 (0.036-0.06); $p=0.7$.

#### 4.1.11.1.5 Standard group (Pre observation) versus Standard group (Post observation)

In standard group, eight weeks of standard follow-up did not result in any change in $CD34^+$ cells count: SG V1 and standard group post observation (SG V2): median (IQ): 0.047 (0.036-0.06) vs. 0.048 (0.038-0.065) % leukocytes; p=0.9.

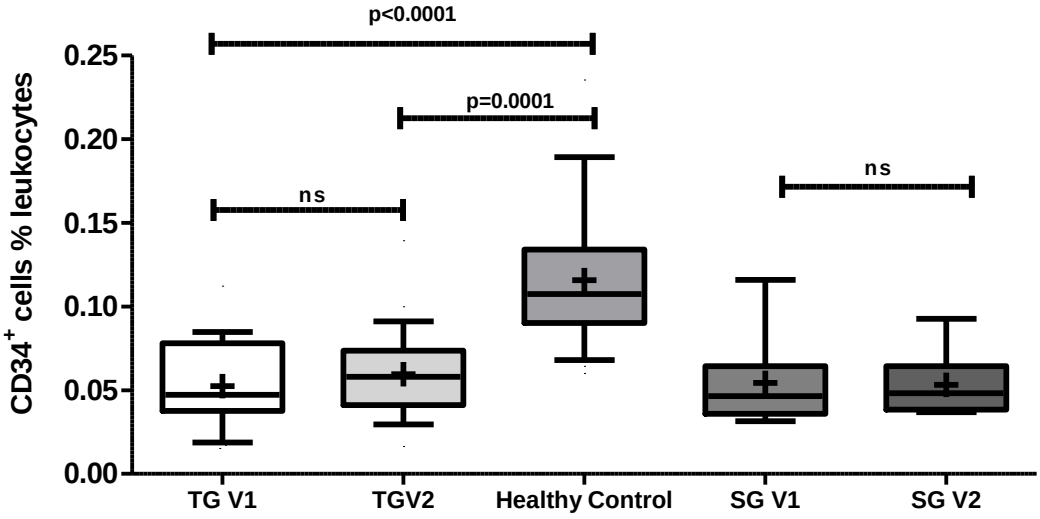


Figure 21: $CD34^+$ cells are comparing all groups (mean+/-SE). Pre-treatment (TG V1) and Post-treatment (TG V2), Healthy control and standard group: Pre observation (SG V1) and Post observation (SG V2). Results are given as per 100 leukocytes.

#### *4.1.11.2 $CD45^{dim}CD34^{+}CD133^{+}$*

Result is presented in Figure 22.

##### 4.1.11.2.1 Treatment group (Pre metformin) versus Healthy control

$CD45^{dim}CD34^{+}CD133^{+}$ cells were significantly lower in TG compared with HC: TG V1 vs HC; mean+/-SE: 0.019+/-0.002 vs 0.03+/-0.002 % leukocytes; p<0.0001).

##### 4.1.11.2.2 Treatment group (Pre metformin) versus Treatment group (Post metformin)

Eight weeks of metformin treatment did not result in any change in $CD45^{dim}CD34^{+}CD133^{+}$ cells (TG V1 vs TG V2; mean+/-SE: 0.0019+/-0.002 vs 0.02+/-0.002 % leukocytes; p=0.4).

##### 4.1.11.2.3 Treatment group (Post metformin) versus Healthy control

After metformin treatment, $CD45^{dim}CD34^{+}CD133^{+}$ cells remained significantly lower in TG when compared with HC (TG V2 vs HC); mean+/-SE: 0.02+/-0/002 vs 0.03+/-0.002 % leukocytes; p=0.0001.

##### 4.1.11.2.4 Treatment group (Pre metformin) versus Standard group (Pre-observation)

At baseline, $CD45^{dim}CD34^{+}CD133^{+}$ cell count was similar in TG and the SG: (TG V1 vs SG V1; mean+/-SE: 0.019+/-0.002 vs 0.023+/-0.004; p=0.3).

#### 4.1.11.2.5 Standard group (Pre observation) versus Standard group (Post observation):

In standard group, eight weeks of standard follow-up did not result in any change in $CD45^{dim}CD34^{+}CD133^{+}$ cells count (SG V1 vs SG V2: mean+/-SE: 0.023+/-0.004 vs. 0.02+/-0.0039 % leukocytes; p=0.3).

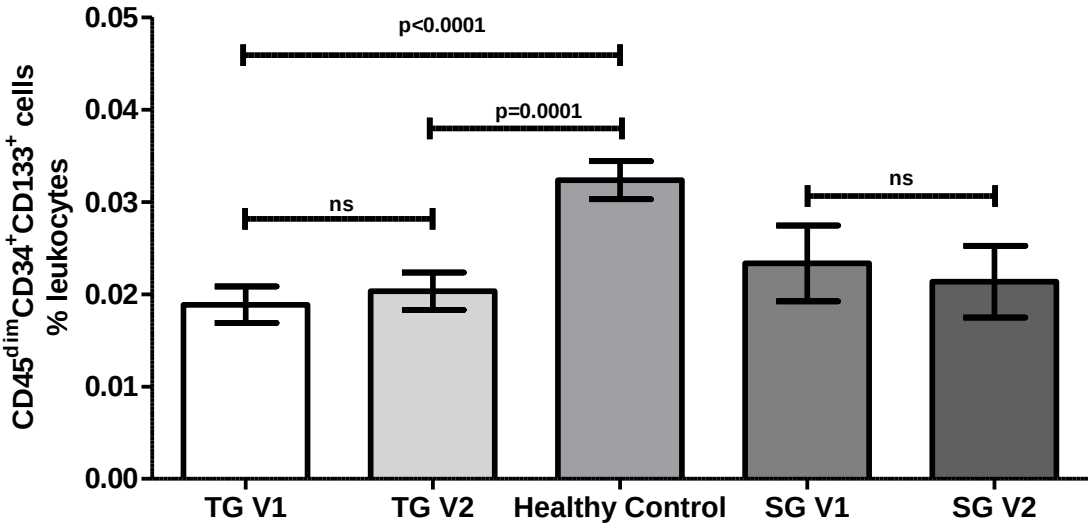


Figure 22: $CD45^{dim}CD34^{+}CD133^{+}$ cells comparing all groups (mean+/-SE). Pre-treatment (TG V1) and Post-treatment (TG V2), Healthy control and standard group: Pre observation (SG V1) and Post observation (SG V2). Results are given as per 100 leukocytes.

#### *4.1.11.3 $CD45^{dim}CD34^{+}VEGFR\text{-}2^{+}$*

Combined result is presented in Figure 28.

##### 4.1.11.3.1 Treatment group (Pre metformin) versus Healthy control

At baseline, cEPCs ($CD45^{dim}CD34^{+}VEGFR\text{-}2^{+}$) were significantly lower (60%) in TG compared with HC (TG V1 vs HC; median (IQ): 0.0028 (0.0016-0.006) vs 0.0068 (0.006-0.009) % leukocytes; $p<0.0001$).

##### 4.1.11.3.2 Treatment group (Pre metformin) versus Treatment group (Post metformin)

Eight-week metformin treatment significantly increased cEPCs in TG by more than 75% (TG V1 vs TG V2; median (IQ): 0.0028 (0.0016-0.006) vs 0.005 (0.0035-0.0085) % leukocytes; $p=0.0004$).

##### 4.1.11.3.3 Treatment group (Post metformin) versus Healthy control

As mentioned above, metformin treatment improved cEPCs number in TG group and normalised the levels of cEPCs count when compared to HC (TG V2 vs HC); median (IQ): 0.0028 (0.0016-0.006) vs. 0.0068 (0.006-0.009) % leukocytes). After treatment, the cEPCs count is only 28% lower in TG when compared to HC versus 60% lower in TG pre-metformin treatment when compared to HC. After metformin treatment, significance disappeared ($p=0.065$).

##### 4.1.11.3.4 Treatment group (Pre metformin) versus Standard group (Pre observation)

At baseline, cEPCs count was similar in TG and the SG (TG V1 vs SG V1; median (IQ): 0.0028 (0.0016-0.006) vs 0.003 (0.0022-0.004); $p=0.9$).

#### 4.1.11.3.5 Standard group (Pre observation) versus Standard group (Post observation)

In standard group, eight weeks of standard follow-up did not result in any change in the cEPCs count (SG V1 SG V2: median (IQ): 0.003 (0.0022-0.004) vs. 0.0035 (0.003-0.005) % leukocytes; p=0.55).

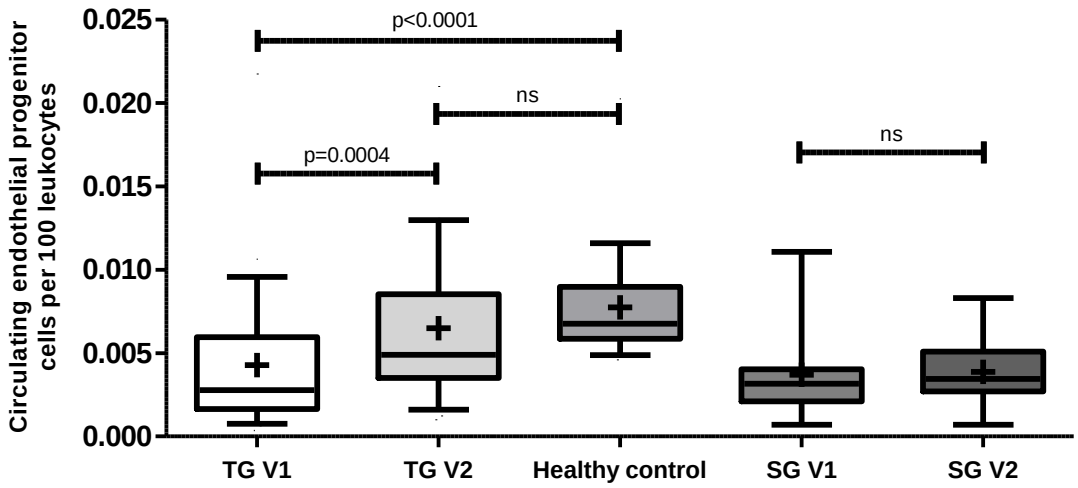


Figure 23: cEPC-CD45$^{dim}$CD34$^{+}$VEGFR-2$^{+}$ comparing treatment group: Pre-treatment (TG V1) and Post-treatment (TG V2), Healthy control and standard group: Pre observation (SG V1) and Post observation (SG V2). Results are given as per 100 leukocytes. Line denotes in each box as median and + in each box denotes mean value.

#### *4.1.11.4 Effect of metformin at four weeks on cEPCs and progenitor phenotypes*

In TG, four weeks of metformin treatment did not change $CD45^{dim}CD34^{+}VEGFR\text{-}2^{+}$ (Figure 29) and $CD45^{dim}CD34^{+}CD133^{+}$ (Figure 30) cell numbers in treatment group. However, the CD34+ cell levels increased by 78% (Figure 31) (Table 7).

| N=7 | **Pre-metformin (TG V1)** | **4-week metformin (TG V1.5)** | **Post-metformin (TG V2)** |
|---|---|---|---|
| **$CD45^{dim}CD34^{+}VEGFR\text{-}2^{+}$** median (IQ) | 0.0034 (0.002-0.006) | 0.0039 (0.0023-0.009) | 0.007 (0.005-0.009) |
| **$CD34^{+}$** mean+/-SE | 0.06+/-0.01 | 0.11+/-0.01* | 0.067+/-0.01^ |
| **$CD45^{dim}CD34^{+}CD133^{+}$** mean+/-SE | 0.02+/-0.004 | 0.02+/-0.0045 | 0.02+/-0.003 |

Table 7: cEPC and CPC levels at three times points (pre-metformin, 4-week metformin and post-metformin). * and ^ denotes significance on Post hoc Bonferroni's multiple comparison tests).

Friedman test did not show significance between three-time points measurement for $CD45^{dim}CD34^{+}VEGFR\text{-}2^{+}$ (p=0.0515). Levels more than doubled between TG V1 and TG V2.Lack of significance is likely due to small sample size.

One-way ANOVA analysis was significant for CD34+ cell (p=0.0019) Post hoc (Bonferroni's multiple comparison tests) was significant for TG V1 vs. TG V1.5 and TG 1.5 vs. TG 2. It was not significant between TG 1 vs. TG 2.

One-way ANOVA analysis was not significant for $CD45^{dim}CD34^{+}CD133^{+}$ cells.

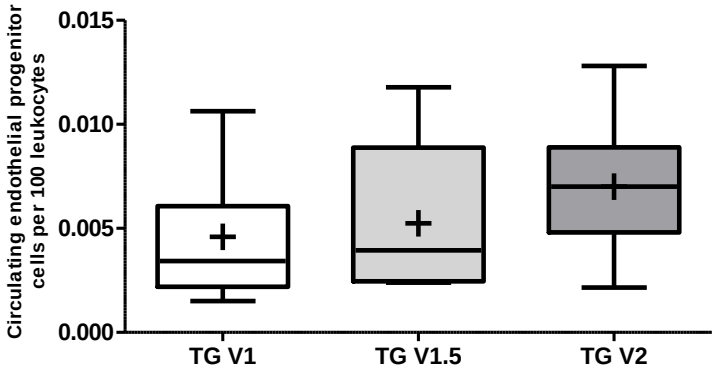


Figure 24: cEPC levels at three-time point (TG V1, TG V1.5 and TG2) (median (IQ).

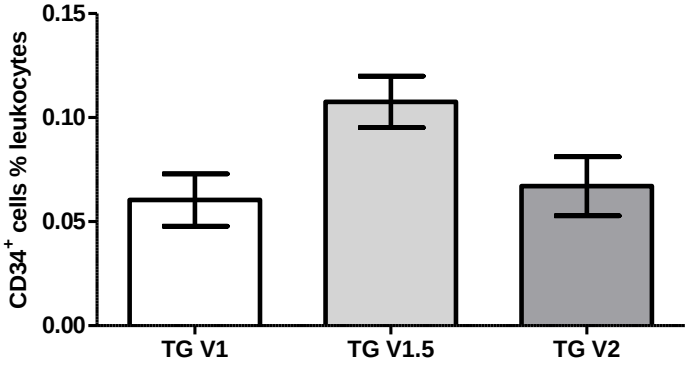


Figure 25: $CD34^{+}$ cell levels at three-time point (TG V1, TG V1.5 and TG2) (mean+/-SE).

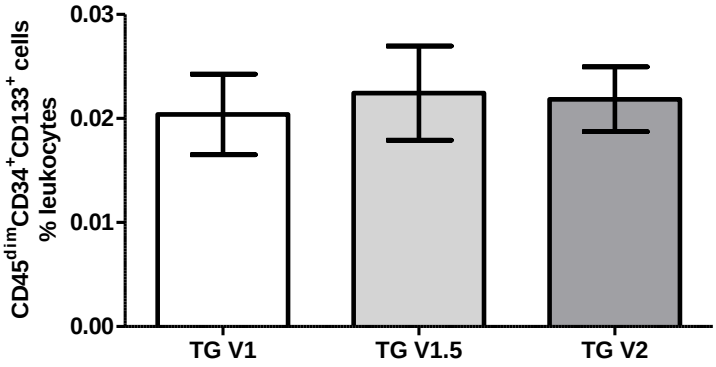


Figure 26: $CD45^{dim}CD34^{+}CD133^{+}$ cell levels at three-time point (TG V1, TG V1.5 and TG2) (mean+/-SE).

#### *4.1.11.5 Proangiogenic cells*

Result is presented in Figure 32.

##### 4.1.11.5.1 Treatment group (Pre metformin) versus Healthy control

At baseline, PAC numbers were significantly lower in TG when compared with HC (TG V1 vs. HC): mean+/-SE: 16.6+/-1.9 vs. 40.3+/-4.2 per hpf: p<0.0001).

##### 4.1.11.5.2 Treatment group (Pre metformin) versus Treatment group (Post metformin)

Eight-week metformin treatment significantly increased PAC number in TG; (TG V1 vs. TG V2) mean+/-SE: 16.6+/-1.9 vs. 28.4+/-2.7 per hpf: p<0.0001).

##### 4.1.11.5.3 Treatment group (Post metformin) versus Healthy volunteer

After metformin treatment, PAC numbers remained significantly different between TG V2 and HC (TG V2 vs. HC); mean+/-SE: 28.4+/-2.7vs 40.3+/-4.2 per hpf: p=0.008.

##### 4.1.11.5.4 Treatment group (Pre metformin) versus Standard group (Pre observation)

At baseline, PAC numbers were similar in TG and the SG (TG V1 vs. SG V1 (Pre); mean+/-SE; (TG V1 vs. SG V1: 16.6+/-1.9 vs. 17.6+/-4 per hpf; p=0.8).

##### 4.1.11.5.5 Standard group (Pre observation) versus Standard group (Post observation)

After eight weeks of observation, PAC numbers remained unchanged (SG V1 and SG V2: mean+/-SE (SG V1 vs. SG V2: 17.6+/-4 vs. 15+/-3.7: p=0.6).

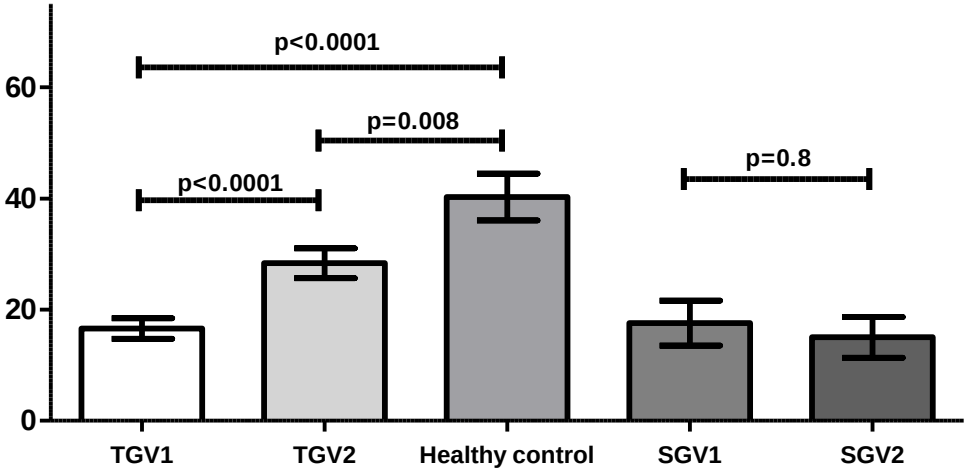


Figure 27: PAC comparing all the groups (mean+/-SE). Results are given as per hpf.

#### *4.1.11.6 Colony forming unit (CFU-Hill colonies)*

Result is presented in Figure 33.

##### 4.1.11.6.1 Treatment group (Pre metformin) versus Healthy control

At baseline, CFU-Hill colonies were significantly lower in TG when compared with HC (TG V1 vs. HC): mean+/-SE: 8.3+/-1.4 vs. 20.6+/-2.3: p<0.0001).

##### 4.1.11.6.2 Treatment group (Pre metformin) versus Treatment group (Post metformin)

Eight-week metformin treatment significantly increased CFU-Hill colonies in TG; (TG V1 vs. TG V2) mean+/-SE: 8.3+/-1.4 vs. 13.8+/-1.9: p<0.0001).

##### 4.1.11.6.3 Treatment group (Post metformin) versus Healthy control

After metformin treatment, CFU-Hill colonies number remained significantly different between TG V2 and HC (TG V2 vs. HC); mean+/-SE: 13.8+/-1.9 vs. 20.6+/-2.3: p=0.02.

##### 4.1.11.6.4 Treatment group (Pre metformin) versus Standard group (Pre observation)

At baseline, CFU-Hill colonies were similar in TG and the SG (TG V1 vs. SG V1; mean+/-SE; 8.3+/-1.4 vs. 10.4+/-2.2; p=0.12).

##### 4.1.11.6.5 Standard group (Pre observation) versus Standard group (Post observation)

After eight weeks of observation, CFU-Hill colonies number remained unchanged (SG V1 vs. SG V2): mean+/-SE: 10.4+/-2.2 vs. 11.1+/2.1: p=0.8).

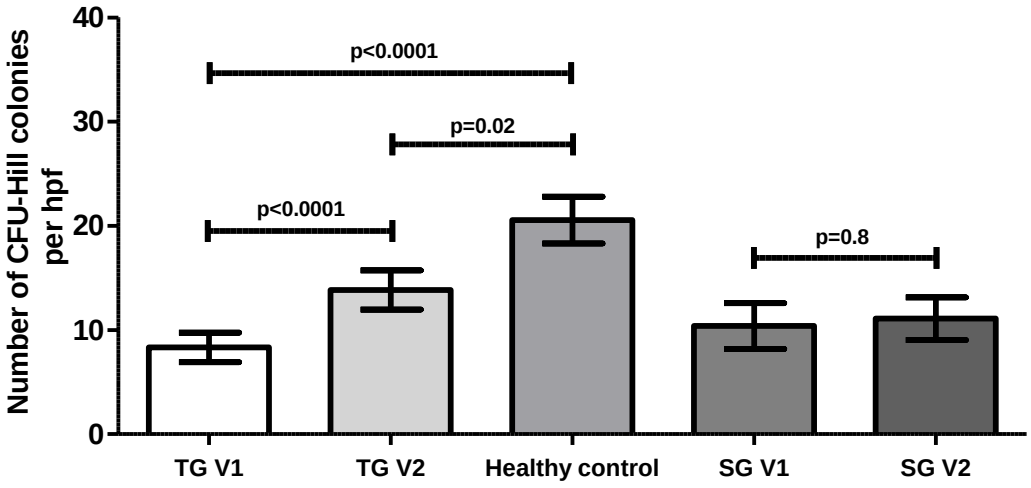


Figure 28: CFU-Hill colonies all the groups (mean+/-SE). Results are given as per hpf.

#### *4.1.11.7 Proangiogenic cell Function-Adhesion*

Result is presented in Figure 34.

##### 4.1.11.7.1 Treatment group (Pre metformin) versus Healthy control

At baseline, adhesion of PACs was significantly lower in TG when compared with HC (TG V1 vs. HC): mean+/-SE: 26.9+/-4.5 vs. 67+/-6: p<0.0001).

##### 4.1.11.7.2 Treatment group (Pre metformin) versus Treatment group (Post metformin)

Eight-week metformin treatment significantly increased adhesion of PACs in TG; (TG V1 vs. TG V2) mean+/-SE: 26.9+/-4.5 vs. 61+/-8.8: p<0.0001).

##### 4.1.11.7.3 Treatment group (Post metformin) versus Healthy control

After metformin treatment, adhesion of PACs in TG became similar to HC (TG V2 vs. HC); mean+/-SE: 61+/-8.8 vs. 67+/-6: p=0.6.

##### 4.1.11.7.4 Treatment group (Pre metformin) versus Standard group (Pre observation)

At baseline, adhesion of PACs number was similar in TG and the SG (TG V1 vs. SG V1; mean+/-SE; 8.3+/-1.4 vs. 35.9+/-5; p=0.12).

##### 4.1.11.7.5 Standard group (Pre observation) versus Standard group (Post observation)

After eight weeks of observation, adhesion of PACs number remained unchanged (SG V1 and SG V2: mean+/-SE: 35.9+/-5 vs. 37.3+/-5.4: p=0.9).

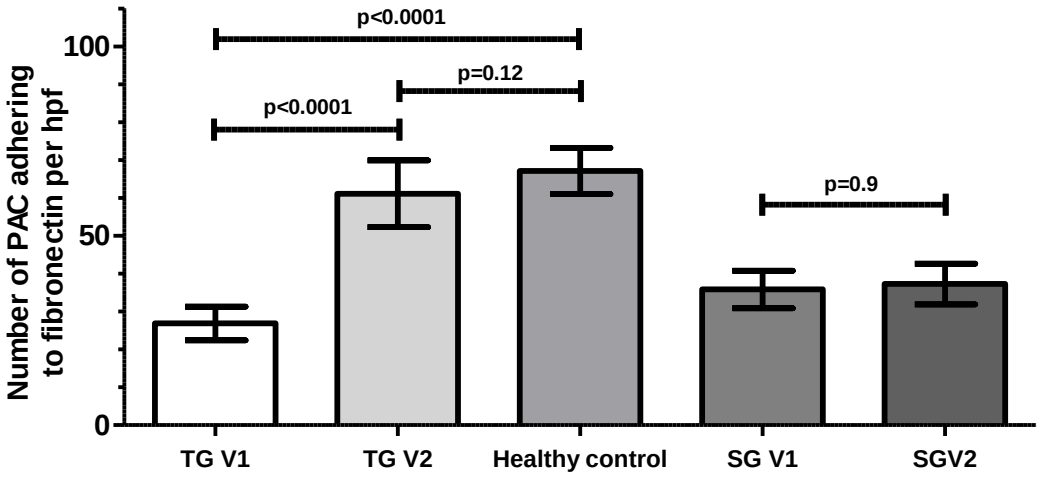


Figure 29: PAC adhesion function comparing all the groups (mean+/-SE). Results are given as per hpf.

#### *4.1.11.8 Subgroup analysis – Effect of metformin in smokers and patients taking ACE/Statins*

Smoking, statin and ACE inhibitors and has been shown to effect EPC numbers and function. This has been discussed above in section 4.1.6.1, 4.1.8.1 and 4.1.8.2. We explored if metformin will affect EPCs, PACs, CFU-Hill colonies numbers and PAC function in patients who smoked or were on statin and/or ACE inhibitors.

In TG who smoked (n=4), metformin resulted in similar or higher increase in cEPC, PAC, CFU-Hill colonies or adhesion properties of PACs (73%; (median (IQ)) 0.0038 (0.002-0.018 vs 0.0066 (0.0048-0.018) % leukocytes, 185%; 9.6 (2.5-13) vs 27.4 (22.6-28.6) per hpf, 133%; 8.4 (5.8-24.8) vs 19.9 (10-37.5) per well and 137%; 18.2 (2.3-49.9) vs 42.4 (18-64.3) per hpf respectively) or were on ACE and/or Statins (n=11) (122%, 69%, 111% and 81% respectively).

#### *4.1.11.9 Correlation*

Correlation is presented in Appendix A. Below, we present important correlations.

At the baseline, PAC numbers were inversely related to glucose variability (CONGA: $r=-0.56$; $p<0.01$; AUC: $r=-5$; $p<0.05$). At the baseline, PAC adhesion function was inversely related to glucose variability (CONGA: $r=-0.43$; $p<0.05$).

In univariate analysis in TG, there was no correlation between changes in HbA1c, BMI, insulin dosage, total cholesterol, HDL cholesterol, LDL cholesterol, cEPCs, PACs, CFU-Hill's colonies levels and PACs adhesion.

### *4.1.12 Discussion*

Markers of vascular repair were assessed at baseline and after four and eight weeks of metformin treatment. These were compared with healthy volunteers. Similar markers were evaluated in T1DM cohort group to explore if any changes in above-mentioned markers can be observed with routine follow up without any treatment with metformin.

#### *4.1.12.1 Decreased cEPC and progenitor cell phenotypes (CPC) in type 1 diabetes*

##### 4.1.12.1.1 Main finding

cEPC and CPC levels in T1DM individuals are significantly lower than healthy controls.

##### 4.1.12.1.2 Prior work

Above mentioned finding is similar to the results of previous work (Sibal *et al.*, 2009a; Hernandez *et al.*, 2014). However, in a younger group of patients with T1DM, we have found EPC numbers to be similar between patients and healthy controls (West *et al.*, 2015). Individual with T1DM in West et a (2015) was much younger, lower BMI and high cardio-respiratory fitness ($VO_2$max 50 ml.kg.min$^{-1}$ - average $VO_2$max 42 ml.kg.min$^{-1}$). It can be speculated that higher cardio-respiratory fitness can be a contributory factor compared to HC.

##### 4.1.12.1.3 Mechanism

Our finding of decreased EPC numbers suggests impaired endothelial repair mechanism and the risk of future development of cardiovascular disease. We know that T1DM patients with CVD or peripheral vascular disease (PVD) had lower EPC when compared with patients without CVD and PVD (Hernandez *et al.*, 2014).

Lower EPCs and CPCs levels in our study could be due to decreased cell survival, increased senescence and/or decreased recruitment from the bone marrow. An animal

model has shown that long-term diabetes mellitus can cause bone marrow microangiopathy (Ferraro *et al.*, 2011; Westerweel *et al.*, 2013). This could be one of the reasons of impaired recruitment in T1DM.

Furthermore, research has shown impaired mobilisation of cEPCs in response to the administration of the recombinant granulocyte colony-stimulating factor in patients with diabete (Fadini *et al.*, 2013). We have also shown that the bone marrow release of cEPCs in response to an exercise is also blunted in T1DM versus healthy controls (West *et al.*, 2015).

We can speculate that the underlying cause of lower EPC numbers in our patients may be due to impaired bone marrow function. DM is known to cause bone marrow microangiopathy. This has been well established in animal models. Microcirculation of the diabetic animal model has shown evidence of rarefaction, reduced circulation and increased permeability. These changes are accompanied by impairment of nerve signalling (sympathetic and nociceptive nerve fibre) increased inflammatory cells and adipocytes deposition (Oikawa *et al.*, 2010; Mangialardi *et al.*, 2013; Albiero *et al.*, 2014; Albiero *et al.*, 2015). Some of these changes in bone marrow have been confirmed in human studies (Spinetti *et al.*, 2013). Fadini and co-workers (2006) observed that EPCs were not mobilised from bone marrow of T1DM rats in response to ischemia/reperfusion injury. There was suboptimal response to growth factor stimulation in these diabetic rats (Fadini *et al.*, 2006b). Impaired mobilisation of progenitor cells due to bone marrow dysfunction was observed in a mouse model of diabetes (Ferraro *et al.*, 2011; Westerweel *et al.*, 2013). Human studies have shown impaired mobilisation of EPC from bone marrow. Ferraro *et al.* (2013) demonstrated reduced mobilisation of progenitor cells in response to G-CSF in patients with diabetes with the haematological disorder. Fadini and co-workers (2013) demonstrated impaired mobilisation of progenitor cells after G-CSF stimulation in patients with DM (Fadini *et al.*, 2013). These findings were corroborated in a meta-analysis by the same group demonstrated reduced $CD34^{+}$ cell response to G-CSF in CVD patients with diabetes (Fadini and Avogaro, 2013). It is interesting to note that HbA1c did not correlate with G-CSF effect on progenitor cell mobilisation (Fadini *et al.*, 2013). Therefore, it is likely other mechanisms may account for an impaired response.

Fadini and group (2013) has demonstrated that SDF-1α gradient was affected in patients with diabetes. SDF-1α is a substrate of CD26/DDP-4. CD26/DDP-4 is a protease, and its activity is impaired in patients with diabetes. G-CSF failed to upregulate the activity of CD26/DDP-4 in bone marrow derived from patients with diabetes. As a result, SDF-1α gradient did not change (Fadini *et al.*, 2013).

There is a need to develop strategies to improve mobilisation of EPC in patients with diabetes. Studies in diabetic mice models have identified that CXCR4 receptor antagonist ameliorated impaired bone marrow mobilisation of progenitor cells (Tepper *et al.*, 2010). This finding was confirmed in a small human study (10/group). In this study, plerixafor (CXCR4 antagonist) was able to equally able to increase CD34+ mobilisation in both groups (with or without DM) after G-CSF stimulation (Fadini *et al.*, 2015a).

#### *4.1.12.2 Decreased PAC, CFU-Hill colonies and PAC adhesion function in type 1 diabetes*

##### 4.1.12.2.1 Main finding

Main findings were:

1. T1DM had lower PAC numbers when compared to HC.
2. Number and adhesion function of PACs were inversely correlated with glucose variability but not correlated with HbA1c.
3. CFU-Hill colonies were lower in T1DM when compared to HC.

##### 4.1.12.2.2 Prior work

Our data was consistent with a previous report by Looman *et al.* (2004). However, in contrast to Looman *et al.* (2004), PAC numbers in our subjects were not associated with HbA1c but were inversely related to glucose variability. In our study, T1DM patients had better diabetes control than patients in above-mentioned study (8.3+/-1.5 vs. 7.3%). Our finding could be relevant for understanding that even with good diabetes control T1DM patients were at high risk of impaired vascular repair.

Our results corroborated findings of seminal work by Hill *et al.* (2003), who showed that the numbers of CFU-Hill colonies were significantly lower in subjects with diabetes compared to individuals without diabetes. Our data showing lower CFU-Hill colonies provide additional evidence that T1DM were at increased risk of development of future CVD in our patients.

#### 4.1.12.2.3 Mechanism

Low levels of PAC in T1DM in our study could be due to increased cell death and apoptosis or poor functional capacity of PACs. It has been shown that apoptosis may not be involved in lower EPC numbers in patients with DM (Jung *et al.*, 2010). An alternative mechanism could be impaired function capacity of PAC to proliferate, differentiate and/or adhere to culture condition. We had demonstrated that the adhesion function of PACs was impaired in T1DM when compared to HC. PACs dysfunction in adhesion in T1DM could result in impaired cEPCs homing, cell-cell contact and transmigration events which were necessary neovascularisation and vascular repair. The lower number of CFU-Hill colonies in our study could also be due to decreased adhesive or migratory properties of cells or due to the decreased fraction of PBMC capable of colony forming properties.

Our data demonstrated that with good glycaemic control, HbA1c may not impact on PAC function. Nevertheless, glucose variability still can play some role in PAC dysfunction resulting in reduced vascular regenerative potential in T1DM and contributing to the pathogenesis of vascular complications. Glucose fluctuations had been shown to cause more endothelial damage than chronic hyperglycaemia (Cavalot, 2013). It is thought that oxidative stress and damage plays an important role. This has been shown to be true in type 2 diabetes (Monnier *et al.*, 2006). However, this has not been shown to be the case with T1DM (Wentholt *et al.*, 2007). It has been shown that reducing glucose variability improved cEPC numbers (Maiorino *et al.*, 2016). Thus, the effect of improved glucose variability on cEPC numbers and PAC function can be another way to improve the balance between vascular repair and damage. This needs to be confirmed by exploring if glucose variability has any effect on endothelial function.

#### *4.1.12.3 Metformin treatment increases cEPC numbers*

##### 4.1.12.3.1 Main finding

Our main findings were:

1. Metformin improved cEPC numbers significantly in T1DM and brought the number closer to HC.
2. After four weeks of metformin treatment, CD34+ cell increased. $CD45^{dim}CD34^{+}VEGFR2^{+}$ did not change after four weeks of metformin treatment. However, after eight weeks of metformin treatment, CD34+ cell levels came down to baseline and $CD45^{dim}CD34^{+}VEGFR2^{+}$ numbers increased.

##### 4.1.12.3.2 Novelty

We have shown for the first time that 8 weeks of metformin treatment increased cEPC numbers in T1DM without altering overall diabetes control as assessed by HbA1c and glucose variability. We believe that this increase was caused by metformin, not by the clinical care given during eight weeks of treatment. SG did not show any change in cEPC numbers. Our SG underwent similar clinical care without treatment with metformin. Other progenitor cell phenotypes did not change after metformin treatment.

In addition, we have shown for the first time that metformin initially increased $CD34^{+}$ cell numbers. This was followed by an increase in differentiation of $CD34^{+}$ cells into more mature cEPCs. This finding provides an important information regarding a possible mechanism of increased cEPC numbers after treatment with metformin.

##### 4.1.12.3.3 Prior work

Previous work had shown that metformin improved cEPC numbers. However, all of the evidence is available in T2DM. There was a significant improvement in HbA1c (Chen *et al.*, 2010; Liao *et al.*, 2010; Esposito *et al.*, 2011). Thus, the change in cEPCs

number could have been attributed to improved diabetic control. We have demonstrated that improvement in cEPC is independent of glycaemic control in TG.

#### 4.1.12.3.4 Mechanism

We speculate that the observed effect of metformin on cEPCs in our study may be due to improved cell survival, decreased senescence, increased recruitment from the bone marrow and/or increased differentiation of $CD34^{+}$ hematopoietic precursor cells into EPCs.

*In-vitro* work has shown that metformin's effect on improved EC survival by reducing premature senescence and apoptosis (Arunachalam *et al.*, 2014b). It had been shown that metformin exerts its protective effect on endothelium through SIRT-1. Does metformin exert a similar effect on cEPC? This will need to be further studied.

It had been shown that in DM, EPC mobilisation could be impaired (Fadini *et al.*, 2013). Multiple mechanisms including eNOS dysfunction (Gallagher *et al.*)(Gallagher et al. 2007) or alteration in cytokines has been shown to play a role in impaired EPC mobilisation (Fadini and Avogaro, 2013). We will discuss this further in chapter 6. Metformin is a potent activator of AMPK pathway. It has been shown that AMPK activation improved EPC mobilisation and differentiation (Li *et al.*, 2008; Li *et al.*, 2012a; Yu *et al.*, 2016).

#### *4.1.12.4 Metformin treatment improves PAC, CFU-Hill colonies and PAC adhesion function*

##### 4.1.12.4.1 Main finding

Our main findings were:

1. Metformin significantly improved PAC numbers independent of glycaemic control in T1DM.
2. Metformin enhanced the function of PAC adhesion and increased the ability to form colonies (CFU-Hill colonies) independent of glycaemic control in T1DM.

##### 4.1.12.4.2 Novelty

We have shown for the first time that the cell fraction of PBMC taken from patients with T1DM after metformin treatment continued to show significant improvement in PACs, CFU-Hill colonies number and PAC adhesion function in a euglycaemic culture environment when compared to the cells taken at the pre-treatment stage.

##### 4.1.12.4.3 Mechanism

We postulate that metformin improves functional capacity of PAC to proliferate, differentiate and/or adhere to culture condition. We have demonstrated that adhesion capacity of PAC improved after metformin treatment and brought it closer to healthy volunteers. PACs adhesion function improvement after metformin treatment in T1DM can improve cEPCs homing, cell-cell contact and transmigration events for neovascularisation and vascular repair. AMPK activator had been shown to improve EPC function (Yu *et al.*, 2016). The beneficial effect of metformin can be in part mediated by its activation of AMPK. It would have been ideal if we had assessed whether AMPK pathway inhibition would abrogate this improvement in PAC adhesion function and ability to form colonies. This is one of the limitations of our study.

Change in PACs and CFU-Hill colonies number and PAC adhesion function were not correlated with changes in insulin dosage. This is interesting, as insulin has been

shown to improve cEPCs number (Humpert *et al.*, 2005; Fadini *et al.*, 2011) and function in type 2 diabetes (Humpert *et al.*, 2008; Oikonomou *et al.*, 2014). However, the improvement in cEPCs number in Fadini *et al*. (2011) could be attributed to improvement in HbA1c. This is in contrast with Humpert *et al.* (2005), who showed that the effect of insulin on the cEPCs number is independent of HbA1c. If the former was likely, given the reduction in insulin dosage in our study, cEPCs, PACs and CFU-Hill colony number and PACs adhesion should have decreased, but this is not the case. This suggests that the insulin dose reduction did not have any effect on improving cEPCs or PACs function.

### *4.1.13 Conclusion*

In conclusion, eight weeks of metformin treatment increased cEPC, PAC, CFU Hill colonies number and PAC adhesion function in T1DM independent of glycaemic control. Our data also corroborated findings from previous work showing lower cEPCs, PACs, CFU-Hill colonies number and PAC function in T1DM. Future work is needed to explore the mechanisms by which metformin improved cEPCs, PAC, CFU-Hill colonies number and PAC function in T1DM.

## 4.2 Circulatory endothelial cells (cECs)

Endothelial cell (EC) lines the vasculature as a monolayer. They not only form a barrier but plays a vital role in vascular haemostasis (Lerman and Zeiher, 2005). In normal condition, EC turnover is very low (0-1% daily). These cells are very rare in the blood of healthy individual (between 5-10 cells/ml) (Woywodt *et al.*, 2006). It is thought that these cells are cleared by the reticuloendothelial system (Boos *et al.*, 2006). cECs are approximately 15-50µm in diameter (Erdbruegger *et al.*, 2006). Vascular damage results in the release of circulating endothelial cells (cECs) from the vascular intima into circulation. The mechanism of cECs detachment from vascular intima is complex and is due to irreversible loss of integrity as a response to endothelial activation by mechanical stress, inflammatory cytokines, growth factors, infectious agents, lipoprotein, and oxidative stress (Boos *et al.*, 2006) (Figure 35). It is not clear if EC apoptosis or detachment occurs in which order, however, the current evidence point towards detachment preceding apoptosis (Erdbruegger *et al.*, 2006).

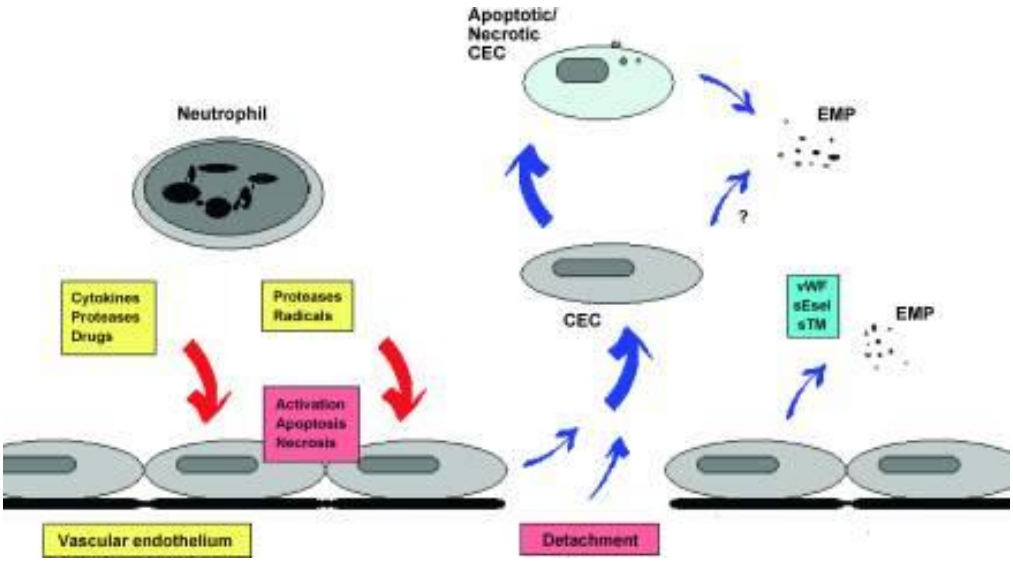


Figure 30: Potential mechanisms for EC detachment and microparticle formation. CEC, circulating endothelial cells; EMP, endothelial microparticles; sTM, soluble thrombomodulin; sE-Sel, soluble E-selectin; vWF, von Willebrand factor. Adapted from (Goon *et al.*, 2006).

### *4.2.1 Enumeration of cECs*

Accurate enumeration of cECs remains difficult due to low numbers of cECs in circulation Boos et al. 2006). Two major methods are used:

1. Immunomagnetic separation (George *et al.*, 1992; Blann *et al.*, 2005)

2. Flow cytometry (Mancuso *et al.*, 2001; Khan *et al.*, 2005)

Immunomagnetic separation involves the use of ferrous beads bound to the anti-CD146 monoclonal antibodies. cECs are characterised by the presence of CD146. Anti-CD146 antibodies are mixed with blood. Anti-CD146 binds to cells with CD146 antigen. The mixture is placed in front of a magnet. Cells bound to the beads are separated by the magnet. The magnet is washed to remove unbound cells. The bound cells can be labelled with endothelial specific fluorescent markers like Ulex europaes lectin 1 and counted under a fluorescent microscope (Boos *et al.*, 2006).

Flow cytometry is more extensively used to enumerate cECs. The procedure is currently not standardised. Multiple markers are used to identify cECs. cECs are characterised by the presence of endothelial cell surface markers like CD144 and absence of haematopoietic (e.g., CD45) and progenitor cell markers (e.g., CD133). CD144 is important for maintaining endothelium integrity through the cell to cell adhesion (Goon *et al.*, 2006). The absence of CD133 has been used to distinguish it from EPCs. Published studies have used surface markers like CD31, CD62e, CD54, CD146, CD141, CD106, CD105, vWF and eNOS to identify cECs. CD34 is also present on EC (Table 8). However, there is a lack of consensus regarding a precise antigen for cECs identification (Boos *et al.*, 2006; Burger and Touyz, 2012; Flores-Nascimento *et al.*, 2015).

| References | cEC markers |
|---|---|
| (Lampka *et al.*, 2010) | CD146+, CD45−, CD31+ |
| (Li *et al.*, 2013) | CD146+, CD45−, Hoechst 33342+, CD31+, and CD133−FWD/SSC |
| (Mourino-Alvarez *et al.*, 2013) | CD45−, CD31bright, CD34+, CD 133−, and FWD/SSC |
| (Flores-Nascimento *et al.*, 2015) | CD45-, CD133-, CD144+, CD146+, VEGFR2+ |
| (Ozdogu *et al.*, 2007) | CD34-, CD117-, CD146+, CD144+ |
| (Machalinska *et al.*, 2010) | CD133-, CD144+ |
| (Shaffer *et al.*, 2006) | CD146+, CD146+CD31+, CD146+CD34+, CD146+CD34+CD31+, CD34+CD31+CD133- |
| (Lombardo *et al.*, 2012) | CD45-, CD31+CD146+ CD106 |

Table 8: Overview of markers used in various publications regarding cECs.

Various studies have described endothelial cells as resting and activated. Clinical and research significance of the activated or resting EC remains to be evaluated. Activated ECs have increased procoagulant activity, increased leukocyte adhesion and phagocytosis. These cells produce markers of oxidative stress like nitric oxide, prostacyclin and oxygen radicals. Activated ECs have been shown to express CD54 (intracellular adhesion molecules), CD62e (E-selectin), CD106 (vascular cell adhesion-1) and CD105 (endoglin) (Mancuso *et al.*, 2001; Khan *et al.*, 2005).

cECs have been shown to reflect endothelial status. Thus, the increase in cECs reflects increased vascular damage.

### *4.2.2 cECs in vascular disease*

Several studies have consistently demonstrated that cECs levels are high in patients with myocardial infarction and unstable angina (Mutin *et al.*, 1999; Makin *et al.*, 2004). cECs levels have been shown to be 400% higher in patients with MI than healthy control (Damani *et al.*, 2012). In stable angina, levels are either normal or minimally raised (Mutin *et al.*, 1999; Lee *et al.*, 2005a). Levels of cECs correlate with markers of endothelial dysfunction (Makin *et al.*, 2004).

Similarly, patients with an active peripheral vascular disease had higher ECs levels when compared to healthy controls or patients with symptoms of intermittent claudication (Makin *et al.*, 2004; Shaffer *et al.*, 2006). cECs levels are raised in patients with acute stroke when compared with healthy controls (Freestone *et al.*, 2005; Nadar *et al.*, 2005; Woywodt *et al.*, 2006). Levels of cECs correlated with makers of endothelial function like (vWF or plasma tissue factor or E-Selectin) (Makin *et al.*, 2004; Nadar *et al.*, 2005). It is interesting to note that patients with hypertension have been shown to have normal cEC levels (Nadar *et al.*, 2005). It is likely that appropriate vascular insult is needed for release of EC. This is evidenced by the fact that stable vascular diseases like stable angina or intermittent claudication did not result in an increase in cECs (Lee *et al.,* 2005 and Makin *et a.,* 2004).

Other conditions like pulmonary hypertension (Smadja *et al.*, 2009), acute and chronic heart failure have shown to be associated with higher cEC levels when compared to healthy control (Chong *et al.*, 2006).

cECs have been shown to predict cardiovascular events in high-risk groups. Koc *et al*. 2005 demonstrated that high cEC levels predicted future CV events in haemodialysis patients (Koc *et al.*, 2005). Similarly, Lee and co-workers 2005 demonstrated that high cECs levels at 48 hours predicted future coronary events and mortality in patients with ACS (Lee *et al.*, 2005a). High cECs levels have been shown to predict increased mortality in a non-cardiac condition like septic shock (Mutunga *et al.*, 2001).

### *4.2.3 cECs and diabetes mellitus*

Relationship of DM on cECs are discussed more in depth in the discussion. Briefly, cECs levels have been shown to be high in both T1DM and T2DM compared to healthy control. Table 9 provides a summary of available evidence published regarding DM and cECs.

| **Reference** | **Population studied** | **Number of cECs** |
|---|---|---|
| (Asicioglu *et al.*, 2010) | T1DM vs HC | 7.5+/-5.4 vs 2.1+/-1 |
| (McClung *et al.*, 2005) | T2DM vs HC | 69+/-30 vs 10+/-5 |
| (Lombardo *et al.*, 2012) | T2DM with or without complication vs. HC | 3427+/-3901 (3400% higher than HC)<br>vs 2483+/-1828 (2500% higher than HC)<br>vs 99+/-76 |

Table 9: cECs and available evidence in patients with diabetes mellitus.

### *4.2.4 Method*

We have discussed method for enumeration and compensation of cEC in section 4.1.9.1 and 4.1.9.2.

#### *4.2.4.1 Optimisation of gating strategy for flow cytometric analysis of circulating Endothelial Cells (cEC)*

BD FACSDiva™ software was used to quantify cEC events. cEC events are defined as: $CD45^{dim}$, $CD133^{-}$, $CD34^{+}$, and $CD144^{+}$ events (West *et al* 2015, Ahmed *et al* 2016). Lymphocytes and monocytes were gated on the side vs. forward-scatter plot. $CD45^{dim}$ population were gated on side-scatter vs. CD45. $CD45^{dim}$ events were further gated to obtain $CD45^{dim}$, $CD133^{-}$, $CD34^{+}$ population. Then the remaining events were gated for $CD144^{+}$ (Figure 36).

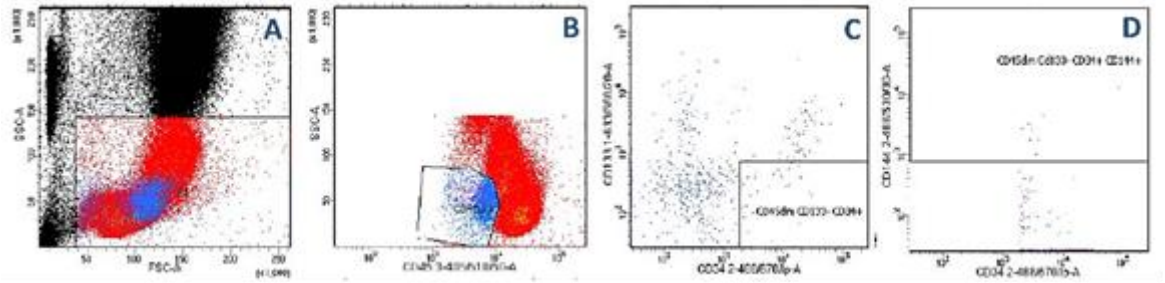


Figure 31: Gating strategy to identify cEC population.

**A.** Lymphocytes and monocytes were gated on side vs. forward-scatter plot; B. CD45 dim population were gated on side-scatter vs. CD45, gate was done by tracing demarcation seen between CD45dim and CD45+ population; **C**. events were further gated to obtain $CD45^{dim}CD133^{-}CD34^{+}$ population; **D.** the remaining events were gated for $CD144^{+}$.

#### *4.2.4.2 Determination of cECs per ml*

cECs per ml were calculated using the following formula:

$$\frac{Events}{ml} = \frac{number\ of\ events\ in\ a\ certain\ gate}{number\ of\ beads} \times \frac{total\ number\ of\ beads}{vollume\ of\ sample\ (ml)}$$

### *4.2.5 Specific aim*

In this section, we aimed to evaluate if metformin improved the numbers of cECs in type 1 diabetes when controlled for glycaemic changes.

### *4.2.6 Results*

Data is presented in Figure 37.

#### *4.2.6.1 Treatment group (Pre metformin) versus Healthy control*

cECs were significantly higher (74.6%) in TG compared with HC (TG V1 vs HC; median (IQ): 74.4 (46.4-221) vs. 42.6 (12.7-65.9) per ml; p=0.006).

#### *4.2.6.2 Treatment group (Pre metformin) versus Treatment group (Post metformin):*

Eight-week metformin treatment significantly decreased cECs in TG by more than 36% (TG V1 vs. TG V2; median (IQ): 74.4 (46.4-221) vs. 47.6 (21.8-76.7) per ml; p=0.009).

#### *4.2.6.3 Treatment group (Post metformin) versus Healthy control*

Metformin treatment decreased the cECs number in TG group and normalised the levels of the cECs count when compared to HC (TG V2 vs. HC; median (IQ): 47.6 (21.8-76.7) vs. 42.6 (12.7-65.9) per ml). After treatment, the cECs count is only 11.7% higher in TG when compared to HC versus 74.6% lower in TG pre-metformin treatment when compared to HC. After metformin treatment, significance disappeared (p=0.7).

#### *4.2.6.4 Treatment group (Pre metformin) versus Standard group (Pre observation)*

At baseline, cECs were similar in TG and the SG (TG V1 vs. standard group SG V1; median (IQ): 74.4 (46.4-221) vs. 99.8 (59.4-210.5) cells per ml; p=0.68).

#### *4.2.6.5 Standard group (Pre observation) versus Standard group (Post observation)*

In standard group, eight weeks of standard follow-up did not result in any change in the cEC count (SG V1 and SG V2: median (IQ): 99.8 (59.4-210.5) vs. 119.5 (80.5-212.6 cells per ml; p=0.46).

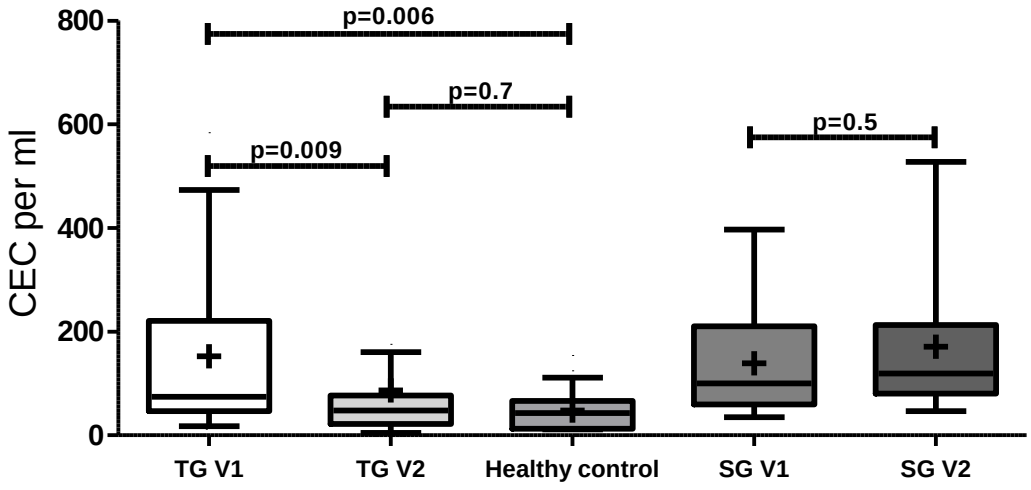


Figure 32: cEC comparing all groups. Results are given as per ml. Line denotes in each box as median and + in each box denotes mean value.

#### *4.2.6.6 Subgroup analysis – Effect of metformin in smokers and patients taking ACE/Statins*

Smokers in TG had 66% (155 (65-497) vs 51.4 (15.6-151) per ml) reduction of cECs. Whilst in TG, individuals on ACE and/or statin led to only 26.6% reduction.

#### *4.2.6.7 Correlation*

In TG at baseline, cECs were not correlated with HbA1c ($r=-0.23$ $p>0.05$). In TG, at baseline, cECs number were inversely correlated with PAC numbers and PAC adhesion function ($r=-0.48$; $p<0.05$; $r=-0.51$; $p<0.05$). In TG, changes in cECs were inversely correlated with changes in CFU-Hill colonies ($r=-0.6$; $p<0.01$).

### *4.2.7 Discussion*

Markers of vascular damage were assessed at baseline and after eight weeks of metformin treatment. These were compared with healthy volunteers. Similar markers were evaluated in T1DM cohort group to explore if any changes in above-mentioned markers can be observed with routine follow up without any treatment with metformin. Individual findings will be discussed first followed by a summary of overall findings.

#### *4.2.7.1 Higher cEC in type 1 diabetes*

##### 4.2.7.1.1 Main findings

Our main findings were:

1. cECs were significantly higher in T1DM when compared to healthy controls.
2. In TG, PAC numbers and PAC adhesion function at the baseline was inversely correlated with cECs levels.

##### 4.2.7.1.2 Novelty/importance

To our knowledge, this is the first study to date quantifying both cEC and PAC numbers and function in the same patient population. PACs play an important role in vascular/endothelial repair. Lower PAC numbers and reduced function may infer that there is reduced endothelial/vascular repair leading to the shedding of cECs.

##### 4.2.7.1.3 Prior work

Our finding is in line with another group (Asicioglu *et al.*, 2010). However, in contrast to Asicioglu *et al.* (2010), our study did not show any correlation between HbA1c and cECs (Appendix A-page 346). This difference could be due to better diabetic control and subject characteristics with more CVD risk factors such as longer duration of diabetes, older age group and higher BMI in our study. Higher BMI in our subjects could lead to slightly higher insulin resistance, thereby causing inflammation mediated endothelial damage. Indeed, patients with type 2 diabetes have been shown to have

higher cEC with no correlation with the level of HbA1c. However, this is speculative and needs further work to confirm.

#### 4.2.7.1.4 Mechanisms

Endothelial damage and generation of cEC in individuals with diabetes mellitus are multifactorial. EC exposed to the diabetic environment results in an increase in reactive oxygen species (ROS) and advanced-glycated end-product (AGE) production (Yao and Brownlee, 2010). Hyperglycaemic conditions have been shown to decrease eNOS expression (Srinivasan *et al.*, 2004). These can result in EC injury causing detachment of EC. Thus, we can infer that in our study, increased level of cEC in T1DM could be mediated by a similar mechanism.

### *4.2.7.2 Metformin reduces cEC numbers*

#### 4.2.7.2.1 Main findings

Our main findings were:

1. Metformin therapy improved the cECs count in T1DM.
2. There was an inverse correlation between changes in cECs and CFU-Hill colonies after metformin treatment.

#### 4.2.7.2.2 Novelty/importance

Our study for the first time showed that metformin therapy improved the cECs count in T1DM and brought it closer to the matched HC. Even though cECs improved significantly, we believe that our study did not show the full effect of metformin on cECs, as some of our patients were using cardio-protective drugs already: statins and ACE inhibitors. Indeed, in TG subjects on ACE inhibitors and/or statins, less reduction of cECs was observed. In addition, our findings of an inverse correlation between changes in cECs and CFU-Hill colonies after metformin treatment demonstrated that changes in markers of vascular/endothelial damage are linked inversely with a marker of CVD risk (CFU-Hill-colonies).

#### 4.2.7.2.3 Prior work

To date, no prior study has explored the in vivo effect of metformin on cECs in T1DM. Previous work has explored the effect of metformin on cECs in *in vitro* conditions. However, in these studies supra-physiological levels of metformin was used. In both studies, metformin improved cEC function via increased availability of eNOS.

#### 4.2.7.2.4 Mechanisms

Metformin has shown to improve vascular health in both T2DM and T1DM (Mather *et al.*, 2001; Pitocco *et al.*, 2013b). This is thought to be mediated by AMPK activation by metformin. Calvert *et al.* (2008) has shown that metformin has cardio-protective properties through AMPK activation (Calvert *et al.*, 2008). Davies *et al.* (2006) demonstrated that metformin-mediated AMPK activation increases NOS activity and NO bioavailability through increased phosphorylation of eNOS. Metformin at supra-physiological concentration inhibits glucose-mediated activation of respiratory chain complex and mitochondrial permeability transition pore (mPTP) in EC. Thereby, limiting ROS dependent damage and apoptosis of EC (Detaille *et al.*, 2005). The additional mechanism includes the reduction of AGE-related damage mediated through the reduced expression of receptor of AGE (RAGE) and Lectin-like oxidized low-density lipoprotein (LDL) receptor-1 (LOX-1) receptors in EC (at supra-physiological concentration of metformin) (Ouslimani et al., 2005). Metformin has been shown to reduce monocyte adhesion to EC (at physiological concentration) (Mamputu *et al.*, 2003). Given the above-mentioned evidence, we can infer that a similar mechanism likely mediated improvement in cEC after metformin treatment in type 1 diabetes.

### *4.2.8 Conclusion*

In conclusion, we have demonstrated that metformin treatment in T1DM reduced cEC numbers. This could likely be mediated via reduced EC injury or increased EC repair. Future studies are needed to explore the mechanism and specific pathways modulated by metformin at physiological concentrations in T1DM.

## 4.3 Microparticles

Microparticles (MPs) are enucleated submicron vesicles released from the plasma membrane of various cells in response to different stimuli causing activation and/or apoptosis (Jimenez *et al.*, 2003). The outer membrane of the MP is characterised by the presence of phosphatidylserine (PS) as well as surface antigens they inherit from their parental cells like platelet, endothelial, leukocytes, erythrocytes, etc. (Piccin *et al.*, 2006).

### *4.3.1 Historical perspective*

Particles shed from activated platelets was first described in 1967 by Wolf using electron microscopy. These particles had procoagulant activity and were called 'platelet dust' (Wolf, 1967). Prior to this, it was known that there are circulating plasma factors which play a role in coagulation. Chargaff and West in 1946 described prolonged plasma coagulation if plasma was centrifuged at high speed. Coagulation time reduced when centrifuged pellet was added (Chargaff and West, 1946). Further work over time provided evidence about the generation of membrane particles released from various cells like endothelial cells, leukocytes, erythrocytes, etc. In an *in vitro* study, Warren and Vales in 1972 demonstrated that there was generation of MPs when the activated platelets interacted with the vascular wall (Warren and Vales, 1972). Khan and colleagues showed for the first-time involvement of MPs in a disease process. There was an increase in platelet fragments in patients with idiopathic thrombocytopenic purpura (Kahn I, 1975). Subsequently, circulating MPs of different cellular origins have been demonstrated including in healthy volunteers (Berckmans *et al.*, 2001; Dignat-George and Boulanger, 2011).

### *4.3.2 Nomenclature*

Defining circulatory vesicles have been problematic. International Society for Extracellular Vesicles (ISEV) has been trying to reach consensus (ISEV: www.isev.org/). However, it has not been possible to develop a uniform method to describe vesicles (van der Pol *et al.*, 2012). Broadly, circulatory vesicles can be

classified by the cellular origin or mechanism of biogenesis or size. Examples of cellular classification are; prostasomes are vesicles released from prostate epithelium (Aalberts *et al.*, 2013), dexosomes originated from dendritic cells (Delcayre *et al.*, 2005); adiposomes derived from adipocytes (Muller *et al.*, 2010) etc.

Circulatory vesicles can be classified based on their properties (size, density, lipid composition, subcellular origin-intracellular or membrane-derived and protein marker). These vesicles can be classified as exosomes, apoptotic bodies, exosomes like vesicles and MPs (Table 10) (Mattson *et al.*, 2012).

| **Vesicles** | **Size** | **Features** | **Markers** |
|---|---|---|---|
| **Exosomes** | 0.05-0.1 µm | • Generated by budding<br>• Intracellularly derived<br>• Contain cytosolic component<br>• Phospholipid bilayer | LAMP-1, CD9, CD63 and CD81 |
| **Apoptotic bodies** | 1-5 µm | • Released by blebbing<br>• Externalise phosphatidylserine<br>• Increased membrane permeability<br>• Contain nuclear material | Annexin V +ve<br>Stains with PI |
| **Microparticles** | 50nm to 1µm | • Phospholipid bilayer<br>• Budding/ Shedding<br>• Intracellular and membrane derived | Cell surface marker of cell origin<br>Annexin V +ve |

Table 10: Features of vesicles. Adapted from (Dignat-George and Boulanger, 2011; György *et al.*, 2011)

Vesicles are a group of extracellular particles which are diverse and heterogeneous in nature. Above mentioned terminologies have been used interchangingly in many

published literatures. However, for the purpose of this thesis microparticle (MP) will be used unless stated otherwise.

### *4.3.3 Microparticle generation*

*In vivo* formation of MPs are not clearly understood. Most of our understanding comes from in vitro experiments (Schock *et al.*, 2013). Eukaryotic cells contain a semi-selective permeable membrane consisting of a double layer of phospholipid with various embedded proteins. Under normal micro-environment, there is the asymmetrical distribution of phospholipid (Mohandas *et al.*, 2002). Phosphatidylcholine and sphingomyelin are located on the external layer whereas aminophospholipid PS and phosphatidylethanolamine is located on the internal layer (Devaux and P, 1984).

Maintenance of this phospholipid membrane asymmetry is ensured by the balance between three main transmembrane enzymes; flippase, floppase and scramblase. Flippase, an ATP-dependent protein transports PS and phosphatidylethanolamine back to the inner layer from the external layer through amino-phospholipid translocation (Schock *et al.*, 2013). Floppase, another ATP-dependent protein is responsible for disruption of membrane asymmetry when cells are activated by dynamic outward translocation of PS from internal to the external layer (Bevers *et al.*, 1999). Scramblase is a lipid transporter which plays an important role in lipid randomisation across the plasma membrane. Activation of scrambalase results in a bidirectional flow of phospholipid across the membrane; that is a movement of the internal layer phospholipid to external layer and vice versa (Bevers *et al.*, 1999; Schock *et al.*, 2013).

MP release is dependent on $Ca^{2+}$ mediated enzyme activation and inhibition. Stimuli causing cellular activation or apoptosis leads to a rapid increase in cellular $Ca^{2+}$ concentration. The increase in Ca2+ concentration results in kinase and caplain activation and phosphatase inhibition. Furthermore, there is activation of floppase and inhibition of flippase activity. Caplain is a Ca2+ dependent proteases involved in cytoskeleton reorganisation. Above mentioned process leads to externalisation of PS,

blebbing and release of MP into the microenvironment (Yano *et al.*, 1993; Schock *et al.*, 2013) (Figure 38).

Rho activation has been shown to play an important role in endothelial microparticle (EMP) formation (Tramontano *et al.*, 2004). This can be mediated through activation of caspases. Under apoptotic signals, ROCK I, a Rho kinase isoform is activated by caspase 3 mediated cleavage. This leads to myosin light chain phosphorylation induced cell membrane contraction and MP formation. Non-apoptotic stimuli like thrombin have been shown to cause caspase 2 mediated ROCK II (Rho-kinase) activation. Rho kinase inhibitors like fluvastatin and Y26732 has been shown to reduce EMP formation (Sebbagh *et al.*, 2001; Tramontano *et al.*, 2004).

As mentioned above, one of the steps in the formation of MP is externalisation of PS. PS on MP membrane serves essential processes. It plays an important role in the procoagulant activity of MP through binding of the coagulation factor II, Va and Xa (Furie *et al.*, 2004). One of the properties of PS is binding to Annexin V via Ca-dependent manner (Gerke and Moss, 2002; Dignat-George and Boulanger, 2011). Annexin V can be used to quantify a total number of circulatory MP. Annexin V will not discriminate MP into differential cell origin and stimuli. MP surface protein can be used to discriminate cellular origin and stimuli leading to the generation of MP.

Initially, it was thought that all MPs were Annexin V positive. However, the presence of Annexin V negative MPs are now well established. Connor *et al*. demonstrated that more than 80% of platelets derived microparticles (PMPs) in healthy volunteers are Annexin V negative. Importantly, these Annexin V negative MPs lack procoagulant activity (Connor *et al.*, 2010). It is not fully clear why MPs are Annexin V negative.

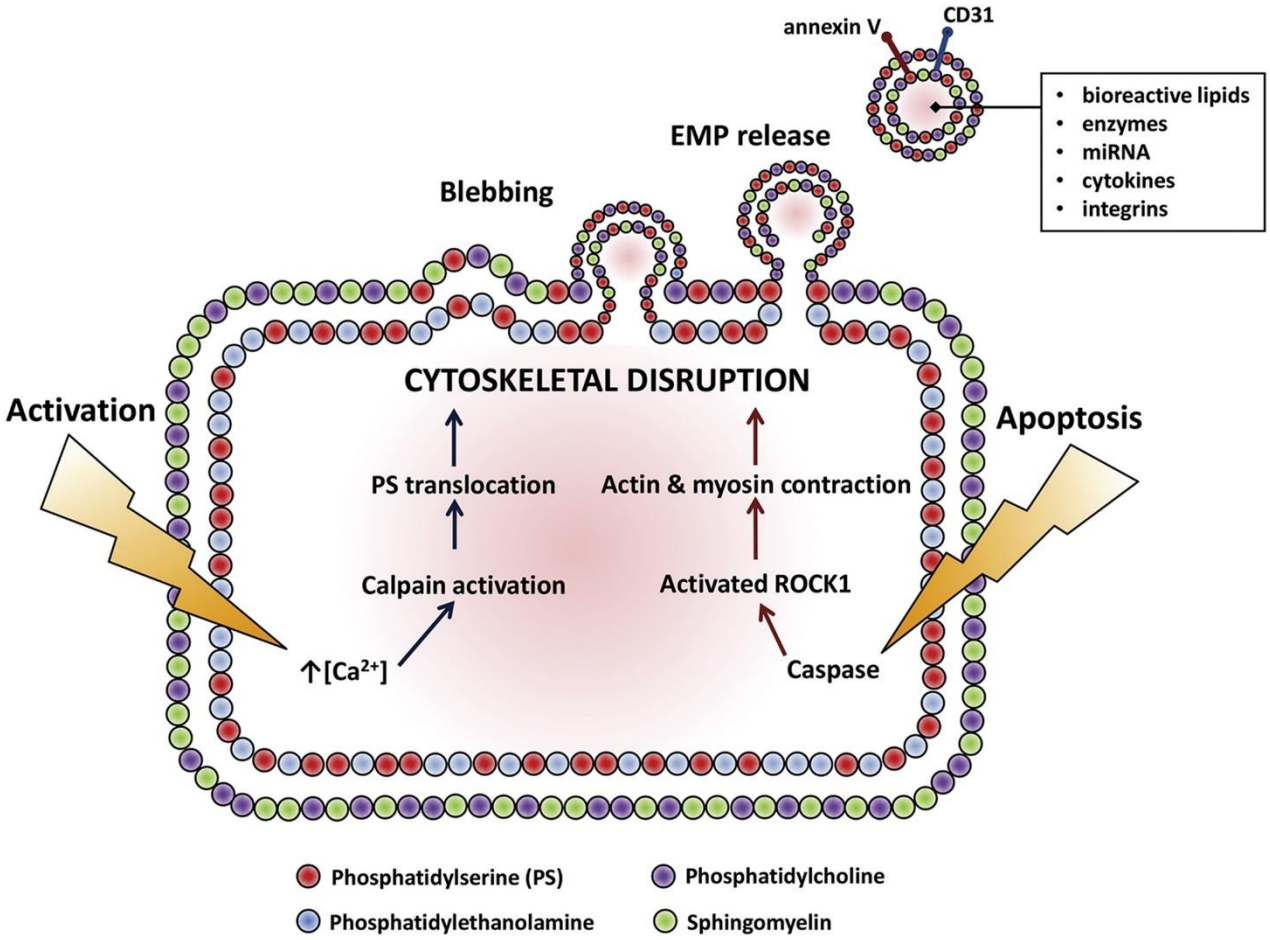


Figure 33:Schematic representation of MP generation from endothelial cells.

Taken from (Schiro *et al.*, 2014)

### *4.3.4 Microparticle characterization*

Circulatory MPs can be characterised by the cells they originate from. These MPs have markers which are cell specific. In this thesis, we will be discussing following circulatory MPs.

#### *4.3.4.1 Platelet-derived microparticles (PMP)*

The majority of circulatory MPs in healthy individuals are derived from platelets. It accounts for 70 to 90% of circulatory MPs (Berckmans *et al.*, 2001). PMPs play an important role in physiological and pathological function (Figure 39). Physiological function includes endothelial repair, angiogenesis, blood clotting, inflammatory response and intracellular signal transduction. PMPs are involved in the pathogenesis of atherosclerosis via playing a role in adhesion, inflammation, pro-coagulation and lipid deposition (Thushara *et al.*, 2015). PMPs deliver the growth factor which may promote angiogenesis. PMP levels are increased in diseases Table 12. Therefore, it can be used as a biomarker.

The PMPs have been identified by the presence of glycoprotein receptor like PECAM (CD31) and intergrinα11bB3 (CD41) (Piccin *et al.*, 2006). PMP derived from activated platelets have been shown to express P-selectin-CD62P (Table 11) (Flaumenhaft *et al.*, 2009). It was thought that most of CD41+ MP were derived from activated platelet. However, Flaumenhaft et al. in 2009 demonstrated that CD41+ MP is also derived from megakaryocytes. Megakaryocyte-derived MP does not express CD62P or LAMP1 (a marker of granule fusion) or cytoskeletal degradation (filamin A) (Flaumenhaft *et al.*, 2009).

| Surface marker | Available literature |
|---|---|
| CD31+CD41+ | (Amabile *et al.*, 2005) |
| CD31+CD42+ | (Jung *et al.*, 2012) |
| CD41+ | (Dignat-George *et al.*, 2002) |
| CD42a | (Connor *et al.*, 2010) |
| CD61+ | (Connor *et al.*, 2010) |

Table 11: Surface markers for platelets microparticles.

| Disease | Available literature |
|---|---|
| **T2DM** | (Nomura *et al.*, 1995; Omoto *et al.*, 2002; Ogata *et al.*, 2005) |
| **T1DM** | (Dignat-George *et al.*, 2002; Salem *et al.*, 2015) |
| **ESRF** | (Amabile *et al.*, 2005) |
| **Uraemia** | (Ando *et al.*, 2002) |
| **Sleep apnoea** | (Maruyama *et al.*, 2011) |
| **CAD** | (Bernal-Mizrachi *et al.*, 2003; Jung *et al.*, 2012) (Porto *et al.*, 2012) |
| **Atrial fibrillation** | (Choudhury *et al.*, 2007; Azzam and Zagloul, 2009) |
| **Pulmonary embolism** | (Bal *et al.*, 2010) |
| **Cerebrovascular disease** | (Jung *et al.*, 2009) |
| **Obesity** | (Esposito *et al.*, 2006) |

Table 12: Raised platelets microparticles and some of available evidence.

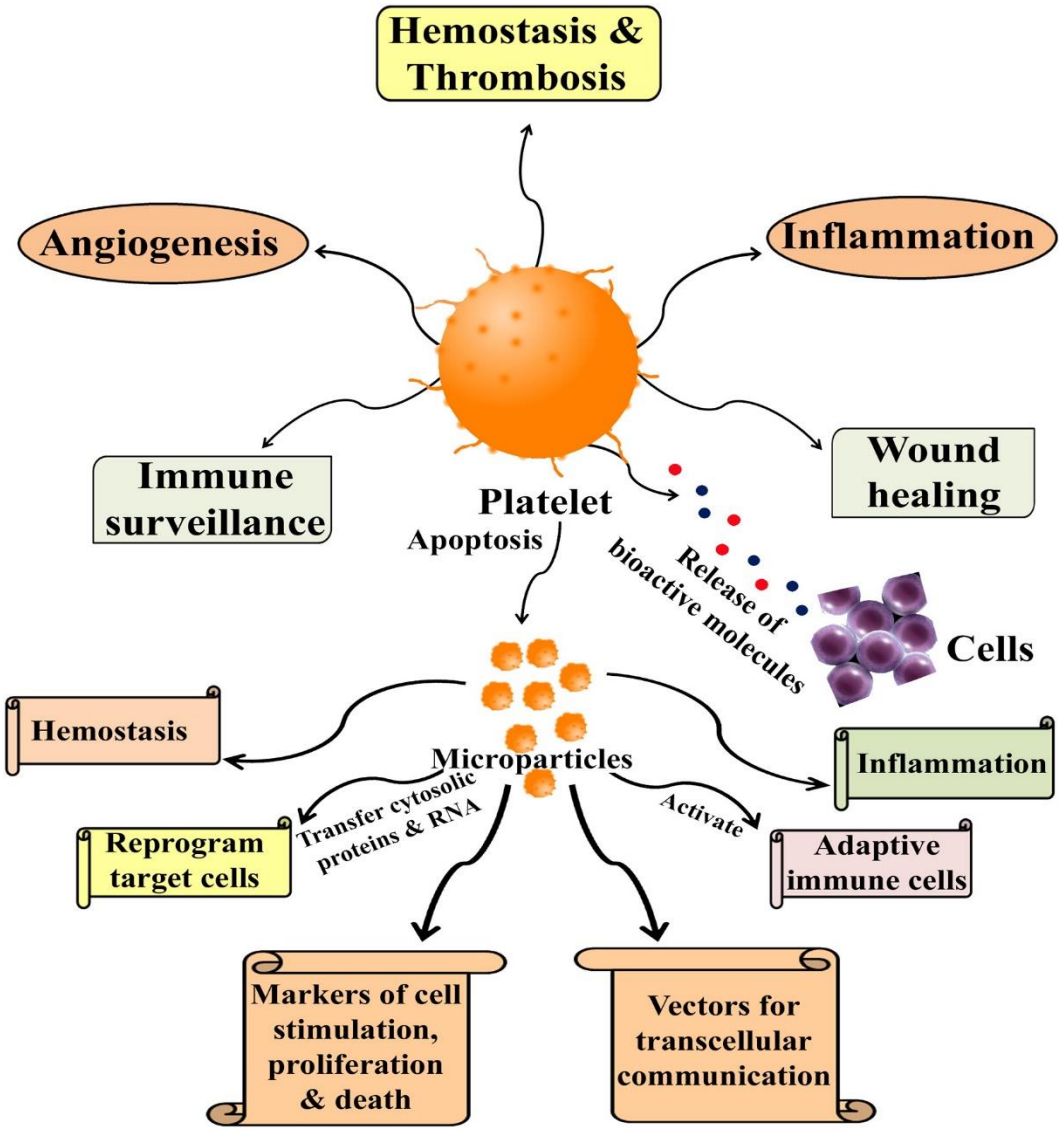


Figure 34: Physiological and pathological role of platelet microparticle.

Adapted from (Thushara *et al.*, 2015)

#### *4.3.4.2 Endothelial-derived microparticles (EMP)*

EMPs, as the term implies, are released by endothelial cells as a result of various stimuli causing endothelial cell activation or apoptosis. EMP levels are much lower in a healthy individual when compared to PMPs (Berckmans *et al.*, 2001). EMP plays many biological processes medicated via active surface markers and transportation of biologically active molecules.

EMP are associated with vascular disease and levels reflect endothelial dysfunction (Amabile *et al.*, 2005; Nickenig *et al.*, 2006). Increased EMP levels are associated with cardiovascular disease and cardiovascular risk factors (Amabile *et al.*, 2014). It has been shown that even second-hand smoking in healthy volunteers result in increased EMP levels and impaired endothelial function (Heiss *et al.*, 2008). Thus, EMPs are potential biomarkers of vascular health. In addition to being a biomarker of cardiovascular disease, EMP has been shown to have prognostic properties (Dignat-George and Boulanger, 2011). Levels of EMP have been demonstrated to predict cardiovascular outcome in renal failure patients (Amabile *et al.*, 2012). In heart failure patients, circulatory EMP levels predicted future cardiovascular events (Nozaki *et al.*, 2010).

EMP plays an important role in vascular disease pathogenesis and progression. This is mediated via pro-inflammatory, pro-angiogenic and pro-coagulant effects (Dignat-George and Boulanger, 2011). EMPs have been shown to play an important role in the development and progression of atherosclerosis (Schiro *et al.*, 2014).

EMPs are identified by the surface marker. They express PECAM (CD31), VE-Cadherin (CD144), MCAM (CD146), VCAM-1 (CD106), Endoglin CD105) and E-Selectin (CD62E). These cell surface markers have been used to count the levels of EMPs. Researchers have used different marker combinations for EMP evaluation (Table 13). This is due to the fact that most markers are not exclusive to EMPs. CD62e and CD144 are specific for EMPs. Whereas, CD31 is expressed by platelets and leukocytes in addition to endothelial. Similarly, CD146 is expressed by activated T cell lymphocytes (Dignat-George and Boulanger, 2011).

As mentioned above, EMPs are generated either by activation or apoptosis of EC. It has been demonstrated EMP constitutive markers are distinct in activation versus activation. Exploring phenotypical distinctive EMP will yield further insight into the nature of endothelial injury in a disease process. Apoptosis leads to increase in expression of CD31 and CD105 while activation leads to increase in expression of CD54 or CD106 and CD62E. In order to distinguish between activation or apoptosis, researchers have studied the ratio of CD62E/CD31 population of EMPs instead of absolute numbers. A ratio of ≤ 1 is suggestive of apoptosis whereas a ratio of ≥ 10 is suggestive of activation. EMP phenotype in ACS is suggestive of EC apoptosis whereas in the acute phase of thrombotic thrombocytopenic purpura (TTP) the phenotype is indicative of EC activation. In diabetes mellitus levels of CD31+, CD105+ and CD106+ EMP are significantly higher. This EMP phenotype in diabetes mellitus is reflective of EC apoptosis.

| EMP surface markers | References |
|---|---|
| **CD31** | (Mallat *et al.*, 2000; Bernal-Mizrachi *et al.*, 2003; Jung *et al.*, 2009) |
| **CD31+/CD41-** | (Amabile *et al.*, 2005; Amabile *et al.*, 2008) |
| **CD31+/CD42-** | (Pirro *et al.*, 2006; Giugliano *et al.*, 2011) |
| **CD144** | (Amabile *et al.*, 2008; Gunduz *et al.*, 2013; Amabile *et al.*, 2014) |
| **CD31+/CD144+** | (Berezin *et al.*, 2016) |
| **CD51** | (Bernal-Mizrachi *et al.*, 2003; Zielinska *et al.*, 2005; Chironi *et al.*, 2009; Hu *et al.*, 2014) |
| **CD31+/CD51+** | (Zielinska *et al.*, 2005) |
| **CD62E** | (Amabile *et al.*, 2008; Amabile *et al.*, 2009; Tramontano *et al.*, 2010) |
| **CD105** | (Chironi *et al.*, 2009; Tramontano *et al.*, 2010; Breen *et al.*, 2014) |
| **CD146** | (Mallat *et al.*, 2000; Gunduz *et al.*, 2013) |
| **CD105+CD41a-CD45-** | (Simak *et al.*, 2006) |
| **CD105+CD144+** | (Simak *et al.*, 2006) |
| **CD105+CD54+CD45-** | (Simak *et al.*, 2006) |
| **CD105+PS+CD41a-** | (Simak *et al.*, 2006) |
| **CD106** | (Jimenez *et al.*, 2003; Tramontano *et al.*, 2010) |

Table 13: Surface markers for endothelial microparticles.

EMP levels are associated with vascular injury and cardiovascular risk factors. Table 14 provides some available evidence of elevated EMPs levels in various cardiovascular pathological conditions.

| Cardiovascular pathology | Reference |
|---|---|
| **T1DM** | (Dignat-George *et al.*, 2002) |
| **T2DM** | (Dignat-George *et al.*, 2002; Tramontano *et al.*, 2010; Jung *et al.*, 2011; Berezin *et al.*, 2016) |
| **Hypertension** | (Preston *et al.*, 2003; Wang *et al.*, 2008a) |
| **Metabolic syndrome** | (Arteaga *et al.*, 2006) |
| **Erectile dysfunction** | (Esposito *et al.*, 2006; Heiss *et al.*, 2008) |
| **Hypertriglyceridemia** | (Ferreira *et al.*, 2004) |
| **Hypercholesterolemia** | (Pirro *et al.*, 2006) |
| **Renal failure** | (Amabile *et al.*, 2005; Faure *et al.*, 2006; Gurgoze *et al.*, 2009) |
| **Obesity** | (Giugliano *et al.*, 2011; Gunduz *et al.*, 2013) |
| **Coronary calcification** | (Jayachandran *et al.*, 2008) |
| **Acute coronary syndrome** | (Mallat *et al.*, 2000; Bernal-Mizrachi *et al.*, 2003) |
| **Stable coronary artery disease** | (Bernal-Mizrachi *et al.*, 2003) |
| **Carotid atherosclerosis** | (Chironi *et al.*, 2009) |
| **Acute stroke** | (Simak *et al.*, 2006; Jung *et al.*, 2009) |
| **Pulmonary hypertension** | (Amabile *et al.*, 2008) |

Table 14: Raised endothelial microparticles and some of available evidence.

#### *4.3.4.3 Other microparticles*

Circulatory cell like leucocytes and red blood cells also generate MPs. Leucocyte-derived microparticles (LMPs) are generated from lymphocytes, granulocytes or monocytes (Angelillo-Scherrer, 2012). In healthy individual levels of LMP are very low. CD45 is the main marker used to identify LMPs (Nieuwland *et al.*, 2000; Gelderman and Simak, 2008; Ayers *et al.*, 2009; Macey *et al.*, 2011). It has been shown that CD45 is not expressed on all LMPs (Macey *et al.*, 2011). Granulocytes, monocytes and lymphocyte fraction of LMPs express markers from their parental cells (Table 15).

| Cellular Origin of MPs | Markers |
|---|---|
| **Granulocytes** | CD15<br>CD64<br>CD66b<br>CD66e |
| **Monocytes** | CD14<br>CD11A<br>CD18 |
| **Lymphocytes** | CD2<br>CD4<br>CD8<br>CD19<br>CD20 |

Table 15: Markers of leukocyte-derived microparticles. (Adapted from Angelilo-Scherrer 2012)

Levels of LMP are raised in inflammatory conditions like rheumatoid arthritis (Berckmans *et al.*, 2002). LMP plays a role in the development of atherosclerosis plaques. Levels of LMP are raised at the site of plaque formation (Leroyer *et al.*, 2007). LMP levels are raised by three-fold in diabetes mellitus (Dignat-George *et al.*, 2002).

Red blood cell-derived microparticles (RBC-MPs) are present in a small amount in healthy individuals. Levels of RBC-MP are elevated in condition causing haemolysis of RBCs like Sickle cell disease (Shet *et al.*, 2003). Other condition includes nephrotic syndrome (Gao *et al.*, 2012) and patients with graft-versus-host disease after haematopoietic stem-cell transplantation (Rank *et al.*, 2011). CD235a (monoclonal antibody against glycophorin A), a membrane spanning protein is used to detect RBC MP (Amabile *et al.*, 2005).

### *4.3.5 Microparticle clearance*

Our knowledge regarding the mechanism of microparticle clearance is very limited. It is speculated that the MP levels depends on the rate of clearance. In vitro work has

demonstrated that MPs are cleared by phagocytosis (Distler *et al.*, 2005). Currently, it is thought that liver or spleen is involved in clearance of MP. It is speculated that phagocytic cells directly bind to PS or opsonisation proteins on the MP (Flaumenhaft, 2006; Davila *et al.*, 2008). *In vivo* animal study by Willekens *et al.* 2004 demonstrated that RBC-MPs are engulfed by phagocytic cells of the liver, that is the reticuloendothelial system (Willekens *et al.*, 2004). Rand *et al.* 2006 demonstrated that infused PMPs are rapidly cleared after being injected in the rabbit (Rand *et al.*, 2006). In mice, it is shown to be cleared in 30 minutes (Flaumenhaft, 2006). In humans, procoagulant PMPs and EMPs are cleared within one hour. It was noted that RBC-MPs were not cleared to the same proportion to other MPs. It was speculated that other MPs, being more procoagulant needed to be cleared rapidly due to thrombotic risk (Augustine *et al.*, 2013).

EC is also thought to play a role in MP clearance. An *in vitro* experiment demonstrated that PMPs are endocytosed and internalised by cultured EC (Faille *et al.*, 2011). An EC-associated protein called developmental endothelial locus-1 (Del-1) plays a major role in this process. In vitro and in vivo animal experiments have shown that inhibition of Del-1 reduced internalisation of PMP by EC (Dasgupta *et al.*, 2012).

### *4.3.6 Microparticle function*

Depending on the mode of generation and the microenvironment, MPs play various physiological and pathophysiological functions. MPs can act locally or circulate and act as a vehicle for intercellular communication and exchange via the transfer of biological signals and genetic information, membrane-associated receptor, the release of protein and bioactive lipids. In addition to the physical transfer of information, MPs can act a circulatory signalling molecules. Bioactive membrane molecular can induce a specific response in a target cell. An example includes, MPs with exposed CD40L can promote intraplaque neovascularization and plaque vulnerability via endothelial cell proliferation (Leroyer *et al.*, 2008). All these processes can result in a multifaceted response of MPs including inflammation, thrombosis and bleeding and endothelial dysfunction (VanWijk *et al.*, 2003) (Figure 40).

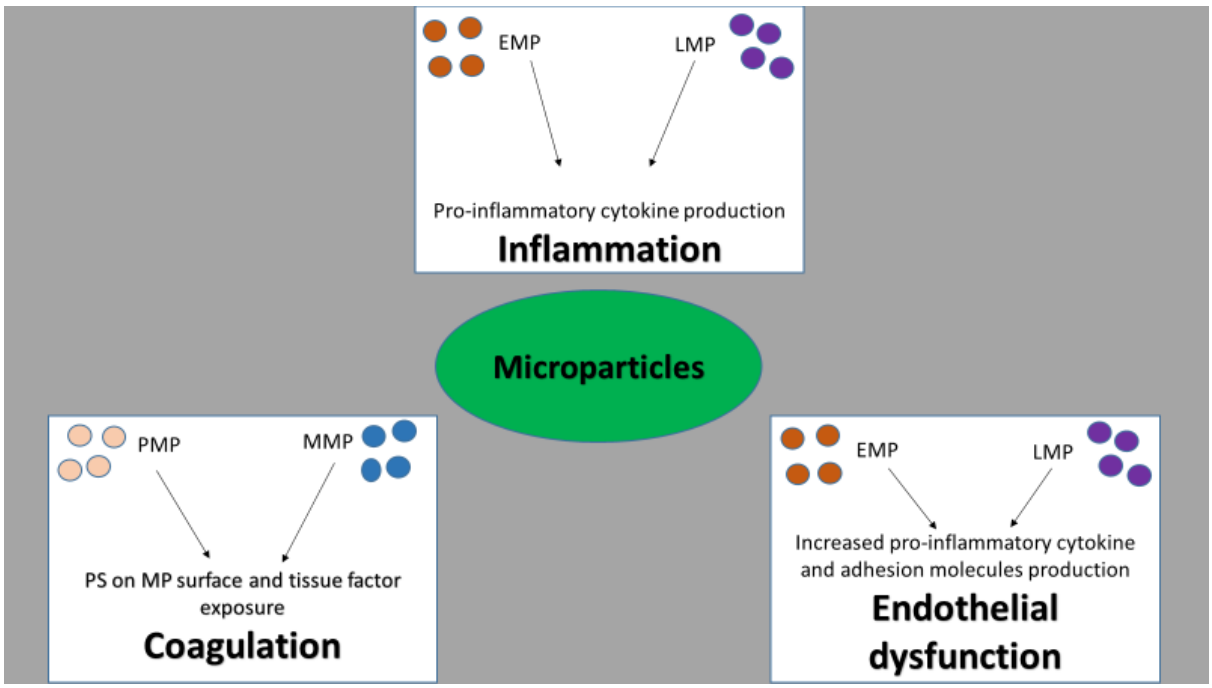


Figure 35: Schematic presentation of microparticle function. (Adapted from (VanWijk *et al.*, 2003)

### *4.3.7 Pre-analytical and analytical protocols for microparticle characterization*

Understanding in the role of MPs in the physiology and pathology of a disease process has been gaining interest. However, MP isolation and characterization has remained problematic due to lack of standardisation. Various studies have used multiple methods. These variations have shown to affect MP analysis and quantification (Jayachandran *et al.*, 2012). There has been a call by International Society on Thrombosis and Haemostasis (ISTH) to address these issues. However, significant variation still remained in the pre-analytical and analytical (Lacroix *et al.*, 2013).

It has been recognised that it is important to prevent the production of MP generation after blood collection by preventing ex vivo activation. It has been recommended to use a large bore needle ranging 19-22 Gauge and to avoid prolonged use of a tourniquet. First, few millilitres of blood should be discarded (Yuana *et al.*, 2010).

Sodium citrate (3.2% or 3.8%) is the most common anticoagulant used for blood collection. Other anticoagulation used are ethylenediaminetatraacetic acid (EDTA), heparin and Acid-Citrate-Dextrose (ACD) (Yuana *et al.*, 2010). Annexin V positive MPs

has been shown to be higher in samples collected in heparin than blood collected in sodium citrate or EDTA (Jayachandran *et al.*, 2012). The blood should be processed immediately after collection to prevent generation of MP (Lacroix *et al.*, 2013).

Next important step is plasma isolation. It is important to remove platelets from plasma to avoid generation of MP via cellular activation. Two-step centrifugation method has been proposed to collect platelet-free plasma (PFP). As seen in Table 16, various studies have used a different protocol. Standardising this step is important. Higher centrifugation rate can be too vigorous leading not only to depletion of platelets but also some MP. A lower centrifugation rate will result in a plasma sample still containing platelets (Lacroix *et al.*, 2013). The samples should be centrifuged at room temperature (20-25), especially for citrated blood. Lower temperature can activate citrated blood leading to elevated levels of factor VII and factor VIII. Unfortunately, this has not been investigated adequately (Yuana *et al.*, 2010). Therefore, there is a need to explore the effect of temperature on MP during centrifugation.

It is ideal to use freshly isolated PFP for MPs measurement (Yuana *et al.*, 2010). However, it is not feasible in most cases to perform MP enumeration at the same time. Similar was the case in our study. We had to plan for blood sampling logistics, transport time and most importantly processing of different assays like cell culture. We used available evidence and directly froze our samples at -80$^0$C (Yuana *et al.*, 2010). However, other publications suggested to snap freeze the plasma first in liquid nitrogen followed by storing the samples at -80$^0$C (Lacroix *et al.*, 2013).

There is concern that freezing-thawing of plasma can affect the enumeration of MPs. Mobarrez *et al.* (2010) have demonstrated that freezing and thawing can increase PMP count by ten folds. However, samples used was platelet-poor plasma (Mobarrez *et al.*, 2009). Studies using PFP have shown no major effect on MP count due freezing and thawing of the PFP samples (Jayachandran *et al.*, 2012).

Various qualitative and quantitative methods have been reported in the available literature. All these methods vary widely and have their benefits and limitations. The

commonly used method is flow cytometry. It allows for the enumeration of MP numbers and also exploring the cellular origins including the subtypes. Furthermore, the analysis is quicker (Yuana *et al.*, 2010). In order to improve defining MP population by size, beads of 1 um in diameter are used to define the upper limit of MPs. This helps in differentiating MP from bigger plasma constituents like platelets (Kim *et al.*, 2002). However, use of flow cytometry needs to be done with understanding the limitations of this technique. The variable includes the use of laser, antibody, instruments and its calibration (Yuana *et al.*, 2010). Our analysis was repeatedly interrupted by the maintenance of the instrument. We were unable to analyse one of our group due to changes in the machine.

Other methods include electron and fluorescence confocal laser scan microscopy, capture based assay, proteomic analysis, impedance-based flow cytometry, dynamic light scattering and nanoparticle tracking analysis and atomic force microscopy. Each method has some advantages and limitations. Therefore, the research question should guide the choice of technique. (Yuana *et al.*, 2010).

| Blood collection | Plasma processing | | | Second centrifugation steps | | | Storage | Reference |
|---|---|---|---|---|---|---|---|---|
| **Anticoagulation** | **Centrifugation (g)** | **Time (Min)** | **Temp (C)** | **Centrifugation (g)** | **Time (Min)** | **Temp (C)** | | |
| **Citrate** | 1500 | 10 | 20 | 13000 | 10 | 20 | -80 | (Shet *et al.*, 2003) |
| **EDTA** | 1200 | 15 | 20 | 12000 | 12 | 20 | -70 | (Aras *et al.*, 2004) |
| **Citrate** | 1500 | 15 | RT | 13000 | 2 | RT | -80 | (Dignat-George *et al.*, 2002) |
| **Citrate** | 160 | 10 | | 1000 | 6 | | | (Pirro *et al.*, 2006) |
| **EDTA** | 1500 | 15 | | 4000 | 10 | | -80 | (Bernal-Mizrachi *et al.*, 2003) |
| **Citrate** | 1500 | 10 | | | | | | (Preston *et al.*, 2003) |
| **Citrate** | 500 | 15 | RT | 10000 | 5 | RT | | (Amabile *et al.*, 2008) |
| **Citrate** | 11000 | 2 | | | | | -80 | (Amabile *et al.*, 2005; Boulanger *et al.*, 2005) |
| **Citrate** | 160 | 10 | | 1000 | 10 | | | (Esposito *et al.*, 2006) |
| **Citrate** | 860 | 15 | 4 | 1700 | 5 | 20 | -80 | (Amabile *et al.*, 2014) |
| **Citrate** | 160 | 10 | | 15000 | 30 | | | (Venable *et al.*, 2014) |
| **EDTA** | 2500 | 15 | | | | | -80 | (Hachulla *et al.*, 2010)Duval et al. 2010 |

Table 16: Different methods used to extract platelet free and platelet poor plasma.

### *4.3.8 Microparticle in healthy individuals and physiological variation*

Level of MPs are lower in healthy individuals when compared to individuals with the metabolic or cardiovascular disease. PMPs form around 70% of the circulating MPs (Berckmans *et al.*, 2001). It is important to note that MPs in healthy individuals have an anticoagulant function. It was hypothesised that PMP causes low levels thrombin generation leading to activation of protein C (Berckmans *et al.*, 2001). MP levels are higher in women when compared to men. The level of MPs is modulated with the menstrual cycle (Toth *et al.*, 2007). In contrast to this, Nielsen *et al.* 2014 showed that men had higher MP levels (especially EMP and MMP) than women (Nielsen *et al.*, 2014). Women in Nielsen *et al.* (2014) were older and in peri-menopausal range. Furthermore, there was the methodological difference in the analysis of MP. These differences could explain the conflicting results. Post-menopausal women have lower PMP levels than pre-menopausal women. EMP levels are similar in both pre and post-menopausal women (Rank *et al.*, 2012). During pregnancy, MP levels increase gradually during each trimester. MPs are the highest in the third trimester (Radu *et al.*, 2015).

Few studies have analysed differences in MP levels due to age. Forrest *et al.* (2019) demonstrated lower EMP levels in elderly whereas levels of PMP, RBC-MP or total MP were similar (Forest *et al.*, 2009). (Emmerechts *et al.*, 2012) results were in agreement with Forrest *et al.* 2010. They did not evaluate EMP levels.

Moderate and strenuous exercise has been shown to alter MP levels. PMP and LMP levels are increased in strenuous exercise (Chaar *et al.*, 2011). Acute exercise has shown to increase EMPs in men and CD34+MP in women (Lansford *et al.*, 2015). Sossdorf *et al.* 2010 demonstrated that there was a difference in MP kinetic and dynamic release and the clearance between trained and untrained individuals. At baseline levels of MP and their pro-coagulant activity was similar. An acute bout of exercise raised levels of PMP, EMP and LMP in trained individuals but levels returned to normal after 2 hours. In an untrained individual, only PMP levels increased significantly. Furthermore, levels did not return to the baseline (Sossdorf *et al.*, 2010).

### *4.3.9 Microparticle and diabetes mellitus*

It is well established that MP levels are altered in DM. From the available evidence, we can infer that MP levels are elevated in diabetes mellitus. Though, studies have reported different MP profile in DM. We could only identify two studies conducted in patients with T1DM (Dignat-George *et al.*, 2002; Salem *et al.*, 2015). T1DM and T2DM have a different profile of altered MP levels. In T2DM, only total microparticle (TMP) levels are raised. However, TMP, PMP and EMP are raised in T1DM (Dignat-George *et al.*, 2002). MP levels have been shown to correlate with diabetes-related complications like nephropathy or retinopathy or foot disease (Nomura *et al.*, 2004; Ogata *et al.*, 2005; Tsimerman *et al.*, 2011). EMP levels have been shown to be an independent predictor of CAD in patients with DM (Koga *et al.*, 2005). Table 17 summarise finding of studies in diabetes mellitus.

| Published evidence | Study subjects | Results |
|---|---|---|
| (Berezin *et al.*, 2016) | T2DM with and without atherosclerosis vs. HC | No change between DM vs HC: (CD41a+, CD64+, CD144+, CD144+/CD31+, Annexin V+, CD144+/annexin V+ and CD144+/CD31+/annexin V+).<br>Lower number of MPs with immune phenotypes CD62E+, CD105E+ and higher numbers of CD31+/annexin V+ MPs in DM vs HC<br>Increased circulating CD41a+ MPs, CD144+/CD31+ MPs, CD31+/annexin V+ MPs, and decreased CD62E+ MPs in T2DM patients with asymptomatic coronary atherosclerosis<br>Apoptotic EMP higher in DM vs HC<br>Activated EMP lower in DM vs HC<br>CD31+/Av increased and CD62E decreased in DM with asymptomatic atherosclerosis |
| (Chen *et al.*, 2012) | T2DM with and without HTN vs. HC | EMP, PMP, Total MP and LMP higher in T2DM vs. HC<br>EMP higher in T2DM with HTN. EMP is associated with impaired artery elasticity in DM. |
| (Omoto *et al.*, 2002) | T2DM vs. HC | MMP and PMP higher<br>MMP is higher in patients with vascular complications. esp. nephropathy |
| (Nomura *et al.*, 2004) | T2DM vs. HC | EMP and PMP higher<br>EMP higher with complication esp. nephropathy |
| (Nomura *et al.*, 2009) | HTN with and without T2DM | PMP, EMP and MMP higher in T2DM |
| (Jung *et al.*, 2011) | T2DM with and without macroangiopathy and microangiopathy | EMP higher in macroangiopathy |
| (Dignat-George *et al.*, 2002) | T1DM and T2DM | PMP, EMP and TMP in T1DM<br>TMP in T2DM |
| (Salem *et al.*, 2015) | T1DM vs. HC | PMP elevated<br>Early marker of micro-vascular complications and subclinical atherosclerosis |

| Published evidence | Study subjects | Results |
|---|---|---|
| (Omoto *et al.*, 1999) | T2DM | PMP higher |
| (Ogata *et al.*, 2005; Ogata *et al.*, 2006) | T2DM with retinopathy | PMP is higher. Levels increased with retinopathy progression<br>MMP higher with progression of retinopathy |
| (Nomura *et al.*, 1995) | DM | PMP higher<br>Higher in DM patients with high LDL |
| (Feng *et al.*, 2009) | T2DM | PMP, EMP, LMP TMP higher in T2DM<br>EMP correlated negatively with FMD and pulse wave velocity |
| (Bernard *et al.*, 2008) | T2DM with coronary non-calcified plaque | EMP increased in T2DM with ACS. EMP is higher in non-calcified disease vessel. |
| (Koga *et al.*, 2005) | T2DM | EMP levels are higher in T2DM especially in patients at risk of CAD |
| (Diamant *et al.*, 2007) | T2DM and three high fat mixed meal | EMP increased with meals and correlated negatively with EF. |
| (Esposito *et al.*, 2006) | T2DM with erectile dysfunction | EMP higher in T2DM with erectile dysfunction when compared to HC without erectile dysfunction |
| (Tsimerman *et al.*, 2011) | T2DM with CAD, retinopathy and foot ulcer vs. HC | EMP and PMP highest in patients with a severe diabetic foot ulcer. |
| (Lumsden *et al.*, 2013) | T2DM with ACS vs. HC | 40% lower EMPs in patients. |
| (Diamant *et al.*, 2002) | T2DM vs. HC | TF+MP 2X higher in T2DM. Higher % of TF+MP comes from platelets, granulocytes and T-helper cells. |

Table 17: Some of the available evidence relating T1DM and T2DM and microparticles.

### *4.3.10 Method*

#### *4.3.10.1 Platelet-free plasma isolation*

After an overnight fast, peripheral blood was collected in citrate vacutainer tubes. Blood samples were processed within four hours of collection. Samples were centrifuged for 15 minutes at 500 x*g*. The upper, clear fraction (platelet rich plasma, PRP) was collected without disturbing the bottom cellular layer and transferred to 1.5mL polypropylene tubes. These PRP samples were further centrifuged for 5 minutes at 13000 x *g* in a microcentrifuge. After centrifugation, the clarified platelet-free plasma (PFP) was transferred to fresh 1.5mL polypropylene tubes and stored at -80ºC for subsequent analysis. The platelet pellet was discarded.

#### *4.3.10.2 Flow cytometry protocol for enumeration of microparticles*

BD LSR II instrument with five customised lasers (blue 488nm, red 635nm, violet 405nm, green 532nm and UV 355nm) was used for MPs enumeration.

PFP was thawed and went for ultracentrifugation at 13000g x 5 minutes before antibody incubation. Two sets of Trucount tubes, each containing 200µl of the PFP was stained with Antibodies set 1 (Table 18) for EMP and PMP and Antibodies set 1 (Table 19) for PC-derived MP and EPC MP. Both tubes underwent 30 minutes incubation in the dark.

| **Specimen: Platelet-free plasma** | | | |
|---|---|---|---|
| **Antibodies** | **Fluorochrome** | **Volume added** | **Manufacturer** |
| **Annexin V** | PE | 5µl | BD Biosciences |
| **CD31** | AlexaFlour 647 | 20µl | BD Biosciences |
| **CD41a** | PerCPCy5.5 | 20µl | BD Biosciences |
| **CD144** | FITC | 20µl | BD Biosciences |
| **CD62e** | BV421 | 5µl | BD Biosciences |

Table 18: Antibodies set 1 for EMP and PMP.

| Specimen: Platelet-free plasma | | | |
|---|---|---|---|
| **Antibodies** | **Fluorochrome** | **Volume added** | **Manufacturer** |
| **Annexin V** | PE | 5µl | BD Biosciences |
| **CD45** | V500 | 5µl | BD Biosciences |
| **CD34** | PerCPCy5.5 | 20µl | BD Biosciences |
| **CDVEGFR2 (KDR)** | FITC | 20µl | R&D systems |

Table 19: Antibodies set 2 for PC-derived MP ($CD34^+$ MP), EPC MP and LMP.

Prior to analysing the study samples, optimisation was carried out. Compensation and gating strategy for were refined with support from flow cytometry department, which is discussed below.

#### *4.3.10.3 Optimising compensation strategy for enumeration of microparticles*

The MP gate was established via preliminary experiment using 1 micron fluorescent beads plotted on forward and side scatter. Threshold was set up to acquire Annexin V+ events. True events for MPs were defined as particles that were positive for Annexin V and less than 1 µm in diameter. Compensation was done to make sure there is no overlap of the florescence channels (e.g., FITC can spill over into the PE channel). Once the compensation was achieved using each florescence antibody with Annexin V than all the fluorochromes were added and analysed together. Adequate compensation was deemed once each fluorochromes florescence was similar to fluorochromes florescence run separately.

Samples were run with stopping gate for atleast 20,000 <1micron events. Analysis was also performed using BD FACS DIVA™ software, with EMP events defined as: Annexin-$V^+CD31^+CD41a^-$. Annexin-$V^+CD31^+$, Annexin-$V^+CD62e^+$ and Annexin-$V^+CD144^+$ events were also gated for EMP phenotype study. PMP were defined as Annexin-$V^+CD31^+CD41a^+$. PC-derived MP and EPC MP events were defined as Annexin-$V^+CD34^+$, and Annexin$^-V^+CD34^+VEGFR2^+$. Lymphocyte derived MPs were defined as Annexin-$V^+CD45^+$.

Gating strategy to define above mentioned MP population is presented in Figure 41 and 42.

MP enumeration for standard group (SG) was not performed due to changes in the setting and lasers of BD LSR II instrument. If we had analysed the sample then we would not have been able to compare SG data with TG and/or HC.

#### *4.3.10.4 Determination of MP per µl*

Events per µl were counted using the following formula:

$$\frac{Events}{\mu l} = \frac{number\ of\ events\ in\ a\ certain\ gate}{number\ of\ beads} \times \frac{total\ number\ of\ beads}{volume\ of\ sample\ (\mu l)}$$

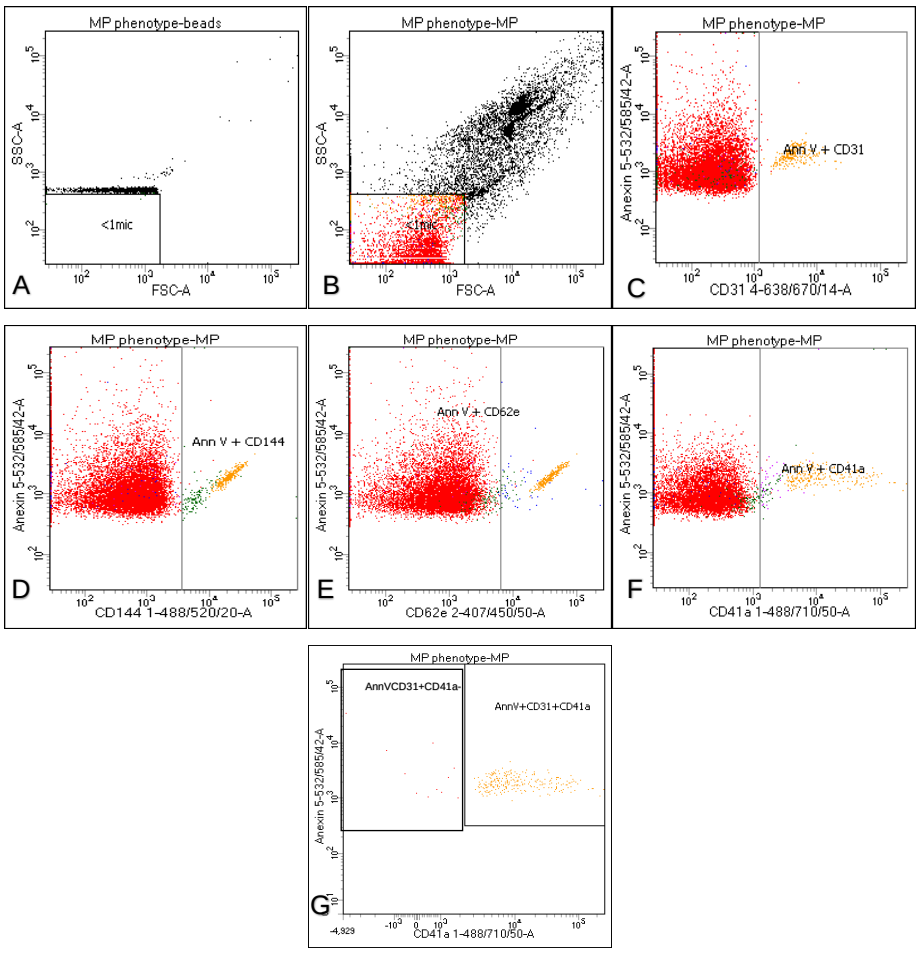


Figure 36: Gating strategy for EMP and PMP quantification.

**A.** 0.88 micron latex beads were ran to estimate the gating for <1µm events; **B.** <1µm events gated; **C.** threshold were set for Annexin V positive events and were gated for CD31+; **D and E.** Endothelial MPs (EMP) events were further gated for CD144+ and CD62e+; **F and G.** Annexin V+, CD31+ and CD41a+ showing platelet-derived MPs (PMP) contributing to majority of microparticle events and events positive for Annexin V and CD31 were gated for CD41a- to exclude PMP population.

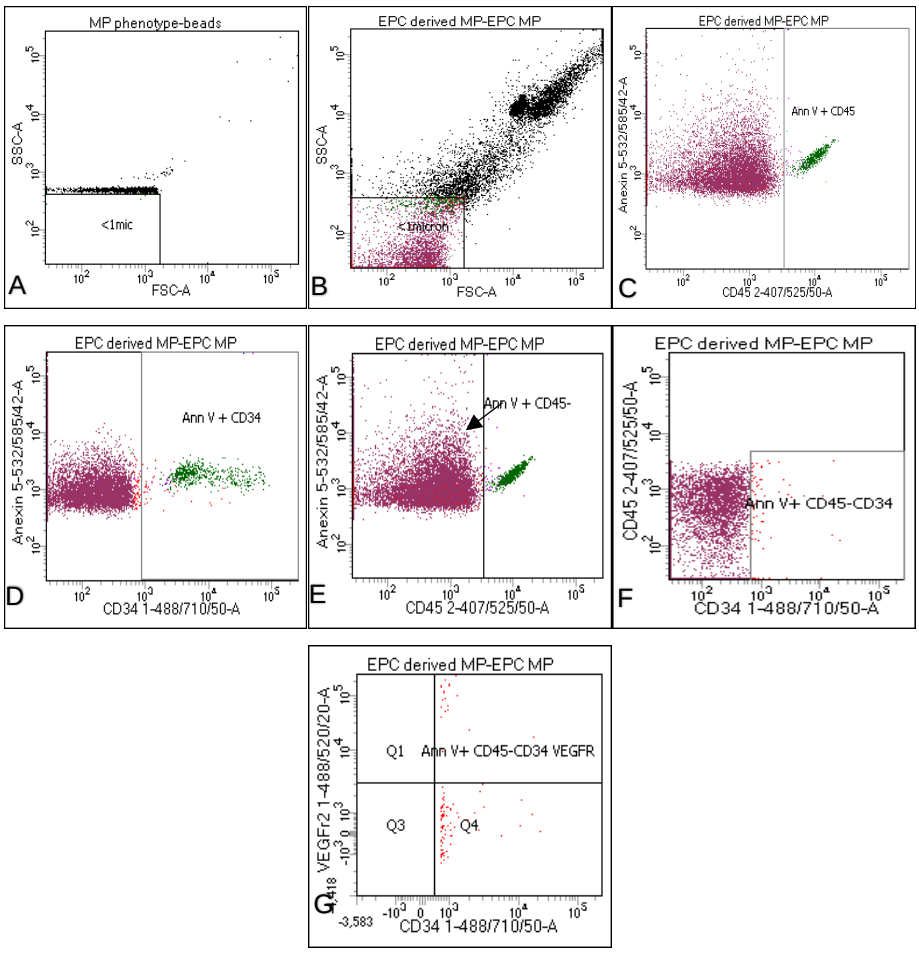


Figure 37: Gating strategy for EMP and PMP quantification.

**A.** 0.88 micron latex beads were ran to estimate the gating for <1µm events; **B.** <1µm events gated; **C.** threshold were set for Annexin V positive events and were gated for CD45+ (Lymphocytic MP); **D** CD34+ events were further gated; E**.** Annexin V positive events and were gated for CD45-; **F.** AnnexinV$_+$CD45$_+$ was plotted against CD34 to gate for CD34$_+$ events; **G**. AnnexinV$_+$CD45$_+$CD34$_+$ was plotted against CD34 and VEGFR2 and gated for AnnexinV$_+$CD45$_+$CD34$_+$VEGFR2$_+$.

### *4.3.11 Specific aim*

In this section, we aimed to evaluate if metformin improved the following in type 1 diabetes when controlled for glycaemic changes:

1. Numbers of EMP,
2. Numbers of PMP,
3. Numbers of LMP, and
4. Numbers of $CD34^{+}$ and EPC MP.

### *4.3.12 Results*

All the EMP phenotypes (AnnexinV$^{+}$CD31$^{+}$, AnnexinV$^{+}$CD31$^{+}$CD41$^{-}$, AnnexinV$^{+}$CD62e$^{+}$ and AnnexinV$^{+}$CD144$^{+}$), PMPs (AnnexinV$^{+}$CD31$^{+}$CD41$^{+}$), LMP (AnnexinV$^{+}$CD45$^{+}$), AnnexinV$^{+}$CD34$^{+}$ and AnnexinV$^{+}$CD45$^{dim}$CD34$^{+}$VEGFR2$^{+}$ were significantly higher in treatment group (TG V1) at baseline when compared to healthy control. In treatment group eight weeks of metformin treatment did not change any of the EMP phenotypes, PMPs, LMPs, AnnexinV$^{+}$CD34$^{+}$ and AnnexinV$^{+}$CD45$^{dim}$CD34$^{+}$VEGFR2$^{+}$. After metformin treatment, EMP phenotypes, PMPs, LMPs, AnnexinV$^{+}$CD34$^{+}$ and AnnexinV$^{+}$CD45$^{dim}$CD34$^{+}$VEGFR2$^{+}$ remained significantly higher when compared to healthy control (Table 20).

| | **TG V1** | **TG V2** | **TG v1 vs TG V2 p-value** | **HC** | **HC vs TG V1 p-value** | **HC vs TG V2 p-value** |
|---|---|---|---|---|---|---|
| **AnnexinV$^{+}$CD31$^{+}$** per µl mean+/-SE | 3.2+/-0.3 | 3+/-0.35 | 0.5 | 0.8+/-0.15 | <0.0001 | <0.0001 |
| **AnnexinV$^{+}$CD31$^{+}$CD 41$^{-}$** per µl median (IQ) | 0.24 (0.17-0.75) | 0.17 (0.1-0.3) | 0.3 | 0.08 (0.06-0.1) | <0.0001 | 0.0003 |
| **AnnexinV$^{+}$CD62e$^{+}$** per µl mean+/-SE | 3.7+/-0.4 | 3.4+/-0.4 | 0.4 | 0.9+/-0.2 | <0.0001 | <0.0001 |
| **AnnexinV$^{+}$CD144$^{+}$** per µl mean+/-SE | 3.9+/-0.5 | 3.6+/-0.4 | 0.4 | 0.9+/-0.15 | <0.0001 | <0.0001 |
| **AnnexinV+CD31+C D41+** per µl mean+/-SE | 2.9+/-0.3 | 2.7+/-0.3 | 0.4 | 0.7+/-0.2 | <0.0001 | <0.0001 |
| **AnnexinV+CD45+** per µl mean+/-SE | 5.9+/-0.8 | 5.5+/-0.8 | 0.7 | 2+/-0.4 | 0.0003 | 0.0001 |
| **AnnexinV+CD34+** per µl mean+/-SE | 5.8+/-0.8 | 5.5+/-0.8 | 0.7 | 2.2+/-0.4 | 0.0001 | 0.0008 |
| **AnnexinV+CD45di mCD34+VEGFR2+** per µl median+/-SE | 0.09 (0.03-0.3) | 0.09 (0.03-0.2) | 0.4 | 0.07 (0.035-0.09 | 0.1 | 0.3 |

Table 20: Microparticle comparing treatment group pre-metformin (TG V1), post-treatment and healthy control (HC). Results are given as per µl.

#### 4.3.12.1.1 CD62e$^{+}$ to CD31$^{+}$ ratio

The ratio of CD62E+/CD31+ EMP populations has been used to identify if generation of EMPs were caused via activation versus apoptosis. A ratio ≥10 identifies activation while ratio ≤1.0 identifies apoptosis (Tramontano *et al.,* 2010). CD62e$^{+}$ to CD31$^{+}$ ratio was similar in TG and healthy control (1.3+/-0.1, 1.2+/-0.02 and 1.4+/-0.1 respectively: TG V1 vs. HC: p=0.1; TG V1 vs. TG V2: p=0.4; TG V2 vs. HC: p=0.2).

#### 4.3.12.1.2 EMP to cEPC ratio (AnnexinV$^{+}$CD31$^{+}$CD41$^{-}$ to CD45$^{dim}$CD34$^{+}$VEGFR2$^{+}$ ratio)

The ratio of EMP to cEPC ratio (AnnexinV$^{+}$CD31$^{+}$CD41$^{-}$ to CD45$^{dim}$CD34$^{+}$VEGFR2$^{+}$ ratio) provides information regarding balance between vascular injury and repair (Pirro *et al.,* 2006). Increased ratio is associated with early atherosclerosis (Pirro *et al.,* 2006). EMP to cEPC ratio was significantly higher in treatment group (TG V1) at baseline when compared to healthy control (TG V1 vs HC: 2.3+/-0.5 vs 0.3+/-0.04 per µl: p=0.0005). In treatment group eight weeks of metformin treatment significantly reduced EMP to the cEPC ratio (TG V1 vs. TG V2: 2.3+/-0.5 vs. 1.3+/-0.5 per µl; p=0.04). Even after reduction of EMP to the cEPC ratio in the treatment group after eight weeks of metformin, EMP to cEPC ratio remained significantly higher when compared to healthy control (TG V2 vs. HC: 1.3+/-0.5 vs. 0.3+/-0.04 per µl: p<0.05).

### *4.3.12.2 EPC microparticles to cEPC ratio*

The ratio of EPC MP to cEPC (AnnexinV$^{+}$CD45$^{dim}$CD34$^{+}$VEGFR2$^{+}$ to CD45$^{dim}$CD34$^{+}$VEGFR2$^{+}$ ratio) provides information regarding proportion of cEPC destruction. EPC to cEPC ratio were significantly higher in treatment group (TG V1) at baseline when compared to healthy control (TG V1 vs. HC: 0.6 (0.17-1.6) vs. 0.14 (0.08-0.35) per µl: p=0.002). In treatment group, eight weeks of metformin treatment reduced the EPC MP to cEPC ratio significantly (TG V1 vs. TG V2: 0.6 (0.17-1.6) vs. 0.4 (0.07-0.9) per µl; p=0.009). After metformin treatment, EPC MP to cEPC ratio phenotypes became non-significant when compared to healthy control (TG V2 vs. HC: 0.09 (0.03-0.2) vs. 0.07 (0.035-0.09) per µl: p=0.17).

#### *4.3.12.3 Correlation*

In this section, we will only present important correlation data discussed below. Full correlation data is presented in Appendix A.

At baseline, $CD34^+$ EMP was directly associated with $CD31^+$ EMP ($r=0.5$; $p<0.05$) and PMP in TG ($r=0.54$; $p<0.01$).

At baseline, PMP was directly associated with LMP in TG ($r=0.54$; $p<0.01$).

### *4.3.13 Discussion*

Markers of vascular damage were assessed at baseline and after eight weeks of metformin treatment. These were compared with healthy controls. MPs were not evaluated in standard treatment group-SG to explore if any changes in above-mentioned markers could be observed with the routine follow up without any treatment with metformin. Individual findings will be discussed first followed by a summary of overall findings.

#### *4.3.13.1 Increased EMP phenotypes, PMPs and EMP/cEPC ratio in type 1 diabetes*

We assessed different EMP phenotypes (AnnexinV+CD31+, Annexin V+CD31+CD41-, Annexin V+CD144+ and Annexin V+CD62e+ MP), PMP (Annexin V+CD34+CD41+ MP), LMP (Annexin V+CD45+ MP) and PC-MP and EPC-MP (Annexin V+CD34+, Annexin V+CD45dimCD34+VEGFR2+ MP) in TG and HC.

##### 4.3.13.1.1 Main findings

Main findings were:

1. All EMP phenotypes, PMPs, LMPs and EMP/cEPC ratio were higher in TG when compared to healthy control.
2. Progenitor cell MPs in T1DM were higher when compared to healthy controls. Though, EPC MP levels in T1DM were similar to healthy controls. However, EPC MPs and the cEPC ratio were significantly higher in type 1 diabetes than in healthy controls.
3. AnnexinV$^{+}$CD34$^{+}$ MPs were directly associated with AnnexinV$^{+}$CD31$^{+}$ and PMPs in TG (Appendix A).
4. PMPs were directly associated with AnnexinV$^{+}$CD45$^{+}$ MPs in TG.

#### 4.3.13.1.2 Novelty

For the first time, we have studied an extensive array of MPs including phenotypes of EMP, PMP, LMP, PC-MP, EPC MP in T1DM and compared with HC. This make our study unique in providing broad information regarding MP levels in T1DM. We have shown for the first time that EPC MP levels were similar in T1DM and HC. However, the ratio of EPC MP and cEPC was significantly higher demonstrating an increased generation of EPC MP in T1DM when compared to HC.

#### 4.3.13.1.3 Prior work

Our data was consistent with the previous report of Sabatier *et al.* (2002) who showed increased EMPs and PMPs in type 1 diabetes. Different studies in diabetes have shown that not all the different phenotypes of EMP were higher. However, all of these studies included type 2 diabetes patients. It had been shown that different phenotypes of MPs were elevated in type 1 and type 2 diabetes (Dignat-George *et al.*, 2002). This difference in EMP phenotype might show that there were different mechanisms of MP production in T1DM.

PMP and LMP have been shown to be higher in T1DM (Dignat-George *et al.*, 2002; Salem *et al.*, 2015). PMPs have been shown to be higher in patients with diabetes-related microvascular complication and microalbuminuria. Furthermore, PMP levels are directly related to the severity of diabetic retinopathy.

#### 4.3.13.1.4 Mechanism

High EMP levels in T1DM as shown in our study not only demonstrated ongoing endothelial damage but put these patients at risk of developing vasculopathy as demonstrated by the extensive available evidence in diabetes and other conditions (Table 10 and 15). EMPs are associated with increased macroangiopathy in type 2 diabetes (Jung *et al.*, 2011) and positively correlated with CV risk factors (Amabile *et al.*, 2014). Furthermore, high Annexin $V^+CD31^+$ MPs are linked with increased CV events in stable CAD patients (Sinning *et al.*, 2011). Annexin $V^+CD31^+CD41^-$ MPs is an independent predictor of CV mortality in ESRF (Amabile *et al.*, 2012). Annexin

$V^+CD144^+$ MP is negatively associated with impaired FMD (Tushuizen *et al.*, 2007). Annexin $V^+CD62e^+$ numbers are negatively correlated with endothelial function in Kawasaki disease (Ding *et al.*, 2014). Annexin $V^+CD62e^+$ levels are linked with poor outcome in pulmonary hypertension and stroke (Amabile *et al.*, 2009; Lee *et al.*, 2012).

It has been shown that apoptosis is an important mechanism in production of EMPs in T2DM (Tramontano *et al.*, 2010). However, our data did not show that apoptosis was the major factor in MP production. High $CD31^+$ and $CD62e^+$ in our study suggested that in addition to apoptosis, activation played an important role.

MPs in poorly controlled T1DM patients have a higher pro-coagulant activity. This may account for increased thrombogenicity in T1DM. HbA1c has been positively associated with the pro-coagulant activity of MP (Dignat-George *et al.*, 2002). Our T1DM patients had good overall diabetes control. There is a need to explore if good diabetes control has an effect on pro-coagulant activity of MP in T1DM.

We have shown that PMP and LMP were higher in T1DM than in HC. It has been proposed that PMPs can stimulate coagulation cascade by increasing adhesion of leukocytes and endothelial cells (Ogata *et al.*, 2005). Our data showed that PMPs are directly associated with Annexin$V^+CD45^+$ MP. The interaction between activated platelets, PMPs, LMPs and endothelial cells are important in the early stages of atherosclerosis (Puddu *et al.*, 2010). Patients with carotid atherosclerotic plaques have been shown to have higher PMP (Michelsen *et al.*, 2009). In children and adolescents with T1DM, PMPs are directly related to CIMT (Salem *et al.*, 2015).

EMPs are not only related to endothelial dysfunction but also has been shown to have an effect on EPC. In an *in vitro* condition, it has been demonstrated that EMPs from patients with hypercholesterolemia significantly increased EPCs apoptosis and reduced colony forming capacity (Pirro *et al.*, 2006). Similarly, EMPs from hypertensive with microalbuminuria/microalbuminuria reduced EPC function and increased EPC death/apoptosis (Huang *et al.*, 2010a). EMPs and PMPs have been shown to be inversely related to EPCs (Pirro *et al.*, 2006). This is in contrast to our finding of no

correlation between EMP and PMPs with cEPC in T1DM. Even though there was no correlation, our data of high EMPs and low cEPCs complements Pirro *et al.* (2006) finding in patients with CVD risk factor. Thereby, causing an imbalance between endothelial repair and damage. Furthermore, we have shown that increased EPC MP to cEPC ratio in T1DM than in HC. This finding demonstrated an increased microparticle production from cEPCs and progenitor cells.

#### *4.3.13.2 Metformin has no effect on EMPs, PMP and cEPC MPs but decreases EMP to cEPC and EPC MP to cEPC ratio*

##### 4.3.13.2.1 Main findings

Eight weeks of metformin treatment

1. Did not decrease EMP and their phenotypes, PMP, LMPs and EPC-derived MPs in T1DM.
2. Showed a significant reduction in EMP to cEPC ratio in TG.

##### 4.3.13.2.2 Novelty

For the first time, we explored the effect of metformin treatment on the array of MPs in T1DM independent of glycaemic control. Our data provided evidence that eight weeks of metformin treatment in T1DM shifted the focus towards vascular repair from vascular damage. However, future studies need to explore if this yield long term CV benefit in T1DM.

##### 4.3.13.2.3 Prior work

Our results are similar to Esposito *et al.* 2011 who showed that 24 weeks of metformin treatment in newly diagnosed type 2 diabetes did not improve EMPs. However, no change in the cEPC count was also noted (Esposito *et al.*, 2011). As mentioned above, we demonstrated a significant increase in cEPC numbers. So far, no study has explored the effect of metformin on the wide array of MPs in T1DM.

#### 4.3.13.2.4 Mechanism

Our study was not powered to explore MPs. This is the most likely reason we did not see the difference in MPs in our study. Other reasons for no change in EMPs after metformin treatment can be explained by the method of MP enumeration and the retrospective nature of MP assessment. We gated all our MPs on Annexin V. This meant that we did not enumerate Annexin V negative MPs. It has been shown previously that not all MP are Annexin V positive. It is speculated that some MPs do not externalise phosphatidylserine or not bind Annexin V (Shet *et al.*, 2003). MP can be altered at any stage of enumeration including centrifugation, storage and thawing (Piccin *et al.*, 2006). It has been shown that freezing and storage duration can effect MP numbers and phenotypes (van Ierssel *et al.*, 2010). Centrifugation, storage condition and thawing was similar during pre- and post-treatment. Each patient samples pre- and post-treatment were analysed together. The difference in the duration of storage between pre- and post-treatment is eight weeks. We believe that stages of enumeration cannot completely explain our results of no effect of metformin on EMP numbers.

In our study, cEPC numbers increased significantly after metformin treatment. As a consequence, EMP to cEPC ratio showed a significant reduction. Thereby, shifting the balance from endothelial damage to improved repair capacity. A previous intervention study in type 2 diabetes patients had demonstrated that pioglitazone decreased EMP to the cEPC ratio (Esposito *et al.*, 2011). Furthermore, our assessment of cEPC MP to cEPC ratio showed a significant reduction. We can infer from these finding that metformin not only increased cEPC numbers but also reduced MP generation from EPCs.

### *4.3.14 Conclusion*

In conclusion, MPs numbers are higher in T1DM except for EPC-MP. Metformin treatment did not change the levels of MP in T1DM. It may be the metformin can have an effect on MP biological function by shifting the focus from vascular damage to repair. However, this theory needs to be robustly investigated. This can include in vitro experiment exploring if MP from metformin-treated patients can improve endothelial

cell survival. Further extension of this work can include an animal model to assess if MP from metformin-treated endothelial cells can improve angiogenesis and mobilisation of MPs.

## 4.4 Summary

In this section strengths, limitations and clinical/research implications will be discussed.

### *4.4.1 Strengths*

In our knowledge, this is the first study exploring all of the above-mentioned markers in one group of patients with type 1 diabetes. This is a major strength of our work. Most of the studies on type 1 diabetes have looked at one or two specific markers (Sibal *et al.*, 2009a; Esposito *et al.*, 2011). Our data gives a much better overview of underlying vascular repair and damage imbalance in patients with type 1 diabetes. Our study has corroborated finding of previous studies that the markers of vascular damage are significantly higher, and markers of vascular repair are significantly lower in type 1 diabetes compared to healthy match control (Sibal *et al.*, 2009a; Asicioglu *et al.*, 2010; Salem *et al.*, 2015).

### *4.4.2 Limitations*

Our work in this section could have been further strengthened if we had randomised equal numbers of SG and TG. In SG, we were not able to study MPs due to changes made to BD LSR II laser. This would have made comparing TG and SG impossible. Our data showed that overall diabetes control as assessed by HbA1c did not have any effect on the results of all the above markers in TG and SG. Glucose variability remained unchanged in TG. However, we did not study glucose variability in SG. If we had studied glucose variability in SG, it would have provided a better design to explore the effect of glucose variability on above-discussed markers.

### *4.4.3 Clinical and research implication*

We demonstrated that in patients with relatively well-controlled type 1 diabetes mellitus (mean HbA1c 7.3% or 56.4 mmol/mol), metformin therapy improved markers of vascular damage (cECs) and repair (cEPCs). Further evidence that our patients might benefit from metformin comes from the fact that CFU-Hills' colonies, PACs number and

adhesion properties improved significantly. It is well established that CFU-Hill colonies number are inversely related to Framingham risk score, and therefore CFU-Hill colonies are yet another predictor of CVD (Hill *et al.*, 2003b). In addition, PACs adhesion function is an important factor in cEPCs homing, cell-cell contact and transmigration events for neovascularisation and vascular repair (Urbich and Dimmeler, 2004b). Metformin not only improved the level of cEPCs but also brought PACs number/ adhesion and CFU-Hill colonies closer to HC levels. In addition, an inverse correlation between changes in cECs and CFU-Hill colonies shows that changes in markers of vascular/endothelial damage are linked inversely with a marker of CVD risk (CFU-Hill-colonies).

EMP levels did not improve with metformin treatment. Our results are similar to another study on type 2 diabetes patients (Esposito *et al.*, 2011). However, in contrast to Esposito *et al.* (2011), the EMPs/EPC ratio after metformin treatment reduced significantly. This could reflect a shift in balance towards endothelial repair capacity from endothelial damage. We also noted the reduction in cEPC MP numbers relative to increase in cEPC numbers. This could either mean increased consumption or decreased the production of cEPC MP. Increased consumption of cECP MP can be due to increase in interaction with EC. Thereby, increasing vascular repair and leading to reduction in cECs. However, this is speculative and needs further work to understand the mechanism of relative reduction of cEPC MPs.

Data in chapter 4 yields important information regarding the effect of metformin on cardiovascular markers. Our work can be meaningfully expanded to design a randomised controlled trial to further evaluate above studied markers. Our data can be used to calculate the number of subjects needed for the study to have adequate power. Furthermore, future work should define the upstream and downstream pathways effected by the physiological dose of metformin leading to changes in above-studied CV markers.

### *4.4.4 Conclusion*

We believe that this study may have positive clinical implication for patients with T1DM. Metformin treatment may provide potential CVD benefit by rebalancing the emphasis in their management from limiting damage alone to also improving vascular repair.

# Chapter 5: Effect of metformin on microRNA

## 5.1 Introduction

MicroRNAs (miR) are a family of small highly conserved endogenous non-coding single-stranded RNA that is produced in every animal and plant cells (Ambros, 2004; David *et al.*, 2004). They are approximately 22 (19-24) nucleotides in length (David *et al.*, 2004; Bhaskaran and Mohan, 2014). They play a significant role in the regulating post-transcriptional gene expression. miR binds the complementary target on messenger RNA leading to their degradation on inhibition of transcription. Also, this results in dysregulation of subsequent protein production. However, the exact mechanism is unknown (Bhaskaran and Mohan, 2014). miRs are thought to regulate approximately 30% of mRNA (Filipowicz *et al.*, 2008).

Before 1993, our understanding of protein synthesis was limited to transcription of DNA into RNA. This was then translated into protein. In 1993, the first miRNA lin-4 was discovered, and the importance of miR was revealed (Lee *et al.*, 1993).

Rasalind Lee and Rhonde Finbaum from Ambros laboratories discovered the first miRNA, Lin-4 in the nematode, Caenorhabditis elegans. Lin-4 controlled the larval transition from stage 1 and stage 2 thus affecting larval development. Lin-4 was not transcribed into mRNA, thus did not code for any protein. Lin-4 is transcripted into two single strands of 61 nucleotides (nt) and 21 nt lengths. Ambrose together with Ruvkun's laboratory demonstrated that Lin-4 transcripts were complementary in the 3' UTR of Lin-14. Lin-4 is a negative regulator of Lin-14 and reduces the amount of Lin-14 proteins (Lee *et al.*, 1993).

Finding of this new regulatory product spurred new research in this area of molecular biology. However, it took seven years till the second miR; Let-7 was discovered (Reinhart *et al.*, 2000). Let-7 controlled the larval transition from stage 4 to the adult stage. Let-7 miR family was found in other species including humans (Reinhart *et al.*, 2000). The presence of miR in other species resulted in a revolution in miR research. It was estimated that more than 800 miR genes are present in humans (Bentwich *et al.*, 2005). So far, 2588 miRs have been identified in humans as per miRbase database

(http://www.mirbase.org/) in their latest version 21 released in June 2014. Across all species so far, 28645 miRs have been discovered (Kozomara and Griffiths-Jones, 2014).

The reason behind the rapid expansion in numbers of miRNA is the fact that miRNA is now thought to play a pivotal role in a disease like cancer, CV disease, autoimmune disease, neurodegenerative disease and diabetes mellitus, etc. As a result of rapid expansion, there was the need to develop a database. This has led to multiple databases being set up like miRbase (Griffiths-Jones, 2004), Pictar (Krek *et al.*, 2005), microRNA.org (Betel *et al.*, 2008), Target Scan (Agarwal *et al.*, 2015), etc. To facilitate miRNA target prediction and interpretation of their role in disease, tools and software like miRanda (John *et al.*, 2004), DIANA Tool (Kiriakidou *et al.*, 2004), etc have been developed.

### *5.1.1 microRNA nomenclature*

So far a large number of microRNA has been discovered. Naming and identification of microRNA could have been a problem. However, to have consistent naming technique, researchers have agreed on a guideline (Ambros *et al.*, 2003).

Each microRNA is identified by a number attached to prefix miR like miR-126, miR-129, etc. Three or four-letter prefixes are used to identify the organism (example hsa in homa sapiens). Therefore, microRNA in humans will be named as hsa-miR-126. microRNA (miRNA) is named after the manuscript is accepted for publication. So for example, at the time of acceptance of manuscript last published, miRNA was miR-1011. Then the new miRNA in the manuscript will be named miR-1012. In case, a miRNA has been discovered in more than one species than it is advised that the numbers are kept the same (Ambros *et al.*, 2003).

'miR' is used to designate mature miRNAs. In some case, a letter is attached after the unique number. This letter suffixes relationship between mature sequence (example has-miR-133a is related to miR-133b). The miRNA gene is also named in a similar

way, but they can have three letter prefix ‘mir’ written in italics, capitalization or hyphenated (like MIR-156 or *mir-156*) (Ambros *et al.*, 2003).

In some cases, two miRNA sequences are identified. The functional sequence is assigned as miR, and the non-functional sequence is identified as miR*. However, in certain cases, it is not clear which sequence is functional. 5p and 3p suffixes are added after unique numbers to identify 5’ and 3’ arm respectively (Ambros *et al.*, 2003).

### *5.1.2 miRNA Biogenesis*

miRNA biogenesis starts in the nucleus and continues in the cytoplasm of the cells (Lee *et al.*, 2002; Lee *et al.*, 2004; Borchert *et al.*, 2006) (Figure 51 and 52).

miRNA gene is transcribed into primary miRNA transcript called primary miRNA (pri-miRNA). These pri-miRNA can produce multiple miRNAs (Lee *et al.*, 2002). However, it has been demonstrated that a pri-miRNA transcribed from a genome containing miRNA coding cluster can only express single miRNA (Ramalingam *et al.*, 2013).

Next step is processing of Pri-miRNA by a microprocessor into 70 nucleotide hair-pin like structure called Pre-miRNA. The microprocessor is a multiprotein complex consisting of Dorsha; RNase III enzyme and DiGeorge Syndrome Critical Region 8 (DGCR8/Pasha) (Lee *et al.*, 2003). DGCR8 is a double-stranded blinding protein also known as Pasha (Diederichs *et al.*, 2009).

Pre-miRNA is exported from the nucleus by nuclear transport domain called Expotin 5 and RAN-GTP to the cytoplasm (Diederichs *et al.*, 2009).

In the cytoplasm, pre-miRNA is spliced by an enzyme called Dicer. It is another RNase III. The resulting product is 22nt RNA duplexes called miRNA duplex (miRNA and miRNA*). Dicer forms complex with the double-stranded RNA-binding protein (TRBP),

a protein activator of PKR (PACT) and Argonaute (Ago) 2. This is called RICS loading complex (RLC) (Diederichs *et al.*, 2009).

Next step is splitting and unwinding of the duplex. This is mediated through an enzyme called helicase. However, the specific enzyme has not been identified. In theory, both strands of the duplex can result in a functional miRNA. However, in most cases, strand bound to Ago leads to a functional mature miRNA. Whereas other strand is degraded. Depending on the strand's stability, one strand of the duplex is loaded with Ago protein. Strand with the least stable 5' region is incorporated into Ago protein. In most cases, another strand (passenger strand-miRNA*) is degraded. In some tissues, both strands are retained. In this case, both, mature miRNA and passenger miRNA may have a functional role (Ro *et al.*, 2007).

miRNA once incorporated into Ago protein has a long half-life. Nearly 97% of miRNA in cells are tightly bound to RISC. Binding to RISC not only provides stability but also is the first step towards the functional activity of miRNA (Diederichs *et al.*, 2009).

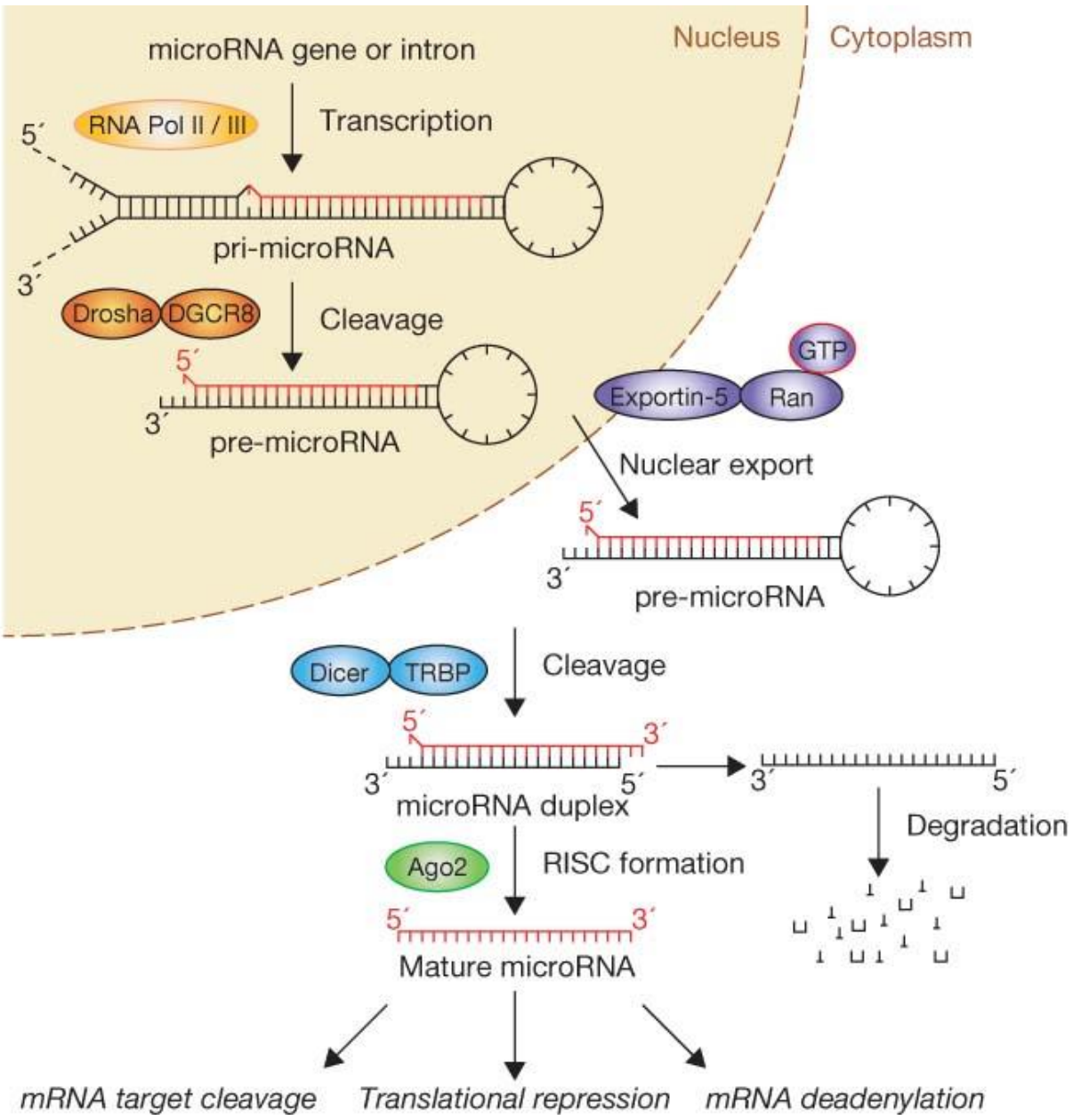


Figure 38: Biogenesis of miRNA.

Adapted from (Diederichs *et al.*, 2009)

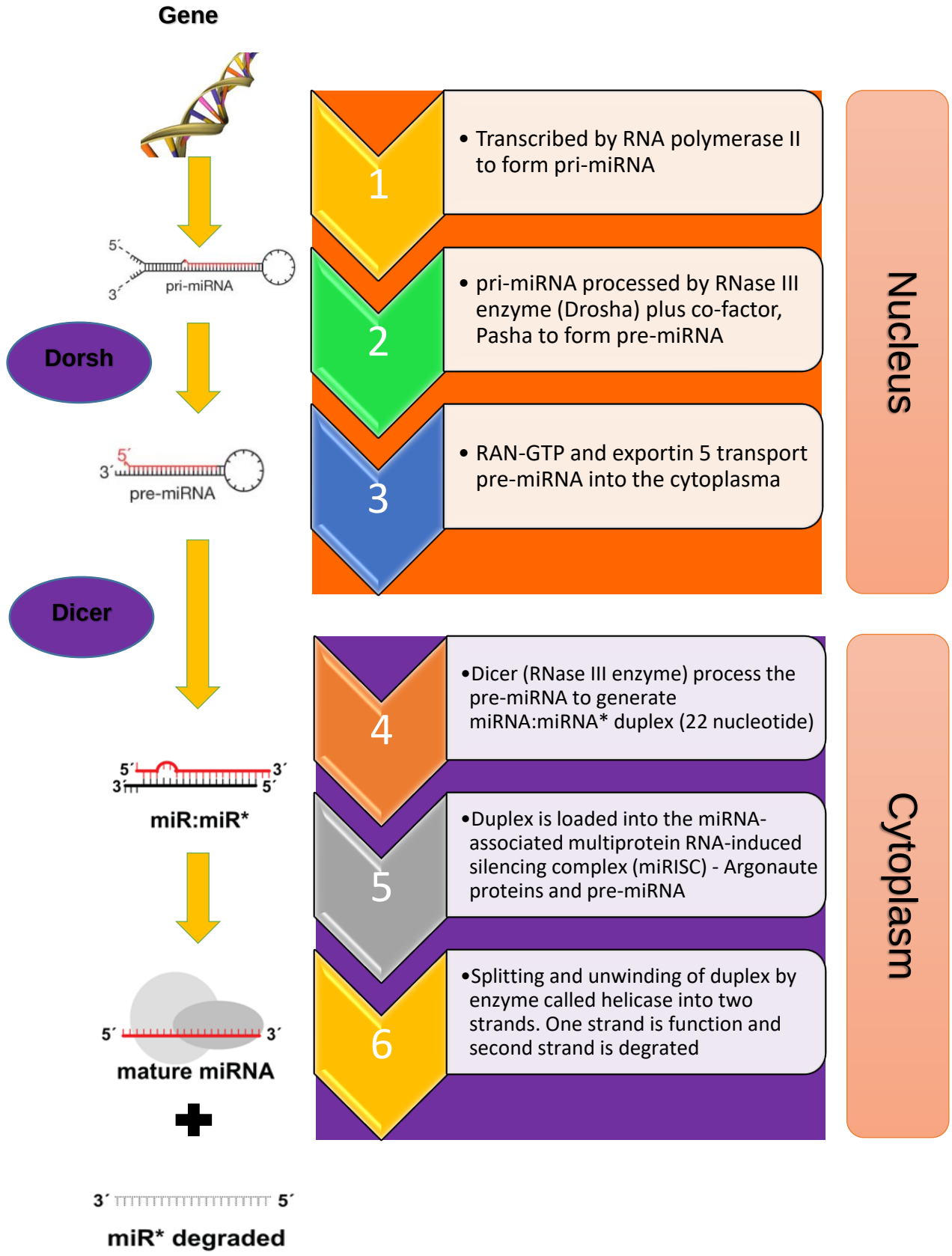


Figure 39: Simplified mechanism of miRNA biosynthesis.

### *5.1.3 miRNA target recognition*

miRNA interacts with its target mRNA through RNA-RNA base pairing. The miRNA guides RISC to the target messenger RNA and downregulates gene expression. This was first observed by (Wightman *et al.*, 1993) between 3'UTRs of the target mRNA (lin14) and 5' end of lin4 (miRNA).

The mechanism by which gene regulation is downregulated depends on the complementarity. Full complementarity between miRNA and mRNA results in suppression of gene expression through mRNA cleavage (Zeng and Cullen, 2003). RISC is important in mRNA cleavage. Ago 2 have mRNA splicing property. Perfect complementarity is rare in animals. However, in animal/humans, most of the complementarity are imperfect. As a result, there is translational repression instead of miRNA cleavage. Thus, protein synthesis is inhibited (Guo *et al.*, 2010). Two translational repression models have been suggested. In first proposed model, translation is repressed at a later stage after initiation of translation (Mathonnet *et al.*, 2007). In the second model, initiation is suppressed. These theories are still under investigation (Behm-Ansmant *et al.*, 2006). (Figure 53).

Recently, it has been found that miRNA can also activate translation of target mRNA. (Vasudevan *et al.*, 2007) demonstrated that miR-369-3 could upregulate translation of TNF-α. Similarly, (Place *et al.*, 2008) showed that miR-373 acts as gene promoter and increases expression of E-cadherin and CSDC2.

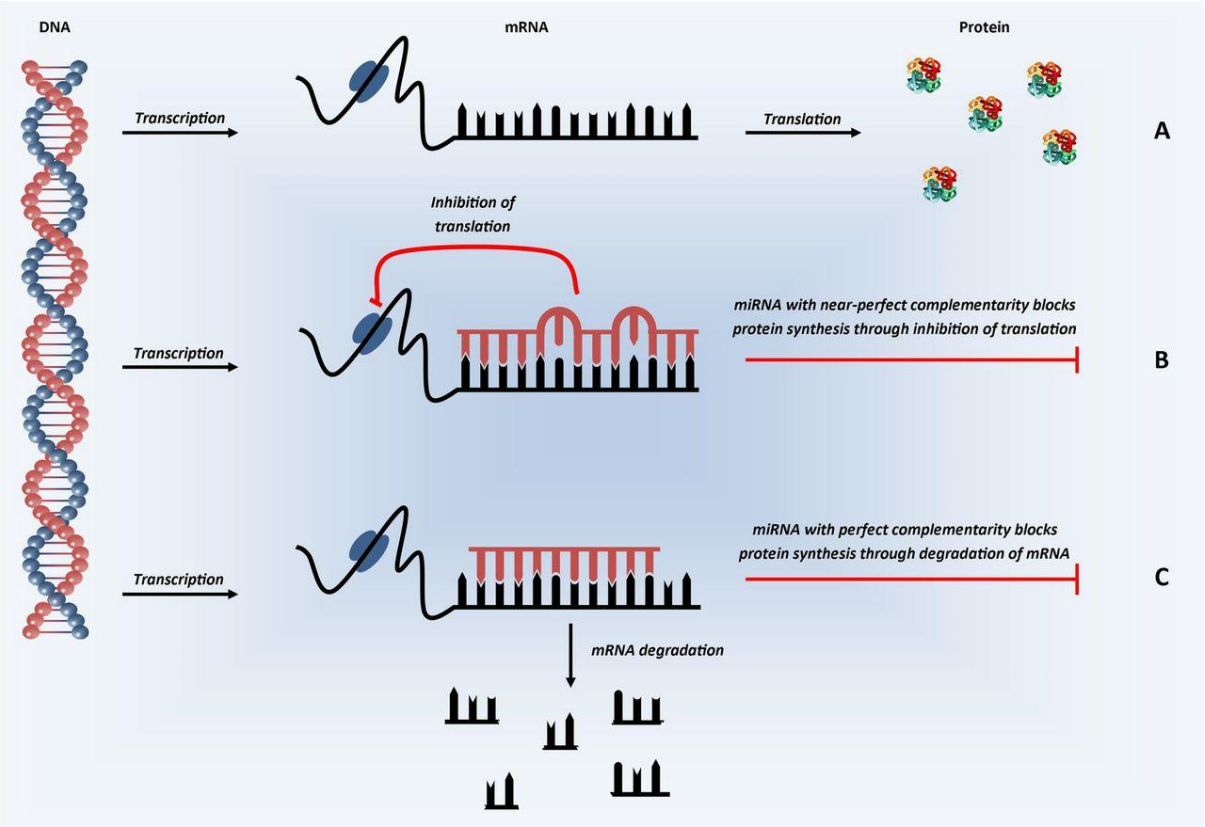


Figure 40: microRNA mechanism of action.

A: mRNA transcripts are translated into protein. B: miRNA represses translation, thereby inhibiting protein synthesis. C: miRNA can cause mRNA degradation, thereby inhibiting protein synthesis.

Adapted from (Romaine *et al.*, 2015)

### *5.1.4 miRNA and cardiovascular disease*

With advances in miRNA field, we are now able to detect miRNA in plasma (Mitchell *et al.*, 2008). This has opened many possibilities including the role of miRNA as a biomarker of cardiovascular disease. Creatinine kinase and troponin T are used as biomarkers for diagnosis of the acute cardiac ischaemic injury. microRNA have emerged as a new potential biomarker not only for diagnosis but also for prognosis of acute cardiac ischaemic injury (Devaux *et al.*, 2015).

Even in stable coronary artery disease, miRNA levels have been noted to be affected. miR-126 and cluster miR-17-90 have been demonstrated to be reduced while miR-133a and miR-208 levels were increased (Fichtlscherer *et al.*, 2010). In a prospective population-based study, Zampetaki *et al.*, 2012 found that miR-126, miR-197 and miR-223 could predict future ischaemic cardiac events in asymptomatic adults over a period of 10 years follow up.

miRNA during post-ischemia phase have been noted to play an important role. miR-21 is cardio-protective (Gu *et al.*, 2015), miR-133 and miR-21 is anti-apoptotic (Xu *et al.*, 2007; Cheng *et al.*, 2010c), miR-1 is antiarrhythmic (Yang *et al.*, 2007) and miR-1 and miR-320 is apoptotic (Xu *et al.*, 2007; Ren *et al.*, 2009). However, our understanding is limited and further work needs to be carried out to understand mechanisms by which microRNA play a role in cardiovascular disease.

### *5.1.5 microRNA and diabetes*

The role of miRNA in diabetes has been acknowledged recently. miR-126 has been identified as major miRNA associated with type 2 diabetes. miR-126 levels were inversely related to blood glucose levels during glucose tolerance test. miR-126 levels are inversely correlated with the future development of peripheral vascular disease in diabetes mellitus (Zampetaki *et al.*, 2010). miR-21, miR-218 and miR-211 has been shown to dysregulated in patients with diabetes induced atherosclerosis (Zhang *et al.*,

2016). Thus, these miRs in future might serve as a novel biomarker of vascular dysfunction in diabetes patients. However, this will need to be confirmed.

There is limited evidence regarding role of miRNAs in type 1 diabetes. Evidence is pointing towards distinct dysregulation of miRNAs in the blood. Osipova *et al.*, 2014 demonstrated that plasma miR-21 and miR-210 was upregulated in plasma and urine of individuals with type 1 diabetes mellitusas miR-126 levels were reduced. miR-126 levels correlated negatively with HbA1c. In individuals with newly diagnosed type 1 diabetes, twelve miRNAs were demonstrated to be upregulated (miR-152, miR-30a-5p, miR-181a, miR-24, miR-148a, miR-210, miR-27a, miR-29a, miR-26a, miR-27b, miR-25, miR-200a). Plasma miR-25 was directly related to HbA1c (Nielsen *et al.*, 2012).

Role of miRNA as a biomarker for diabetes related complication in type 1 diabetes is still being explored. Osipova *et al*. (2014) concluded that urinary miR-21 might help identify patients with renal injury. As mentioned above, urinary miR-21 was elevated in type 1 diabetes patients. miR-21 has been known to cause fibrosis in kidney (Chau *et al.*, 2012). This has been demonstrated in type 1 and type 2 diabetes animal model (Dey *et al.*, 2011a; Zhong *et al.*, 2013). Levels of urinary miRNAs varies with different stage of diabetes related nephropathy in type 1 diabetes (Argyropoulos *et al.*, 2013).

Our understanding of the mechanism of action of miR in diabetes is limited. Further work still needs to be conducted to explore the role of miR in development of CVD in DM.

## 5.2 Method

### *5.2.1 Platelet-free plasma isolation*

After an overnight fast, peripheral blood was collected in EDTA vacutainer tubes. Blood samples were processed within four hours of collection. Samples were centrifuged for 15 minutes at 500 x*g*. The upper, clear fraction (platelet rich plasma, PRP) was collected without disturbing the bottom cellular layer and transferred to 1.5mL polypropylene tubes. These PRP samples were further centrifuged for 5 minutes at 13000 x *g* in a microcentrifuge. After centrifugation, the clarified platelet-free plasma (PFP) was transferred to fresh 1.5mL polypropylene tubes and stored at -80ºC for subsequent analysis. The platelet pellet was discarded.

### *5.2.2 Quantifying microRNAs by reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR)*

miRNA levels were assayed directly from PFP using the Taqman real-time RT-PCR platform (Life Technologies). Control samples (RT control) for each miRNA were prepared in parallel for each patient, where the reverse transcriptase enzyme was omitted from the reaction mix to discount false-positive results due to DNA contamination. The PFP were thawed on ice then diluted 1:3 with nuclease-free water (Ambion). The diluted PFP were heated to 95°C for 10 minutes then cooled to 4°C. MicroRNA-specific primer (Life Technologies) was added (3µL) and the mixture incubated at 65°C for 5 minutes then cooled to 4°C to allow primer annealing. Reaction mastermix (reverse transcription buffer, 1mM dNTPs, RNase inhibitor, reverse transcriptase and nuclease-free water) (Table 21) was added to the primer/PFP mix to a final volume of 15µLand incubated at 16°C for 30 minutes followed by 42°C for 30 minutes and 85°C for 5 minutes. The RT mixture was then cooled to 4°C.

Aliquots of 2µL of the reverse transcription reaction product was combined with 18µL of PCR master mix (10µL 2x Taqman Fast Advanced mastermix, 1µL 20x miRNA Assay, 7µL nuclease-free water) in a 96-well reaction plate (Life Technologies) (Table 22). No template controls were included for each miRNA to discount false-positive results due to DNA contamination. Each sample was amplified in triplicate. The

amplification reactions were performed using the Applied Biosystems® 7900HT Fast Real-Time PCR system as follows: 50°C for 2 minutes, 95°C for 20 seconds followed by 50 cycles of 95°C for 1 second and 60°C for 20 seconds. Amplification was monitored by the SDS 2.3 software (Life Technology).

| **Reagent** | **RT** | **RTC** |
|---|---|---|
| | **Volume (µl)** | **Volume (µl)** |
| 10x RT buffer | 1.50 | 1.50 |
| 100mM dNTPs | 0.15 | 0.15 |
| RNase inhibitor | 0.19 | 0.19 |
| Multiscribe RT | 1.00 | |
| Nuclease-free $H_2O$ | 4.16 | 5.16 |
| **Total** | **7.00** | **7.00** |

Table 21: Master Mix reagents volumes required for single sample of RT and RTC reactions. Reverse transcription: RT; Reverse transcription negative control: RTC.

| **Reagent** | **Volume (µl)** |
|---|---|
| 2x TaqMan fast master mix | 10.0 |
| 20x miRNA assay mix | 1.00 |
| Nuclease-free $H_2O$ | 7.00 |
| **Total** | **18.0** |

Table 22: Master Mix reagents volumes required for single sample of PCR.

### *5.2.3 Plasma microRNA determination*

MicroRNA levels were determined using the PCR kinetics equation described in Equation 1.

***Equation 1*** $Nc = N_0 \times 2^c$

where $N_C$ is the template quantity at cycle $C$, $N_0$ is the initial template quantity prior to amplification and $C$ is the amplification cycle.

This is rearranged to give the initial level of target in a sample (Equation 2).

***Equation 2*** $N_0 = N_c \times 2^{-C}$

In real-time PCR, the quantity of target template at the quantification cycle is directly proportional to the emitted fluorescence at that point. Therefore, by substituting $N$ for fluorescence, $F$, and $C$ for quantification cycle, $Cq$, the starting levels of microRNA can be determined using equation 3.

***Equation 3*** $F_0 = F_{Cq} \times 2^{-Cq}$

The parameters required to quantify target genes using this equation, namely $F_{Cq}$ and $Cq$, were obtained from multiparameter models fit to the fluorescence data using the programming package, qpcR, designed for the R statistical programming environment (version 2.15.1., GNU General Public Lisence). The fluorescence data were exported following completion of amplification and the model-fitting process executed in R. The calculated amplification parameters were used to determine the initial microRNA levels as given by Equation 3.

*C. elegans* miR-39 was tried as endogenous control in our assay. However, after repeated experiments, we found that *C. elegans* could not be used in experiment as it interfered with levels of microRNAs that we measured. Thus, the obtained miRs results were controlled for plasma volume.

### *5.2.4 Choice of miRNA to be analysed*

We decided to explore twenty miRNAs in the first instance. These miRNAs were chosen based on available evidence with regards to their role in endothelial function, atherosclerosis, angiogenesis and anti-angiogenic properties. miR-34a, miR-133a, miR-125b, miR-126, miR-21, miR-10a, miR-155, miR-130a, miR-145, miR-223, miR-33a, miR-210, miR-195, miR-503, miR-320, miR-222, miR-92a, miR-221 and miR-296 were studied (Table 23).

| **miRNA** | **Function** | **Evidence** |
|---|---|---|
| miR-21 | Anti-angiogenic | (Sabatel *et al.*, 2011) |
| miR-222 | Anti-angiogenic<br><br>Neointimal formation | (Poliseno *et al.*, 2006)<br>(Suárez *et al.*, 2007)<br>(Liu *et al.*, 2009) |
| miR-195 | Anti-angiogenic in cancer<br>Anti neointimal formation | (Wang *et al.*, 2013b) (Wang *et al.*, 2012) |
| miR-210 | Angiogenic | (Fasanaro *et al.*, 2008; Pulkkinen *et al.*, 2008) |
| miR-126 | Angiogenic | (Fish *et al.*, 2008)<br>(Wang *et al.*, 2008b) |
| miR-320 | Anti-angiogenic | (Wang *et al.*, 2009) |
| miR-92a | Anti-angiogenic | (Bonauer *et al.*, 2009; Fang and Davies, 2012) |
| miR-223 | Anti-angiogenic | (Shi *et al.*, 2013) |
| miR-34a | Anti-angiogenic | (Arunachalam *et al.*, 2014a) |
| miR-133 | Anti-angiogenic | (Soufi-Zomorrod *et al.*, 2016) |
| miR-125b | Anti-angiogenic | (Muramatsu *et al.*, 2012) |
| miR-10a | Angiogenic | (Hassel *et al.*, 2012) |
| miR-155 | Neointimal formation | (Nazari-Jahantigh *et al.*, 2012) |
| miR-130a | Angiogenic | (Chen and Gorski, 2008) |
| miR-145 | Neointimal formation | (Santovito *et al.*, 2013) |
| miR-503 | Angiogenic | (Caporali *et al.*, 2011) |
| miR-296 | Angiogenic | (Würdinger *et al.*, 2008) |
| miR-33a | Neointimal formation | (Rayner *et al.*, 2011) |

Table 23: Studied miRNAs and some available evidence regarding their function.

## 5.3 Result

### *5.3.1 Choice of miRNA based on $C_q$ value*

We evaluated if we can measure twenty miRNAs (miR-34a, miR-133a, miR-125b, miR-126, miR-21, miR-10a, miR-155, miR-130a, miR-145, miR-223, miR-33a, miR-210, miR-195, miR-503, miR-320, miR-222, miR-92a, miR-221 and miR-296) reliably in our plasma samples. We used five recruit samples to study plasma levels of twenty miRNAs. Cq values of eight miRNAs (miR-126, miR-21, miR-223, miR-210, miR-195, miR-503, miR-320, miR-222 and miR-92a) was <35 (Figure 54). Therefore, these eight miRNAs could be reliably measured in the plasma.

$C_q$ values for miR-125b, miR-130a, miR-34a, miR-133a, miR-33a, miR-10a, miR-145, miR-221, miR-155, miR-503, and miR-296 were high (>35.00) (Figure 55) Therefore, plasma levels of above mentioned twelve miRNAs were not measured reliably by the method mentioned above. High $C_q$ values indicated that plasma levels of these miRNAs are very low and cannot be reliably measured in plasma. This also resulted in high coefficient of variation (CV%). For example, CV% for miR-125b was 43.01%.

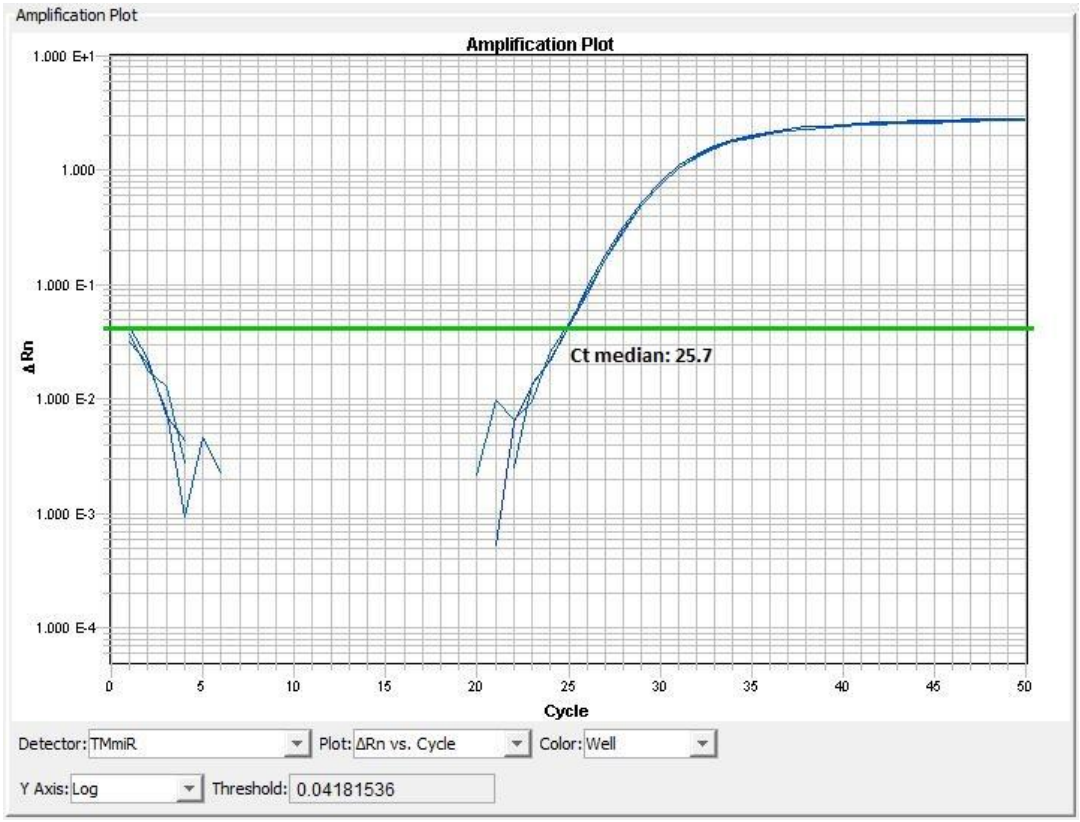


Figure 41: Amplification plot demonstrating Ct value of miR-92a.


Figure 42: Amplification plot demonstrating Ct value of miR-125b.

### *5.3.2 Coefficient of variation*

Our previous unpublished work on miRNA evaluation demonstrated that miR-92a, miR-126, miR-222, and miR-320 had coefficient of variation (CV%) of 8.0%, 6.1%, 4.9%, 6.8% respectively.

Intra-assay coefficient of variation for eight miRNAs was < 20% except for miR-210 (21.7% CV). We aimed to evaluate miRNA with CV of <25%.

### *5.3.3 Statistical analysis*

We have discussed statistical analysis in section 3.2. We have analysed eight miRNAs. We took advice from the statistician regarding correcting for multiple testing. The statistician advised us that based on the aims and objectives of the study, multiple testing is not recommended. MERIT study's aim was to evaluate and discover biomarkers of interest. Significant biomarkers will be needed to be studied further to confirm the finding. Furthermore, small numbers of hypothesis were tested (less than ten).

Based on the advice, we have not performed correction for multiple testing.

### *5.3.4 Specific aims*

We aimed to evaluate if metformin treatment, independent of glycaemic control in type 1 diabetes

1. Increased levels of angiogenic circulatory miRNAs, and
2. Decreased levels of anti-angiogenic circulatory miRNAs

### *5.3.5 miRNA 21a*

Results presented in Figure 56.

#### *5.3.5.1 Treatment group (pre metformin) versus healthy control*

At baseline, plasma miR-21a levels were significantly higher in TG when compared with HC (TG V1 vs HC) mean+/-SE: $1.8x10^{-7}$+/-$1.6x10^{-8}$ vs $8.9x10^{-7}$+/-$6x10^{-9}$: p=0.0001.

#### *5.3.5.2 Treatment group (pre metformin) versus treatment group (post metformin)*

Eight-weeks metformin treatment significantly reduced plasma miR-21a levels in TG; (TG V1 vs TG V2) mean+/-SE: $1.8x10^{-7}$+/-$1.6x10^{-8}$ vs $1.3x10^{-7}$+/-$1.3x10^{-8}$: p=0.0012.

#### *5.3.5.3 Treatment group (post metformin) versus healthy control*

After metformin treatment, plasma miR-21a levels in TG V2 remained significantly higher when compared to HC (TG V2 vs HC); mean+/-SE: $1.3x10^{-7}$+/-$1.3x10^{-8}$ vs $8.9x10^{-7}$+/-$6x10^{-9}$: p=0.02.

#### *5.3.5.4 Treatment group (pre metformin) versus standard group (pre-observation)*

At baseline, plasma miR-21a levels were similar in TG when compared with SG (Treatment group pre-metformin TG V1 vs standard group SG V1 (pre-observation); mean+/-SE; (TG V1 vs ST V1): $1.8x10^{-7}$+/-$1.6x10^{-8}$ vs $1.6x10^{-7}$+/-$3.6x10^{-8}$; p=0.5.

#### *5.3.5.5 Standard group (pre-observation) versus standard group (post-observation)*

After eight weeks of observation, plasma miR-21a remained unchanged (standard group pre-observation (SG V1) and standard group post-observation (SG V2): mean+/-SE (SG V1 vs SG V2): $1.6x10^{-7}$+/-$3.6x10^{-8}$ vs $1.7x10^{-7}$+/-$4x10^{-8}$: p=0.6.

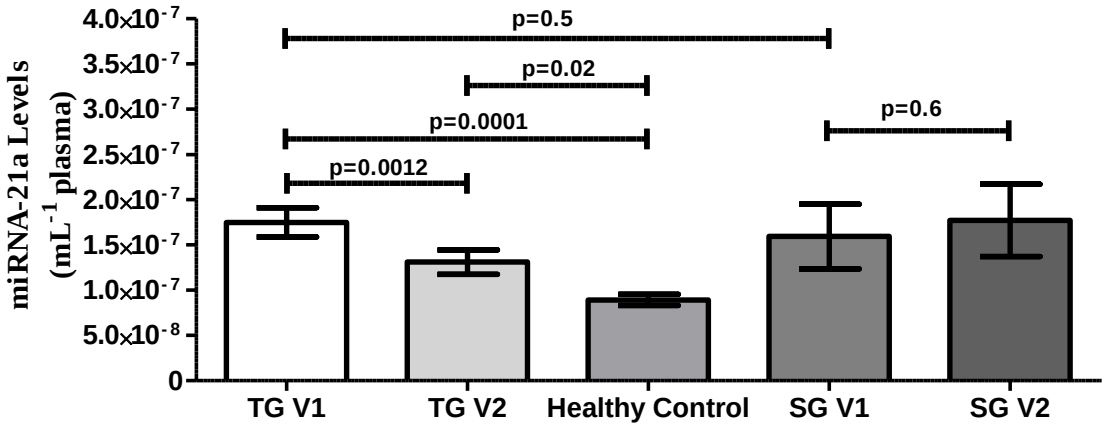


Figure 43: Comparing miR-21a level (per ml) in plasma in all groups. Results are presented as mean±SE. Treatment group pre-metformin (TG V1), treatment group post-metformin (TG V2), standard group pre-observation (SG V1) and standard group post-observation (SG V2).

### *5.3.6 miRNA 222*

Results presented in Figure 57.

#### *5.3.6.1 Treatment group (pre metformin) versus healthy control*

At baseline, plasma miR-222 levels were significantly higher in TG when compared with HC (TG V1 vs HC) mean+/-SE: $8.9x10^{-8}$+/-$8.6x10^{-9}$ vs $5.8x10^{-8}$+/-$3.5x10^{-9}$: p=0.009.

#### *5.3.6.2 Treatment group (pre metformin) versus treatment group (post metformin)*

Eight-weeks metformin treatment significantly reduced plasma miR-222 levels in TG; (TG V1 vs TG V2) mean+/-SE: $8.9x10^{-8}$+/-$8.6x10^{-9}$ vs $7x10^{-8}$+/-$5.8x10^{-9}$: p=0.007.

#### *5.3.6.3 Treatment group (post metformin) versus healthy control*

After metformin treatment, plasma miR-222 levels were similar in TG and HC. (TG V2 vs HC); mean+/-SE: $7x10^{-8}$+/-$5.8x10^{-9}$ vs $5.8x10^{-8}$+/-$3.5x10^{-9}$: p=0.2.

#### *5.3.6.4 Treatment group (pre metformin) versus standard group (pre-observation)*

At baseline, plasma miR-222 levels were similar in TG when compared with SG pre-observation mean+/-SE; (TG V1 vs. ST V1): $8.9x10^{-8}$+/-$8.6x10^{-9}$ vs. $8x10^{-8}$+/-$1.8x10^{-8}$; p=0.5.

### *5.3.6.5 Standard group (pre-observation) versus standard group (post-observation)*

After eight weeks of observation, plasma miR-222 remained unchanged in standard group (SG V1 and SG V2): mean+/-SE (SG V1 vs. SG V2): $8x10^{-8}$+/-$1.8x10^{-8}$ vs. $8.4x10^{-8}$+/-$1.8x10^{-8}$: p=0.99.

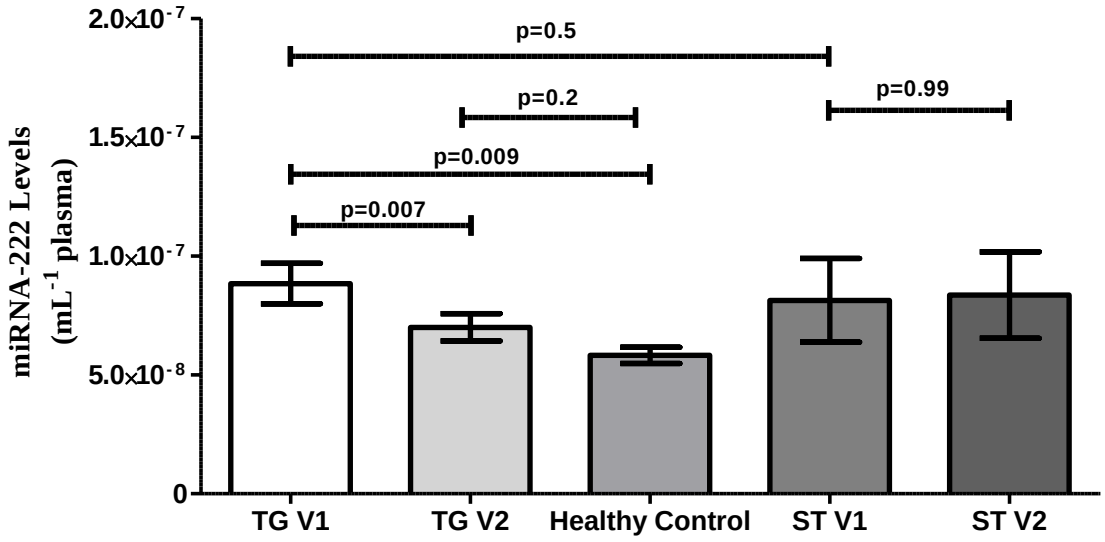


Figure 44: Comparing miR-222 level (per ml) in plasma in all groups. Results are presented as mean±SE. Treatment group pre-metformin (TG V1), treatment group post-metformin (TG V2), standard group pre-observation (SG V1) and standard group post-observation (SG V2).

### *5.3.7 miRNA 195*

Results presented in Figure 58.

#### *5.3.7.1 Treatment group (pre metformin) versus healthy control*

At baseline, plasma miR-195 levels were significantly higher in TG when compared with HC (TG V1 vs. HC) mean+/-SE: $6.6x10^{-7}$+/-$1.2x10^{-7}$ vs. $7.1x10^{-8}$+/-$1.1x10^{-8}$: p<0.0001.

#### *5.3.7.2 Treatment group (pre metformin) versus treatment group (post metformin)*

Eight-weeks metformin treatment significantly reduced plasma miR-195 levels in TG; (TG V1 vs. TG V2) mean+/-SE: $6.6x10^{-7}$+/-$1.2x10^{-7}$ vs. $4.4x10^{-7}$+/-$8x10^{-8}$: p=0.002.

#### *5.3.7.3 Treatment group (post metformin) versus healthy control*

After metformin treatment, plasma miR-195 levels remained significantly higher when compared to HC (TG V2 vs. HC); mean+/-SE: $4.4x10^{-7}$+/-$8x10^{-8}$ vs. $7.1x10^{-8}$+/-$1.1x10^{-8}$: p<0.0001.

#### *5.3.7.4 Treatment group (pre metformin) versus standard group (pre-observation)*

At baseline, plasma miR-195 level was significantly higher in TG when compared with SG: mean+/-SE; (TG V1 vs. SG V1): $6.6x10^{-7}$+/-$1.2x10^{-7}$ vs. $2.8x10^{-7}$+/-$8x10^{-8}$; p=0.03.

### *5.3.7.5 Standard group (pre-observation) versus standard group (post-observation)*

After eight weeks of observation, plasma miR-195 remained unchanged in the standard group (SG V1 and SG V2): mean+/-SE (SG V1 vs. SG V2): $2.8x10^{-7}$+/-$8x10^{-8}$ vs. $2.8x10^{-7}$+/-$7.2x10^{-8}$: p=0.97.

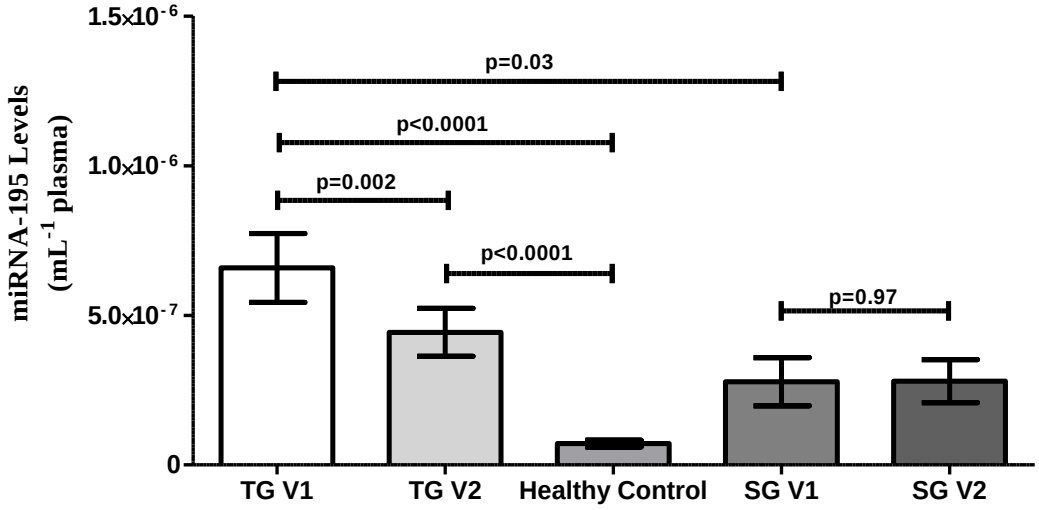


Figure 45: Comparing miR-195 level (per ml) in plasma in all groups. Results are presented as mean±SE. Treatment group pre-metformin (TG V1), treatment group post-metformin (TG V2), standard group pre-observation (SG V1) and standard group post-observation (SG V2).

### *5.3.8 miRNA 210*

Results presented in Figure 59.

#### *5.3.8.1 Treatment group (pre metformin) versus healthy control*

At baseline, plasma miR-210 levels were significantly higher in TG when compared with HC (TG V1 vs. HC) mean+/-SE: $1.3x10^{-8}$+/-$1.2x10^{-9}$ vs. $7.9x10^{-9}$+/-$8.9x10^{-10}$; p=0.003.

#### *5.3.8.2 Treatment group (pre metformin) versus treatment group (post metformin)*

Eight-weeks metformin treatment significantly reduced plasma miR-210 levels in TG; (TG V1 vs. TG V2) mean+/-SE: $1.3x10^{-8}$+/-$1.2x10^{-9}$ vs. $9.7x10^{-9}$+/-$1.2x10^{-9}$: p=0.004.

#### *5.3.8.3 Treatment group (post metformin) versus healthy control*

After metformin treatment, plasma miR-210 levels in TG became similar to HC (TG V2 vs. HC); mean+/-SE: $9.7x10^{-9}$+/-$1.2x10^{-9}$ vs. $7.9x10^{-9}$+/-$8.9x10^{-10}$: p=0.4.

#### *5.3.8.4 Treatment group (pre metformin) versus standard group (pre-observation)*

At baseline, plasma miR-210 level was similar in TG and SG: mean+/-SE; (TG V1 vs. SG V1): $1.3x10^{-8}$+/-$1.2x10^{-9}$ vs. $1.3x10^{-8}$+/-$1.9x10^{-9}$; p=0.98.

#### *5.3.8.5 Standard group (pre-observation) versus standard group (post-observation)*

After eight weeks of observation, plasma miR-210 remained unchanged in the standard group (SG V1 and SG V2): mean+/-SE (SG V1 vs. SG V2): $1.3x10^{-8}$+/-$1.9x10^{-9}$ vs. $1.2x10^{-8}$+/-$1.7x10^{-9}$: p=0.5.

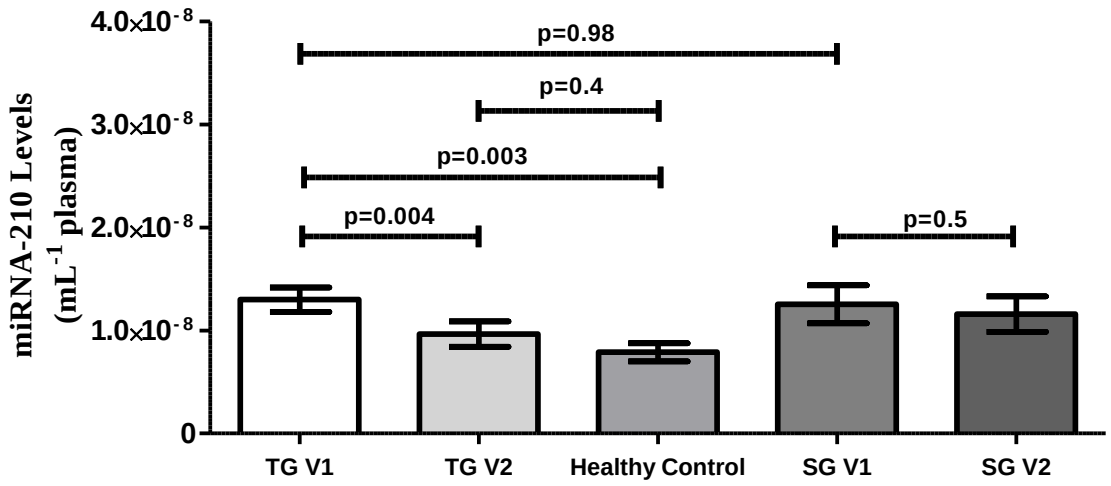


Figure 46: Comparing miR-210 level (per ml) in plasma in all groups. Results are presented as mean±SE. Treatment group pre-metformin (TG V1), treatment group post-metformin (TG V2), standard group pre-observation (SG V1) and standard group post-observation (SG V2).

### *5.3.9 miRNA 223*

Results presented in Figure 60.

#### *5.3.9.1 Treatment group (pre metformin) versus healthy control*

At baseline, plasma miR-223 levels were significantly higher in TG when compared with HC (TG V1 vs. HC) mean+/-SE: $1.1x10^{-6}$+/-$1.4x10^{-7}$ vs. $3.8x10^{-7}$+/-$3.4x10^{-8}$; p<0.0001.

#### *5.3.9.2 Treatment group (pre metformin) versus treatment group (post metformin)*

Eight-weeks metformin treatment did not change plasma miR-223 levels in TG; (TG V1 vs. TG V2) mean+/-SE: $1.1x10^{-6}$+/-$1.4x10^{-7}$ vs. $9.9x10^{-7}$+/-$1.5x10^{-7}$: p=0.4.

#### *5.3.9.3 Treatment group (post metformin) versus healthy control*

After metformin treatment, plasma miR-223 levels in TG remained significantly higher when compared to HC (TG V2 vs. HC); mean+/-SE: $9.9x10^{-7}$+/-$1.5x10^{-7}$ vs. $3.8x10^{-7}$+/-$3.4x10^{-8}$: p<0.0001.

#### *5.3.9.4 Treatment group (pre metformin) versus standard group (pre-observation)*

At baseline, plasma miR-223 level was similar in TG and SG: mean+/-SE; (TG V1 vs. SG V1): $1.1x10^{-6}$+/-$1.4x10^{-7}$ vs. $7.9x10^{-7}$+/-$1.6x10^{-7}$; p=0.18.

#### *5.3.9.5 Standard group (pre-observation) versus* standard *group (post-observation)*

After eight weeks of observation, plasma miR-223 remained unchanged in the standard group: mean+/-SE (SG V1 vs. SG V2): $7.9x10^{-7}$+/-$1.6x10^{-7}$ vs. $1.2x10^{-6}$+/-$2.8x10^{-7}$: p=0.09.

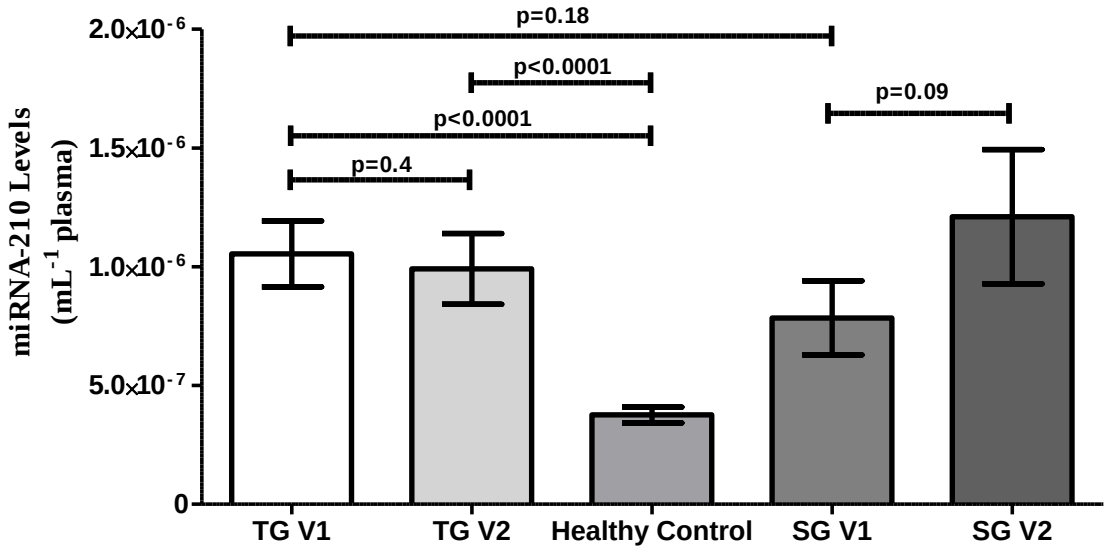


Figure 47: Comparing miR-223 level (per ml) in plasma in all groups. Results are presented as mean±SE. Treatment group pre-metformin (TG V1), treatment group post-metformin (TG V2), standard group pre-observation (SG V1) and standard group post-observation (SG V2).

### *5.3.10 miRNA 320*

Results presented in Figure 61.

#### *5.3.10.1 Treatment group (pre metformin) versus healthy controls*

At baseline, plasma miR-320 levels were significantly higher in TG when compared with HC (TG V1 vs HC) mean+/-SE: $3.6x10^{-7}$+/-$3.8x10^{-8}$ vs $2.6x10^{-7}$+/-$2x10^{-8}$: $p<0.05$.

#### *5.3.10.2 Treatment group (pre metformin) versus treatment group (post metformin)*

Eight-weeks metformin treatment non-significantly reduced plasma miR-320 levels in TG; (TG V1 vs TG V2) mean+/-SE: $3.6x10^{-7}$+/-$3.8x10^{-8}$ vs $3x10^{-7}$+/-$2.8x10^{-8}$: $p=0.06$.

#### *5.3.10.3 Treatment group (post metformin) versus healthy control*

After metformin treatment, plasma miR-320 levels in TG V2 were similar to HC (TG V2 vs HC); mean+/-SE: $3x10^{-7}$+/-$2.8x10^{-8}$ vs $2.6x10^{-7}$+/-$2x10^{-8}$: $p=0.6$.

#### *5.3.10.4 Treatment group (pre metformin) versus standard group (pre-observation)*

At baseline, plasma miR-320 levels were similar in TG and SG; mean+/-SE; TG V1 vs SG V1: $3.6x10^{-7}$+/-$3.8x10^{-8}$ vs $5.5x10^{-7}$+/-$7.8x10^{-8}$, $p=0.03$.

#### *5.3.10.5 Standard group (pre-observation) versus Standard group (post-observation)*

After eight weeks of observation, plasma miR-320 remained unchanged in standard group: mean+/-SE (SG V1 vs SG V2): $5.5x10^{-7}$+/-$7.8x10^{-8}$ vs $5.5x10^{-7}$+/-$6.8x10^{-8}$: $p=0.98$.

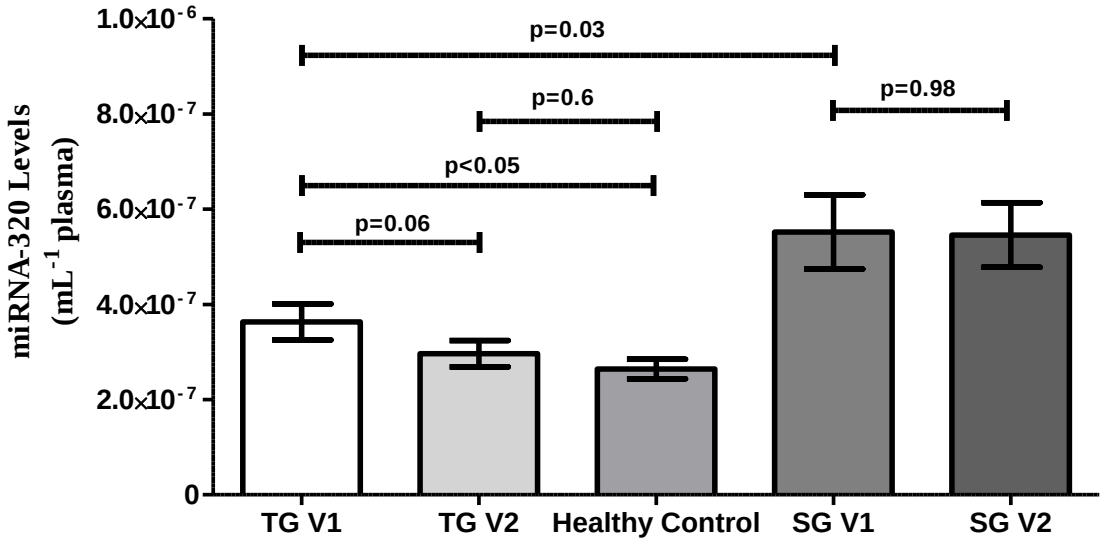


Figure 48: Comparing miR-320 level (per ml) in plasma in all groups. Results are presented as mean±SE. Treatment group pre-metformin (TG V1), treatment group post-metformin (TG V2), standard group pre-observation (SG V1) and standard group post-observation (SG V2).

### *5.3.11 miRNA 126*

Results presented in Figure 62.

#### *5.3.11.1 Treatment group (pre metformin) versus healthy control*

At baseline, plasma miR-126 levels were non-significantly higher in TG when compared with HC (TG V1 vs HC): $1.4x10^{-7}$ +/- $1.7x10^{-8}$ vs $9.4x10^{-8}$ +/- $7.3x10^{-9}$: p=0.06).

#### *5.3.11.2 Treatment group (pre metformin) versus treatment group (post metformin)*

Eight-weeks metformin treatment did not change plasma miR-126 levels in TG; (TG V1 vs TG V2) mean+/-SE: $1.4x10^{-7}$ +/- $1.7x10^{-8}$ vs $1.37x10^{-7}$ +/- $1.7x10^{-8}$: p=0.8).

#### *5.3.11.3 Treatment group (post metformin) versus healthy control*

After metformin treatment, plasma miR-126 levels remained not significantly higher in TG V2 when compared to HC (TG V2 vs HC); mean+/-SE: $1.37x10^{-7}$ +/- $1.7x10^{-8}$ vs $9.4x10^{-8}$ +/-$7.3x10^{-9}$: p=0.14.

#### *5.3.11.4 Treatment group (pre metformin) versus standard group (Pre-observation)*

At baseline, plasma miR-126 levels were similar in TG and the SG (Treatment group pre-metformin TG V1 vs standard group SG V1 (Pre-observation); mean+/-SE; (TG V1 vs ST V1: $1.4x10^{-7}$ +/- $1.7x10^{-8}$ vs $1.7x10^{-7}$+/- $3.3x10^{-8}$; p=0.45).

#### *5.3.11.5 Standard group (pre-observation) versus standard group (post-observation)*

After eight weeks of observation, plasma miR-126 remained unchanged (standard group pre-observation (SG V1) and standard group post-observation (SG V2): mean+/- SE (SG V1 vs SG V2: $1.7x10^{-7}$+/- $3.3x10^{-8}$ vs $2.1x10^{-7}$ +/- $4.5x10^{-8}$: p=0.3).

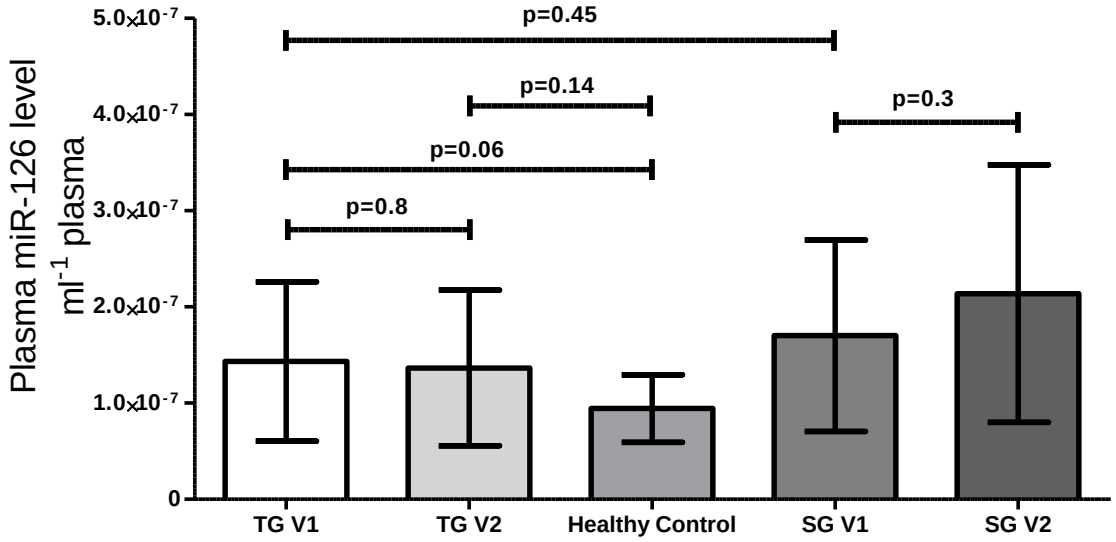


Figure 49: Comparing miR-126 level (per ml) in plasma in all groups. Results are presented as mean±SE. Treatment group pre-metformin (TG V1), treatment group post-metformin (TG V2), standard group pre-observation (SG V1) and standard group post-observation (SG V2).

### *5.3.12 miRNA 92a*

Results presented in Figure 63.

#### *5.3.12.1 Treatment group (pre metformin) versus healthy control*

At baseline, plasma miR-92a levels were non-significantly higher in TG when compared with HC (TG V1 vs HC) mean+/-SE: $9.7x10^{-7}$+/-$1.1x10^{-7}$ vs $7.6x10^{-7}$+/-$8x10^{-8}$: p=0.33.

#### *5.3.12.2 Treatment group (pre metformin) versus treatment group (post metformin)*

Eight-weeks metformin treatment did not change plasma miR-92a levels in TG; (TG V1 vs TG V2) mean+/-SE: $9.7x10^{-7}$+/-$1.1x10^{-7}$ vs $7.5x10^{-7}$+/-$9x10^{-8}$: p=0.16.

#### *5.3.12.3 Treatment group (post metformin) versus healthy control*

After metformin treatment, plasma miR-92a levels were not significantly different between TG V2 and HC (TG V2 vs HC); mean+/-SE: $7.5x10^{-7}$+/-$9x10^{-8}$ vs $7.6x10^{-7}$ +/-$8x10^{-8}$: p=0.7.

#### *5.3.12.4 Treatment group (pre metformin) versus standard group (pre-observation)*

At baseline, plasma miR-92a levels were similar in TG and the SG (TG V1 vs SG V1; mean+/-SE; (TG V1 vs ST V1: $9.7x10^{-7}$+/-$1.1x10^{-7}$ vs $1.3x10^{-6}$+/-$2.3x10^{-7}$; p=0.18).

#### *5.3.12.5 Standard group (pre-observation) versus standard group (post-observation)*

After eight weeks of observation, plasma miR-92a remained unchanged in standard group: mean+/-SE (SG V1 vs SG V2): $1.7x10^{-7}$+/- $3.3x10^{-8}$ vs $2.1x10^{-7}$ +/- $4.5x10^{-8}$: p=0.96).

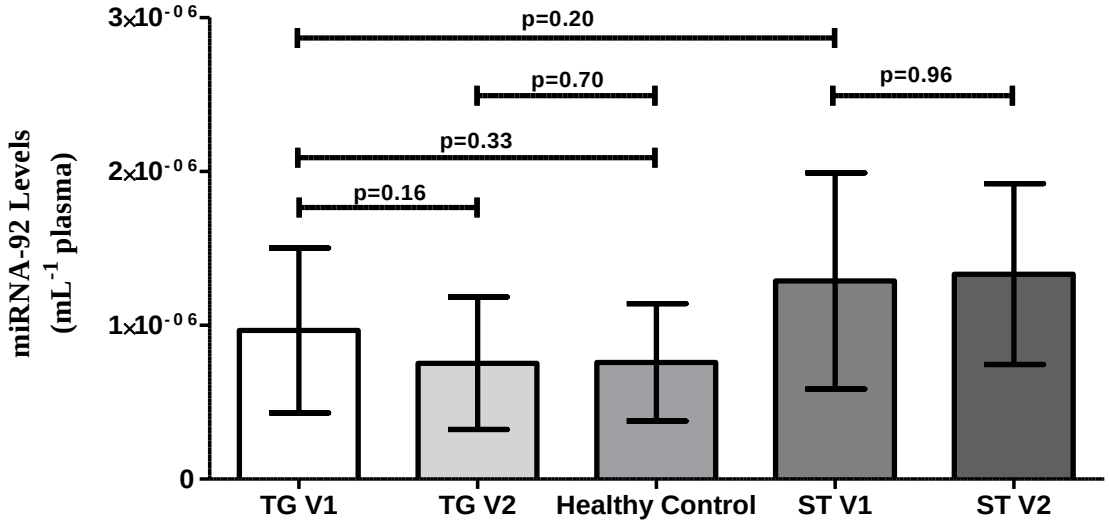


Figure 50: Comparing miR-92 level (per ml) in plasma in all groups. Results are presented as mean±SE. Treatment group pre-metformin (TG V1), treatment group post-metformin (TG V2), standard group pre-observation (SG V1) and standard group post-observation (SG V2).

### *5.3.13 Correlation*

Correlation is presented in Appendix A. Below, we are presenting important correlations.

- In TG, there was a direct correlation between changes in plasma miR-222 and cECs levels ($r=0.42$; $p<0.05$).
- In TG at baseline, there was direct correlation between numbers of cECs and miR-320 levels ($r=0.51$; $p<0.05$).
- In TG at baseline, there was an inverse correlation between weight and miR-195 levels ($r=-0.43$; $p<0.05$).
- In TG, there was a direct correlation between levels of miR-126 and HDL cholesterol ($r=-0.53$; $p<0.05$).
- In TG, there was no correlation between circulating miR-126 and HbA1c and/or glucose variability in our type 1 diabetes patients.
- In TG, there was an inverse correlation of EMPs and plasma miR-92a $r=-0.52$; $p<0.05$).
- In TG, four miRNAs (miR-21a, miR-222, miR-320 and miR-195) except miR-223 were positively correlated with each other.
- In TG, we did not demonstrate correlation between plasma miR-210 levels and anti-angiogenic miRNAs.

## 5.4 Discussion

We focused on eight miRNAs which has emerged to play a major role in the pathogenesis of CVD and diabetes-related complication. Plasma miRNA levels were assessed at baseline and after eight weeks of metformin treatment. These were compared with healthy controls. Furthermore, miRNA plasma levels were evaluated in type 1 diabetes cohort group (standard treatment group-SG) to explore if any changes in above mentioned markers can be observed with routine follow up without any treatment with metformin.

The discussion is divided into two main sections. The first section discusses each miRNA separately. The second section summarises those findings of our work and its significance.

### *5.4.1 Plasma miRNA levels in type 1 diabetes and effect of metformin*

This section discusses the studied plasma miRNA levels in type 1 diabetes and compares the levels with healthy controls. Effect of eight weeks of metformin on each studied miRNA levels in type 1 diabetes is assessed. SG underwent similar follow-up except for treatment with metformin. At baseline, all the miRNA levels were similar in treatment and standard group. Plasma levels of all the measured miRNA did not change after eight weeks of follow-up in the standard group.

#### *5.4.1.1 miR-21*

##### 5.4.1.1.1 Main finding

We have demonstrated the plasma levels of miR-21 were significantly higher in type 1 diabetes when compared to healthy volunteers. We have for the first time shown that metformin decreased miR-21 levels in the plasma of type 1 diabetes independent of glycaemic control.

#### 5.4.1.1.2 Novelty

miR-21 is an anti-angiogenic miR. Decrease in miR-21 by metformin can result in the reduction of anti-angiogenic signal. Thereby, indirectly promoting angiogenesis and vascular repair. However, this is speculative and will need to be confirmed in future studies. Prior to our work, no study has explored the effect of metformin in type 1 or type 2 diabetes.

#### 5.4.1.1.3 Prior work

Our data complements another study by Osipova and group who demonstrated the circulatory miR-21 was higher in patients with type 1 diabetes (Osipova *et al.*, 2014). Increased miR-21 levels in type 1 diabetes served as an indicator for established vascular damage or ongoing vascular damage (Osipova *et al.*, 2014). Levels of plasma miR-21 in type 2 diabetes patients were shown to be lower when compared to healthy controls (Zampetaki *et al.*, 2010). Thus, type 1 diabetes and type 2 diabetes have been shown to have different plasma miR-21 levels.

#### 5.4.1.1.4 Mechanism

miR-21 has been shown to be involved in the pathogenesis of vascular disease like atherosclerosis (Raitoharju *et al.*, 2011; Cengiz *et al.*, 2015). It has been shown in an animal model that miR-21 levels were five-fold higher in balloon injured carotid arteries (Ji *et al.*, 2007). Up-regulation of miR-21 inhibits PTEN (Ji *et al.*, 2007) and PDCD4 (Lin *et al.*, 2009), thereby inhibiting apoptosis and promoting vascular smooth muscle proliferation. Similarly, in endothelial cells under stress, increased miR-21 expression decreases apoptosis through PTEN and the PI3K/Akt survival pathway and enhanced NO production (Weber *et al.*, 2010). This is suggestive that increased miR-21 should decrease endothelial cell and VSMC apoptosis. Decreased vascular repair with ongoing damage is involved in the pathogenesis of atherosclerosis. It has been shown that inhibiting effect of miR-21 decreases neointimal lesion formation after angioplasty (Ji *et al.*, 2007). Overexpression of miR-21 promotes inflammation by directly targeting and downregulating peroxisome proliferators activated receptor-α (PPAR-α) (Zhou *et*

*al.*, 2011). miR-21 has been shown to reduce RhoB mediated endothelial cell proliferation, migration and tube formation (Sabatel *et al.*, 2011).

*In vivo* and *in vitro* data has suggested an inhibitory effect of miR-21 on EPC proliferation and angiogenesis function. miR-21 levels in CAD patients was directly correlated with impaired EPC migratory capacity. Inhibition of miR-21 improved EPC migratory function (Fleissner *et al.*, 2010). Another recent study showed increased expression of miR-21 in EPC of patients with atherosclerosis. There was an elevation of miR-21 when EPCs were exposed to hypoxia. Inhibiting miR-21 reversed hypoxia induced growth arrest in EPC. This has been shown to be mediated by TGF-β (Zuo *et al.*, 2015). Furthermore, suppression of miR-21 improved EPC survival via suppressing high-mobility group A2 (hmga2) (Zhu *et al.*, 2013). miR-21 expression in EPC of type 2 diabetes is much higher in patients with previous major CV event (Domenico *et al.*, 2015). Above mentioned data infers an inhibitory effect of miR-21 on angiogenesis and vascular repair. However, in contrast, a study involving type 2 diabetes patients without CVD showed reduced expression of miR-21 in the PAC (Meng *et al.*, 2012). There is no published evidence regarding miR-21 expression in cultured EPCs in type 1 diabetes.

Salas-Perez and group demonstrated that miR-21 levels were reduced in PBMC collected from type 1 diabetes patients compared with healthy controls. Incubating PBMCs at different glucose concentrations increased miR-21 expression. However, when compared to healthy control, miR-21 expression in type 1 diabetes was still lower (Sala-Perez *et al.* 2013). It was hypothesised that reduced miR-21 expression in PBMC of T1DM could lead to increased survival of PBMC due to decreased apoptosis of these cells. This might lead to a continued pro-inflammatory environment maintained by apoptosis resistant PBMC (Salas-Pérez *et al.*, 2013).

miR-21 can have tissue specific effects. miR-21 dysregulation plays an important role in diabetes-related complication, especially renal impairment. In type 1 diabetes animal models, hyperglycaemia induced increased expression of miR-21 in mesangial cells (Dey *et al.*, 2011b). This had been shown *in vitro* studies where high glucose-induced increased expression of miR-21 by endothelial cells (Zeng *et al.*, 2013). miR-21 plays

an important role in renal fibrosis and inflammation by upregulating TGF-β and NF-κB signalling pathway (Dey *et al.*, 2011b). Urinary miR-21 levels are elevated in type 1 diabetes (Osipova *et al.*, 2014). This is indicative of ongoing fibrotic renal damage in young type 1 diabetes population. Ongoing inflammatory process with direct correlation of CPR and urinary miR-21 is speculated to play a role in kidney damage (Osipova *et al.*, 2014). miR-21 may have a functional role in wound healing in patients with diabetes mellitus. miR-21 has shown to be highly expressed in diabetes skin. In contrast to the inhibitory effect of miR-21 on endothelial cell migration, it has been demonstrated that miR-21 promotes fibroblast migration in the skin (Madhyastha *et al.*, 2011).

From the above-mentioned evidence, we can see that miR-21 expression in different cells may have a contrasting response. However, miR-21 expression in different cells is likely playing a role in the development of vascular disease (e.g. high miR-21 expression in EPC correlating with impaired migration capacity or lower expression of miR-21 in PBMCs pf type 1 diabetes individuals). We believe that high plasma miR-21 levels in our type 1 diabetes cohort may be suggestive of an ongoing process of diabetes-related vascular complication.

Based on the upper mentioned evidence, reduced expression of miR-21 is suggestive of decreased ongoing vascular damage. We hypothesised that decreased expression of plasma miR-21 could improve endothelial cell functions of proliferation, migration and tube formation. This effect can be mediated through attenuating RHO-kinase activity (Liu *et al.*, 2014a). Furthermore, metformin has been shown to reduce TGF-β signalling pathway (Xiao *et al.*, 2010). Metformin has been demonstrated to inhibit NFκB signalling pathway in endothelial cells (Hattori *et al.*, 2006). Both are upregulated by increased expression of miR-21. Reduced expression of miR-21 in vascular smooth muscle will lead to increased expression of PTEN (Ji *et al.*, 2007). Metformin has been shown to activate AMPK induced expression of PTEN leading to decreased inflammation in vascular smooth muscle cells. However, metformin concentrations used were higher than clinically relevant and increased the expression of PTEN regulated inflammatory response of vascular smooth muscle (Kim and Choi, 2012). Based on the current available evidence, we can infer that metformin can reduce the

inflammatory response and improve vascular repair through reducing miR-21 expression.

The flow diagram below gives a pictorial proposed pathway of action miR-21 in the development of vascular pathogenesis in type 1 diabetes (Figure 64A). We have shown that metformin reduced circulatory miR-21 levels thereby attenuating pathways leading to the development of diabetes-related complication (Figure 64B). However, this will need to be confirmed.

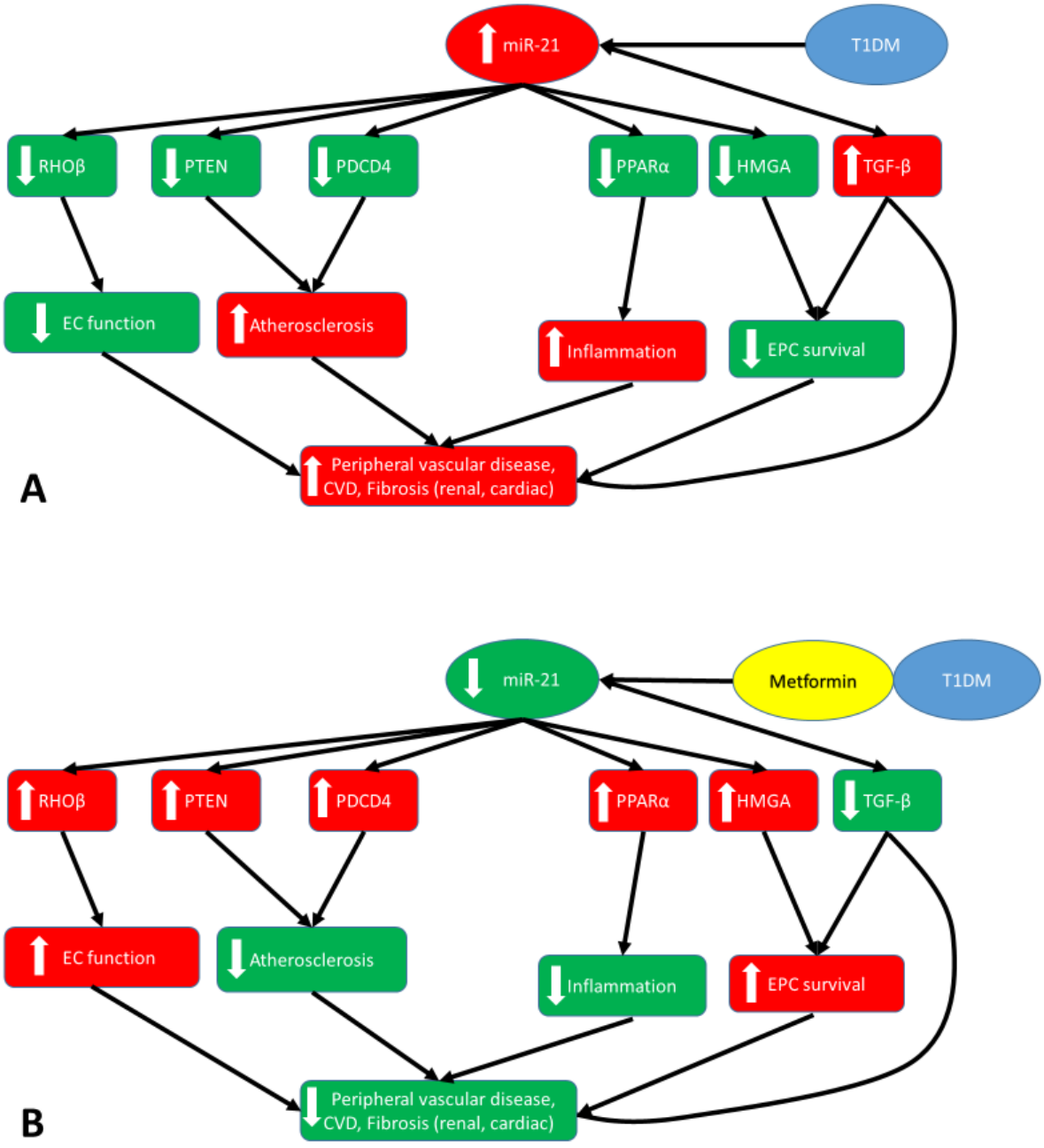


Figure 51: A- Flowchart proposing the effect of miR-21 on the development of vascular pathology in type 1 diabetes patients. B- Effect of metformin on circulatory miR-21 levels. Green colour represents downregulation and red represent upregulation.

#### *5.4.1.2 miR-222*

We studied plasma levels of miR-222 in type 1 diabetes patients. We also investigated miR-221. miR-222 and miR-221 are located on the same gene separated by only a distance of 726 base pair (Chistiakov *et al.*, 2015). They both play a vital role in vascular haemostasis. Most of the studies have evaluated miR-221 and miR-222 together. However, in our study miR-221 coefficient of variation was high. Therefore, we have only evaluated miR-222.

##### 5.4.1.2.1 Main finding

We demonstrated that plasma levels of miR-222 were higher in type 1 diabetes when compared to healthy controls. We also demonstrated that 8 weeks of metformin treatment significantly reduced the plasma miR-222 levels in type 1 diabetes.

##### 5.4.1.2.2 Novelty

We have shown for the first time that miR-222 levels were significantly higher in type 1 diabetes than HC. In addition, we have shown that metformin treatment can reduce miR-222. miR-222 is an anti-angiogenic miRNA. Decrease in miR-222 by metformin can result in the reduction of anti-angiogenic signal. Thereby, indirectly promoting angiogenesis and vascular repair. However, this is speculative and will need to be confirmed in future studies.

##### 5.4.1.2.3 Prior work

Our findings of higher miR-222 in type 1 diabetes is similar to Ortega *et al.* 2014, who showed that miR-222 levels were elevated in type 2 diabetes. It is likely that metabolic factors like insulin resistance in type 2 diabetes play a role in miR-222 levels. In our study, TG cohort were overweight. Thus, insulin resistance may have played a role in higher miR-222 levels in type 1 diabetes. However, insulin resistance was not evaluated in our study. Therefore, future studies should evaluate if changes in insulin resistance have any effect of miR-222 levels.

We demonstrated that eight weeks of metformin reduced miR-222 levels in type 1 diabetes. Our findings complement another study on type 2 diabetes which showed how metformin treatment reduced miR-222 levels in the internal mammary artery (Coleman *et al.*, 2013). Coleman and team measured miR-222 levels in the smooth muscle cells and endothelial cells (Coleman *et al.*, 2013). Therefore, from our work and Coleman et al, 2013, we can infer that metformin can have an effect on miR-222 levels in plasma and at cellular level.

#### 5.4.1.2.4 Mechanism

miR-222 is pro-atherogenic and anti-angiogenic properties and plays a role in endothelial dysfunction. miR-222 is highly expressed in EPC of CAD patient (Chang *et al.*, 2014), vascular smooth muscle cells (Liu *et al.*, 2012) and endothelial cells (Liu *et al.*, 2012). miR-222 mechanism of action has been shown to be variable and dependent on the tissue. miR-222 in VSMC stimulates proliferation, migration and attenuates apoptosis (Liu *et al.*, 2012). At the site of vascular injury, platelets and EC releases platelet derived growth factor (PDGF) (McNamara *et al.*, 1996). PDGF induces expression of miR-222 and miR-221. Increased expression of miR-222/miR-221 reduced levels of $p27^{kip1}$ (Liu *et al.*, 2012). $p27^{kip1}$ is known to attenuate atherosclerosis development (Shahzad *et al.*, 2011). Reduced expression of $p27^{kip1}$ leads to the development of atherosclerotic plaque (DÍEZ-JUAN and ANDRÉS, 2001; Akyürek *et al.*, 2010).

Metformin has been observed to reduce miR-222 expression in EC *in vitro* in type 2 diabetes (Coleman *et al.*, 2013). Our data showed that metformin reduced miR-222 levels in type 1 diabetes plasma. miR-222 levels were reduced to levels similar to healthy volunteers. We have shown a positive correlation between changes in plasma miR-222 and cECs levels). We can postulate that metformin may decrease EC damage through improving miR-222 levels. However, further functional studies need to be conducted to confirm this finding and elucidate this mechanism. Firstly, *in vitro* work should be conduction studying the effect of metformin at the physiological concentration on miR-222. This should be extended further to study downstream pathways e.g. eNOS pathway. Further work using miR-222 gain and loss of function in culture microenvironment should be conducted to evaluate downstream pathways.

The flow diagram below gives a pictorial proposed pathway of action miR-222 in the development of vascular pathogenesis in type 1 diabetes (Figure 65A). We have shown that metformin reduced circulatory miR-222 levels thereby attenuating pathways leading to the development of diabetes-related complication (Figure 65B). However, this will need to be confirmed.

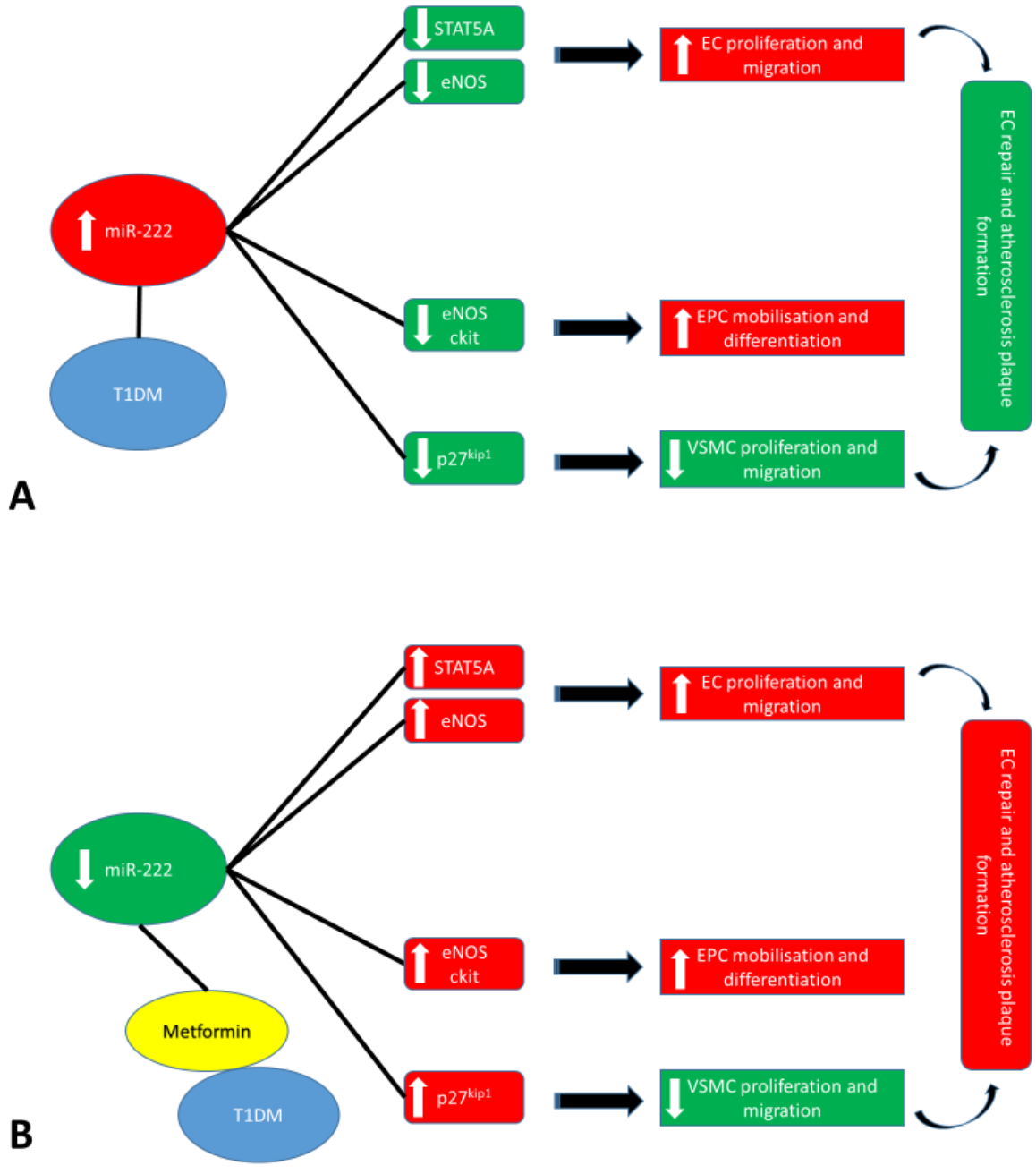


Figure 52: A- Flowchart proposing the effect of miR-222 on the development of vascular pathology in type 1 diabetes patients. B- Effect of metformin on circulatory miR-222 levels. Green colour represents downregulation and red represent upregulation.

#### *5.4.1.3 miR-195*

##### 5.4.1.3.1 Main Finding

Main findings of our study included:

1. Plasma levels of miR-195 were significantly higher in type 1 diabetes individual when compared to healthy controls.
2. There was an inverse correlation between weight and miR-195 levels in type 1 diabetes.
3. Eight weeks of metformin reduced miR-195 levels in plasma significantly in type 1 diabetes mellitus. However, the level of plasma miR-195 remained significantly higher than healthy volunteers.

##### 5.4.1.3.2 Novelty

So far, no study has specifically found evidence regarding dysregulation of miR-195 in type 1 diabetes. We have for the first time demonstrated that miR-195 levels were higher in type 1 diabetes when compared to HC. In addition, metformin lowered miR-195 levels after eight weeks of metformin treatment. It is for the first time, we have shown that anti-angiogenic miR-195 is reduced along with miR-21a. Thereby, reducing anti-angiogenic signals.

##### 5.4.1.3.3 Prior work

In contrast to our data, miR-195 levels in plasma were observed to be significantly lower in type 2 diabetes (Ortega *et al.* 2014). This might be due to the different disease population in our study and Ortega *et al.* (2014). Furthermore, plasma collection protocol was different (500g for 15 minutes followed by 13000g for 5 minutes (our protocol) vs 1000g for 15 minutes followed by 2000g for 5 minutes (Ortega *et al.* 2014). Type 2 diabetes has more of metabolic disturbance whereas type 1 diabetes is mainly due to insulin deficiency and less metabolic dysfunction. There may be different mechanisms and factor causing miR-195 dysregulation. Higher weight or BMI does increase insulin resistance even in type 1 diabetes. Lower miR-195 expression in type 2 diabetes and our data of inverse correlation between weight and BMI is suggestive of insulin resistance playing an important role in miR-195 dysregulation.

#### 5.4.1.3.4 Mechanism

miR-195 is a marker of vascular injury. It is raised in patients with acute myocardial ischemia (Long *et al.*, 2012). There is developing evidence that miR-195 plays a role in vascular repair and damage. It has been shown in the model of balloon-injury carotid artery model that miR-195 reduced proliferation, migration and pro-inflammatory biomarkers (Wang *et al.*, 2012).

In diabetes animal model, miR-195 has been linked with diabetes-related complication. In type 1 diabetes animal model, miR-195 has been shown to play a major role in microvessels damage in diabetic retinopathy through it downregulating SIRT1 (Mortuza *et al.*, 2014). miR-195 has been shown in the diabetic animal model to augment myocardial hypertrophy, decrease coronary blood flow and reduce myocardial function (Zheng *et al.*, 2015). miR-195 plays a role in oxidative damage, apoptosis and reducing angiogenesis. miR-195 has been shown to be anti-angiogenic through reducing the expression of VEGF (Wang *et al.*, 2013b). miR-195 has been demonstrated to play an important role in palmitate-induced cardiomyocytes apoptosis and sepsis-induced apoptosis, and multi-organ injury though its effect on SIRT1 (Zhu *et al.*, 2011). miR-195 has also been shown to downregulate HMGA-1 expression. Downregulation of HMGA-1 expression is involved in isoprenaline-induced cardiomyocyte hypertrophy (You *et al.*, 2014). Based on the evidence mentioned above, we can infer that high miR-195 levels in type 1 diabetes can be linked with the development of diabetes-related complication and CVD.

Based on above mentioned result, metformin effect in reducing miR-195 expression may lead to a partial decrease in risk of development of diabetes-related complication in type 1 diabetes. We hypothesised that SIRT-1 will play a role in this. We know that hyperglycaemia has been shown to decrease SIRT1 expression. Restoring SIRT1 expression has been demonstrated to play a protective role in diabetes vasculopathy (Orimo *et al.*, 2009). Metformin has been shown to attenuate hyperglycaemia related endothelial senescence due to partly restoring SIRT-1 expression (Arunachalam *et al.*, 2014b). Concentration of metformin in Arunachalam and group is higher than physiological concentration of metformin (50mM). Therefore, effect of physiological concentration of metformin on miR-195 and SIRT-1 will need to be confirmed.

The flow diagram below gives a pictorial proposed pathway of action miR-195 in the development of vascular pathogenesis in type 1 diabetes (Figure 66A). We have shown that metformin reduced circulatory miR-195 levels thereby attenuating pathways leading to the development of diabetes-related complication (Figure 66B). However, this will need to be confirmed.

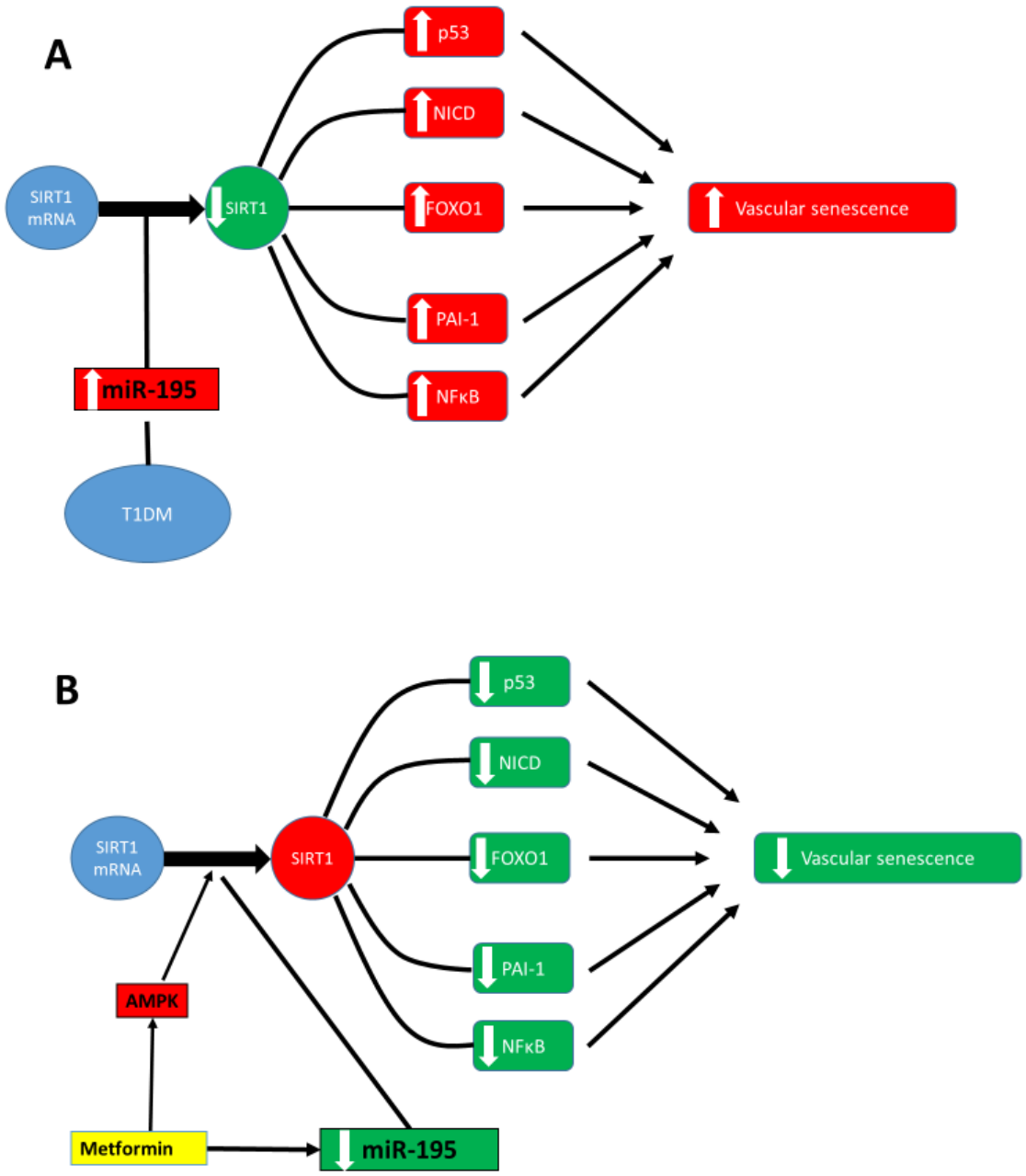


Figure 53: A-Flowchart proposing the effect of miR-195 on the development of vascular pathology in type 1 diabetes patients. B-Effect of metformin on circulatory miR-195 levels. Green colour represents downregulation and red represent upregulation.

#### *5.4.1.4 miR-210*

##### 5.4.1.4.1 Main finding

We demonstrated that miR-210 plasma levels were higher in type 1 diabetes when compared to healthy controls. Furthermore, for the first time, we have shown that metformin significantly reduced miR-210 levels. However, after metformin treatment, levels remained significantly higher in type 1 diabetes individual when compared to healthy volunteers.

##### 5.4.1.4.2 Novelty

As the only study looking at effect of metformin in type 1 diabetes, we have shown that eight weeks of metformin treatment significantly reduced miR-210 in type 1 diabetes. This provides novel information which can be used to elucidate the mechanistic pathways affected by metformin.

##### 5.4.1.4.3 Prior work

Our results are similar to Osipova *et al.* (2014), who also showed increased levels of miR-210 in plasma and urine of individuals with type 1 diabetes. Circulating levels of miR-210 is also upregulated even in children with newly diagnosed type 1 diabetes (Nielsen *et al.*, 2012). To the best of our knowledge, there is currently no evidence on the effect of metformin in type 1 or type 2 diabetes.

##### 5.4.1.4.4 Mechanism

miR-210 has been established as hypoxia responsive “master” miRNA (Kulshreshtha *et al.*, 2008; Huang *et al.*, 2010b). There is an extensive body of evidence developing regarding the role of miR-210 in tumour initiation (Huang *et al.*, 2009c) and progression. Rapidly expanding tumours are known to have hypoxic microenvironment (Huang *et al.*, 2010b). miR-210 is increased in patients with malignancy (Giannakakis *et al.*, 2007; Camps *et al.*, 2008). miR-210 can serve as potential biomarkers in renal cell carcinoma (Zhao *et al.*, 2013a). Based on cancer work, (Huang and Zuo, 2014)

summarised miR-210 functions, which included promoting angiogenesis, protection from apoptosis, a regulator of cell cycle progression, repressing DNA damage and regulating mitochondrial metabolism. Hypoxia induced factor-α (HIF-α) has been identified as an important regulator of miR-210. HIF-α is upregulated under hypoxic condition. HIF-α is involved in regulating gene expression associated with promoting angiogenesis and preventing apoptosis under hypoxic condition, thereby promoting protection (Huang *et al.*, 2010b). HIF-1α and HIF-2α, both have been shown to be involved in regulating miR-210 (Camps *et al.*, 2008; Huang *et al.*, 2009c; Zhang *et al.*, 2009b). However, in comparison to HIF-2α, HIF-1α plays a major role in increasing expression of miR-210 (Crosby *et al.*, 2009; Huang *et al.*, 2009c).

miR-210 promotes angiogenesis and cell migration in EC even in normoxic condition. miR-210 is up-regulated in response to hypoxia in EC. This is mediated through HIF-1α activation (Fasanaro *et al.*, 2008). miR-210 has been shown to protect EC and cardiomyocytes from apoptosis (Cicchillitti *et al.*, 2012). miR-210 levels have been shown to remain elevated even after disappearance of hypoxia (Fasanaro *et al.*, 2008). This means that miR-210 protective response is maintained to improve cell survival. Angiogenic and cell migration signal by miR-210 in response to hypoxia is modulated through downregulating EFNA3 (Fasanaro *et al.*, 2008). Growth factors like VEGF upregulated miR-210 in CD34+ cells (Alaiti *et al.,* 2012). miR-210 has been shown to enhance *ex vivo* angiogenesis in normoxic condition when there was no VEGF in the medium. Silencing miR-210 attenuated VEGF mediated angiogenesis (Alaiti *et al.*, 2012). miR-210 has been demonstrated to improve cardiomyocyte survival in murine ischaemic models (Hu *et al.*, 2010).

The above-mentioned properties of miR-210 are interesting in the light of our results and other evidence of high miR-210 plasma levels in type 1 diabetes patients (Osipova *et al.*, 2014). Diabetes microenvironment has been shown to increase vascular damage. In vitro studies have demonstrated increased miR-210 expression due to high glucose (Greco *et al.*, 2012). Increased miR-210 levels could be a response to ongoing vascular injury leading to counter-regulatory enhanced vascular repair and endothelial protection through promoting angiogenesis and preventing apoptosis. However, this could also be a marker of increased vascular injury. miR-210 is a marker of

atherosclerosis (Li *et al.*, 2011b). The miR-210 expression has been shown to be higher in atherosclerotic plaque (Raitoharju *et al.*, 2011). It is hypothesised that increased angiogenic signal of miR-210 affects VSMC phenotypes and plays a critical role in atherosclerosis (Li *et al.*, 2011b). (Chen *et al.*, 2015b) demonstrated that oxidised LDL resulted in HIF-1α mediated increase in miR-210. miR-210 inhibits sprouty-related EVH1 domain 2 (SPRED2) expressions. SPRED2 inhibition is linked with the development of vascular disease. HIF-1α is associated with the development of atherosclerosis by regulating development of foam cells (Vink *et al.*, 2007). Expression of HIF-1α is directly correlated with diabetic retinopathy progression (Yan and Su, 2014). The HIF-1α expression is an independent risk factor of coronary artery calcification in patients with type 2 diabetes (Li *et al.*, 2014). In an animal model, the HIF-1α expression has been shown to mediate diabetes-related kidney disease (Nayak *et al.*, 2016).

In summary, HIF-1α mediated miR-210 expression is important in vascular repair. However, excess expression of HIF-1α and downstream target (e.g. miR-210) may lead to the development of a vascular disease like atherosclerosis.

As mentioned above, metformin treatment reduced miR-210 in type 1 diabetes. Reduction in miR-210 after eight weeks of metformin treatment could be due to a reduction in ongoing vascular damage and/or less inflammatory microenvironment. Metformin effect on plasma miR-210 levels could be mediated through HIF-1α. Metformin is known to reduce HIF-1α expression (Wheaton *et al.*, 2014). HIF-1α inhibition has been shown to prevent development and progression of diabetic retinopathy in type 1 diabetes animal model (Chen *et al.*, 2013). Plasma miR-210 levels in our type 1 diabetes patient after eight weeks of metformin treatment did not reach levels similar to healthy volunteer's levels. As mentioned above, the excess angiogenic signal can lead to the development of atherosclerosis and diabetes-related complication. Therefore, reduction of miR-210 levels by metformin may be rebalancing excess angiogenic signal to a more balanced vascular repair. Plasma miR-210 levels were still significantly higher in type 1 diabetes than healthy control suggesting presence of microenvironment causing vascular damage and increased need for

vascular repair and.  This hypothesis needs to be confirmed by designing *in vitro* and *in vivo* studies.

#### *5.4.1.5 miR-223*

##### 5.4.1.5.1 Main finding

We have for the first time shown that plasma miR-223 levels were significantly higher when compared to healthy controls. Eight weeks of metformin treatment did not have any effect on miR-223 levels.

##### 5.4.1.5.2 Novelty

Our result of raised miR-223 in type 1 diabetes when compared to healthy control is unique to our study. We need to explore and elucidate the role of miR-223 in pathogensis of diabetes-related complication.

##### 5.4.1.5.3 Prior work

Our findings of raised miR-223 levels in type 1 diabetes were in contrast to studies exploring plasma miRNA levels in type 2 diabetes (Zampetaki *et al.*, 2010), patients with coronary heart disease (Finn et al. 2013) and risk of ischaemic heart disease (Zampetaki *et al.*, 2012). Zampetaki *et al.* 2010 demonstrated that plasma miR-223 levels were not only lower in type 2 diabetes, but reduced levels could be detected well before the presentation of type 2 diabetes. This could in part be explained by levels of EMPs and PMPs. miR-223 is known to be transported through MP (Finn *et al.*, 2013). It has been shown previously that EMPs and PMPs are significantly higher in type 1 diabetes when compared with healthy control (Dignat-George *et al.*, 2002). Whereas in type 2 diabetes, EMPs and PMPs are similar to healthy volunteers (Dignat-George *et al.*, 2002). This can explain why plasma miR-223 levels were raised in our type 1 diabetes cohort. Additionally, in type 2 diabetes, decreased miR-223 could be due to decreased loading in MPs.

#### 5.4.1.5.4 Mechanism

miR-223 levels vary depending on the disease process. It has been established that miR-223 expression is dysregulated in malignancy. It belongs to a class of miRNA called OncomiRs (Taïbi *et al.*, 2014). In the breast, gastric and ovarian cancer, the miR-223 expression is upregulated and promotes invasiveness of malignancy (Laios *et al.*, 2008; Li *et al.*, 2011c; Yang *et al.*, 2011). However, miR-223 levels are reduced in other cancers like hepatocellular carcinoma and AML (Wong *et al.*, 2008; Eyholzer *et al.*, 2010). Thus, up- or downregulation of miR-223 in different cancers have been shown to promote carcinogenesis and metastasis. It is only in 2013 when Shi *et al.* published their work in Circulation Research, miR-223 became recognised for its significant role in the development of vascular pathophysiology (Thum, 2013). Shi *et al.*, 2013 demonstrated that miR-223 attenuated EC angiogenic potential. Furthermore, miR-223 inhibited growth factors (VEGF and bFGF), mediated EC proliferation, migration and sprouting. This is partly mediated through downregulation of β1integrin. Similarly, (Pan *et al.*, 2014) demonstrated that platelet-derived miR-223 promoted EC apoptosis. It has been demonstrated that miR-223 increased osteoblast apoptosis. miR-223 mediated this effect through downregulating insulin life growth factor receptor 1 (Qin *et al.*, 2016).

We can infer from the above-mentioned evidence that miR-223 is antiangiogenic. High levels of miR-223 in our type 1 diabetes patients are a further proof suggestive of the antiangiogenic signal in this group of patients.

Metformin has shown to have no effect on plasma miR-223 levels. This could be due to unchanged MP levels in our TG after eight weeks of metformin treatment. Our result is similar to previous work which showed that metformin has no effect on miR-223 plasma levels in type 2 diabetes (Ortega *et al.* 2014). miR-223 overexpression does not affect AMPK or PIK3 pathway (Lu *et al.*, 2010). Both of these pathways are activated by metformin (Pernicova and Korbonits, 2014). Thus, it is likely that metformin mediates its effects through other mechanisms without affecting miR-223 expression. However, this needs to be further confirmed through in vitro and in vivo studies using metformin for a longer duration of time.

#### *5.4.1.6* miR-320

##### 5.4.1.6.1 Main finding

We have demonstrated that plasma miR-320 was significantly higher in type 1 diabetes patients when compared to healthy controls. Eight weeks of metformin treatment non-significantly reduced plasma miR-320 levels in type 1 diabetes. However, after metformin treatment, plasma miR-320 levels in type 1 diabetes and healthy controls were not significantly different.

##### 5.4.1.6.2 Novelty

We have for the first time demonstrated that miR-320 was significantly higher in type 1 diabetes. Our data provides additional evidence that anti-angiogenic signals plays an important role in type 1 diabetes. Our data can be used to understand the miR-320 upstream and downstream pathways in type 1 diabetes.

##### 5.4.1.6.3 Prior work and mechanism

Plasma miR-320 levels have been shown to be lower in type 2 diabetes (Zampetaki *et al.*, 2010). Similarly, *in vitro* animal model data has demonstrated that high glucose concentration would result in a reduction of miR-320 expression in EC and kidney (Feng and Chakrabarti, 2012). Therefore, it is likely that there is another mechanism other than hyperglycaemia resulting in differential expression of miR-320 in type 2 diabetes. miR-320 plasma levels are reduced in other metabolic condition like PCOS (Sørensen *et al.*, 2014). However, there is conflicting evidence regarding miR-320 levels in metabolic syndrome associated with type 2 diabetes (Karolina *et al.*, 2012). It was demonstrated that miR-320 levels were high in patients with metabolic syndrome with type 2 diabetes (Karolina *et al.*, 2012).

miR-320 levels have been demonstrated to be high in patients with acute myocardial ischemia (Devaux *et al.*, 2015). Similarly, miR-320 levels are upregulated in patients with coronary artery disease or high-risk individuals (Chen *et al.*, 2015a). *In vitro* and *in vivo* experiments have revealed that miR-320 played a role in the development of

atherosclerosis through inhibiting EC proliferation and inducing apoptosis (Chen *et al.*, 2015a). Furthermore, miR-320 expression in cardiomyocytes has been shown to be detrimental to their survival during ischemia/reperfusion injury (Ren *et al.*, 2009; Song *et al.*, 2014). Therefore, increased plasma miR-320 levels in type 1 diabetes is an additional factor suggestive of a microenvironment not favourable for endothelial repair.

Metformin reduced the plasma miR-320 levels in type 1 diabetes to levels similar to healthy controls. However, this reduction was not statistically significant. Is this reduction clinically significant? We have demonstrated a positive correlation between changes in plasma miR-320 levels with markers of endothelial injuries (cECs). Currently, available evidence and our data point towards a protective effect of this reduction in plasma miR-320. *In vitro* and *in vivo* experiments have demonstrated that blocking miR-320 during ischemia in cardiomyocytes was protective (Ren *et al.*, 2009; Song *et al.*, 2014). This will need to be confirmed by studying the effect of metformin on miR-320 and its downstream pathways.

#### *5.4.1.7 miR-126*

##### 5.4.1.7.1 Main finding

Our main finding includes:

1. We demonstrated that plasma miR-126 levels were non-significantly higher in type 1 diabetes when compared to healthy volunteers.
2. Plasma miR-126 levels were directly correlated with HDL cholesterol.
3. Eight weeks of metformin treatment did not change plasma miR-126 levels.

##### 5.4.1.7.2 Novelty

There is a lot of focus on miR-126 due to its angiogenic properties. We for the first time had demonstrated that metformin did not have any effect on circulatory miR-126 levels.

##### 5.4.1.7.3 Prior work and mechanism

Plasma miR-126 levels have been demonstrated to be lower in type 2 diabetes patients (Zampetaki *et al.*, 2010). Reduced plasma miR-126 levels have been shown to be present many years prior to the occurrence of type 2 diabetes (Zampetaki *et al.*, 2010). Plasma miR-126 levels are lower in patients with impaired fasting glucose and impaired glucose tolerance (Liu *et al.*, 2014b). In healthy individuals, plasma miR-126 levels increase with age. However, this effect is lost in type 2 diabetes. Levels are lower in patients with poorly controlled diabetes. This has been demonstrated in vitro where high glucose reduced miR-126 expression in EC (Domenico *et al.*, 2015). Treatment with diet or insulin improved plasma miR-126 levels in type 2 diabetes (Liu *et al.*, 2014b). Circulatory angiogenic cells in type 2 diabetes also expressed low levels of miR-126 (Domenico *et al.*, 2015). In contrast to type 2 diabetes, patients with type 1 diabetes has shown similar levels of plasma miR-126 levels when compared to healthy controls (Osipova *et al.* 2014 and Nielsen et al. 2012). However, urinary miR-126 levels are lower and correlated negatively with HbA1c in type 1 diabetes (Osipova *et al.* 2014). Cultured PAC from type 1 diabetes demonstrated an increased miR-126 expression (Garcia de la Torre *et al.*, 2015).

Our data of non-significantly raised miR-126 levels in our type 1 diabetes patients is in line with another study on type 1 diabetes (Osipova *et al.* 2014). However, our and Osipova *et al.* (2014) results are in contrast with type 2 diabetes studies. In type 2 diabetes studies (Liu *et al.*, 2014b; Domenico *et al.*, 2015), it has been proposed that hyperglycaemia may be a factor in dysregulation of plasma miR-126 levels. Both type 1 diabetes and type 2 diabetes patients are exposed to hyperglycaemia. However, we have not shown any correlation between circulating miR-126 and HbA1c and/or glucose variability in our type 1 diabetes patients. Thus, there is likely another mechanism for the dysregulation of miR-126 levels in type 2 diabetes.

Our patients with type 1 diabetes had higher HDL cholesterol when compared to healthy volunteers. This could be due to some of our patients being treated with statins. Circulatory miR-126 is known to be transported on HDL particles (Wagner *et al.*, 2013). This could explain a slight non-significantly higher plasma miR-126 levels in our patients.

Eight weeks of metformin treatment did not change plasma miR-126 levels. This is in line with data from type 2 diabetes study. Three months of metformin treatment did not alter the expression of miR-126 plasma levels (Ortega *et al.* 2014). Therefore, we can conclude that metformin most likely has no effect on plasma miR-126 levels.

miR-126 has been shown to have angiogenic properties and essential for vascular haemostasis. It has been proved in *in vitro* and *in vivo* experiments. miR-126 mediates its effect through repressing SPRED-1 expression which in turn activate MAP kinase pathway. In addition to SPRED-1, miR-126 inhibits PIK3 pathway. This, in turn, activates VEGF-mediated angiogenesis (Fish *et al.*, 2008). This is most likely the mechanism in non-diabetic individuals. As mentioned above, miR-126 levels are lower in type 2 diabetes. This reduced the functions of EPC through affecting VEGF-mediated pathway (Wu *et al.*, 2016). In contrast to this, (van Solingen *et al.*, 2011) had shown that damaged endothelium had lower miR-126 expression leading to increase in SDF-1 and VCAM-1 levels. This resulted in the increased mobilisation of EPC. However, increased PIK3 activity due to reduced miR-126 can lead to EPC-mediated intimal hyperplasia (Zhang *et al.*, 2013). This can contribute to atherogenesis.

Therefore, miR-126 levels are necessary to keep an equilibrium between angiogenesis, mobilisation of EPC and vascular repair.

#### *5.4.1.8 miR-92a*

miR-92a is a proatherogenic and anti-angiogenic miRNA. It is essential for endothelial cell function (Iaconetti *et al.*, 2012). We demonstrated that plasma miR-92a levels were similar in type 1 diabetes and healthy volunteers. This is in line with other available evidence that miR-92a expression does not change in both type 1 diabetes and type 2 diabetes (Nielson *et al.* 2012 and Ortega *et al.* 2014). Furthermore, eight weeks of metformin treatment did not significantly reduce the plasma levels in type 1 diabetes. Limited evidence from a type 2 diabetes study demonstrated similar results where three months of metformin treatment did not change plasma miR-92a levels (Ortega *et al.* 2014).

miR-92a is thought to be endothelial in origin (Fichtlscherer *et al.*, 2010; Ren *et al.*, 2013). miR-92a is upregulated in EMPs of CAD patients (Ren *et al.* 2013). However, circulatory levels are not significantly higher in our cohort despite increased levels of EMPs. Furthermore, we have demonstrated an inverse correlation of EMPs and plasma miR-92a (Appendix A). This is suggestive that most of the miR-92a are not carried by MP in type 1 diabetes. Indeed, our data is in line with current evidence which is suggestive that most of the circulatory miR-92a are outside vesicles. MP isolated from patients with diabetes did not show any differential expression of miR-92a (Jansen *et al.*, 2016). Hyperglycaemia did not demonstrate any change in expression of miR-92a from EC (Jansen *et al.* 2016).

Our data is suggestive that plasma miR-92a may not play a major role in the pathogenesis of vascular disease and endothelial dysfunction in type 1 diabetes. Furthermore, evidence from our data and type 2 diabetes have shown that metformin does not regulate miR-92a expression.

### *5.4.2 Summary*

We have studied eight microRNAs. These can be divided into two major groups: 1) angiogenic and 2) antiangiogenic. miR-126 and miR-210 are angiogenic miRNAs whereas miR-21a, miR-222, miR-320, miR-92a, miR-223 and miR-195 are anti-angiogenic miRNAs. We have shown that miR-21a, miR-222, miR-320, miR-223 and miR-195 are significantly raised in type 1 diabetes when compared to age- and gender-matched healthy volunteers. Four miRNAs (miR-21a, miR-222, miR-320 and miR-195) except miR-223 positively correlated with each other. Thus, it is suggestive that microenvironment in type 1 diabetes patients increases the release of antiangiogenic and proapoptotic miRNAs in the plasma.

These changes in plasma miRNA levels could be reflective of a clinical condition in the microenvironment of our type 1 diabetes patients. Circulating miRNAs have been reported as biomarkers for diagnosis and prognosis in patients with CVD. Each disease process provides a unique feature which may cause differences in the profile of circulating microRNAs. Furthermore, expression/levels may differ in each tissue.

In addition to being a biomarker and reflecting clinical condition, circulating miRNAs has been shown to play a role in gene expressions of exposed tissues. Multiple studies have shown the functional capacity of circulating miRNAs in either development of the disease or vascular repair. Raised antiangiogenic miRNA levels in patients with type 1 diabetes individuals provide an environment leading to impaired vascular repair.

We explored which pathways will be affected by raised antiangiogenic miRNAs (Vlachos *et al.*, 2015). We analysed the pathway using DIANA TOOLS mirPATH v.3 online software. The analysis of the pathway demonstrates that angiogenesis, proliferative, migration, adhesion, cell cycle and apoptosis pathways are dysregulated. Few of the pathways which are dysregulated includes PIK3-AkT signalling, focal adhesion, TGF-β signalling, cell cycle and p53 pathways, etc (Figure 67).

One of the angiogenic miRNAs (miR-210) was significantly raised in patients with type 1 diabetes. We have complemented findings of a previous study showing raised circulating miR-210 in type 1 diabetes. This could represent a compensatory mechanism trying to mount a regenerative response to ongoing vascular damage. However, it is important to note that plasma miR-210 levels do not correlate with any of the anti-angiogenic miRNAs. miR-210 is an angiogenic miRNA. It may be that as a part of reparative response, there is an excess expression of miR-210. We have discussed previously that excess expression of miR-210 has been shown to play a role in the development of diabetes complication and development of atherosclerosis. The role of excess miR-210 expression needs to be studied in the presence of anti-angiogenic miRNAs *in vitro* and *in vivo* studies. Similarly, miR-126, another angiogenic miRNA studied did not show increased plasma levels when compared to healthy controls. To our knowledge, there is no published evidence exploring the effect of the intervention on plasma miRNA in type 1 diabetes. Only one study explored the effect of metformin on plasma miRNA levels in patients with diabetes. This study included patients with type 2 diabetes (Ortega *et al.*, 2014). In this study, circulatory levels of miR-222 and miR-140 were significantly decreased whereas circulatory levels of miR-142 and miR-192 were significantly increased. Insulin resistance, weight and HbA1c decreased significantly. These factors could play a role in modifying levels of circulatory miRNAs levels. HbA1c and weight did not change in our study. Furthermore, glucose variability did not change after eight weeks of metformin treatment. Effect of glucose variability and HbA1c on plasma miRNA levels needs to be explored further.

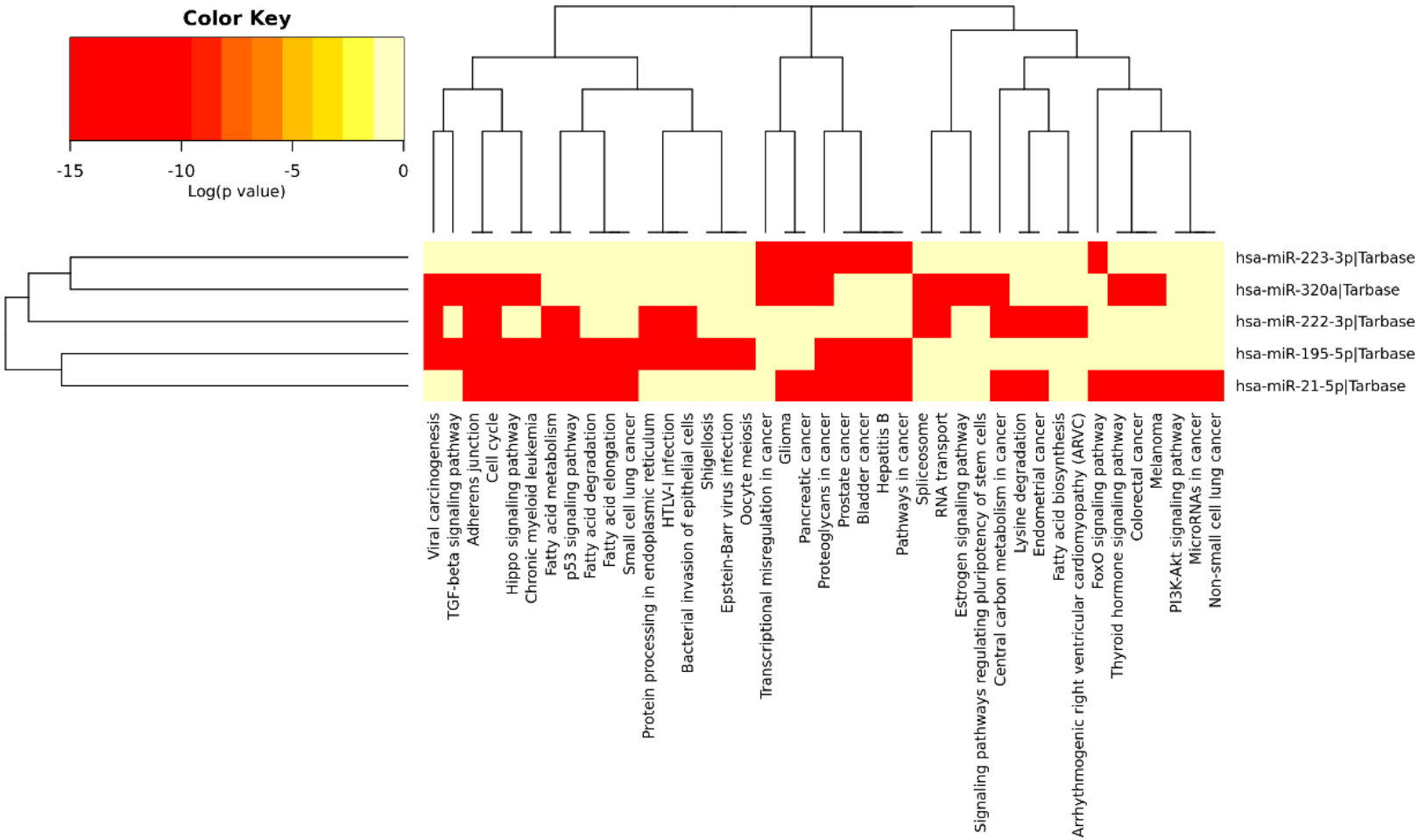


Figure 54: miRNAs (miR-21-5p, miR-5p, miR-222-3p, miR-320, miR-223-5p) versus pathways heat map (clustering based on significance levels). Darker colours represent higher significance values and light colour represents lower significance value. The attached dendrograms on both axes depict hierarchical clustering results for miRNAs and pathways, respectively.

miR-222, miR-21a, miR-320 and miR-195 are anti-angiogenic miRNAs. Reduction of these miRNA levels may provide an environment conducive to vascular repair. Effect of metformin on circulatory miR-210 levels is interesting. It seems that metformin is encouraging to normalise angiogenic signal towards normal. This could be compensatory as anti-angiogenic miRNA are being reduced. Alternatively, this could represent the effect of metformin on excess angiogenic signal and balance the repair process. This will need to be explored further in *in-vitro* and *in vivo* studies. Before this could be done, further work needs to be done in improving the validity of the miR-210 analysis.

We explored which pathways will be affected by improvement of two significantly reduced (miR-21a and miR-195) antiangiogenic miRNAs (Vlachos *et al.* 2015). The analysis of the pathway demonstrates that reduction in plasma levels of these miRNA will improve angiogenesis, proliferation, migration, adhesion and cell cycle (Figure 68).

Our data has yielded important information regarding angiogenic and antiangiogenic signal in the plasma. Our work can be meaningfully extended by exploring miRNA, miRNA targets and downstream protein expression. Gain of function and loss of function experiment (in vitro and in vivo) experiments will yield information regarding individual miRNA and their functions. This will be important in not only generating circulatory biomarkers but also prognostic markers and future treatment.

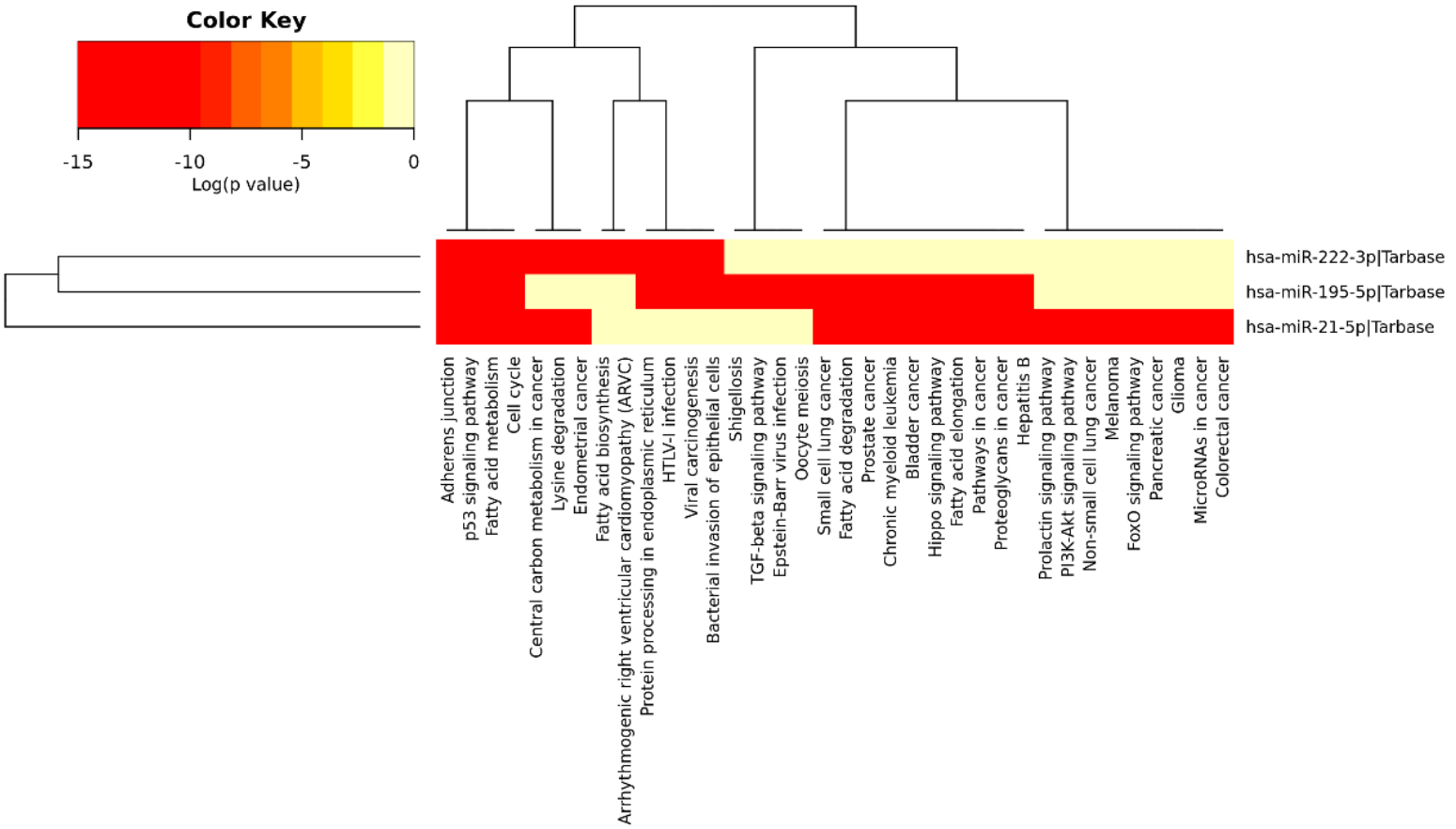


Figure 55: miRNAs (miR-21, miR-195, miR-222) versus pathways heat map (clustering based on significance levels). Darker colours represent higher significance values and light colour represents lower significance value. The attached dendrograms on both axes depict hierarchical clustering results for miRNAs and pathways, respectively.

# Chapter 6: Effect of metformin on cytokines

## 6.1 Introduction

Cytokines are a diverse group of mediators released by inflammatory cells or tissues. Cytokines play a vital role in physiological and pathological processes. Cytokines are involved in inflammatory, vascular, immune (innate and adaptive) and haematopoiesis response. The effects of cytokines are mediated by binding to specific cell surface receptors and modulating intracellular transduction pathways (Kofler *et al.*, 2004). This can ultimately lead to activation of transcription mediators like NF-κB and STAT (Kishimoto *et al.*, 1994; O'Shea *et al.*, 2002).

Cytokines play an integral role in vasculopathy. Cytokines act on EC via paracrine and autocrine manner. Circulatory cytokines like TNF-α act on EC, leading to EC activation. Activation of a transcription mediator like NF-κB leads to increased production of pro-inflammatory cytokines like IL-6, IL-1 and TNF-α by EC (Kofler *et al.*, 2004). Cytokines like IL-6 and TNF-α have been shown to cause endothelial dysfunction (Wassmann *et al.*, 2004; Zhang *et al.*, 2009a). Cytokines cause vascular cell activation, increased adhesion molecule expression, leukocyte activation, foam cell formation and oxidative stress. All these are steps in the formation of atherosclerotic plaque (Figures 69 and 73). Cytokines also contribute to atherosclerotic plaque rupture and thrombosis by increasing macrophages and smooth muscle cell (SMC) apoptosis, inhibition of collagen synthesis, increased MMP activity, reduced TIMP-1 activity and increased coagulation activation (Ait-Oufella *et al.*, 2011).

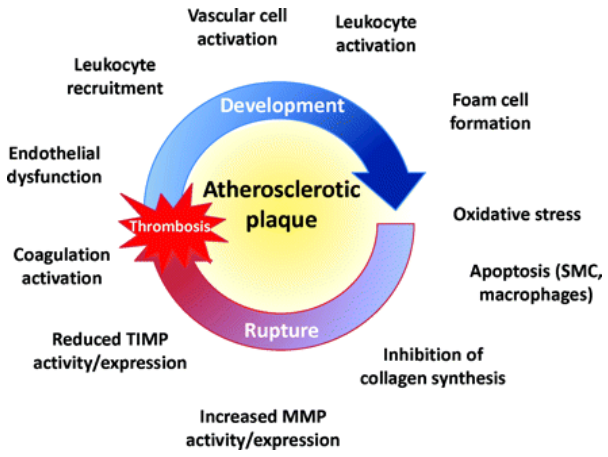


Figure 56: Role of cytokines in atherosclerosis development and plaque disruption.

(Reproduced from Ait-Oufella *et al.* 2011).

Cytokines play a major role in the development of complications in patients with diabetes mellitus. Hyperglycaemia is a major issue in both type 1 and type 2 diabetes. Even with good control, intermittent glucose excursion cannot be controlled. Therefore, hyperglycaemia results in a modulating myriad of metabolic and haemodynamic changes leading to diabetic vasculopathy. Hyperglycaemia-induced inflammation and oxidative stress is a major mechanism in the pathophysiology of development of a diabetes-related complication. The release of inflammatory cytokines and chemokines contributes to a vicious cycle of continued vascular damage (King, 2008; Elmarakby and Sullivan, 2010) (Figure 70).

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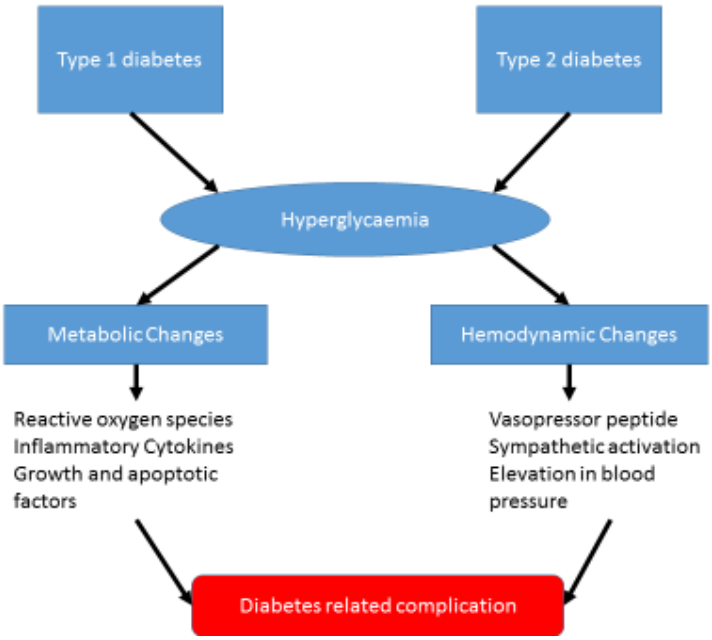


Figure 57: Role of hyperglycaemia and in type 1 and type 2 diabetes-related complications.

Elmarakby and Sullivan 2010

Below we discuss selected cytokines which have been shown to play a role in the pathogenesis of atherosclerosis and diabetes-related complication.

## 6.2 TIMP-1

TIMP-1 or tissue inhibitor of metalloproteinases-1 is one of the four inhibitors that control the activity of matrix metalloproteinases (MMP). TIMP-1 plays a role in extracellular matrix turnover via its effect on MMP (Brew and Nagase, 2010). The role of MMP and TIMP-1 in EPC mobilisation is still being elucidated. Based on current evidence, we understand that MMP most likely plays a role in the mobilisation of EPC from bone marrow through the release of cKIT. cKIT, in turn, facilitate the release of EPC (Huang *et al.*, 2009b). Increased TIMP activity will reduce MMP function (Brew and Nagase, 2010), thereby limiting EPC mobilisation (Figure 71).

TIMP-1 levels are higher in type 1 and type 2 diabetes than in thehealthy control (Maxwell *et al.*, 2001; Lee *et al.*, 2005b). Higher TIMP-1 is associated with CVD and albuminuria in type 1 diabetes and type 2 diabetes (Peeters *et al.*, 2015). TIMP-1 and its target, MMPs, is speculated to be involved in diabetic nephropathy (Dimas *et al.*, 2015). High glucose concentration has been shown to increase TIMP-1 levels and decrease MMP levels in renal tissues (Bai *et al.*, 2006). This can lead to glomeruli basement membrane abnormality. miR-21 has been shown to increase TIMP-1 levels and is speculated to play a role in renal fibrosis (Wang *et al.*, 2013a).

Currently, there is no in vivo evidence on effect of the metformin on TIMP-1 levels in type 1 diabetes. Most of the experiments have been done in vitro using a supraphysiological dose of metformin (Hwang *et al*., 2010).

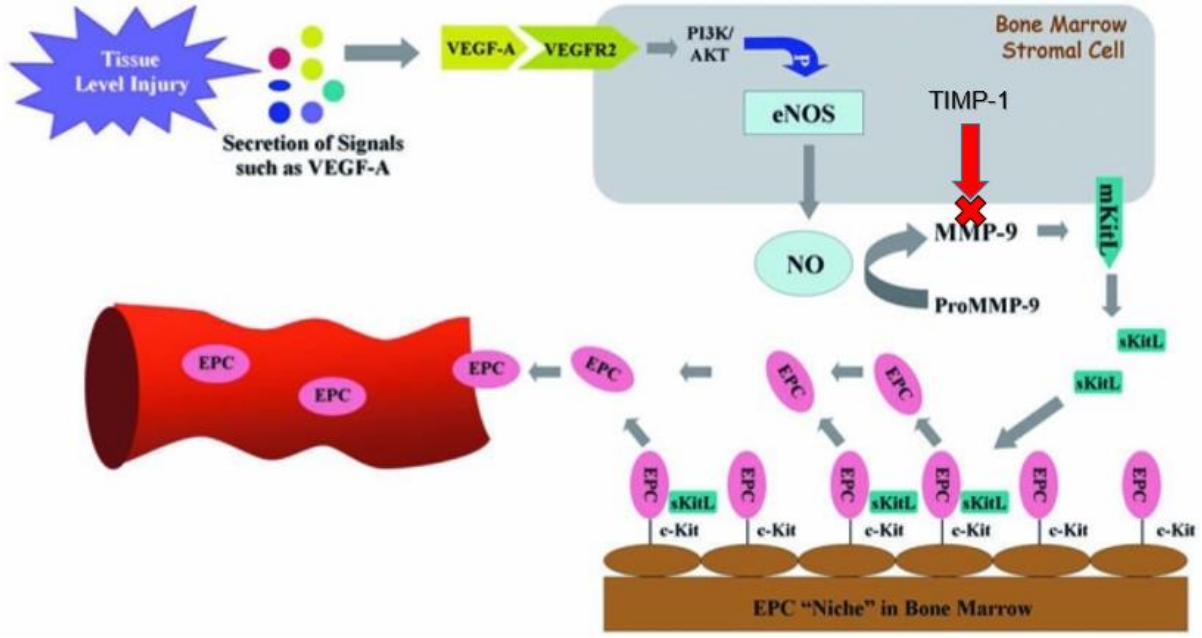


Figure 58: Mobilisation of EPC from bone marrow. Effect of TIMP-1 on MMP-9.

Adapted from (Liu and Velazquez, 2008)

## 6.3 Inflammatory cytokines

### *6.3.1 Tumour necrosis factor alpha (TNFα)*

TNFα is a proinflammatory cytokine involved in acute phase reaction (Moller, 2000). Levels of TNFα are elevated in type 1 and type 2 diabetes (Swaroop *et al.*, 2012; West *et al.*, 2015). Circulatory TNFα is implicated in endothelial dysfunction. This effect can be mediated via its action on the endothelium causing increased expression of adhesion molecules. This can lead to an increase in cell infiltration. TNFα reduces the availability of NO. TNFα acts on NO synthase and reduces the production of NO (Zhang *et al.*, 2009a). Furthermore, removal of NO is increased (Gao *et al.*, 2007). TNFα increases the production of reactive oxygen species (ROS). This effect is mediated via multiple mechanisms. In diabetes mellitus, TNFα increases the activity of NADPH oxidase, leading to increased production of ROS (Guzik *et al.*, 2002).

The effect of TNFα on EPC levels is of considerable interest. It is speculated that the TNFα myelosuppressive effect potentially plays a role in reduced levels of cEPC in patients with CVD (Agnoletti *et al.*, 1999). Anti- TNFα treatment in RA have been shown to improve cEPC numbers (Spinelli *et al.*, 2013).

Furthermore, our group has shown that improvement in cEPC numbers was inversely related to levels of TNFα at baseline (West *et al.* 2015). This might be due to TNFα direct myelosuppressive effect or due to TNFα effect on levels of NO. There is a need to explore the effect of inflammation on cEPC numbers.

*In vitro* studies have shown that metformin decreases TNFα levels (Huang *et al.*, 2009a; Arai *et al.*, 2010; Hyun *et al.*, 2013). Huang *et al*., 2009 demonstrated that metformin exposure reduced the production of TNFα by EC. A similar effect was noted when human monocytes were incubated in a metformin treated medium (Arai *et al.,* 2010). However, this effect has not been replicated in *in vivo* studies. Metformin treatment has been shown to have no effect on TNFα levels in type 2 diabetes (Lithell *et al.*, 1998; Hsieh *et al.*, 2007).

### *6.3.2 IL-6*

IL-6 belongs to the IL-6 cytokines family and is considered to be a pro-inflammatory cytokine. However, we now understand that IL-6 is a pleiotropic cytokine with both pathological and protective properties in type 1 and type 2 diabetes mellitus. IL-6 acts on cells with the gp130 receptor and can modulate the JAK/STAT and MAPK pathway. These pathways play a role in proliferation, differentiation, survival and apoptosis (Kristiansen and Mandrup-Poulsen, 2005).

IL-6 is considered a biomarker of vascular inflammation and CVD (Hou *et al.*, 2008). IL-6 levels have been shown to predict high-risk mortality in older patients with CVD (Tsutamoto *et al.*, 1998). Levels of IL-6 have been demonstrated to be higher in individual with obesity and those who go on to develop T2DM and MI (Ridker *et al.*, 2000b; Pradhan *et al.*, 2001). In addition to being a biomarker, IL-6 plays a central role in the pathogenesis of CVD (Hou *et al.,* 2008).

IL-6 has been shown to play a role in endothelial dysfunction via increased production of ROS. This is mediated via increased expression of the Angiotensin 1 receptor (AT-

1) (Wassmann *et al.*, 2004). Overexpression of AT-1 exacerbates oxidative stress leading to endothelial dysfunction. Furthermore, Ang-II mediated vasoconstriction may be accentuated (Wassmann *et al.,* 2004). IL-6 levels are associated with endothelial dysfunction in healthy individuals (Esteve *et al.*, 2007). Given the available evidence, IL-6 is involved in the pathogenesis of atherosclerosis (Schuett *et al.*, 2009).

*In vitro* studies have demonstrated that metformin reduces IL-6 levels in ECs (Libby *et al.*, 2006). This is likely mediated via PI3K-dependent AMPK phosphorylation (Libby *et al.,* 2006). Effect of metformin in patients with PCOS has produced conflicting results. Lin *et al*., 2011 reported that IL-6 levels were reduced significantly after 12 weeks of metformin treatment (Lin *et al.*, 2011). However, Mohlig *et al.,* 2004 and Jakubowska *et al.,* 2008 demonstrated that plasma IL-6 levels did not change after 6 months of metformin therapy (Mohlig *et al.*, 2004; Jakubowska *et al.*, 2008). IL-6 levels have been shown to be higher in type 1 and type 2 diabetes (Sibal *et al.,* 2009). There is limited evidence on the effect of metformin on plasma levels of IL-6 in type 2 diabetes. Metformin treatment did not reduce IL-6 levels in obese patients with type 2 diabetes (Ersoy *et al.*, 2008).

### *6.3.3 CRP*

CRP is an acute phase reactant and is associated with the inflammatory process contributing to CVD. Levels of CRP are elevated in patients with obesity, impaired glucose tolerance, and type 1 and type 2 diabetes. CRP levels are not only elevated in patients with CVD but they are also a strong predictive marker for future cardiovascular risk in patients without overt CV disease (Tracy *et al.*, 1997; Koenig *et al.*, 1999; Ridker *et al.*, 2002).

CRP is not only a biomarker for CV disease or risk but may also may play a role in the pathogenesis of atherosclerosis (Verma *et al.*, 2004b; Amit Kumar Shrivastava, 2015). The causal relationship between CRP and CVD is controversial. Even with a wealth of evidence, we are still debating whether CRP plays a role in development of CVD or is a by-product of the inflammatory process which causes the development of CVD. It acts not only on EC but also on vascular smooth muscle cells (VSMCs). CRP inhibits

eNOS activity, stimulates endothelin-1 and IL-6 production and increases adhesion molecules expression in EC (Pasceri *et al.*, 2000; Verma *et al.*, 2002a; Verma *et al.*, 2002b). CRP acts on macrophages and augments their ability to take up LDL cholesterol and develop into form cells (Amit Kumar Shrivastava, 2015). CRP has been shown to augment Ang-1 activity in VSMC and promote ROS production and neointimal formation (Wang *et al.*, 2003). CRP has been shown to upregulate NF-κB activity, leading to pro-atherosclerotic genes transcription (Verma *et al.*, 2003). Furthermore, CRP has shown to reduce EPC survival and differentiation (Verma *et al.*, 2004a).

Metformin has been shown to reduce CRP levels in PCOS, individuals at high risk of type 2 diabetes (Chakraborty *et al.*, 2009; Shi *et al.*, 2014). CRP levels have been shown to be influenced by weight and glycaemic control (Goldberg *et al.*, 2014). In a randomised trial, it was demonstrated that the effect of metformin on CRP can be explained by changes in the weight and glycaemic control (Goldberg *et al.,* 2014). To the best of our knowledge, there is no evidence on the effect of metformin on CRP in type 1 diabetes. What we know is that CRP levels are higher in patients with type 1 diabetes (Sibal *et al*., 2009). Our work explored the effect of metformin on CRP in patients with type 1 diabetes.

### *6.3.4 IL-8*

IL-8 is a chemokine and is also known as a neutrophil chemotactic factor. It is produced by many cells including endothelial cells. It is stored in Weibel-Palade bodies in ECs (Utgaard *et al.*, 1998). IL-8 levels are elevated in both type 1 and type 2 diabetes. Being a potent chemoattractant, IL-8 plays a critical role in the development of atherosclerosis (Shin *et al.*, 2002). IL-8 plays an important role in the recruitment of IL-8 at the site of vascular injury. Furthermore, IL-8 facilitates the transmigration of inflammatory cells into subendothelial space (Gerszten *et al.*, 1999). IL-8 plays a role in initial leukocytes adhesion to the EC (DiVietro *et al.*, 2001). However, experimental IL-8 elimination resulted in the prevention of atherosclerosis (Boisvert *et al.*, 1998). The monocyte chemoattractant protein-1 (MCP-1) plays an important role alongside IL-8. Levels of IL-8 have been noted to be high in the atherosclerotic arterial wall (Rus *et al.*,

1996). Macrophages have been identified as the main source of IL-8 in atherosclerotic plaques (Apostolopoulos *et al.*, 1996).

Currently, there is no evidence on the effect of metformin in type 1 diabetes. Evidence on the effect of metformin on IL-8 in type 2 diabetes is limited. We could only find one study exploring the effect of metformin in type 2 diabetes. This study demonstrated that metformin treatment reduced IL-8 levels in type 2 diabetes (Lavrenko *et al.*, 2012). In vitro studies have shown that metformin reduced IL-8 production in EC, smooth muscle cells, macrophages and endometriotic stromal cells (Libby *et al.,* 2006; Takemura *et al.,* 2007). Metformin reduced IL-8 production in a dose dependent manner. This was shown to be significant at physiological concentration of metformin (Takemura *et al.*, 2007).

### *6.3.5 INF-γ*

INF-γ is a pro-inflammatory cytokine and belongs to the INF cytokine family (Mire-Sluis A., 1998). It has been established that INF-γ plays a role in the progression of atherosclerosis (McLaren and Ramji, 2008) (Figure 72). However, it is debated whether it is pro-atherosclerotic or anti-atherosclerotic (Muhl and Pfeilschifter, 2003). INF-γ increases the production of chemokines like MCP-1, leading to increased recruitment of macrophages and T-lymphocytes (Valente *et al.*, 1998). INF-γ, itself increases recruitment of immune cells. INF-γ increases expression of adhesion molecules in EC (Chung *et al.*, 2003). INF-γ has been shown to potentiate the adhesion factor expression by other inflammatory cytokines like TNF-α (Zhang *et al.*, 2011). INF-γ activates monocytes to macrophages and promotes the development of form cells (Schroder *et al.*, 2004). In an animal model, INF-γ promotes SMC proliferation and differentiation (Tellides *et al.*, 2000). INF-γ plays a role in apoptosis of foam cells and plaque disruption (Harvey and Ramji, 2005). In contrast, INF-γ functions have some anti-atherogenic properties. INF-γ has been shown to reduce oxidation of LDL and uptake of oxiLDL by macrophages (Fong *et al.*, 1994). INF-γ increases the production of NO via upregulation of iNOS (Chung *et al.*, 2010). Most INF-γ action is mediated by activating the JAK/STAT pathway (Darnell *et al.*, 1994).

Limited information is available on the effect of metformin in INF-γ. In animal model mimicking inflammatory condition like multiple sclerosis, metformin has been shown to suppress expression of INF-γ (Nath *et al.*, 2009).

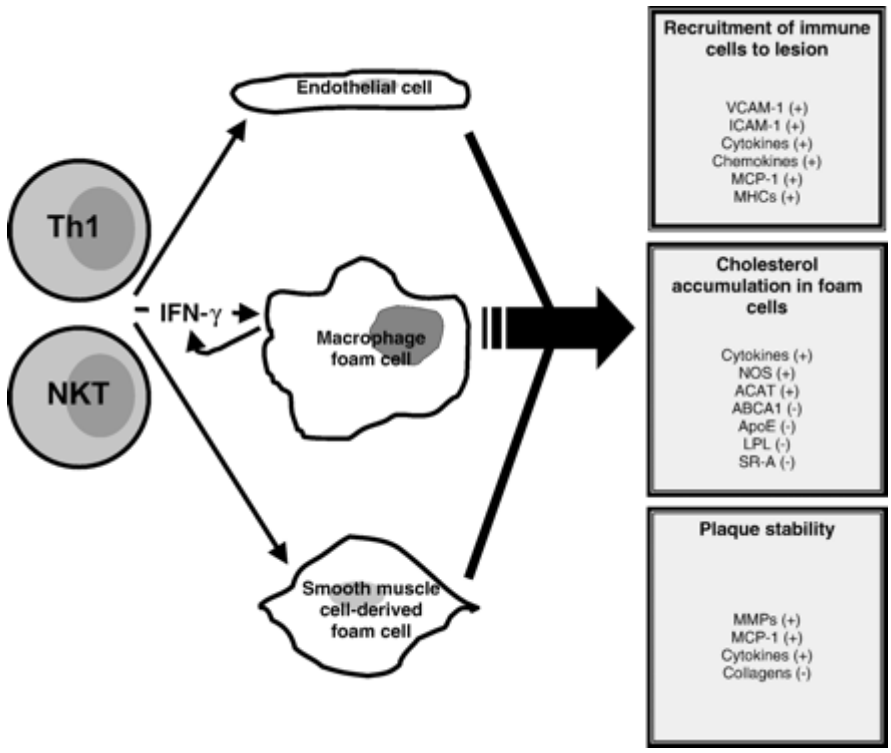


Figure 59: Role of INF-γ in atherosclerosis.

Adapted from Harvey and Ramji, 2005

### *6.3.6 IL-10*

IL-10 is a potent anti-inflammatory and anti-atherogenic cytokine (Han and Boisvert, 2014). IL-10 acts directly on macrophages to control lipid modulation and inhibition of MMP and tissue factor (TFT) production (Kamimura *et al.*, 2005; Han *et al.*, 2010). IL-10 inhibits the release if pro-inflammatory cytokines like IL-8, TNF-α and IL1β (de Waal Malefyt *et al.*, 1991). Furthermore, it reduces the release of chemokines like MCP-1. IL-10 inhibits the action of IL-12 (Uyemura *et al.*, 1996). This leads to reduced activation of the immune cell-like macrophages. IL-10 is present in atherosclerotic plaques. IL-10 plays a vital role in plaque stability. IL-10 inhibits the production of iNOS, thereby protecting the cell in the plaques from increased cell death (Mallat *et al.*, 1999).

In hyperlipidemic mice models, overexpression of IL-10 reduced atherosclerosis (Han *et al.*, 2010).

Levels of IL-10 have been demonstrated to be lower in patients with diabetes mellitus (Yaghini *et al.*, 2011). IL-10 levels are associated with improved clinical outcomes in patients with CVD (Heeschen *et al.*, 2003b).

Metformin has been shown to increase IL-10 production from activated macrophages. (Kelly *et al.*, 2015). The effect of metformin in type 1 diabetes has not been studied so far. Metformin has been shown to increase IL-10 levels in type 2 diabetes (Chen *et al.*, 2016). However, this effect was noted after one year of metformin treatment.

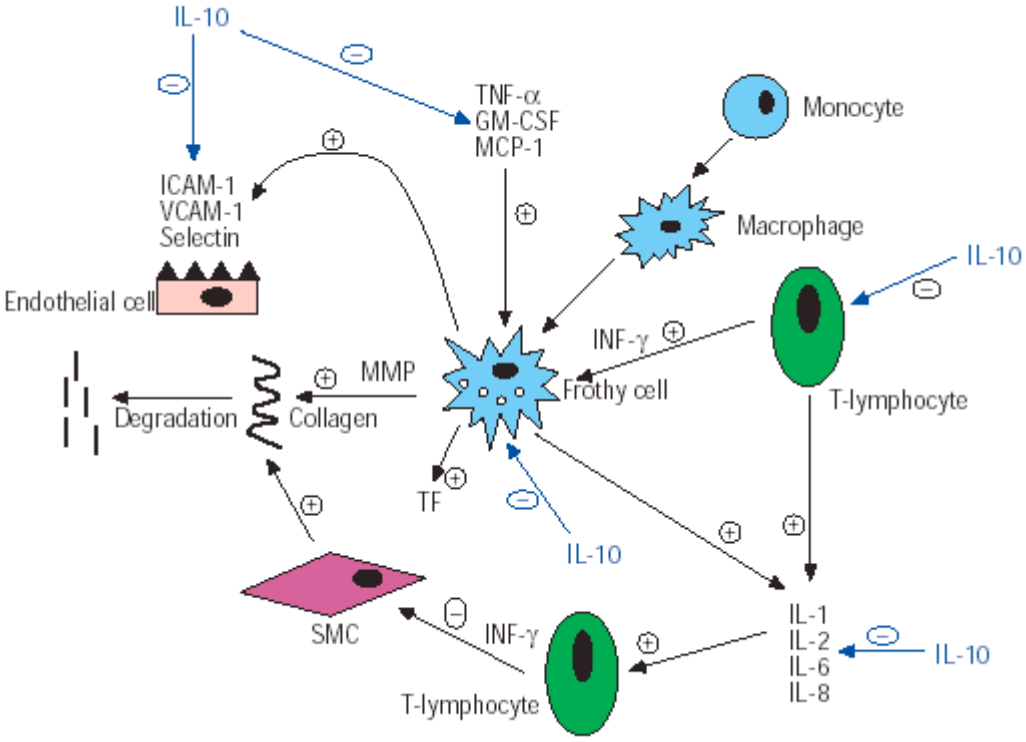


Figure 60: Role of cytokines in atherosclerosis.

Adapted from Pérez Fernández and Kaski, 2002

### *6.3.7 IP-10*

IP-10 is also known as interferon gamma-induced protein 10 or C-X-C motif chemokine 10 (CXCL-10) (Heller *et al.*, 2006). IP-10 secretion from ECs and inflammatory cells are induced by INF-γ (Luster and Ravetch, 1987). IP-10 plays a major role in atherosclerosis. This effect is mediated by VSMC proliferation and inflammatory cell infiltration (Wang *et al.*, 1996). IP-10 has been shown to be expressed in atherosclerotic lesions IP-10 acts as a chemokine and attract immune cells to the site of vascular injury (Niki *et al.*, 2015). In an animal model, blocking IP-10 action has been shown to inhibit atherosclerosis formation (Heller *et al.*, 2006; Zuojun *et al.*, 2011). IP-10 levels are elevated in individuals with type 1 and type 2 diabetes (Shimada *et al.*, 2001; Xu *et al.*, 2005). IP-10 is associated with endothelial dysfunction in type 2 diabetes (Kajitani *et al.*, 2010). Increased IP-10 levels have been demonstrated in CAD patients and seemed to precede cardiac events (Herder *et al.*, 2006).

There is paucity of evidence regarding the effect of metformin on IP-10. Our group has demonstrated that metformin in physiological concentration reduced the expression of IP-10 in CD34+ cells in a model of the diabetic state combined with hypoxia (Bakhashab *et al.,* 2016).

## 6.4 Vascular injury and adhesion markers

### *6.4.1 P-Selectin and E-Selectin*

P-Selectin and E-Selectin belong to the selectin family (Blankenberg *et al.*, 2003). P-Selectin is stored in granules of platelets and ECs. P-Selectin is immediately released once platelets and ECs are activated by an inflammatory response. This response is short lived. However, with continued inflammatory stimuli like TNF-α, P-Selectin is formed and expressed on the cell surface (McEver, 2002). E-Selectin is present on ECs. E-Selectin is not found in resting ECs. Once ECs are activated by the inflammatory response, E-selectin is formed by the ECs and expressed on the cell surface (Wang and Huo, 2010). P-Selectin and E-Selectin play a major role in the initial stages of atherosclerosis. P-Selectin and E-Selectin are involved in leukocyte capture and rolling on activated ECs (McEver, 2002) (Figure 74).

Levels of P-Selectin and E-Selectin are elevated in patients with diabetes mellitus (Jilma *et al.*, 1996; Albertini *et al.*, 1998; Yngen *et al.*, 2001). E-Selectin has been demonstrated to be associated with increased risk of diabetes (Song *et al.*, 2007). P-Selectin is associated with an increased risk of future CVD (Ridker *et al.*, 2001). E-Selectin has been demonstrated to be elevated in individuals with CV risk factors (Demerath *et al.*, 2001). P-Selectin levels are elevated in patients with CAD including AMI (Ikeda *et al.*, 1994; Aref *et al.*, 2005).

Currently, there is no evidence regarding the effect of metformin on P-Selectin and E-Selectin levels in type 1 diabetes. Most of the work has been done in type 2 diabetes and on E-Selectin. Improvement in glycaemic control and lipid profile has been shown to improve E-Selectin levels (Albertini *et al.*, 1998; Yki-Järvinen and Hannele, 2001). Short-term (16 weeks) metformin treatment in type 2 diabetes improved E-Selectin levels (De Jager *et al.*, 2005). However, this finding can be explained by improvement in glycaemic control and lipid profile. When this group was followed for over 4 years, metformin treatment did not lead to any change in E-Selectin levels in type 2 diabetes (de Jager *et al.*, 2014).

There is a paucity of evidence on the effect of metformin in diabetes mellitus. An in vitro and animal study has demonstrated that P-Selectin expression is lower in activated platelets (Xin *et al.*, 2016). Further work is needed to elucidate the effect of metformin on P-Selectin in type 1 diabetes.

### *6.4.2 Immunoglobulin superfamily*

Intercellular adhesion molecules 1 (ICAM-1), ICAM-2, ICAM-3 and Vascular cell adhesion molecules 1 (VCAM-1) belong to the immunoglobulin superfamily. ICAM-1 is expressed by leukocytes and ECs. ECs express VCAM-1 (Blankenberg *et al.*, 2003). ICAM-1 and VCAM-1 plays a major role in the pathogenesis of atherosclerosis. ICAM-1 and VCAM-1 mediate the adhesion of inflammatory cells to EC and are involved in the transmigration of inflammatory cells into intimal space (Wang and Huo, 2010) (Figure 74).

ICAM-1 and VCAM-1 can be measured in plasma and referred to as soluble ICAM-1 (sICAM-1) and soluble VCAM-1 (sVCAM-1). sICAM-1 and sVCAM-1 have been shown to be elevated in children with diabetes mellitus, obesity and hypertension (Glowinska *et al.*, 2005). However, our group has previously demonstrated that ICAM-1 and VCAM-1 were not elevated in patients with type 1 diabetes (Sibal *et al.*, 2009a). In type 2 diabetes, ICAM-1 and VCAM-1 have been demonstrated to be elevated in patients with microalbuminuria (Bruno *et al.*, 2008). Similar findings have been described in type 1 diabetes (Clausen *et al.*, 2000). sICAM-1 has been shown to have strong predictive value for future CAD in healthy individuals (Hwang *et al.*, 1997; Ridker, 1998), whereas sVCAM-1 has been shown to predict future CV events in patients with CAD and diabetes mellitus (Jager *et al.*, 2000; Malik *et al.*, 2001). sICAM-1 has been shown to demonstrate atherosclerotic disease (Pradhan *et al.*, 2002), whereas sVCAM-1 demonstrates disease severity (De Caterina *et al.*, 1997; Blankenberg *et al.*, 2003).

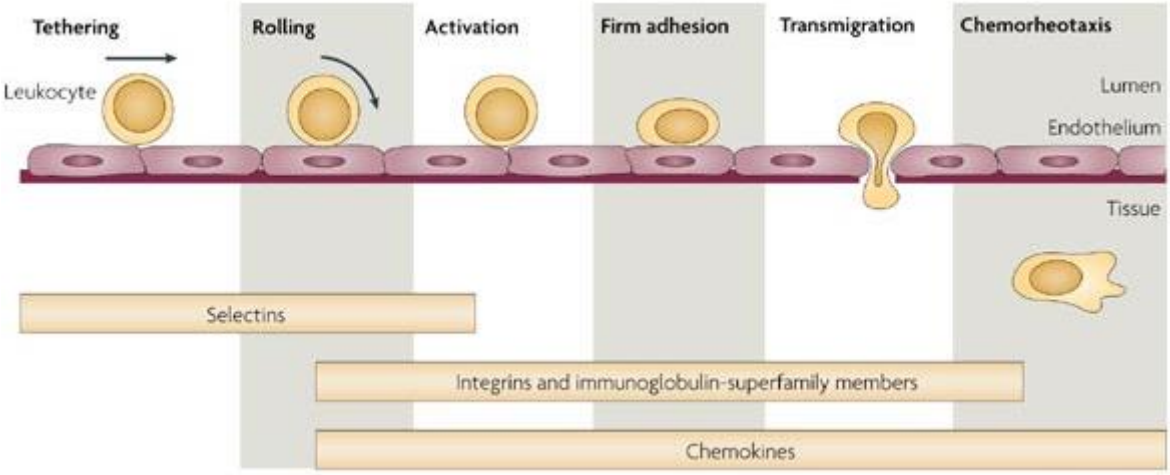


Figure 61: Role of Selectin and immunoglobulin family due atherosclerosis.

Adapted from Weber *et al.*, 2007

Metformin has been shown to reduce sICAM and sVCAM in type 2 diabetes over short and long-term treatment. This effect was shown to be mediated independent of glycaemic control (De Jager *et al.,* 2005; De Jager *et al.,* 2014). Jager *et al.,* 2014 in their double blind randomised controlled trial estimated that sVCAM together with another maker of endothelial dysfunction accounted for 34% reduction of CV morbidity

and mortality. The glucose independent reduction of the above-mentioned markers provides important information that needs to be explored in type 1 diabetes.

### *6.4.3 Thrombomodulin (TM)*

TM is a cell surface protein expressed on ECs. It plays an important role in the coagulation cascade. It binds thrombin and forms the TM-thrombin complex. This complex activates protein C. The complex and protein C have anti-inflammatory and anti-coagulation properties (Li *et al.*, 2012b).

TM levels are elevated in patients with type 1 and type 2 diabetes (Gabat *et al.*, 1996) and atherosclerotic disease (Seigneur *et al.*, 1993). However, (Iwashima *et al.*, 1990) in their study demonstrated that patients with diabetes mellitus without complication have similar TM levels to healthy individuals. Patients with microalbuminuria or renal disease due to diabetes mellitus have been shown to have increased TM levels. The prognostic value of TM in healthy individuals and individuals with diabetes mellitus related complication is different. It has been shown that in healthy individuals, higher levels of TM were associated with lower risk of CV events (Salomaa *et al.*, 1999). However, this is not the case in patients with diabetes mellitus. Higher levels of TM in patients with diabetes mellitus with microalbuminuria have been shown be linked to poor prognostic outcomes (von Scholten *et al.*, 2016). Therefore, levels of TM in diabetes mellitus reflect endothelial injury and progression of atherosclerotic disease (Blann *et al.*, 1997).

To our knowledge, no evidence is available regarding the effect of metformin on thrombomodulin levels *in vivo* and *in vitro*.

## 6.5 Growth factors

### *6.5.1 basic Fibroblast growth factor (bFGF)*

bFGF is produced by ECs, VSMC and macrophages (Cordon-Cardo *et al.*, 1990). It is released as a response to EC injury (Gajdusek and Carbon, 1989). bFGF has mitogenic and angiogenic properties (Lindner *et al.*, 1991; Peyrat *et al.*, 1992). bFGF action is mediated in a paracrine and autocrine manner (Sigala *et al.*, 2010). bFGF plays an important role in the pathogenesis of atherosclerosis. In vitro and animal studies have shown that short-term exposure to bFGF may have an anti-atherosclerotic effect (Six *et al.*, 2004). However, the major effect of bFGF is neointimal formation. This is mediated via SMC migration and proliferation (Lindner *et al.*, 1991; Sato *et al.*, 1991). Furthermore, bFGF is involved in plaque instability. This is due to increased plaque size (SMC migration and proliferation) and weakness (inhibition of collagen synthesis) (Flugelman *et al.*, 1993; Kennedy *et al.*, 1995). This effect is mediated by the bFGF dependent downstream pathway: MAPK and NF-κB pathways (Sigala *et al.*, 2010).

bFGF levels have been shown to be high in patients with CVD and diabetes mellitus especially those with complications (Zimering and Eng, 1996; Zimering *et al.*, 2010; Zimering *et al.*, 2013). Incubation of cardiomyocytes with metformin have been shown to reduce bFGF production (Wang *et al.*, 2010). To the best of our knowledge, there is no *in vivo* study exploring the effect of metformin in diabetes mellitus.

### *6.5.2 Vascular endothelial growth factor (VEGF)*

VEGF is an important growth factor and plays a vital role in the maintenance of endothelium. The VEGF family consists of VEGF-A, VEGF-B, VEGF-C and VEGF-D. These four factors act via three receptors: VEGFR-1, VEGFR-2 and VEGFR-3 (Ferrara, 1999; Rutanen *et al.*, 2003). VEGF has pro-angiogenic and anti-thrombotic properties. It acts on EC and promotes proliferation and migration. This is crucial for endothelium maintenance and renewal. It also reduces EC apoptosis. It acts on SMC and has an anti-proliferative effect (Waltenberger, 2009).

VEGF effect is pleiotropic in diabetes mellitus. It is important for maintaining vascular repair. However, it plays a role in diabetes-related complication (Duh and Aiello, 1999). Levels of VEGF have been shown to be increased in type 1 diabetes with nephropathy when compared to patients with type 1 diabetes without complication (Chiarelli *et al.*, 2000; Hovind *et al.*, 2000). High VEGF levels have been shown to predict future diabetes-related nephropathy (Santilli *et al.*, 2001).

VEGF-A has been demonstrated in atherosclerotic plaques and levels of VEGF-A increased with progression of atherosclerotic disease. VEGF-A has been demonstrated in macrophages in atherosclerotic plaque (Rutanen *et al.*, 2003). In animal studies, high levels of VEGF-A have been shown to induce development of atherosclerotic lesions (Celletti *et al.*, 2001). Rutanen and colleague (2003) demonstrated that VEGF-D was expressed in the normal endothelium. However, levels of VEGF-D decreased in complicated lesions. Interestingly, VEGF-D levels in the macrophages at the lesion site increased leading to plaque neovascularisation. Thus, in addition to an overprotective effect, VEGF-A and VEGF-D are involved in the pathogenesis of atherosclerosis (Rutanen *et al.*, 2003).

We have demonstrated that VEGF-A levels are increased by hyperglycaemia, hypoxia and by metformin in CD34+ cells. However, hyperglycaemia reduces VEGFR-2 expression, thereby reducing VEGF signalling in CD34+ cells (Bakhashab *et al.*, 2016). The effect of metformin in *in vivo* experiments has not produced consistent results. One study did not show any effect on VEGF (Watanabe *et al.*, 2001), whereas another study demonstrated that metformin reduced VEGF levels in obese patients with type 2 diabetes (Ersoy *et al.,* 2008).

## 6.6 Method

### *6.6.1 Platelet-free plasma isolation*

Cytokines were evaluated in TG, HC and SG. After an overnight fast, peripheral blood was collected in citrate vacutainer tubes. Blood samples were processed within 4 hours of collection. Samples were centrifuged for 15 minutes at 500 x*g*. The upper, clear fraction (platelet rich plasma, PRP) was collected without disturbing the bottom cellular layer and transferred to 1.5mL polypropylene tubes. These PRP samples were further centrifuged for 5 minutes at 13000 x *g* in a microcentrifuge. After centrifugation, the clarified platelet-free plasma (PFP) was transferred to fresh 1.5mL polypropylene tubes and stored at -80ºC for subsequent analysis. The platelet pellet was discarded.

### *6.6.2 Cytokines*

Cytokines were measured using a Mesoscale Discovery (MSD) kit as it offers high sensitivity and reproducibility. The protocol is simple and validated. Furthermore, technical support was provided in the form of an adviser during the experiments. Initially, pre-configured V-Plex panels and TIMP-1 kit were used (Table 24).

| | |
|---|---|
| 1 | **Vascular injury panel 1**<br>E-Selectin, P-Selectin, sICAM-3 and thrombomodulin |
| 2 | **Vascular injury panel 2**<br>CRP, SAA, sICAM-1, sVCAM-1 |
| 3 | **Cytokine panel 1**<br>GM-CSF, IL-12p40, IL-15, IL-16, IL-1α, IL-5, IL-7, TNF-β, VEGF |
| 4 | **Pro-Inflammatory panel 1**<br>INF-γ, IL-10, IL-12p70, IL-13, IL-1β, IL-2, IL-4, IL-6, IL-8, TNF-α |
| 5 | **Angiogenesis panel**<br>bFGF, PIGF, sFIT-1, Tie-2, VEGF, VEGF-C, VEGF-D |
| 6 | **Chemokine**<br>Eotaxin, Eotaxin-3, IL-8, IP-10, MCP-1, MCP-4, MDC, MIP-1α, MIP-1alpha, MIP-1β, TARC |
| 7 | **TIMP-1** |

Table 24: MSD panel used for cytokine analysis.

All the MSD assays were conducted according to the manufacturer's instructions. MSD plate reader was used to analyse the plates. MSD software was used to evaluate the levels of cytokines.

Plasma samples were diluted according to the manufacturer's instruction (Table 25).

| **Cytokine panel** | **Dilution** |
|---|---|
| Vascular injury panel 1 | 1X |
| Vascular injury panel 2 | 1000X |
| Cytokine panel 1 | 2X |
| Pro-Inflammatory panel 1 | 2X |
| Angiogenesis panel | 2X |
| Chemokine | 4X |
| TIMP-1 | 100X |

Table 25: Dilution of plasma for each panel.

Initially, 46 cytokines were evaluated. However, 17 cytokines could be reliably assessed in all the samples. For 29 cytokines, not all the samples were in the detection range (Figure 75). Only cytokines, where all the samples were in detection range were evaluated (Figure 76). Therefore, we have presented 17 cytokines in our final analysis. These are given in Table 26. Data were analysed using MSD Discovery Workbench version 2.0 software.

| | |
|---|---|
| 1 | **Vascular injury panel 1**<br>E-Selectin, P-Selectin, sICAM-3 and thrombomodulin |
| 2 | **Vascular injury panel 2** |
| | CRP, sICAM-1, sVCAM-1 |
| 3 | **Pro-Inflammatory panel 1** |
| | INF-γ, IL-10, IL-6, IL-8, TNF-α |
| 4 | **Angiogenesis panel** |
| | bFGF, VEGF, VEGF-D |
| 5 | **Chemokine** |
| | IP-10 |
| 6 | **TIMP-1** |

Table 26: Final list of cytokines studied.

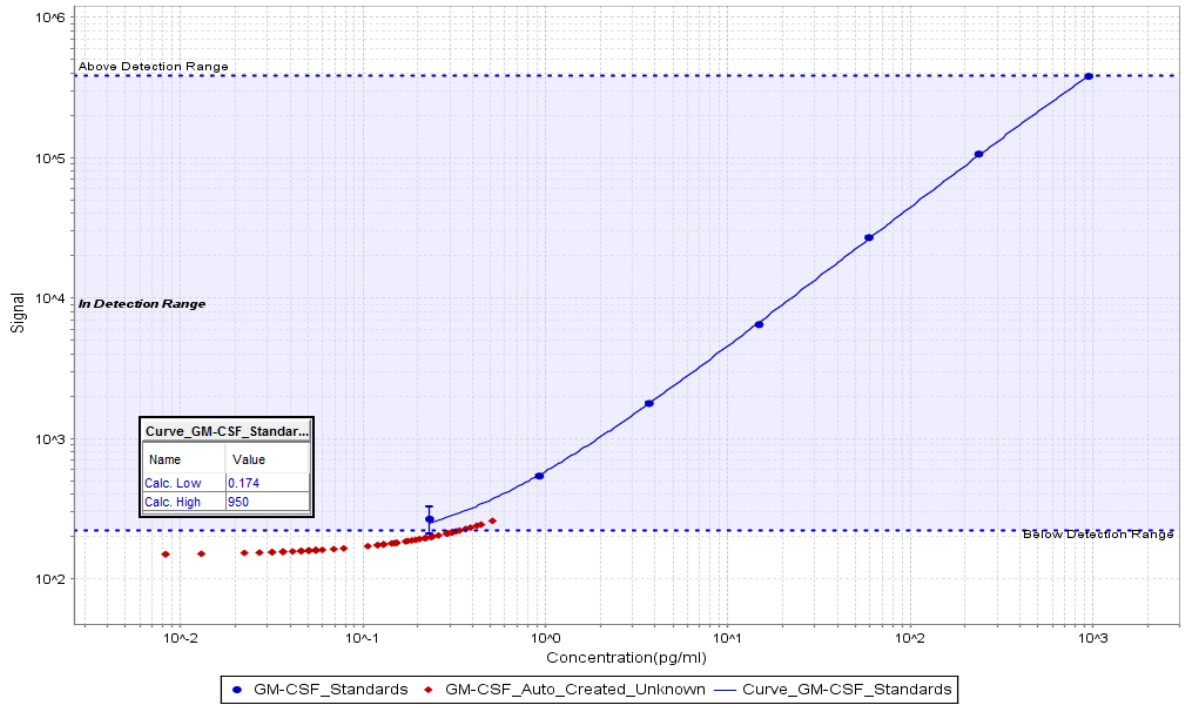


Figure 62: GM-CSF detection curve (blue line). Red dots are the sample detection values.

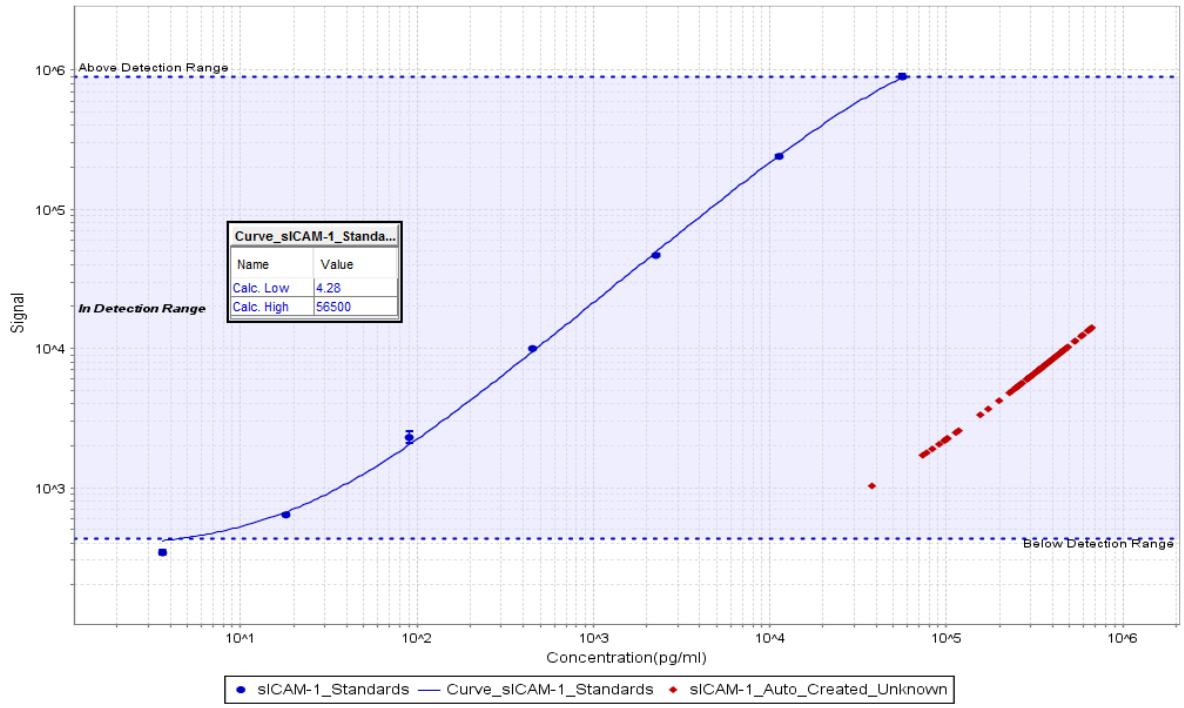


Figure 63: sICAM-1 detection curve (blue line). Red dots are the sample detection values.

#### *6.6.2.1 TIMP-1*

25µl of diluent 2 was placed in each well. The plate was then sealed with an adhesive seal. The plate was incubated for 30 minutes with vigorous shaking at room temperature. 25 µl of samples and calibrator were placed in appropriate wells. The plate was again incubated for 2 hours with vigorous shaking at room temperature. The plate was washed 3 times with phosphate-buffered saline with 0.05% Tween-20 (PBS-T). 25 µl of detection antibody was dispensed into each well. The plate was incubated for 2 hours with vigorous shaking. The plate was washed 3 times again with PBS-T. 150 µl of 2X Read Buffer T was added to each well. The plate was read with MSD Sector Imager 2400.

#### *6.6.2.2 Pro-inflammatory panel 1*

50 µl of samples and calibrator were added to appropriate wells. The plate was sealed with an adhesive plate and incubated with shaking for 2 hours. The plate was washed 3 times with at least 150 µl per well of wash buffer. 25 µl of detection antibody solution was added to each well. The plate was sealed and incubated at room temperature with shaking for 2 hours. The plate was washed 3 times again with wash buffer. 150 µl of 2X Read Buffer T was added to each well. The plate was read with MSD Sector Imager 2400.

#### *6.6.2.3 Vascular injury panel 1*

Blocker A solution (150 µl) was added to each well. The plate was incubated at room temperature for 1 hour on a plate shaker. The plate was then washed 3 times with PBS-T (200 µl per well). 40 µl of Diluent 10 was added. 10 µl of samples and calibrator were added to appropriate wells and incubated for 2 hours with shaking. The plate was then washed 3 times with PBS-T. 25 µl of 1X detection antibody was added to each well and incubated for 1 hour at room temperature with shaking. The plate was then washed 3 times with PBS-T. 150 µl of 1X Read Buffer T was added to each well. The plate was read with MSD Sector Imager 2400.

#### *6.6.2.4 Vascular injury panel 2*

The plate was washed 3 times with 150 µl per well of wash buffer. 25 µl of diluted samples and calibrator were added to appropriate wells. The plate was sealed with an adhesive seal and incubated for 2 hours at room temperature with shaking. The plate was washed 3 times again with wash buffer. 25 µl of detection antibody was added to each well. The plate was sealed and incubated for 1 hour at room temperature with shaking. The plate was washed 3 times again with wash buffer. 150 µl of 1X Read Buffer T was added to each well. The plate was read with MSD Sector Imager 2400.

#### *6.6.2.5 Angiogenesis panel 1*

150 µl of Blocker A solution was added to each well. The plate was sealed with an adhesive seal and incubated at room temperature for 1 hour with shaking. The plate was washed 3 times with 150 µl per well of wash buffer. 50 µl of diluted samples and calibrator were added in appropriate well. The plate was sealed with an adhesive plate and incubated for 2 hours with shaking. The plate was again washed with wash buffer. 25 µl of detection antibody solution was added to each well. The plate was sealed with an adhesive plate and incubated at room temperature for 2 hours with shaking. The plate was washed 3 times with wash buffer and 150 µl of Read Buffer T was added to each well. The plate was read with MSD Sector Imager 2400.

#### *6.6.2.6 Chemokine panel 1 (IP-10)*

50 µl of samples and calibrator were added to appropriate wells. The plate was sealed with an adhesive plate and incubated with shaking for 2 hours. The plate was washed 3 times with at least 150 µl per well of wash buffer. 25 µl of detection antibody solution was added to each well. The plate was sealed and incubated at room temperature with shaking for 2 hours. The plate was washed 3 times again with wash buffer. 150 µl of 2X Read Buffer T was added to each well and incubated at room temperature for 10 minutes. The plate was read with MSD Sector Imager 2400.

## 6.7 Specific aim

In type 1 diabetes, when changes in glycaemia are accounted for, metformin will:

1. Increase cytokines related to EPC mobilisation and angiogenesis.
2. Decrease cytokines representative of vascular injury markers and inflammatory cytokines.

## 6.8 Statistical analysis

General statistical analysis has been discussed in section 3.2. We took advice from the statistician regarding correcting for multiple testing. We were advised that data should be interpreted without multiple corrections due to the discovery nature of the study. However, as we had studied more than 10 cytokines, we were advised that we should present both non-corrected and corrected p values with the results. After Bonferroni correction, the p value of $<0.003$ was assumed to be significant. Nevertheless, for discussion in our thesis, a p value of $<0.05$ was assumed to be significant.

## 6.9 Results

### *6.9.1 TIMP-1*

Result is presented in Figure 77.

#### *6.9.1.1 Treatment group (Pre metformin) versus Healthy control*

TIMP-1 levels were significantly higher in TG when compared with HC (Treatment group pre-metformin (TG V1) vs HC; mean+/-SE: 179665+/-9452 vs 156202+/-4097 pg/ml; p=0.02; after Bonferroni correction: p=0.32).

#### *6.9.1.2 Treatment group (Pre metformin) versus Treatment group (Post metformin)*

Eight-week metformin treatment significantly reduced TIMP-1 levels in TG (TG V1 vs TG V2; mean+/-SE: 179665+/-9452 vs 158503+/-5424 pg/ml; p=0.018; after Bonferroni correction: p=0.3).

#### *6.9.1.3 Treatment group (Post metformin) versus Healthy control*

After metformin treatment, TIMP-1 levels in TG group became similar to HC ((TG V2 vs HC); mean+/-SE: 158503+/-5424 vs 156202+/-4097 pg/ml; p=0.7).

#### *6.9.1.4 Treatment group (Pre metformin) versus Standard group (Pre observation)*

At baseline, TIMP-1 levels were similar in TG and the SG (Treatment group pre-metformin TG V1 vs standard group SG V1 (Pre); mean+/-SE: 179665+/-9452 vs 174529+/-10010 pg/ml; p=0.8).

#### *6.9.1.5 Standard group (Pre observation) versus Standard group (Post observation)*

After eight weeks of standard, TIMP-1 levels remained unchanged (standard treatment group pre (SG V1) and standard treatment group post (SG V2): mean+/-SE: 174529+/-10010 vs 180589+/-7919 pg/ml; p=0.6).

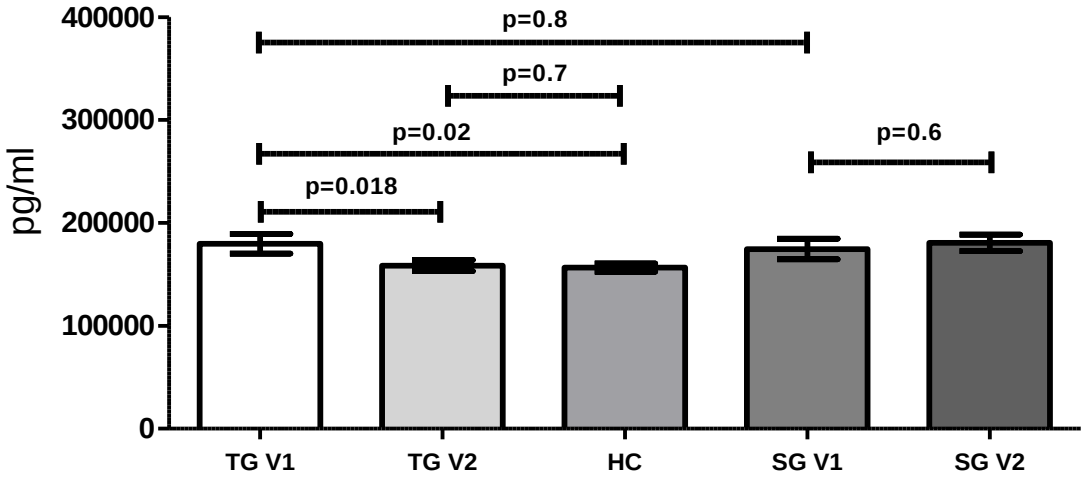


Figure 64: TIMP-1 levels comparing groups. Results given as pg/ml.

TGV1: Treatment group pre metformin; TGV2: Treatment group post metformin; HC: Healthy control; SGV1: Standard group pre observation; SGV2: Standard group post observation.

### *6.9.2 TNF-α*

Result is presented in Figure 78.

#### *6.9.2.1 Treatment group (Pre metformin) versus Healthy control*

TNF-α levels were significantly higher in TG compared with HC (Treatment group pre-metformin (TG V1) vs HC; median (intraquartile range (IQ): 1.97 (1.5-2.5) vs 1.5 (1.28-1.6) pg/ml; p=0.0004; after Bonferroni correction: p=0.064).

#### *6.9.2.2 Treatment group (Pre metformin) versus Treatment group (Post metformin)*

In TG eight-week metformin treatment did not result in any change in TNF- levels (Treatment group pre-metformin (TG V1) vs treatment group post metformin (TG V2); median (IQ): 1.97 (1.5-2.5) vs 1.91 (1.5-2.4) pg/ml; p=0.2).

#### *6.9.2.3 Treatment group (Post metformin) versus Healthy control*

TNF-α level remained significantly higher in TG after metformin treatment when compared to HC (TG V2 vs HC); median (IQ): 1.91 (1.5-2.4) vs. 1.5 (1.28-1.6) pg/ml; p=0.0003; after Bonferroni correction: p=0.0048.

#### *6.9.2.4 Treatment group (Pre metformin) versus Standard group (Pre observation)*

At baseline, TNF-α level was similar in TG and the SG (Treatment group pre-metformin TG V1 vs standard group SG V1 (Pre); median (IQ): 1.97 (1.5-2.5) vs 1.64 (1.5-1.7) pg/ml; p=0.07).

#### *6.9.2.5 Standard group (Pre observation) versus Standard group (Post observation)*

In standard group, eight weeks of standard follow-up did not result in any change in TNF-α level (standard treatment group pre (SG V1) and standard treatment group post (SG V2): median (IQ): 1.64 (1.5-1.7) vs 1.8 (1.5-1.96) pg/ml; p=0.2).

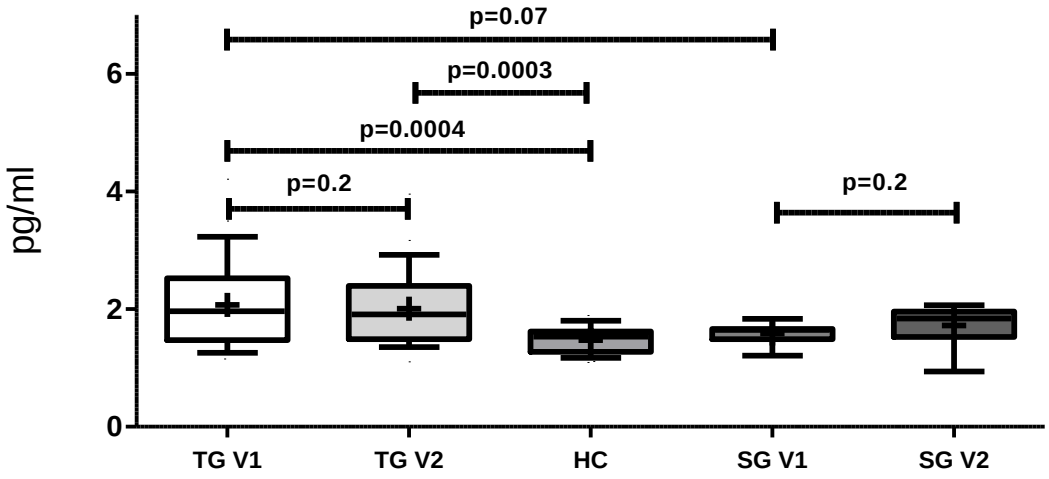


Figure 65: TNF-α levels comparing all groups. Results given as pg/ml.

### *6.9.3 IL-6*

Result is presented in Figure 79.

#### *6.9.3.1 Treatment group (Pre metformin) versus Healthy control*

IL-6 levels were significantly higher in TG compared with HC (Treatment group pre-metformin (TG V1 vs HC; median (intraquartile range (IQ): 0.6 (0.34-1) vs 0.33 (0.22-0.4) pg/ml; p=0.0056; after Bonferroni correction: p=0.09).

#### *6.9.3.2 Treatment group (Pre metformin) versus Treatment group (Post metformin)*

Eight-week metformin treatment significantly reduced IL-6 levels in TG: (TG V2 vs TGV1; median (IQ): 0.6 (0.34-1) vs 0.44 (0.36-0.4) pg/ml; p=0.0093; after Bonferroni correction: p=0.15).

#### *6.9.3.3 Treatment group (Post metformin) versus Healthy control*

Metformin treatment reduced IL-6 levels in TG group and brought it closer to HC ((TG V2 vs HC); median (IQ): 0.44 (0.36-0.4) vs. 0.33 (0.22-0.4) pg/ml). After metformin treatment, significance disapperred (p=0.053).

#### *6.9.3.4 Treatment group (Pre metformin) versus Standard group (Pre observation)*

At baseline, IL-6 level was similar in TG and the SG (Treatment group pre-metformin TG V1 vs standard group SG V1 (Pre); median (IQ): 0.6 (0.34-1) vs 0.54 (0.3-0.8) pg/ml; p=0.6).

#### *6.9.3.5 Standard group (Pre observation) versus Standard group (Post observation)*

In standard group, eight weeks of standard follow-up did not result in any change in IL-6 level (standard treatment group pre (SG V1) and standard treatment group post (SG V2): median (IQ): 0.54 (0.3-0.8) vs 0.54 (0.3-0.9) pg/ml; p=0.7).

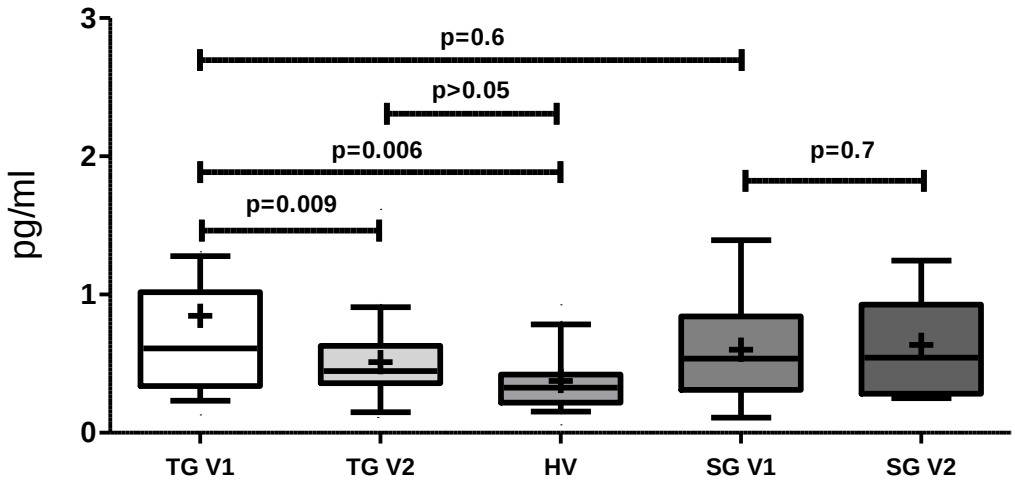


Figure 66: IL-6 levels comparing all groups. Results given as pg/ml.

### *6.9.4 CRP*

Result is presented in Figure 80.

#### *6.9.4.1 Treatment group (Pre metformin) versus Healthy control*

CRP levels were significantly higher in TG when compared with HC (Treatment group pre-metformin (TG V1) vs HC; median (intraquartile range (IQ): $1.9X10^6$ ($1.1X10^6$ – $2.4X10^6$) vs 632829 (374400 – $1.7X10^6$) pg/ml; p=0.0003; after Bonferroni correction: p=0.005).

#### *6.9.4.2 Treatment group (Pre metformin) versus Treatment group (Post metformin)*

In TG eight-week metformin treatment did not change CRP levels (TG V1 vs TG V2); median (IQ): $1.9X10^6$ ($1.1X10^6$ – $2.4X10^6$) vs $1.9X10^6$ ($1.5X10^6$ – $2.5X10^6$) pg/ml; p=0.98).

#### *6.9.4.3 Treatment group (Post metformin) versus Healthy control*

CRP levels in TG after metformin treatment remained similar when compared to HC (TG V2 vs HC); median (IQ): $1.9X10^6$ ($1.5X10^6$ – $2.5X10^6$) vs. 632829 (374400 – $1.7X10^6$) pg/ml; p=0.0008; after Bonferroni correction: p=0.013.

#### *6.9.4.4 Treatment group (Pre metformin) versus Standard group (Pre observation)*

At baseline, CRP level was similar in TG and the SG (Treatment group pre-metformin TG V1 vs standard group SG V1 (Pre); median (IQ): $1.9X10^6$ ($1.1X10^6$ – $2.4X10^6$) vs 913450 (387014 – $3.2X10^6$) pg/ml; p=0.4).

#### *6.9.4.5 Standard group (Pre observation) versus Standard group (Post observation)*

In standard group, eight weeks of standard follow-up did not result in any change in CRP level (standard treatment group pre (SG V1) and standard treatment group post (SG V2): median (IQ): 913450 (387014 – $3.2X10^6$) vs $1.3X10^6$ ($349435 – 3X10^6$) pg/ml; p=0.8).

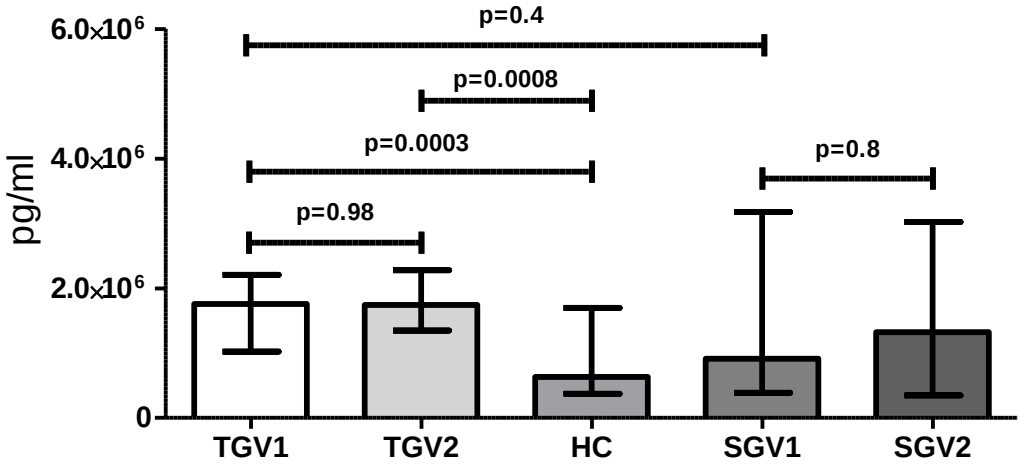


Figure 67: CRP levels comparing all groups. Results given as pg/ml.

### *6.9.5 IL-8*

Result is presented in Figure 81.

#### *6.9.5.1 Treatment group (Pre metformin) versus Healthy control*

IL-8 levels were significantly higher in TG compared with HC (Treatment group pre-metformin (TG V1) vs HC; mean+/-SE: 4.6+/-0.4 vs 3+/-0.15 pg/ml; p<0.0001; after Bonferroni correction: p<0.01).

#### *6.9.5.2 Treatment group (Pre metformin) versus Treatment group (Post metformin)*

In TG eight-week metformin treatment did not result in any change in IL-8 levels (TG V1 vs. TG V2; mean+/-SE: 4.6+/-0.4 vs 4.6+/-0.4 pg/ml; p=0.9).

#### *6.9.5.3 Treatment group (Post metformin) versus Healthy control*

IL-8 level remained significantly higher in TG after metformin treatment when compared to HC (TG V2 vs HC; mean+/-SE: 4.6+/-0.4 vs. 3+/-0.15 pg/ml; p<0.0001; after Bonferroni correction: p<0.01).

#### *6.9.5.4 Treatment group (Pre metformin) versus Standard group (Pre observation)*

At baseline, IL-8 level was similar in TG and the SG (Treatment group pre-metformin TG V1 vs standard group SG V1 (Pre); mean+/-SE: 4.6+/-0.4 vs 4.9+/-0.5 pg/ml; p=0.5).

#### *6.9.5.5 Standard group (Pre observation) versus Standard group (Post observation)*

In standard group, eight weeks of standard follow-up did not result in any change in IL-8 level (standard treatment group pre (SG V1) and standard treatment group post (SG V2): mean+/-SE: 4.9+/-0.5 vs 5.6+/-0.4 pg/ml; p=0.4).

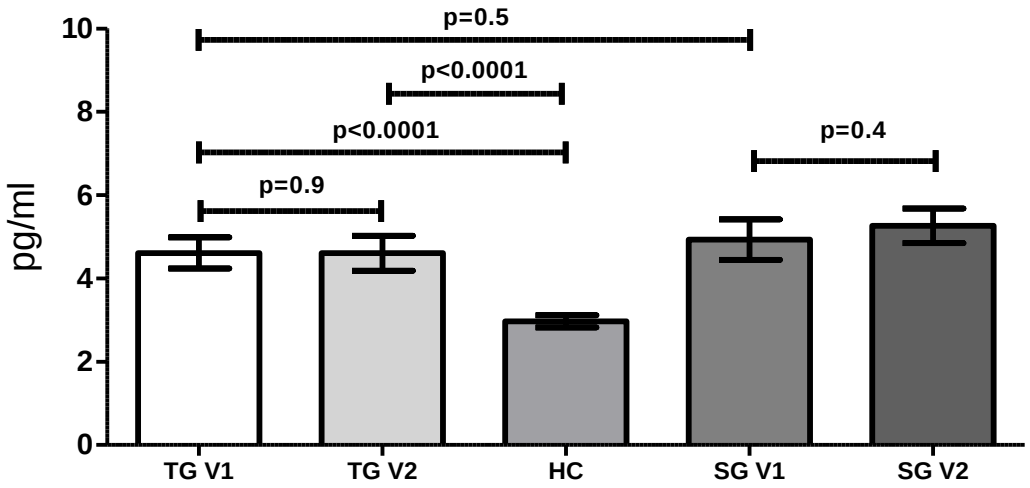


Figure 68:IL-8 levels comparing all groups. Results given as pg/ml.

### *6.9.6 INFγ*

Result is presented in Figure 82.

#### *6.9.6.1 Treatment group (Pre metformin) versus Healthy control*

INFγ levels were similar in TG when compared with HC (Treatment group pre-metformin (TG V1) vs HC; median (intraquartile range (IQ): 3.9 (3.2 – 6) vs 4.7 (2 – 6.3) pg/ml; p=0.9).

#### *6.9.6.2 Treatment group (Pre metformin) versus Treatment group (Post metformin)*

In TG eight-week metformin treatment did not result in any change in INFγ levels ( (TG V1 vs TG V2); median (IQ): 3.9 (3.2 – 6) vs 4.7 (2 – 6.3) pg/ml; p=0.9).

#### *6.9.6.3 Treatment group (Post metformin) versus Healthy control*

INFγ levels in TG after metformin treatment remained similar when compared to HC (TG V2 vs HC); median (IQ): 3.8 (2.9 – 3.8) vs. 4.7 (2 – 6.3) pg/ml; p=0.8.

#### *6.9.6.4 Treatment group (Pre metformin) versus Standard group (Pre observation)*

At baseline, INFγ level was similar in TG and the SG (Treatment group pre-metformin TG V1 vs standard group SG V1 (Pre); median (IQ): 3.9 (3.2 – 6) vs 3.7 (2.7 – 7.8) pg/ml; p=0.97).

#### *6.9.6.5 Standard group (Pre observation) versus Standard group (Post observation)*

In standard group, eight weeks of standard follow-up did not result in any change in INFγ level (standard treatment group pre (SG V1) and standard treatment group post (SG V2): median (IQ): 3.7 (2.7 – 7.8) vs 3.2 (2.5 – 7.9) pg/ml; p=0.7).

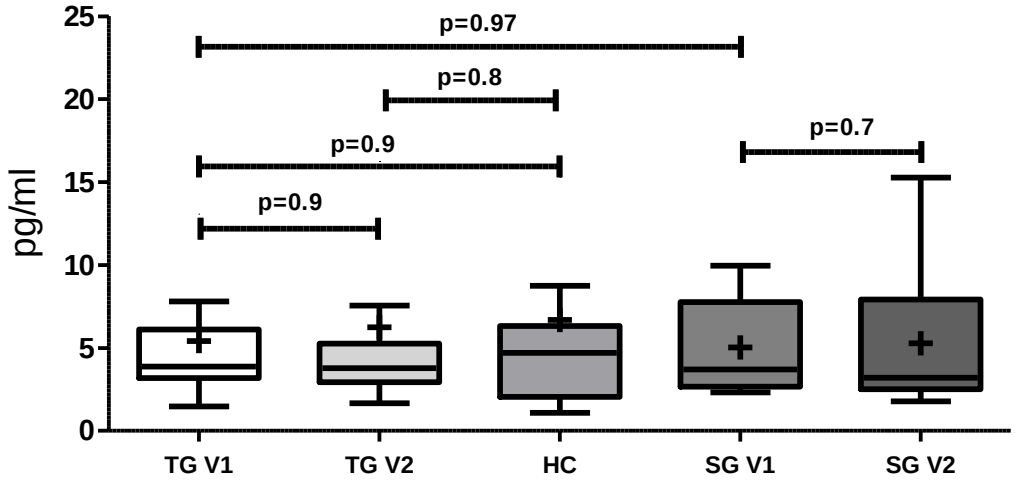


Figure 69: INFγ levels comparing all groups. Results given as pg/ml.

### *6.9.7 IL-10*

Result is presented in Figure 83.

#### *6.9.7.1 Treatment group (Pre metformin) versus Healthy control*

IL-10 levels were significantly higher in TG compared with HC (Treatment group pre-metformin (TG V1) vs HC; median (intraquartile range (IQ): 0.34 (0.24-0.76) vs 0.19 (0.14-0.23) pg/ml; p=0.0003; after Bonferroni correction: p=0.005).

#### *6.9.7.2 Treatment group (Pre metformin) versus Treatment group (Post metformin)*

In TG eight-week metformin treatment did not result in any change in IL-10 levels (TG V1 vs TG V2); median (IQ): 0.34 (0.24-0.76) vs 0.33 (0.23-0.5) pg/ml; p=0.3).

#### *6.9.7.3 Treatment group (Post metformin) versus Healthy control*

IL-10 level remained significantly higher in TG after metformin treatment when compared to HC (TG V2 vs HC; median (IQ): 0.33 (0.23-0.5) vs. 0.19 (0.14-0.23) pg/ml; p=0.0001; after Bonferroni correction: p=0.0016).

#### *6.9.7.4 Treatment group (Pre metformin) versus Standard group (Pre observation)*

At baseline, IL-10 level was similar in TG and the SG (Treatment group pre-metformin TG V1 vs standard group SG V1 (Pre); median (IQ): 0.34 (0.24-0.76) vs 0.22 (0.16-0.34) pg/ml; p=0.06).

#### *6.9.7.5 Standard group (Pre observation) versus Standard group (Post observation)*

In standard group, eight weeks of standard follow-up did not result in any change in IL-10 level (standard treatment group pre (SG V1) and standard treatment group post (SG V2): median (IQ): 0.22 (0.16-0.34) vs 0.29 (0.16-0.38) pg/ml; p=0.7).

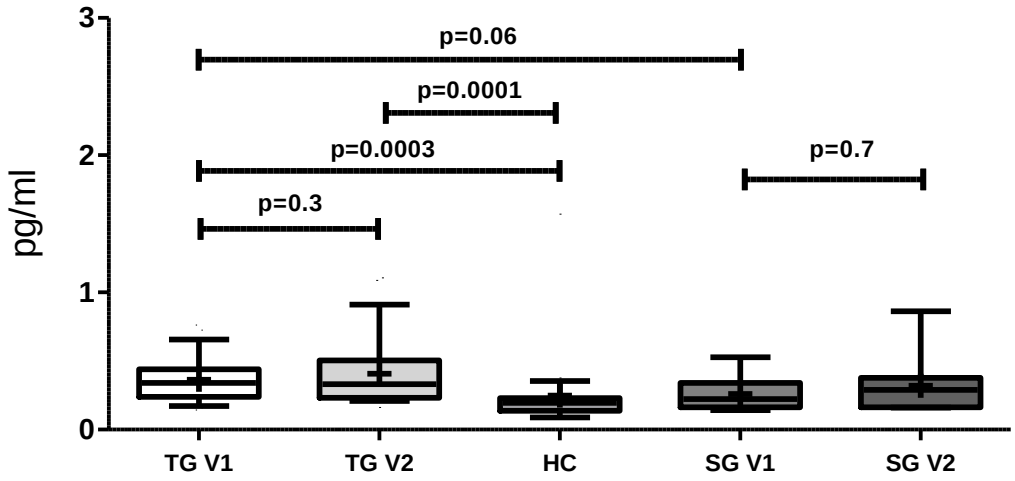


Figure 70: IL-10 levels comparing all groups. Results given as pg/ml.

### *6.9.8 IP-10*

Result is presented in Figure 84.

#### *6.9.8.1 Treatment group (Pre metformin) versus Healthy control*

IP-10 levels were non-significantly higher in TG when compared with HC (Treatment group pre-metformin (TG V1) vs HC; median (intraquartile range (IQ): 245.5 (144-369) vs 232 (151-324) pg/ml; p=0.8).

#### *6.9.8.2 Treatment group (Pre metformin) versus Treatment group (Post metformin)*

Eight-week metformin treatment significantly reduced IP-10 levels in TG (TG V1 vs TG V2); median (intraquartile range (IQ): 245.5 (144-369) vs 192 (127-298) pg/ml; p=0.028; After Bonferroni correction: p=0.45).

#### *6.9.8.3 Treatment group (Post metformin) versus Healthy control*

After metformin treatment, IP-10 levels in TG group became non-significant when compared to HC ((TG V2 vs HC); median (intraquartile range (IQ): 192 (127-298) vs 232.3 (151-324) pg/ml; p=0.35).

#### *6.9.8.4 Treatment group (Pre metformin) versus Standard group (Pre observation)*

At baseline, IP-10 levels were similar in TG and the SG (Treatment group pre-metformin TG V1 vs standard group SG V1 (Pre observation); median (intraquartile range (IQ): 245.5 (144-369) vs 260.4 (204.2-357) pg/ml; p=0.8).

#### *6.9.8.5 Standard group (Pre observation) versus Standard group (Post observation)*

After eight weeks of observation, IP-10 levels remained unchanged (standard treatment group pre observation (SG V1) and standard treatment group post observation (SG V2): median (intraquartile range (IQ): 260.4 (204.2-357) vs 239 (217.2-404) pg/ml; p=0.3).

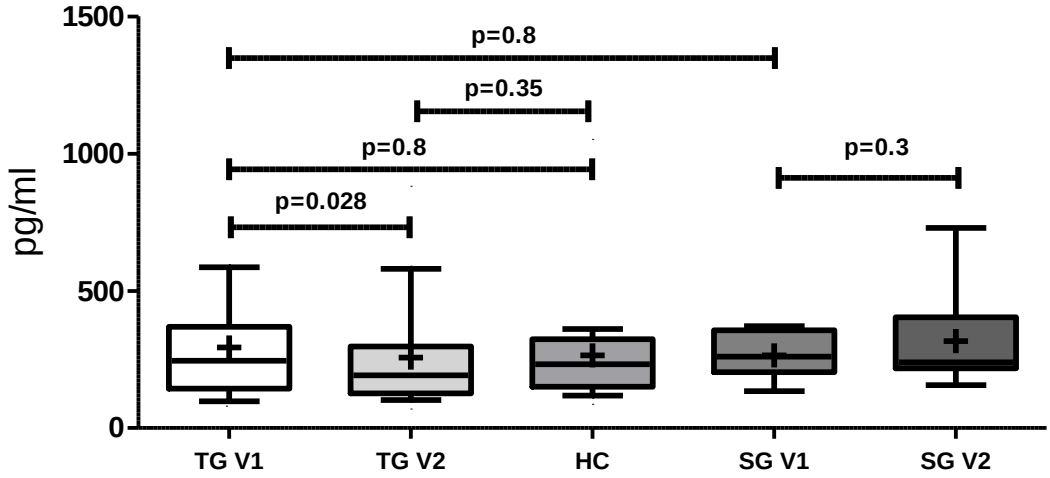


Figure 71: IP-10 levels comparing all groups. Results given as pg/ml.

### *6.9.9 P-Selectin*

Result is presented in Figure 85.

#### *6.9.9.1 Treatment group (Pre metformin) versus Healthy control*

P-Selectin levels were non significantly higher in TG when compared with HC (Treatment group pre-metformin (TG V1) vs HC; mean+/-SE: 43671+/-2882 vs 36167+/-2757 pg/ml; p=0.06).

#### *6.9.9.2 Treatment group (Pre metformin) versus Treatment group (Post metformin)*

Eight-week metformin treatment did not change P-Selectin levels in TG ( (TG V1 vs TG V2); mean+/-SE: 43671+/-2882 vs 41845+/-2715 pg/ml; p=0.2).

#### *6.9.9.3 Treatment group (Post metformin) versus Healthy control*

After metformin treatment, P-Selectin levels in TG group remained non significantly different when compared to HC (TG V2 vs HC); mean+/-SE: 41845+/-2715 vs 36167+/-2757 pg/ml; p=0.1).

#### *6.9.9.4 Treatment group (Pre metformin) versus Standard group (Pre observation)*

At baseline, P-Selectin levels were similar in TG and the SG (Treatment group pre-metformin TG V1 vs standard group SG V1 (Pre); mean+/-SE: 43671+/-2882 vs 40478+/-2982 pg/ml; p=0.5).

#### *6.9.9.5 Standard group (Pre observation) versus Standard group (Post observation)*

After eight weeks of standard, P-Selectin levels remained unchanged (standard treatment group pre (SG V1) and standard treatment group post (SG V2): mean+/-SE: 43671+/-2882 vs 46317+/-5511 pg/ml; p=0.2).

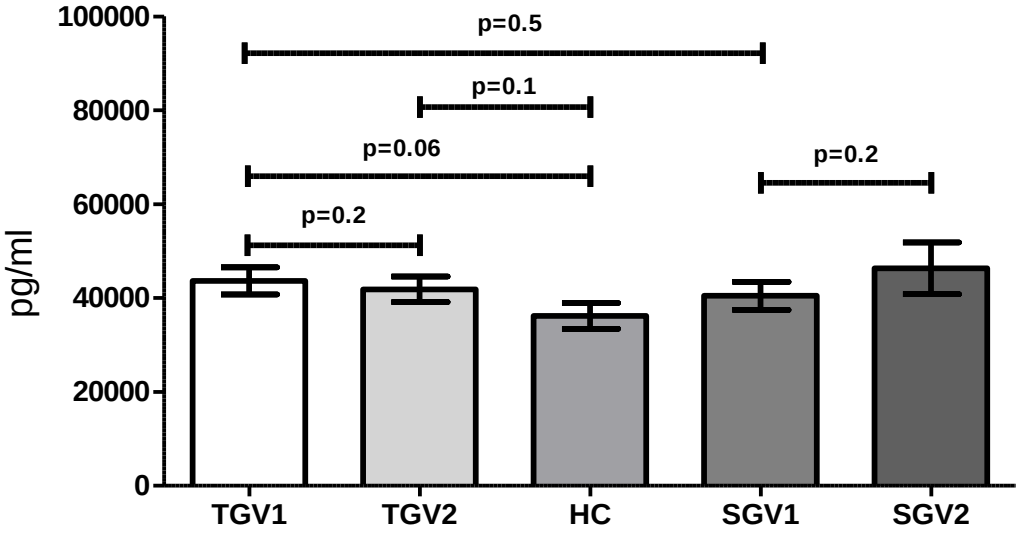


Figure 72: P-Selectin levels comparing all groups. Results given as pg/ml.

### *6.9.10 E-Selectin*

Result is presented in Figure 86.

#### *6.9.10.1 Treatment group (Pre metformin) versus Healthy control*

E-Selectin levels were similar in TG when compared with HC (Treatment group pre-metformin (TG V1) vs HC; mean+/-SE: 15592+/-1438 vs 16697+/-1240 pg/ml; p=0.45).

#### *6.9.10.2 Treatment group (Pre metformin) versus Treatment group (Post metformin)*

Eight-week metformin treatment did not change E-Selectin levels in TG (Treatment group pre-metformin (TG V1 vs TG V2; mean+/-SE: 15592+/-1438 vs 14691+/-1259 pg/ml; p=0.1).

#### *6.9.10.3 Treatment group (Post metformin) versus Healthy control*

After metformin treatment, E-Selectin levels in TG group remained similar when compared to HC ((TG V2 vs HC); mean+/-SE: 14691+/-1259 vs 16697+/-1240 pg/ml; p=0.24).

#### *6.9.10.4 Treatment group (Pre metformin) versus Standard group (Pre observation)*

At baseline, E-Selectin levels were similar in TG and the SG (Treatment group pre-metformin TG V1 vs standard group SG V1 (Pre); mean+/-SE: 15592+/-1438 vs 18154+/-1299 pg/ml; p=0.15).

#### *6.9.10.5 Standard group (Pre observation) versus Standard group (Post observation)*

After eight weeks of standard, E-Selectin levels remained unchanged (standard treatment group pre (SG V1) and standard treatment group post (SG V2): mean+/-SE: 18154+/-1299 vs 17503+/-1145 pg/ml; p=0.4).

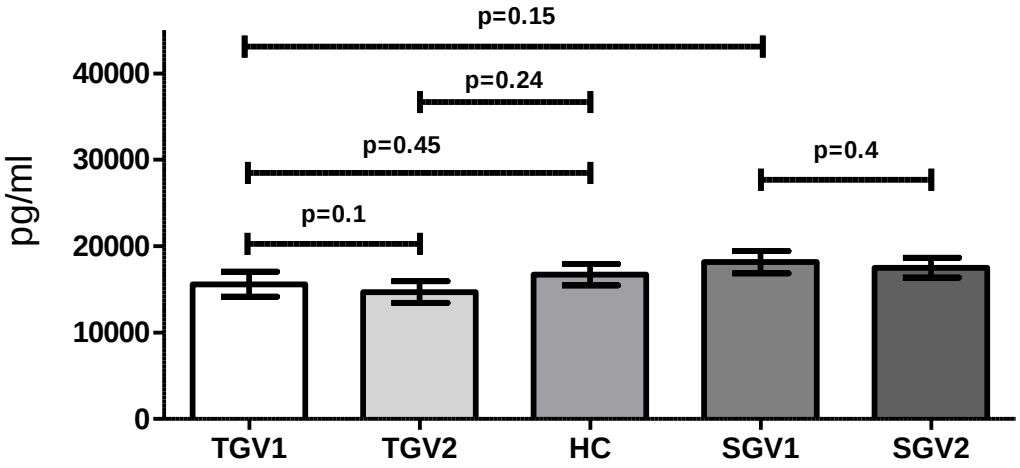


Figure 73: E-Selectin levels comparing all groups. Results given as pg/ml.

### *6.9.11 sICAM-1*

Result is presented in Figure 87.

#### *6.9.11.1 Treatment group (Pre metformin) versus Healthy control*

sICAM-1 levels were non-significantly higher in TG when compared with HC (Treatment group pre-metformin (TG V1) vs HC; mean+/-SE: 323952+/-29749 vs 235393+/-17173 pg/ml; p=0.1).

#### *6.9.11.2 Treatment group (Pre metformin) versus Treatment group (Post metformin)*

Eight-week metformin treatment did not change sICAM-1 levels in TG (Treatment group pre-metformin (TG V1) vs treatment group post metformin (TG V1 vs TG V2; mean+/-SE: 323952+/-29749 vs 319008+/-25289 pg/ml; p=0.9).

#### *6.9.11.3 Treatment group (Post metformin) versus Healthy control*

After metformin treatment, sICAM-1 levels in TG group were significantly higher when compared to HC ((TG V2 vs HC); mean+/-SE: 319008+/-25289 vs 235393+/-17173 pg/ml; p=0.04; after Bonferroni correction: p=0.64).

#### *6.9.11.4 Treatment group (Pre metformin) versus Standard group (Pre observation)*

At baseline, sICAM-1 levels were similar in TG and the SG (Treatment group pre-metformin TG V1 vs standard group SG V1 (Pre); mean+/-SE: 323952+/-29749 vs 294829+/-27450 pg/ml; p=0.6).

#### *6.9.11.5 Standard group (Pre observation) versus Standard group (Post observation)*

After eight weeks of standard, sICAM-1 levels remained unchanged (standard treatment group pre (SG V1) and standard treatment group post (SG V2): mean+/-SE: 294829+/-27450 vs 235574+/-19386 pg/ml; p=0.07).

.

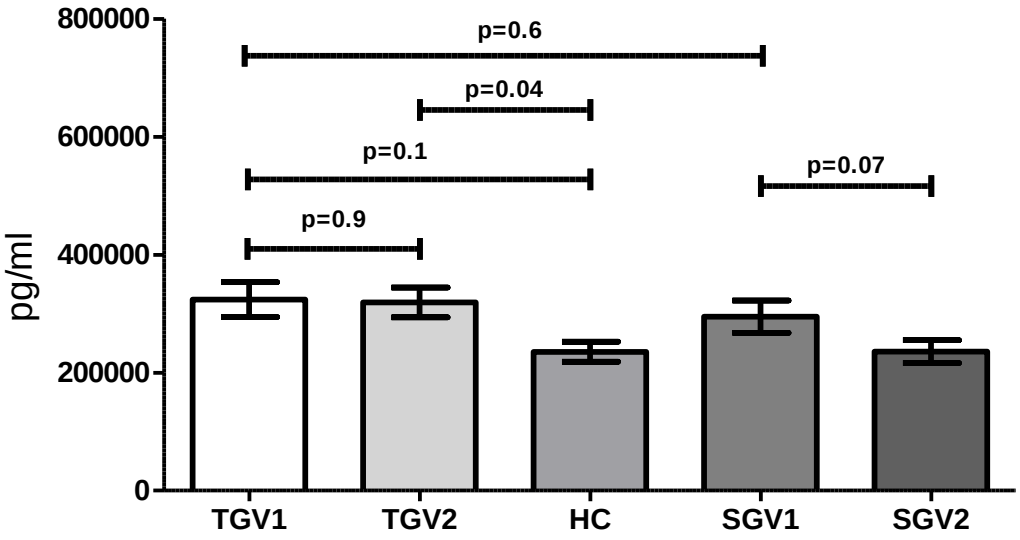


Figure 74: sICAM-1 levels comparing all groups. Results given as pg/ml.

### *6.9.12 sVCAM-1*

Result is presented in Figure 88.

#### *6.9.12.1 Treatment group (Pre metformin) versus Healthy control*

sVCAM-1 levels were non-significantly higher in TG when compared with HC (Treatment group pre-metformin (TG V1) vs HC; mean+/-SE: 898854+/-285166 vs 445233+/-18518 pg/ml; p=0.95).

#### *6.9.12.2 Treatment group (Pre metformin) versus Treatment group (Post metformin)*

Eight-week metformin treatment non-significantly reduced sVCAM-1 levels in TG (TG V1 vs TG V2); mean+/-SE: 898854+/-285166 vs 438643+/-65548 pg/ml; p=0.5).

#### *6.9.12.3 Treatment group (Post metformin) versus Healthy control*

After metformin treatment, sVCAM-1 levels in TG group became similar to HC ((TG V2 vs HC); mean+/-SE: 438643+/-65548 vs 445233+/-18518 pg/ml; p=0.12).

#### *6.9.12.4 Treatment group (Pre metformin) versus Standard group (Pre observation)*

At baseline, sVCAM-1 levels were similar in TG and the SG (Treatment group pre-metformin TG V1 vs standard group SG V1 (Pre); mean+/-SE: 898854+/-285166 vs 625900+/-58664 pg/ml; p=0.5).

#### *6.9.12.5 Standard group (Pre observation) versus Standard group (Post observation)*

After eight weeks of standard, sVCAM-1 levels remained unchanged (standard treatment group pre (SG V1) and standard treatment group post (SG V2): mean+/-SE: 625900+/-58664 vs 503733+/-25669 pg/ml; p=0.1).

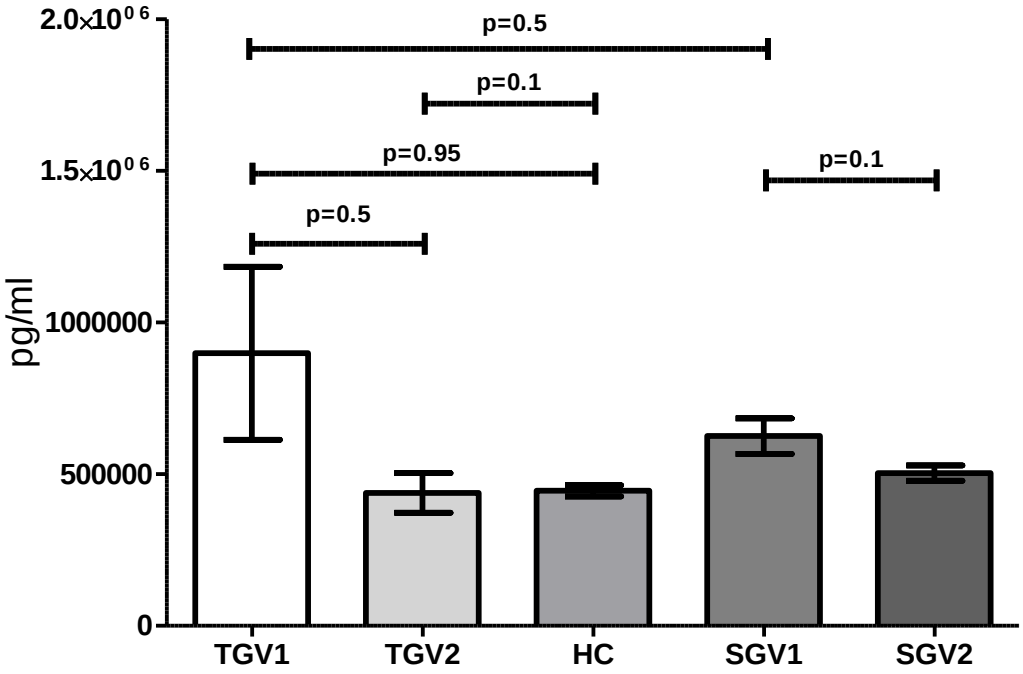


Figure 75: sVCAM-1 levels comparing all groups. Results given as pg/ml.

### *6.9.13 Thrombomodulin*

Result is presented in Figure 89.

#### *6.9.13.1 Treatment group (Pre metformin) versus Healthy control*

Thrombomodulin levels were non significantly higher in TG when compared with HC (Treatment group pre-metformin (TG V1) vs HC; mean+/-SE: 4180+/-227.2 vs 3827+/-174.4 ng/ml; p=0.2).

#### *6.9.13.2 Treatment group (Pre metformin) versus Treatment group (Post metformin)*

Eight-week metformin treatment significantly decreased thrombomodulin levels in TG (Treatment group pre-metformin (TG V1) vs treatment group post metformin (TG V2); mean+/-SE: 4180+/-227.2 vs 3854+/-174.4 ng/ml; p=0.0002; After Bonfferoni correction: p=0.0032).

#### *6.9.13.3 Treatment group (Post metformin) versus Healthy control*

Metformin treatment decreased thrombomodulin levels in TG group and normalised the levels of thrombomodulin levels when compared to HC ((TG V2 vs HC); mean+/-SE: 3854+/-186.8 vs 3827+/-174.4 ng/ml; p=0.92).

#### *6.9.13.4 Treatment group (Pre metformin) versus Standard group (Pre observation)*

At baseline, thrombomodulin levels were similar in TG and the SG (Treatment group pre-metformin TG V1 vs standard group SG V1 (Pre); mean+/-SE: 4180+/-227.2 vs 4243+/-240.1 ng/ml; p=0.9).

#### *6.9.13.5 Standard group (Pre observation) versus Standard group (Post observation)*

Thrombomodulin levels remained unchanged after eight weeks of standard follow-up (standard treatment group pre (SG V1) and standard treatment group post (SG V2): mean+/-SE: 4180+/-217 vs 4018+/-282.5 ng/ml; p=0.14).

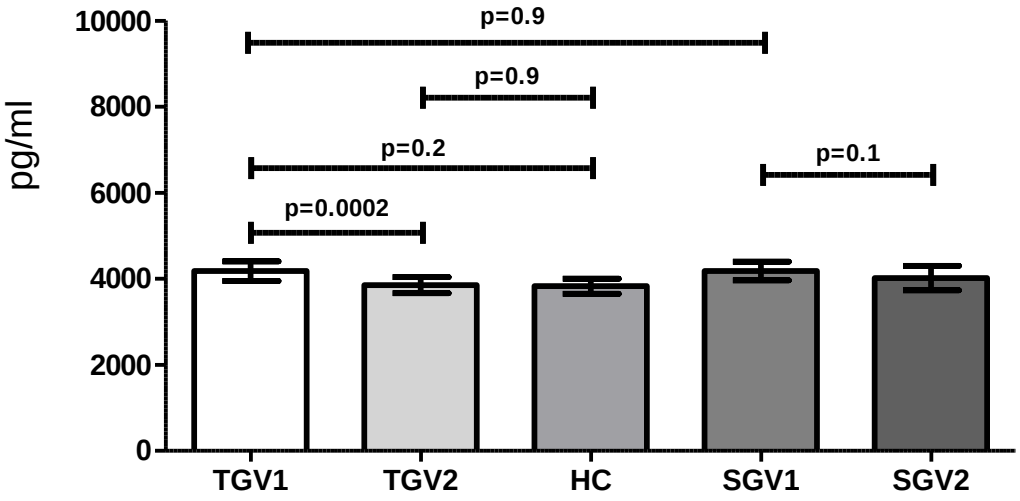


Figure 76: Thrombomodulin levels comparing all groups. Results given as pg/ml.

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### *6.9.14 bFGF*

Result is presented in Figure 90.

#### *6.9.14.1 Treatment group (Pre metformin) versus Healthy control*

bFGF levels were similar in TG and HC (Treatment group pre-metformin (TG V1) vs HC; median (intraquartile range (IQ): 2.92 (1.98-4.55) vs 2.11 (1.15-3.54) pg/ml; p=0.2).

#### *6.9.14.2 Treatment group (Pre metformin) versus Treatment group (Post metformin)*

In TG eight-week of metformin treatment did not result in any change in bFGF levels (TG V1 vs TG V2; median (IQ): 2.92 (1.98-4.55) vs 2.15 (1.6-4) pg/ml; p=0.2).

#### *6.9.14.3 Treatment group (Post metformin) versus Healthy control*

After metformin treatment bFGF levels remained similar in TG and HC (TG V2 vs HC); median (IQ): 2.15 (1.6-4) vs. 2.11 (1.15-3.54) pg/ml; p=0.4.

#### *6.9.14.4 Treatment group (Pre metformin) versus Standard group (Pre observation)*

At baseline, bFGF level was similar in TG and the SG (Treatment group pre-metformin TG V1 vs standard group SG V1 (Pre); median (IQ): 2.92 (1.98-4.55) vs 3.34 (0.75-5) pg/ml; p=0.9).

#### *6.9.14.5 Standard group (Pre observation) versus Standard group (Post observation)*

In standard group, eight weeks of standard follow-up did not result in any change in bFGF level (standard treatment group pre (SG V1) and standard treatment group post (SG V2): median (IQ): 3.34 (0.75-5) vs 4.37 (2.5-9.8) pg/ml; p=0.5).

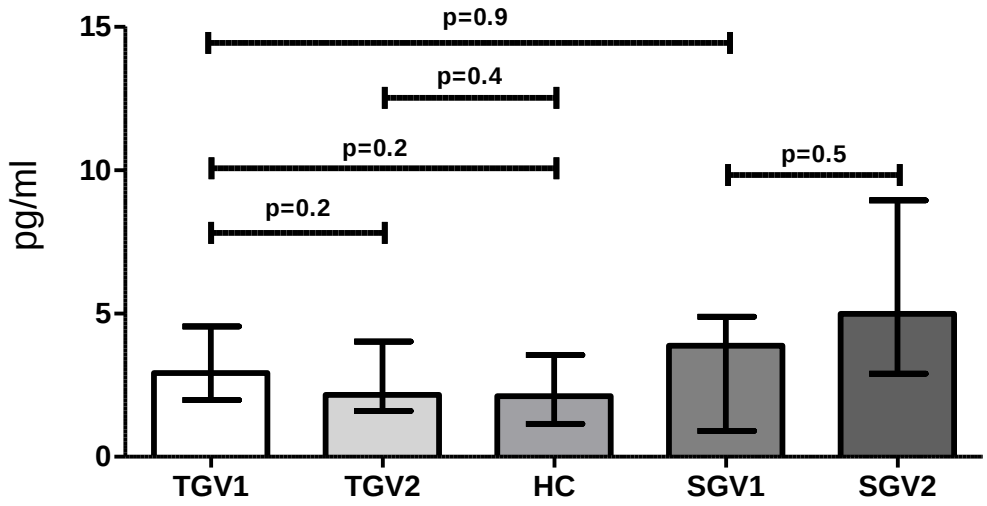



Figure 77: bFGF levels comparing all groups. Results given as pg/ml.

### *6.9.15 VEGF*

Result is presented in Figure 91.

#### *6.9.15.1 Treatment group (Pre metformin) versus Healthy control*

VEGF levels were non-significantly higher in TG when compared with HC (Treatment group pre-metformin (TG V1) vs HC; mean+/-SE: 58+/-11.4 vs 39+/-1.8 pg/ml; p=0.09).

#### *6.9.15.2 Treatment group (Pre metformin) versus Treatment group (Post metformin)*

In TG eight-week metformin treatment did not result in any change in VEGF levels (TG V1 vs TG V2; mean+/-SE: 58+/-11.4 vs 50+/-5 pg/ml; p=0.9).

#### *6.9.15.3 Treatment group (Post metformin) versus Healthy control*

After metformin treatment, VEGF levels remained non-significantly higher in TG when compared to HC (TG V2 vs HC); mean+/-SE: 50+/-5 vs 39+/-1.8 pg/ml; p=0.08.

#### *6.9.15.4 Treatment group (Pre metformin) versus Standard group (Pre observation)*

At baseline, VEGF level was similar in TG and the SG (Treatment group pre-metformin TG V1 vs standard group SG V1 (Pre); mean+/-SE: 58+/-11.4 vs 44.2+/-5.1 pg/ml; p=0.55).

#### *6.9.15.5 Standard group (Pre observation) versus Standard group (Post observation)*

In standard group, eight weeks of standard follow-up resulted in significant increase in VEGF level (standard treatment group pre (SG V1) and standard treatment group post (SG V2): mean+/-SE: 44.2+/-5.1 vs 61.7+/-8.8 pg/ml; p=0.034; after Bonferroni correction: p=0.5).

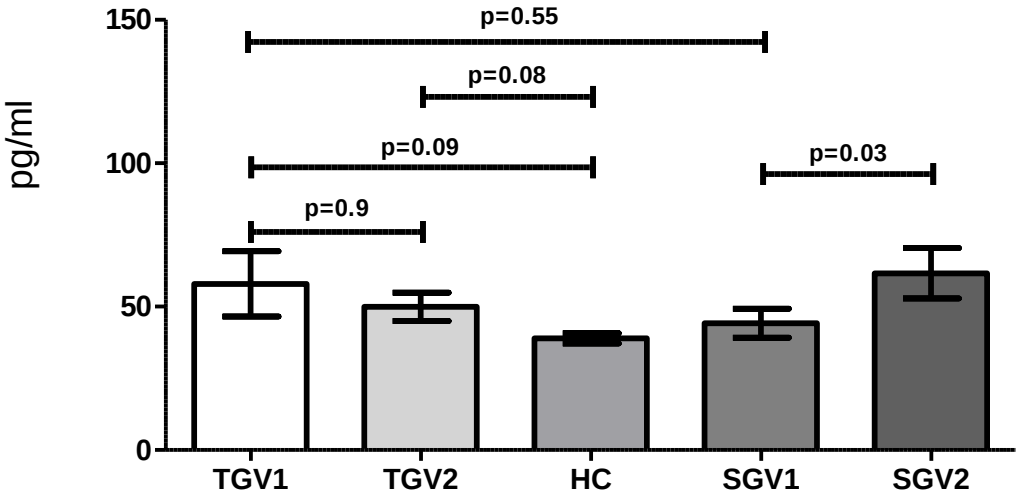



Figure 78: VEGF levels comparing all groups. Results given as pg/ml.

### *6.9.16 VEGF-D*

Result is presented in Figure 92.

#### *6.9.16.1 Treatment group (Pre metformin) versus Healthy control*

VEGF-D levels were significantly higher in TG when compared with HC (Treatment group pre-metformin (TG V1) vs HC; mean+/-SE: 634.7+/-30 vs 498+/-33.6 pg/ml; p=0.003; after Bonferroni correction: p=0.048).

#### *6.9.16.2 Treatment group (Pre metformin) versus Treatment group (Post metformin)*

In TG eight-week metformin treatment did not result in any change in VEGF-D levels (Treatment group pre-metformin (TG V1) vs treatment group post metformin (TG V2); mean+/-SE: 634.7+/-30 vs 629.8+/-30.8 pg/ml; p=0.64).

#### *6.9.16.3 Treatment group (Post metformin) versus Healthy control*

After metformin treatment, VEGF-D levels remained significantly higher in TG when compared to HC (TG V2 vs HC); mean+/-SE: 629.8+/-30.8 vs 498+/-33.6 pg/ml; p=0.005; after Bonferroni correction: p=0.08.

#### *6.9.16.4 Treatment group (Pre metformin) versus Standard group (Pre observation)*

At baseline, VEGF-D level was similar in TG and the SG (Treatment group pre-metformin TG V1 vs standard group SG V1 (Pre); mean+/-SE: 634.7+/-30 vs 628.2+/-74.6 pg/ml; p=0.6).

#### *6.9.16.5 Standard group (Pre observation) versus Standard group (Post observation)*

In standard group, eight weeks of standard follow-up did not result in any change in VEGF-D level (standard treatment group pre (SG V1) and standard treatment group post (SG V2): mean+/-SE: 628.2+/-74.6 vs 617.3+/-54.7 pg/ml; p=0.86).

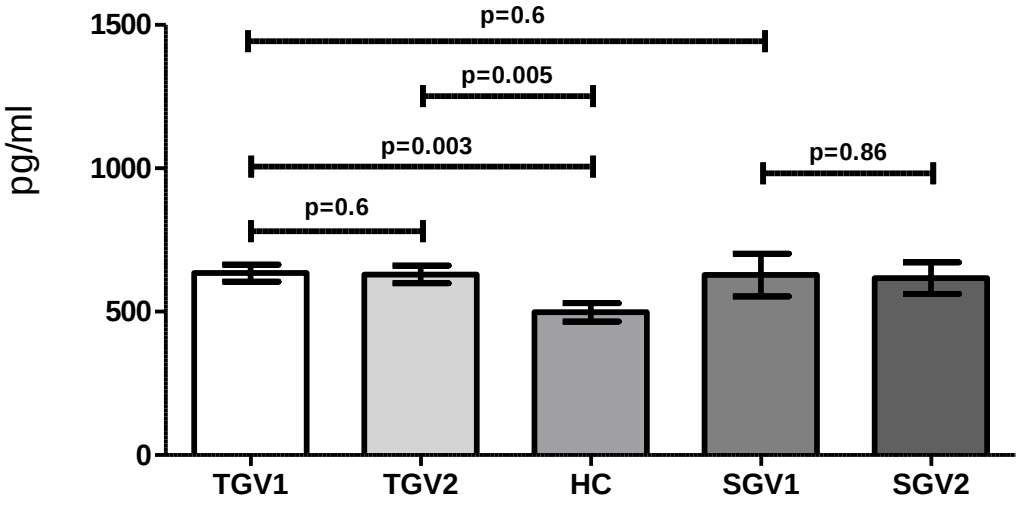



Figure 79: VEGF levels comparing all groups. Results given as pg/ml.

### *6.9.17 Correlation*

In TG, TIMP-1 was inversely correlated with cECs ($r=-0.59$; $p<0.01$) and positively correlated with the PAC adhesion function ($r=0.52$; $p<0.01$). We have demonstrated a positive correlation between CRP and sICAM-1 ($r=0.77$; $p<0.01$) and an inverse correlation of CRP and the PAC adhesion function ($r=-0.43$; $p<0.05$). IP-10 levels in our TG correlated with INF-γ ($r=0.42$; $p<0.05$) and TNF-α ($r=0.53$; $p<0.01$). In TG, there was no correlation between HbA1c and IP-10 levels. In TG, there was a positive correlation between HbA1c and E-Selectin ($r=0.49$; $p<0.05$) and P-Selectin ($r=0.64$; $p<0.01$). We have shown a direct correlation between CRP and sICAM-1 levels ($r=0.82$; $p<0.01$).

In TG, there was no correlation between changes in TIMP-1 and cEPCs levels ($r=-0.23$; $p>0.05$). There is a positive correlation between changes in HDL levels and TIMP-1 levels ($r=0.48$; $p<0.05$).

## 6.10 Discussion

This section of the thesis discusses the effect of metformin on plasma cytokine levels in individuals with type 1 diabetes. Plasma cytokine levels were assessed at baseline and after 8 weeks of metformin treatment. These were compared with healthy volunteers. Furthermore, plasma cytokine levels were evaluated in the type 1 diabetes cohort group (standard treatment group,SG) to explore whether any changes in above-mentioned markers can be observed with routine follow up without any treatment with metformin. The diabetes control remained unchanged over 8 weeks in both the TG and SG groups.

Our work focused on pro-inflammatory, anti-inflammatory, pro-angiogenic, anti-angiogenic and vascular injury markers. We have broken the discussion down into four major sub-sections: 1) extracellular matrix modulator; 2) inflammatory; 3) vascular and 4) growth factors. The extracellular matrix modulator only includes TIMP-1. The inflammatory cytokines discussed are CRP, IL-6, IL-8, IL-10, TNF-α, IP-10 and INF-γ. The vascular markers studied included sVCAM-1, sICAM-1, P-Selectin, E-Selectin and thrombomodulin. The growth factors studied included bFGF, VEGF and VEGF-D.

We were advised by a statistician to present both non-corrected and corrected data to to interpret the data in a more critical manner. However, as our study was designed to identify markers of interest for future research, we were advised that our discussion should be based on non-corrected data.

The discussion is divided into two main parts. The first section discusses each sub-section separately. The second section summarises the findings of our work and its significance.

### *6.10.1 TIMP-1*

We have shown that TIMP-1 levels are higher in individual with type 1 diabetes when compared to healthy volunteers. Our data is in line with most of the available evidence describing elevated TIMP-1 levels in patients with type 1 diabetes and type 2 diabetes (Maxwell *et al.,* 2001 and Lee *et al.,* 2005).

TIMP-1 is an important regulator of matrix metalloproteinases (MMP). MMP plays a major role in angiogenesis. It degrades the extracellular matrix thereby facilitating new vessel formation and expansion (Hiraoka *et al.*, 1998). MMP also plays a role in the mobilisation of EPCs (Heissig *et al.*, 2002; Aicher *et al.*, 2003). In vitro studies have shown increased expression of TIMP-1 in response to hyperglycaemia and hypoxia (Bakhashab *et al.*, 2016). Elevated TIMP-1 levels in type 1 diabetes can be interpreted as a factor limiting vascular repair. Elevated TIMP-1 levels can be postulated to inhibit MMP action thereby resulting in decreased EPC mobilisation and increased basement membrane thickening. It would have been ideal to have measured MMP levels. However, shortage of funding limited us to only measuring TIMP-1. MMP levels are altered in patients with diabetes. In type 1 diabetes patients MMP9 and TIMP-1 levels are elevated (Maxwell *et al.,* 2001), whereas in patients with type 2 diabetes MMP2 and TIMP-1 levels are elevated (Lee *et al.,* 2005). Another study demonstrated an elevated level of MMP9 and TIMP-1 in type 2 diabetes (Tayebjee *et al.,* 2004). Therefore, elevated TIMP-1 levels in type 1 diabetes patients could also be due to increased MMP levels. Another smaller study showed that only MMP2 and MMP9 levels are altered and that there is no change in TIMP-1 levels in type 1 diabetes (Kolset *et al.*, 2009). MMP has been shown to have dual action. In addition to its pro-angiogenic action, it has been shown to suppress angiogenesis through the generation of angiostatin (Chung *et al.*, 2006). This imbalance between MMP and TIMP-1 is thought to play a major role in the development of vascular complications in patients with diabetes mellitus.

We have shown that TIMP-1 was inversely correlated with cECs and positively correlated with the PAC adhesion function (Appendix A). These findings are in contrast to our hypothesis and the available evidence. TIMP-1 has been shown to inhibit endothelial cell migration in in vitro studies. This is mediated through MMP dependent

and MMP independent pathway (Akahane *et al.*, 2004). These need to be assessed in a larger cohort of patients with type 1 diabetes.

There is increasing evidence that TIMP-1 plays a major role in the development of diabetic nephropathy. miR-21 is speculated to be involved in the pathogenesis of diabetic nephropathy via the down-regulation of MMP and upregulation of TIMP-1 (Wang *et al.*, 2013a).

In a large population-based study, TIMP-1 levels have been shown to be elevated in individuals with CVD risk factors. Furthermore, TIMP-1 is associated with increased left ventricular mass, thickness and reduced function. TIMP-1 levels are elevated in patients with CAD and it is linked with poor prognosis in patients with established CVD (Lubos *et al.*, 2005; Cavusoglu *et al.*, 2006). TIMP-1 is related to CV mortality and strokes in elderly men (Hansson *et al.*, 2011).

Given the above evidence, high TIMP-1 levels in type 1 diabetes can be inferred as a marker of ongoing vascular dysfunction. Further work needs to be conducted to explore whether the reduction of TIMP-1 leads to a decrease in the development of diabetes-related complications. ACE-inhibitors have been shown to reduce TIMP-1 levels in patients with hypertension (Laviades *et al.*, 1998). Improvement in metabolic control has been shown to improve levels of TIMP-1 (Tayebjee *et al.* 2004). Around half of our patients have been on a statin, ACE inhibitors and aspirin. Thus, this may a give lower baseline TIMP-1 level in our TG. Insulin has been shown to have no effect on TIMP-1 levels.

We have for the first time shown that 8 weeks of metformin treatment reduced TIMP-1 levels in type 1 diabetes patients to levels similar to healthy volunteers. This may be important as it rebalances vascular repair signals. We have shown that a physiological dose of metformin reduced TIMP-1 levels in vitro under the condition of hyperglycaemia and hypoxia combined. In addition, there is increased tube formation. This effect is not replicated under euglycaemic or euglycaemia and hypoxia condition (Bakhashab *et al.,* 2016). Interestingly, there is an inverse correlation between

changes in levels of TIMP-1 and the PAC adhesion function. Thus, a greater decrease in TIMP-1 levels results in an increased PAC adhesion function. Furthermore, there is a positive correlation between changes in HDL levels and TIMP-1 levels. As mentioned before, HDL levels decreased significantly after metformin treatment. There is some evidence linking high HDL cholesterol with a higher risk of a coronary event. This is an evolving area and further research is needed to get clearer evidence. Other high-risk markers including increased inflammatory markers like CRP and interleukins are discussed below.

Given all the evidence, we believe that the reduction of TIMP-1 levels after 8 weeks of metformin treatment alters the balance more towards vascular repair and angiogenesis. This is independent of glycaemic control. We believe our current work and previously published research (Bakhashab *et al.,* 2016) will be validated in in vivo settings. Furthermore, there is a need to design outcome studies to explore whethersustained reduction of TIMP-1 levels can lead to reduced diabetes-related complications.

### *6.10.2 Inflammatory cytokines*

#### *6.10.2.1 hs-CRP*

CRP is an acute phase protein raised in an inflammatory condition (Amit Kumar Shrivastava, 2015). A hyperglycaemic state in diabetes mellitus leads to the development of an inflammatory microenvironment. CRP levels are higher in patients with diabetes when compared to non-diabetic individuals (Mahajan *et al.*, 2009). In our study, median levels of plasma hs-CRP are significantly higher in type 1 diabetes patients. Our data is similar to a previous type 1 diabetes study showing elevated CRP levels in adults with known type 1 diabetes.

CRP levels have been demonstrated to be related with HbA1c and BMI (King *et al.*, 2003; Kao *et al.*, 2009). We have also shown a positive association between CRP levels and BMI. Indeed, it is established that CRP is higher in overweight and obese when compared to normal weight controls (Kao *et al.*, 2009). Weight loss has been

shown to reduce CRP levels (Selvin *et al.*, 2007). CRP levels have been shown to rise prior to the onset of type 1 diabetes, type 2 diabetes and gestational diabetes (Pradhan *et al.*, 2001; Wolf *et al.*, 2003; Chase *et al.*, 2004). Despite the body of evidence demonstrating high CRP levels in type 1 diabetes patients, Erbagci *et al.*, 2002 showed that CRP levels in children with type 1 diabetes are non-significantly higher when compared to the control group. This could be due to lower BMI and insulin resistance in type 1 diabetes as compared to type 2 diabetes.

CRP is a marker of ongoing low-grade vascular inflammation. It is established as a novel risk marker of development of CVD in healthy individuals (Ridker *et al.*, 2002; Ridker *et al.*, 2003). CRP is a prognostic marker in patients with ischaemic heart disease (Pietllä *et al.*, 1996). Evidence that CRP plays a role in the development of CVD in addition to be a marker of inflammation remains controversial. It is not clear whether CRP causes the development of vascular damage or is a byproduct of ongoing vascular damage. In young individuals with type 1 diabetes increased CRP levels are linked to early stages of carotid atherosclerosis (Hayaishi-Okano *et al.*, 2002).

CRP has been demonstrated to increase expression of other inflammatory cytokines like IL-6, IL-8 and TNF-α (Ballou and Lozanski, 1992). CRP has been shown to increase the expression of vascular injury markers like sICAM-1, sVCAM, etc (Pasceri *et al.*, 2000). We have demonstrated a very strong positive correlation between CRP and sICAM-1. CRP is involved in extenuating infiltration of monocytes into the vessel wall (Torzewski *et al.*, 2000). *In vitro* studies have shown that CRP impairs EC and EPC function, and increases cEC and EMP numbers (Verma *et al.*, 2004a; Devaraj *et al.*, 2011). Our data showed an inverse correlation between CRP and PAC adhesion function (Appendix A). However, ex vivo experiment have not shown similar results. Fasing *et al.*, 2014 demonstrated that in healthy individuals CRP levels do not correlate with the ability of EPC to form colonies, migrate, undergo apoptosis and release angiogenic factors. Therefore, there is a need to explore and clarify if CRP levels play a role in EPC function in vivo.

We explored whether metformin treatment will reduce CRP levels in type 1 diabetes. We showed that metformin did not have any effect on CRP levels in type 1 diabetes.

This could be due to the shorter duration of the treatment and it may be that a longer duration of therapy is needed. Furthermore, our study was not powered to explore the effect of metformin in type 1 diabetes. In a randomised controlled trial using 850mg BD of metformin, Stocker and team (Stocker *et al.*, 2007) demonstrated unchanged CRP levels after 24 weeks of metformin treatment in type 2 diabetes. It was possible that CRP levels could have been reduced if a full dose of metformin (2 grams per day) had been used. In their meta-analysis, Shi *et al.,* 2014 concluded that metformin reduced CRP levels in type 2 diabetes. We have no evidence of the effect of metformin in T1DM. Therefore, a longer duration of study is needed to to establish whether metformin can reduce CRP levels in T1DM.

#### *6.10.2.2 TNF-α and Interleukins*

Diabetes mellitus is associated with chronic low-grade inflammation. This is in part linked to insulin resistance and metabolic syndrome, especially in type 2 diabetes. Hyperglycaemia plays an important role in the development of chronic inflammation. We demonstrated that levels of TNF-α, IL-6 and IL-8 were higher in type 1 diabetes when compared with healthy controls. This is in line with previous evidence showing increased inflammatory markers in type 1 diabetes.

We explored the effect of metformin on inflammatory cytokines. To our knowledge, this is the first study exploring the effect of metformin on inflammatory markers in type 1 diabetes. Metformin treatment did not improve IL-8, TNF-α and IL-10 levels in type 1 diabetes. The major finding in our study is a significant reduction of IL-6 after 8 weeks of metformin treatment.

Our data has demonstrated an increased ongoing inflammatory process in type 1 diabetes. We have shown all three markers to be elevated in individual with type 1 diabetes even with good diabetes control. Sibal *et al.* 2009 showed an increase in CRP and IL-6, whereas TNF-α levels were unchanged in young individuals with type 1 diabetes. IL-6 and TNF-α levels have been shown to be high in T1DM (Targher *et al.*, 2001; West *et al.*, 2015). The EURODIAB study showed increased circulatory IL-6 and TNF-α levels in type 1 diabetes with complications (Schram *et al.*, 2003b).

Similarly, IL-8 levels have been shown to be higher in type 1 diabetes when compared to the healthy control (Lo *et al.*, 2004). In contrast, Purohit *et al.*, 2015 in a large population study, demonstrated that IL-8 levels are lower in type 1 diabetes. TNF-α, IL-6 and IL-8 are implicated in the pathogenesis of diabetes related complications (Şavli *et al.*, 2002; Gonzalez-Clemente *et al.*, 2005; Navarro *et al.*, 2006; Zorena *et al.*, 2007; Lee *et al.*, 2008; Shelbaya *et al.*, 2012).

Elevated inflammatory markers are linked to CVD and CVD risk. Dragut *et al.*, 2014 demonstrated that increased IL-6 and TNF-α are linked with increased CVD risk in patients with metabolic syndrome and type 2 diabetes. Tuomisto *et al.*, 2006 concluded that TNF-α is an independent predictor of CVD in men only. IL-6 and TNF-α are associated with poor prognosis in patients with CAD (Ridker *et al.*, 2000a; Su *et al.*, 2013). Similarly, IL-8 has been shown to be elevated in patients with CAD (Romuk *et al.*, 2002). TNF-α, IL-6 and IL-8 have been linked with the development of atherosclerosis (Apostolopoulos *et al.*, 1996; Ait-Oufella *et al.*, 2011). IL-6 has been associated with endothelial dysfunction in type 1 diabetes (Sibal *et al.*, 2009a). Similarly, TNF-α has been shown to play a role in the development of endothelial dysfunction (Picchi *et al.*, 2006). Our data complemented the above-mentioned studies and demonstrated a positive association between IL-6 and the markers of endothelial injury, sVCAM-1 and EMPs.

In summary, our data complement the available evidence showing increased inflammatory markers in patients with type 1 diabetes.

IL-10 is well established as an anti-inflammatory cytokine (Murray, 2005). It has been shown to have anti-atherogenic properties especially in animal models (Mallat *et al.*, 1999). Given the increased inflammatory markers in type 1 diabetes, one can hypothesise that levels of the anti-inflammatory cytokine, IL-10 will be lower. Contrary to our hypothesis, we have demonstrated increased circulatory IL-10 levels in type 1 diabetes when compared with healthy volunteers.

IL-10 has been shown to be lower in patients with type 2 diabetes (van Exel *et al.*, 2002). Levels of IL-10 have been shown to be even lower in patients with microalbuminuria (Pestana, 2016). In contrast, IL-10 levels are similar in patients with type 1 diabetes and healthy controls, whereas patients with type 1 diabetes and nephropathy had increased IL-10 levels (Mysliwska *et al.*, 2005). The difference between the results of our study and that of Mysliwska *et al*., 2005 can be explained by the assay used and data interpretation. Mysliwska *et al.,* 2005 included data even when it was below the threshold and unmeasurable. The MSD assay used in our study was able to measure IL-10 in all the subjects. Higher IL-10 levels in our study could be a compensatory response to increased inflammatory conditions to counteract high IL-6, TNF-α, IL-8 and CRP. IL-10 has been shown to counteract the effect of IL-6 and TNF-α (Fiorentino *et al.*, 1991). Similarly, Fichtlscherer *et al.,* 2004 demonstrated that higher IL-10 levels improved EF in patients with CAD and counteracted ongoing inflammatory activity (Fichtlscherer *et al.*, 2004).

Against the background of pro-inflammatory melluli in type 1 diabetes, we demonstrated that, IL-6 levels were reduced by metformin treatment in type 1 diabetes. However, metformin did not reduce TNF-α, IL-8 and IL-10 in type 1 diabetes. Our results could be explained by the sample size. Our study was not powered to assess the effect of the above mentioned inflammatory cytokines. Our data could be meaningfully expanded to design a study with an appropriate sample size to answer whether metformin has any effect on TNF-α, lL-6, IL-8 and IL-10.

Metformin has shown contradictory effects on inflammatory cytokines in PCOS and type 2 diabetes. (Mohlig *et al.*, 2004; Kim *et al.*, 2007a; Lund *et al.*, 2008b; Pradhan *et al.*, 2009; Lin *et al.*, 2011; Xu *et al.*, 2014). In vitro studies have shown the anti-inflammatory effect of metformin. Metformin has been shown to increase IL-10 in an *in vitro* study (Kelly *et al.*, 2015). As mentioned above, IL-10 levels in our study were significantly higher than healthy controls. Therefore, there may not be enough capacity to increase IL-10 further.

#### *6.10.2.3 Interferon-γ and Interferon gamma-induced protein 10 (IP-10)*

We have demonstrated that interferon-γ (IFN-γ) and IP-10 levels were similar in type 1 diabetes and healthy volunteers. Eight weeks of metformin treatment did not change IFN-γ and IP-10 concentration in our treatment group.

Levels of INF-γ in type 1 diabetes depends on the duration of diabetes. Levels of INF-γ have been shown to be higher in patients with recent-onset type 1 diabetes (Hussain *et al.*, 1996), whereas, INF-γ levels in patients with established type 1 diabetes have been shown to be similar to healthy controls (Hussain *et al.*, 1996). IFN-γ is involved in the development of type 1 diabetes through β cell destruction. IFN-γ plays a role in progression or remission of type 1 diabetes. High levels of IFN-γ are associated with disease progression whereas low levels are associated with remission of diabetes (Alizadeh *et al.*, 2006). Patients in our group hade type 1 diabetes for more than 20 years. Therefore, complete β cell destruction had already occurred. Furthermore, glycaemic control was good. It is likely that IFN-γ levels reflect completed β cell destruction and good glycaemic control. Furthermore, it is likely that IL-10 mediated suppression of Th1 immune response. As discussed above, IL-10 levels were significantly higher in type 1 diabetes when compared to controls.

IFN-γ acts on many types of cells, including monocytes, neutrophils and ECs, and induces production of IFN-γ inducible protein 10 (IP-10) (Luster and Ravetch, 1987; Cardozo *et al.*, 2003). Other inflammatory cytokines like TNF-α can also induce the release of IP-10 (Gasperini *et al.*, 1999; Narumi *et al.*, 2001; Berthier-Vergnes *et al.*, 2005). IP-10 levels in our TG correlated with INF-γ and TNF-α.

IP-10 is a pro-inflammatory chemokine that enhances adhesion of inflammatory cells to ECs. It has been shown to have anti-angiogenic properties (Yates-Binder *et al.*, 2012) IP-10 has been shown to inhibit EC migration and induces vessel regression (Bodnar *et al.*, 2006; Bodnar *et al.*, 2009). IP-10 levels are higher in patients with type 1 diabetes. This conflicts with our data which showed no difference between circulatory IP-10 concertation in individuals with type 1 diabetes and healthy volunteers. The difference between our data and other studies could be mainly due to the duration of

diagnosis of type 1 diabetes. Most studies have been conducted on patients with a recent diagnosis of type 1 diabetes, whereas our cohort in the TG had an average duration of diagnosis of more than 20 years. IP-10 plays a role in the pathogenesis of type 1 diabetes. It could be that inflammatory mediated β cell destruction had waned completely in our TG. Furthermore, diabetes control in our TG was good. There is no correlation between HbA1c and IP-10 levels.

IP-10 levels were associated with weight in our TG. This is consistent with the available evidence, which demonstrates increased IP-10 levels in patients with obesity. Circulatory levels have been shown to be higher in patients with diabetes mellitus patients with increased BMI (Dalmas *et al.*, 2011). IP-10 levels have been associated with diabetes-related complications. Urinary IP-10 levels are associated with a decline in renal function. However, IP-10 levels are not associated with microalbuminuria (Wolkow *et al.*, 2008).

Eight weeks of metformin treatment non-significantly reduced IP-10 levels in type 1 diabetes. Analysing the data, we could appreciate that data analysis may have yielded better information if our study was powered to explore the effect of metformin on IP-10 levels. Therefore, there is a need to design a study with an adequate sample size and power to explore the effect of metformin on IP-10 levels in type 1 diabetes

The mechanism for lowering IP-10 levels in type 1 diabetes by metformin needs to be explored. We hypothesise that NF-κB plays a role in regulating IP-10 release. NF-κB activity is increased in patients with diabetes (Andreasen *et al.*, 2011). Increased NF-κB activity is involved in macro- and microvascular complications in type 2 diabetes (Kassan *et al.*, 2013). Metformin inhibits NF-κB activation by other pro-inflammatory cytokines like TNF-α (Hattori *et al.*, 2006). NF-κB plays a role in hyperglycaemia induced IP-10 release. A decrease in activity of NF-κB attenuates IP10- release due to hyperglycaemia (Devaraj and Jialal, 2009).

### *6.10.3 Vascular injury markers*

Endothelial dysfunction is well established in young patients with type 1 diabetes before the development of overt CVD (Sibal *et al.,* 2009). We explored plasma markers of ED in type 1 diabetes. The aim was to study the effect of metformin on plasma markers of EF in well-controlled type 1 diabetes mellitus. The biomarkers studied included sICAM-1, sVCAM-1, P-Selectin, E-Selectin and thrombomodulin. sICAM-1 is also a marker of low-grade inflammation.

The major finding of our study was that none of the markers of ED were significantly higher in type 1 diabetes when compared to healthy controls. The circulatory level of P-Selectin, sICAM-1, sVCAM-1 and thrombomodulin were higher in type 1 diabetes but did not achieve statistical significance.

We for the first time demonstrated that 8 weeks of metformin treatment improved thrombomodulin levels in type 1 diabetes. This finding is unique to our study.

There is some conflicting data regarding P-Selectin and E-Selectin. Sibal *et al.,* 2009 demonstrated elevated E-Selectin levels in young individuals with type 1 diabetes. However, Yngen *et al*., 2004 (Yngen *et al.*, 2004) demonstrated that P-Selectin and E-Selectin are not elevated in patients with type 1 diabetes without any microvascular insult. The difference between Yngen *et al.,* 2004 and Sibal *et al.,* 2009 data could be explained by diabetes control. Patients in Yngen *et al*., 2004 had tighter control as evidenced by HbA1c (Yngen *et al.,* 2004 vs. Sibal *et al.,* 2009: 6.5% vs. 8.5%). Our patients had tighter control, with HbA1c of 7.1%. In addition, our individuals with type 1 diabetesshowed a positive correlation between HbA1c and E-Selectin and P-Selectin. However, type 1 diabetes patients with good glycaemic control but with microvascular disease have elevated levels of P-Selectin and E-Selectin (Yngen *et al.,* 2004). Sibal *et al.,* 2009 showed a direct correlation between E-Selectin and ED. Thus, it is likely that levels of E-Selectin and P–Selectin are not raised in individuals with type 1 diabetes without overt endothelial dysfunction and microvascular complications.

Circulatory sICAM-1 and sVCAM-1 levels in type 1 diabetes have been shown to be similar to healthy volunteers (Clausen *et al.*, 2000). Our data is complementary to the available evidence. Furthermore, biomarkers of ED have been shown to be associated with increased risk of CVD mortality in individuals with type 2 diabetes (de Jager *et al.*, 2006). sICAM-1 levels are associated with the development of diabetic nephropathy in type 1 diabetes. It is important to note that overall glycaemic control in these patients was inferior to our patients (8.7% vs. 7.1%) (Lin *et al.*, 2008). Similarly, this putative ED is associated with the development of diabetic nephropathy in type 2 diabetes (Koga *et al.*, 1998; Bruno *et al.*, 2008).

The expression of ICAM-1 and VCAM-1 has been shown to be upregulated by inflammatory cytokines like CRP and TNF-α. An *in vitro* study has shown that CRP induces expression of adhesion molecules (ICAM-1 and VCAM-1) (Pasceri *et al.*, 2000). We have shown a direct correlation between CRP and sICAM-1 levels.

Short-term metformin treatment (16 weeks) has been shown to reduce sVCAM-1 in type 2 diabetes (De Jager *et al.*, 2005). When metformin was continued for more than 4 years, the same group demonstrated that sICAM and sVCAM levels decreased significantly (De Jager *et al*., 2014). This effect on the above-mentioned biomarkers was independent of changes in glycaemic control and weight. In our study, metformin treatment did not change the levels of sICAM and sVCAM. This could be due to the shorter duration of treatment with metformin (8 weeks). Our data suggests that metformin used in the short-term treatment of type 1 diabetes may not affect markers of ED. Longer-term treatment may show improvement in markers of ED. However, this will need to be evaluated in a longer trial.

We have demonstrated that thrombomodulin levels were non-significantly higher in our type 1 diabetes and healthy volunteer group. This is in contrast to previous data which showed that thrombomodulin levels are elevated in type 1 diabetes and type 2 diabetes (Gabat *et al.*, 1996). Levels were higher in individuals associated with diabetes-related complications (Oida *et al.*, 1990). Higher levels in Gabat *et al.,* 1995 could be explained by the fact that levels of thrombomodulin were higher in patients with increasing

numbers of diabetes related complications. In our study, the subjects studied did not have any evidence of diabetes-related complications.

Thrombomodulin levels were directly associated with the EPC and $CD45^{dim}CD34^{+}CD133^{+}$ numbers. This is in line with available evidence that thrombomodulin is released by EPCs (Li *et al.*, 2011a).

We have for the first time demonstrated that 8 weeks of metformin treatment reduced thrombomodulin levels. The reduction in thrombomodulin levels could reflect decreased endothelial damage. Strijbos *et al.*, 2008 demonstrated a direct correlation between cEC numbers and plasma levels of thrombomodulin. However, we have not shown any correlation between thrombomodulin levels or changes in thrombomodulin levels with any markers of endothelial damage. This could be due to the small number of subjects in our TG. This will need to be explored further in a larger trial.

The reduction in thrombomodulin levels after 8 weeks of metformin treatment could be due to improvement in insulin sensitivity. Levels of thrombomodulin were related to insulin sensitivity in type 2 diabetes (Aso *et al.*, 2001). The improvement in insulin sensitivity after metformin treatment in type 1 diabetes is likely to be small when compared to type 2 diabetes. However, the mechanisms by which metformin may reduce thrombomodulin levels in type 1 diabetes needs to be confirmed.

### *6.10.4 Growth factors*

In our study, we have shown that in patients with type 1 diabetes with good glycaemic control, 1) VEGF and FGF levels are similar in type 1 diabetes and healthy volunteers, 2) VEGF-D levels are significantly higher in type 1 diabetes when compared to healthy volunteers, and 3) metformin treatment did not result in any change in growth factor levels.

VEGF is a potent angiogenic growth factor involved in endothelial cell growth and proliferation (Waltenberger, 2009). VEGF levels are elevated in type 2 diabetes (Lim *et al.*, 2004). In patients with type 1 diabetes , VEGF levels are not elevated (Malamitsi-Puchner *et al.*, 1998). Our data is similar to Malamitsi-Puchner *et al.* 1998. However, levels of another family of VEGF, VEGF-D were elevated in our patients in the TG when compared with healthy control. *In vitro* work by our group has shown that VEGF receptors are downregulated in hyperglycaemic environments. This results in a compensatory increase in VEGF levels (unpublished data). VEGF-D acts on VEGFR-3. VEGFR-3 is expressed by ECs. Therefore, we can speculate that VEGFR-3 is downregulated in the ECs of our TG participants, leading to increase an in VEGF-D.

It has been shown that elevated levels of VEGF are involved in the pathogenesis of diabetes-related complications like retinopathy and nephropathy (Adamis *et al.*, 1994; Santilli *et al.*, 2001). Elevated VEGF levels in young type 1 diabetes patients without complications predict future development of microalbuminuria (Santilli *et al.,* 2001). Plasma VEGF levels increase with worsening glycaemic control. Improving glycaemic control improved VEGF levels (Kakizawa *et al.*, 2004). VEGF reduced in type 2 diabetes after multifactorial intervention only in patients with CVD (Lim *et al.*, 2004). Ersoy *et al.,* 2008 demonstrated that metformin treatment for 12 weeks improved VEGF levels in obese patients with type 2 diabetes. In this study, HbA1c and BMI improved significantly but were not associated with VEGF (Ersoy *et al.*, 2008). We have shown in an *in vitro* study that hyperglycaemia raised VEGF-A levels (Bakhashab *et al.,* 2016). However, metformin exposure did not lead to a reduction in VEGF-A levels. Metformin treatment in our type 1 diabetes group did not decrease VEGF levels.

So, in summary, VEGF-A levels are non-significantly higher in our group. Furthermore, VEGF-D levels are significantly higher in our TG. Thus, we can infer that our TG were at risk of development of a diabetes-related complication. Furthermore, our in vitro study (Bakhashab *et al.,* 2016) and current work demonstrate that metformin may not affect VEGF levels.

FGF levels are low in healthy volunteers (Larsson *et al.*, 2003). FGF levels are elevated in patients with type 2 diabetes with microalbuminuria (Zimering *et al.,* 1996) and are

a marker of CVD risk in type 2 diabetes (Zimering *et al.,* 2013). Levels of FGF can be a marker of increased risk for CAD in type 2 diabetes patients (Zimering *et al.,* 2011). Levels of FGF are increased in CVD (Katinioti *et al.*, 2002). In young patients with type 1 diabetes, levels of FGF are not elevated (Malamitsi-Puchner *et al.*, 1998). Similarly, in our study levels of FGF were not elevated. It is likely that in individuals with type 2 diabetes without complications, FGF levels are not elevated. However, this will need to be confirmed in a trial looking at FGF levels in patients with type 1 diabetes and diabetes-related complications.

We also demonstrated that metformin did not have any effect on FGF levels in type 1 diabetes. It is likely that our cohort had lower FGF levels. Thus, metformin may not be able to reduce it further. In addition, it is likely that metformin may not have any effect on FGF levels. This will need to be confirmed in *in vitro* and *in vivo* studies.

## 6.11 Summary

We evaluated sixteen cytokines in our study. They can be divided into the matrix metalloprotease inhibitor (TIMP-1), inflammatory cytokines, vascular injury cytokines and growth factors. TNF-α, IL-6, CRP, IL-8, INF-γ, IL-10 and IP-10 play an important role in inflammatory processes. Except for IL-10, all the cytokines are pro-inflammatory. IL-10 has anti-inflammatory properties. The vascular injury markers studied included E-Selectin, P-Selectin, sVCAM, sICAM and thrombomodulin. The growth factors studied included bFGF, VEGF, VEGF-D. TIMP-1 is an inhibitor of MMP, thereby playing an important role in EPC mobilisation.

### Increased levels of inflammatory cytokines, VEGF-D and TIMP-1 levels in type 1 diabetes

Our study complemented previous work and demonstrated that inflammatory cytokines (IL-6, TNF, CRP and IL-8) were significantly elevated in patients with type 1 diabetes. Even after Bonferroni correction, levels of CRP and IL-8 were significantly elevated. The presence of chronic inflammation in type 1 diabetes would lead to vascular damage and development of vascular disease.

We demonstrated that levels of vascular markers (E-Selectin, P-Selectin, sICAM, sVCAM and thrombomodulin) were not elevated. Our finding complemented previous work demonstrating similar levels of vascular markers in patients with good overall glycaemic control (Yngen *et al*., 2004). With sub-optimal control, it has been shown that E-Selectin was elevated in patients with type 1 diabetes (Sibal *et al.,* 2009).

We have demonstrated that bFGF and VEGF levels were similar in type 1 diabetes and healthy controls. Interestingly, we demonstrated that VEGF-D levels were higher in type 1 diabetes when compared to healthy control. We speculated that VEGF-D in our type 1 diabetes was elevated due to down-regulation of VEGF-D receptors (VEGFR-3). This will need to be confirmed by analysing whether VEGFR-3 levels are lower in type 1 diabetes.

TIMP-1 levels were elevated in our patients with type 1 diabetes when compared to healthy controls. This could lead to poor recruitment of EPCs from bone marrow (Liu and Velazquez, 2008). In addition, increased TIMP-1 levels will decrease MMP activity (Brew and Nagase, 2010). This will lead to thickning of the basement membrane and development of diabetes related complications (Bai *et al*., 2006). However, there is a need to explore the effect of TIMP-1 in type 1 diabetes.

**Metformin treatment reduced IL-6, IP-10, TIMP-1 and thrombomodulin levels in type 1 diabetes**

We evaluated the effect of metformin on a wide array of cytokines in type 1 diabetes. We demonstrated that 8 weeks of metformin treatment significantly reduced TIMP-1, IP-10, IL-6 and thrombomodulin levels in type 1 diabetes. From our results, we can infer that metformin may have an effect in reducing inflammatory conditions in type 1 diabetes. This could potentially lead to reduced vascular damage. We demonstrated that thrombomodulin, a marker of vascular damage, was also reduced after metformin treatment. Other markers of vascular damage, sICAM, sVCAM and E-Selectin, were not reduced. In addition, we demonstrated that metformin significantly reduced TIMP-1 levels. We could infer that reduction in TIMP-1 levels may lead to increased EPC

mobilisation. We have demonstrated that cEPC levels were higher after metformin treatment. However, there was no correlation between changes in TIMP-1 and cEPC levels. We also evaluated our data after Bonferroni correction. Only the reduction in thrombomodulin levels after metformin treatment retained significance. This could be due to the smaller sample size. Our study was not powered to evaluate the effect of metformin on cytokines in type 1 diabetes. This is one of the limitations which should be taken into consideration in evaluating our data. Therefore, our data could be meaningfully used to design a study with adequate power to explore the effect of metformin on cytokines in type 1 diabetes.

Our work has added important information regarding the effect of metformin on cytokines independent of glycaemic control. We have identified cytokines that may play a role in the pathways which may be modified by metformin. Future studies are needed to explore the effect of metformin on inflammatory pathways.

# Chapter 7: Effect of metformin on gene expression of peripheral blood mononuclear cells

## 7.1 Introduction

Peripheral blood mononuclear cells (PBMCs) consist of a group of blood cells which are characterised by a round nucleus. PBMCs consist of two major cells type: lymphocytes and monocytes. Lymphocytes include T lymphocytes (T cells), natural killer cells (NK cells) and B lymphocytes (B cells) (Saša Končarević, 2014). Both lymphocytes and monocytes play an important role in providing immunity (Saša Končarević, 2014). NK cells provide cell-mediated and innate immunity (Paust and von Andrian, 2011). T cells provide cell-mediated and adaptive immunity. B cells provide antibody driven adaptive immunity (Walter *et al.*, 2002a). Monocytes are the largest PBMC and play an important role in both the innate and adaptive immune responses. Monocytes can differentiate into macrophages (Leon and Ardavin, 2008; Parihar *et al.*, 2010).

PBMCs play a central role in providing body protection. They mount an immune response to destroy pathogens. However, in addition to this protective role, PBMCs can contribute to the damage of the human body. PBMCs are central to driving the inflammatory response needed for the development and progression of atherosclerosis. The old dogma for the development of atherosclerosis included the passive deposition of lipids in blood vessel walls (Libby *et al.*, 2002). We now know that in addition to lipids, inflammation plays a major role in the development of atherosclerosis. Both lymphocytes and monocytes are involved in the pathogenesis of vascular damage and atherosclerosis (Moore and Tabas, 2011; Hedrick, 2015).

### *7.1.1 Role of monocytes in development of atherosclerosis*

A number of different stimuli such as hyperglycaemia can lead to endothelial cells (EC) injury and activation of ECs. ECs produce adhesion molecules like E- and P-Selectin (Blankenberg *et al.*, 2003). Activated ECs produce chemoattractants like MCP-1, endothelin-1, etc. and pro-inflammatory cytokines like TNF-alpha, IL-6 and IL-8 (Lucas and Greaves, 2003; Bobryshev, 2006). This leads to recruitment of monocytes at the site EC injury. Monocytes attach to the damage EC via selectin adhesion molecules. Firm attachment of monocytes requires another family of adhesion molecules: VCAM-

1 and ICAM-1. Monocytes transmigrate across the endothelium into sub-endothelial space (Mestas and Ley, 2008; Moore and Tabas, 2011).

Once the monocytes are in sub-endothelial space, they can differentiate into macrophages and dendritic cells (Johnson and Newby, 2009; Paulson *et al.*, 2010). Macrophages can accumulate lipid especially LDL cholesterol and develop into foam cells (Brown *et al.*, 1980). Lipid-rich foam cells and dendritic cells accumulate in the area of EC damage leading to the progression of atherosclerosis. Continued accumulation leads to the development of fibrous plaque. The central area of the plaque leads to the formation of the necrotic core due to increased cell death. Cellular debris and lipid continue to accumulate within the core, thereby increasing the size. Atherosclerotic plaques continue to grow due to continued recruitment of monocytes and differentiation to foam cells (Reviewed by  Bobryshev, 2006; Moore and Tabas, 2011) (Figure 93).

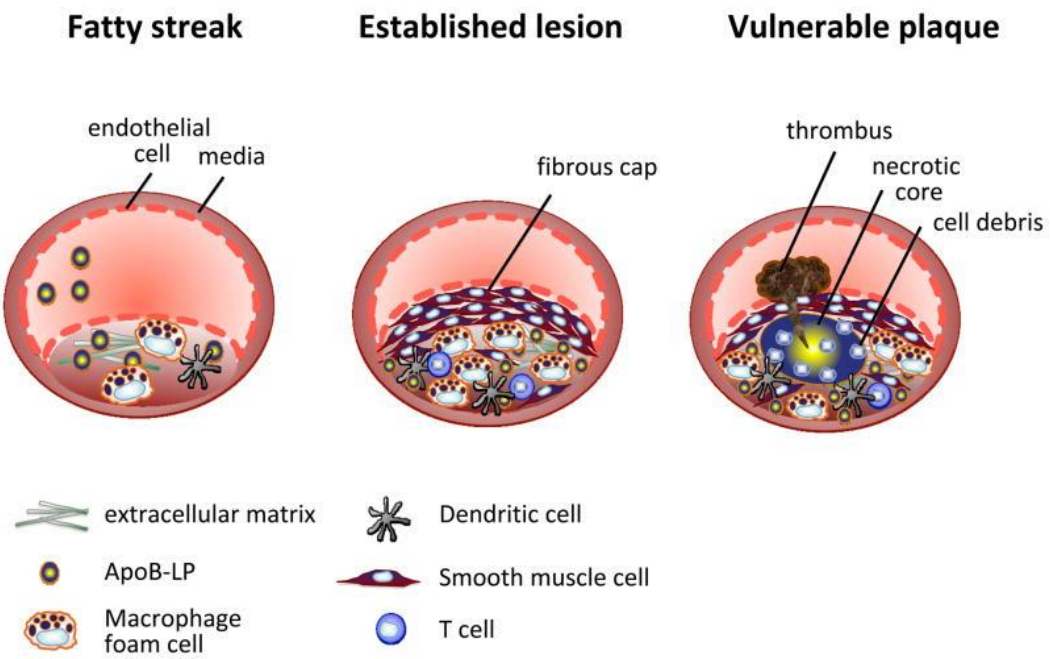



Figure 80: Role of monocytes in development and progression of atherosclerosis.

Adapted from Moore and Tabas 2012

### *7.1.2 Role of lymphocytes in atherosclerosis*

Lymphocytes encompass three major cells. T lymphocytes, B lymphocytes and natural killer cells (NK). They play a major role in atherosclerosis (Wigren *et al.*, 2012).

B lymphocytes have a pleiotropic function. B lymphocytes have been shown to be anti-atherogenic. In a splenectomy mouse model, increased atherosclerosis has been demonstrated. The spleen is a reservoir of B lymphocytes (Caligiuri *et al.*, 2002). B lymphocyte-deficient mice model has shown that hypercholesterolaemia increased development of atherosclerosis (Major *et al.*, 2002). B lymphocytes mount antibody response preventing the development of atherosclerosis. In addition, IgM antibody against oxidised LDL have been shown to be anti-atherogenic (Tsimikas *et al.*, 2007). However, recently B lymphocytes have been shown pro-atherosclerotic properties (Ait-Oufella *et al.*, 2010; Kyaw *et al.*, 2010). B lymphocytes role in atherosclerosis is still under investigation (Tsiantoulas *et al.*, 2015).

The role of T lymphocytes in atherosclerosis appears to be more complex. A subset of T lymphocytes are pro-atherogenic (Th1 and Th17) and another subset anti-atherogenic. (Th2 and Treg). Th1 cells related cytokines like INF-γ have been shown to be in greater quantity in atherosclerotic plaque (Frostegård *et al.*, 1999). Th1 have been shown to increase atherosclerosis (Buono *et al.*, 2005). The role of Treg has been well established in atherosclerosis especially in an animal model. Decrease in Treg has been shown to increased development of atherosclerosis (Mallat *et al.*, 2003; Ait-Oufella *et al.*, 2010). Similarly, NK cells have been shown to have pro-atherosclerotic properties (Li *et al.*, 2015).

### *7.1.3 Role of PBMC in diabetes-related complications*

Diabetes mellitus is associated with increased risk of vascular complications. In addition, inflammation has been shown to play a vital role in the pathogenesis of diabetes-related complications. PBMC, as discussed above plays a critical role in the development of atherosclerosis. There is increasing evidence that PBMCs are involved

in the generation of the inflammatory condition. Hyperglycaemia has been shown to mediate the increase in monocyte adhesion to EC (Kim *et al.*, 1994). Diabetes mellitus patients have shown to have increased adhesion of monocytes to EC (Carantoni *et al.*, 1997). Monocytes adhesion to EC has been demonstrated be related to the diabetes control (Cipolletta *et al.*, 2005). The proatherogenic activity of monocytes has been shown to be increased in diabetes mellitus (increased superoxide anion, IL1β and increased adhesion) (Devaraj and Jialal, 2000).

Macrophages and lymphocytes have been shown to be increased in glomeruli in diabetes mellitus patients (Lim, 2014). T cell infiltration in type 1 diabetes was correlated with albuminuria and renal function (Moriya *et al.*, 2004). In an animal model, T-reg cell (anti-inflammatory cells) has been shown to protect against the development of albuminuria (Eller *et al.*, 2011).

Hyperglycaemia have been shown to effect PBMC cytokine production and function. PBMC's from patients with type 1 diabetes has been shown to be in inflammatory state when cultured. TNF and IL-10 levels were higher in supernatant of PBMC culture from patients with type 1 diabetes when compared to healthy control (Foss-Freitas *et al.*, 2009). Interestingly, PBMC's from patients with type 1 diabetes had poor response to activation by LPS compared to healthy control (Foss *et al.*, 2007). This meant that PBMC's in patients with type 1 diabetes have poor immune response leading to increased incidence of infection.

There is no evidence on the effect of metformin on PBMC in patients with type 1 diabetes. In individual with pre-diabetes, eight weeks of metformin treatment increased expression of genes involved in regulation of longevity, thereby counteract accelerated ageing (Vigili de Kreutzenberg *et al.*, 2015). However, metformin treatment increased insulin sensitivity and decreased weight significantly. Therefore, metabolic factor could have played a part in modulation of gene expression (Vigili de Kreutzenberg *et al.*, 2015). This will need to be confirmed in future studies. Most in vitro studies have used supra-physiological doses of metformin in their experiment. Metformin in supra-physiological concentrations reduced production of pro-inflammatory cytokines. Similarly, metformin (supra-physiological concentration) did not have any effect on

proliferation of non-stimulated PBMC (Djordjic *et al.*). Our group explored the effect of metformin on CD34+ cell (extracted from PBMCs). We demonstrated that metformin at physiological concentration had an inhibitory effect on genes of pro-inflammatory factors like IL-6 and IL-8 (Bakhashab *et al.,* 2017).

## 7.2 Specific aim

We aimed to evaluated if eight weeks of metformin treatment had an effect on the kinase and inflammatory gene expression of PBMCs in patients with type 1 diabetes when compared to healthy control.

## 7.3 Methods

### *7.3.1 Selection of subjects for gene expression*

We only selected subjects from TG and HC. SG was not included. This was due to limitation of funding. In TG, we aimed to select samples from patients not taking any medications for primaryprevention. Statin and ACE inhibitors have been shown to have an effect on gene expression of PBMCs (Wibaut-Berlaimont *et al.*, 2005). Subjects in HC group were selected based on them being matched with TG. Subject's clinical and metabolic characteristics are presented in Table 27.

### *7.3.2 Total RNA extraction*

PBMCs were isolated after ficoll preparation. Ficol preparation was discussed earlier. Cells were plated for PAC. The cells which were left were used for RNA extraction. Approximately, 5 X $10^6$ cells were lysed using the Qiazol lysis reagent (Catalogue number: 79306). 700 µl was used to lyse the cells. The sample was vortexed and kept in the dark for 5 minutes to make sure that the samples were completely lysed. Lysed samples were stored at -$80^0$c.

The RNeasy® Mini kit was used for extraction of total RNA from the lysate. Ten µl β-mercaptoethanol (β-ME) was added to 1 ml Buffer RLT. Buffer RLT with β-ME or DTT can be stored at room temperature for up to 1 month. Four volumes of ethanol (96–100%) were added to Buffer RPE for a working solution.

1 volume of 70% ethanol was added to the lysate and mixed well by pipetting. 700 µl of the sample, including any precipitate, was added to an RNeasy Mini spin column placed in a 2ml collection tube. The sample was centrifuged for 15 s at ≥ 8000 x g. Flow-through was discarded. 350 µl Buffer RW1 was added to the RNeasy spin column. The sample was centrifuged for 15 s at ≥8000 x g. Flow-through was discarded.

10 µl DNase I stock solution was added to 70 µl Buffer RDD. This was mixed gently by inverting the tube. The mixture was centrifuged briefly. 500 µl Buffer RPE was added to the RNeasy spin column. This was then centrifuged for 15 s at ≥8000 x g. Flow-through was discarded. 500 µl Buffer RPE was added to the RNeasy spin column. This was then centrifuged for 2 min at ≥8000 x g. RNeasy spin column was placed in a new 2 ml collection tube. It was than Centrifuge at full speed for 1 min to dry the membrane.

The RNeasy spin column was placed in a new 1.5 ml collection tube. 30–50 µl RNase-free water was added directly to the spin column membrane. We then centrifuged for 1 min at ≥8000 x g to elute the RNA.

| | TG (n=8) | | p value | HC (n=6) | p value |
|---|---|---|---|---|---|
| | TG V1 | TG V2 | TG V1 vs V2 | | HC vs TG V1 |
| Age year | 40.6+/-4 | - | - | 39.7+/-10.3 | 0.9 |
| Sex M/F n | 2/5 | - | - | 3/3 | - |
| DOD years | 21+/-9.5 | - | - | - | - |
| BMI kg/m$^2$ | 27.8+/-6 | 27.6+/-2 | 0.6 | 24+/-3.7 | 0.3 |
| Systolic BP mmHg | 123.7+/-5 | 114+/-2 | 0.06 | 117+/-9.9 | 0.3 |
| Diastolic BP mmHg | 77.4+/-3 | 74+/-5 | 0.3 | 75.5+/-6.2 | 0.6 |
| HbA1c mmol/mol | 59+/-4.7 | 59.6+/-3.5 | | 35.7+/-3.7 | 0.003 |
| Insulin dose Units | 20 (17.7-40) | 16 (15-6-24) | 0.03 | - | - |
| Smoking Y/E/N | 1/0/0 | - | - | 0/0/0 | - |
| Total Cholesterol mmol/l | 4.6+/-1.2 | 4.8+/-1.2 | 0.5 | 4.5+/-0.5 | .9 |
| Triglyceride mmol/l | 0.83+/-0.3 | 0.83+/-0.3 | 0.9 | 1+/-0.4 | 0.4 |
| HDL-cholesterol mmol/l | 1.9+/-0.4 | 1.5+/-0.3 | 0.1 | 1.8+/-0.5 | 0.95 |
| Creatinine umol/l | 73+/-16 | 64+/-7 | 0.04 | 78.2+/-15.3 | 0.6 |
| WCC | 6+/-1.8 | 6.3+/-1.5 | 0.2 | 6.2+/-1.3 | 0.9 |

Table 27**:** Subject's clinical and metabolic characteristics. Values are given as mean+/-SD or * median (Interquartile range (IQ)). kg-kilogram, BMI-body mass index, BP-Blood pressure, M-Male, F-Female, DOD-Duration of diabetes, Y-Yes, E-Ex-smoker, N-No. TG V1: Pre-treatment; TG V2: Post treatment; SG V1: Pre-observation; SG V2: Post observation; WCC: White cell count.

### *7.3.3 nCounter gene expression*

nCounter Gene Expression assay is a highly novel multiplexed method which is sensitive and reproducible. It is based on Nanostring Technology. This includes digital detection and direct molecular barcoding of target molecules. Colour coded probe pairs are used. Probe pair consist of colour coded reporter probe (colour codes at 5' end) and a capture probe (biotin on the 3' end). Reporter probe carries the molecular code for detection. The capture probe is used for immobilisation. Both probes have 35-50 base long target specific region. Once paired probe is added to the sample for evaluation of molecular target, data is collected using nCounter digital analyser. On reporter probe, seven positions are available for four colours to be used as barcodes. This gives availability of thousands of combinations (Figure 94).

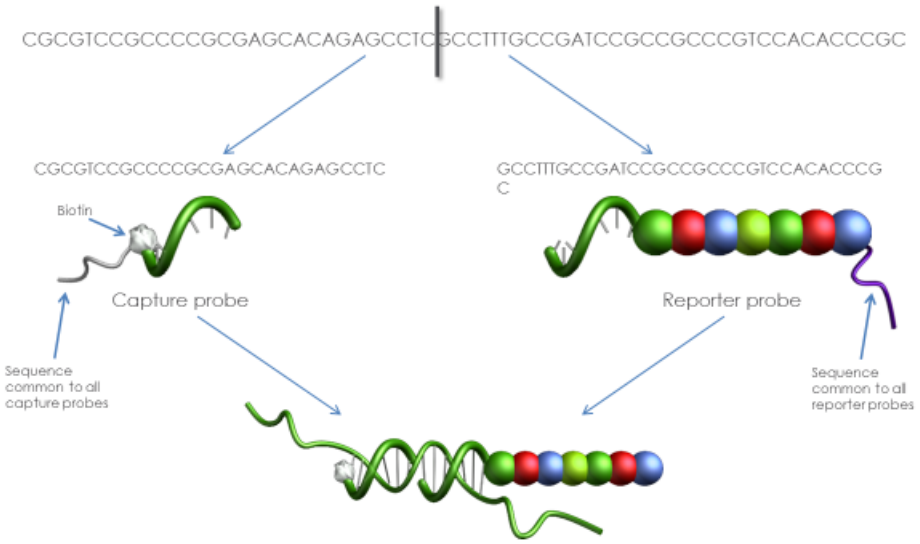



Figure 81: nCounter gene expression. Reporter and Capture probe. (Taken from Kyle Green presentation)

Gene expression using the nCounter system was performed according to the manufacturer's recommendations. Each sample was run as duplicates. The first step was hybridization of the code set to RNA. Reporter probe was added to the extracted total RNA. Capture probe set was added. The mixture was briefly spun down. The sample was placed on pre-heated $65^0$C thermocycler and incubated overnight (minimum 12 hours and maximum 36 hours). Then the sample was loaded to the nCounter Prep Station. This step removes excess reporter, binds the reporter to the

surface and immobilises and aligns reporter probe. Next step was to load the processed sample to the nCounter Digital Analyzer for imaging and data analysis (Figure 95).

Inflammatory and kinase Nano string panels were used. These two-panel covered genes involved in pathways that play an important role in inflammation, vascular dysfunction and vascular repair.

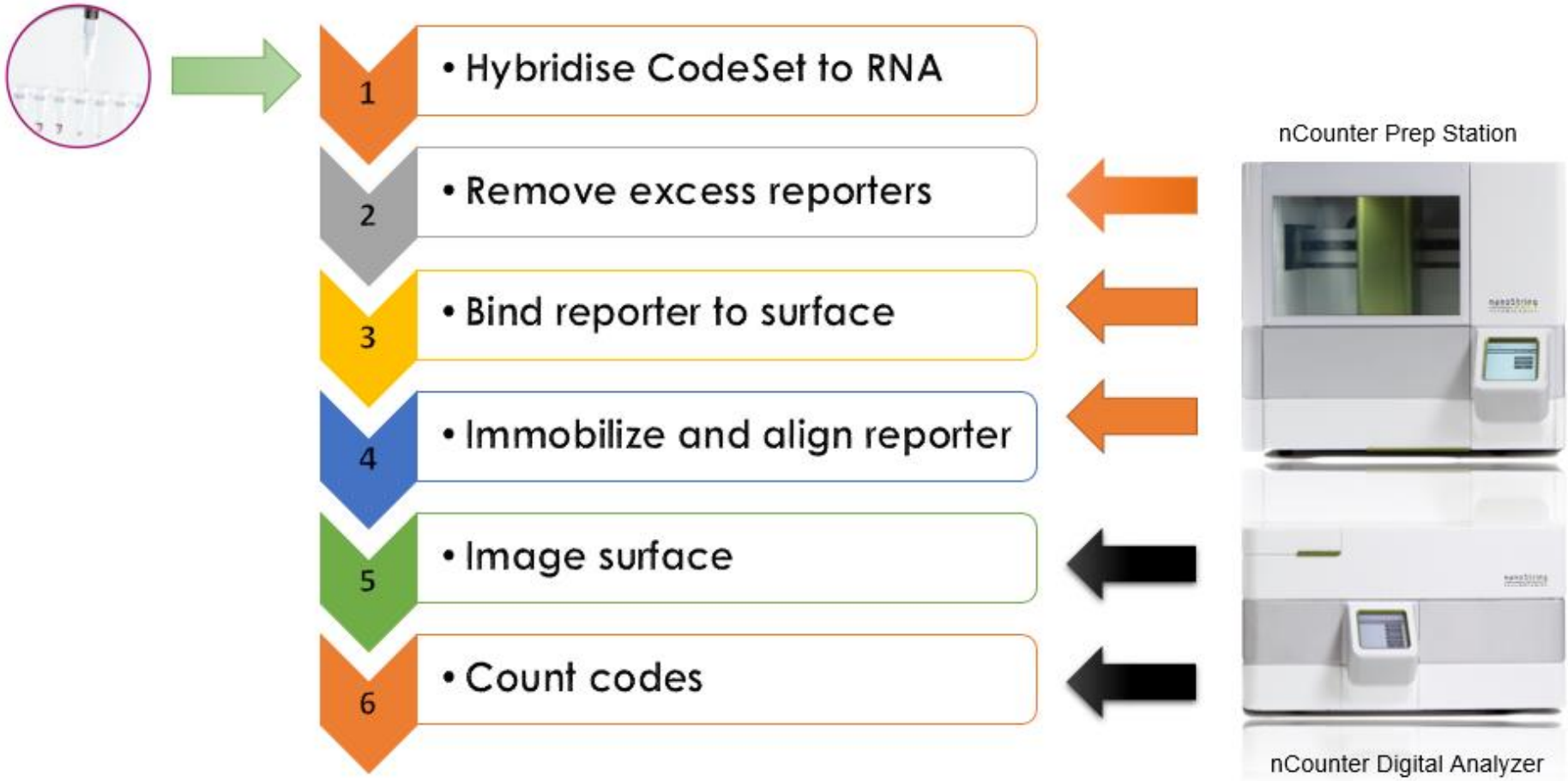



Figure 82: Steps in nCounter gene expression.

### *7.3.4 Data Analysis*

Data was analysed using nSolver software and R. As per NanoString recommendation; the resultant data set was normalised. We used the code sets already present as internal positive controls. This was conducted to eliminate variability unrelated to sample. Inflammatory and kinase panels were analysed individually. However, only 3 and nine genes were differentially expressed on the inflammatory and kinase panel, respectively in the treatment group (TG V1 vs. TG V2).

We evaluated our data more globally and included both the kinase and inflammatory panels together in nSolver software. 28 genes overlapped between the two panels (Figure 96). Ten genes with lowest %CV from the overlapping genes were selected as housekeeping genes (Figure 97). Inflammatory and kinase datasets were normalised using housekeeping genes. Then we manually combined the datasets and calculated the fold change (FC) between treatment group (TGV1 vs TGV2) and between treatment group and healthy control (TGV1 vs HC and TGV2 vs HC).

One treatment group pre-treatment sample gave an error during normalisation step. Therefore, we excluded the sample (both pre and post treatment) from the analysis (Figure 98 and 99). After exclusion of this sample, all other samples passed the quality control (QC) parameters. QC was performed by nSolver software using following parameters:

- Imaging QC – A measure of the percentage of each lane that was successfully scanned.
- Binding Density QC – A measure of barcode density on the scan surface.

- Positive (POS) control linearity QC – A measure of how well the 6 synthetic POS control spike-ins form a dilution series.
- POS control Limit of Detection QC – A measure of whether Positive control spike in E(POS E) is statistically detectable above the NEG control probes.

| Gene Name | Accession # |
|---|---|
| CCL2 | NM_002982 |
| CLTC | NM_004859 |
| GAPDH | NM_002046 |
| GUSB | NM_000181 |
| HPRT1 | NM_000194 |
| MAP2K1 | NM_002755 |
| MAP2K4 | NM_003010 |
| MAP2K6 | NM_002758 |
| MAP3K1 | NM_005921 |
| MAP3K5 | NM_005923 |
| MAP3K7 | NM_145333 |
| MAP3K9 | NM_033141 |
| MAPK14 | NM_001315 |
| MAPK3 | NM_001040056 |
| MAPKAPK2 | NM_004759 |
| MAPKAPK5 | NM_003668 |
| MKNK1 | NM_003684 |
| PGK1 | NM_000291 |
| PRKCA | NM_002737 |
| PRKCB | NM_212535 |
| PTK2 | NM_005607 |
| RAF1 | NM_002880 |
| RIPK1 | NM_003804 |
| RIPK2 | NM_003821 |
| ROCK2 | NM_004850 |
| RPS6KA5 | NM_004755 |
| TGFBR1 | NM_004612 |
| TUBB | NM_178014 |

Figure 83:28 genes common in both kinase and inflammatory panel.

Normalization Codes

| Gene Name | Class Name | Avg Count | %CV |
|---|---|---|---|
| CCL2 | Endogenous | 13.833 | 71.457 |
| CLTC | Endogenous | 698.875 | 56.616 |
| GAPDH | Endogenous | 11,845.5 | 60.375 |
| GUSB | Endogenous | 327.208 | 54.992 |
| HPRT1 | Endogenous | 741.542 | 52.653 |
| MAP2K1 | Endogenous | 1,190.125 | 75.044 |
| PGK1 | Endogenous | 4,639.125 | 63.942 |
| TUBB | Endogenous | 773.583 | 56.931 |
| RAF1 | Endogenous | 1,119.917 | 55.061 |
| MAPK3 | Endogenous | 702.667 | 54.717 |

Figure 84:10 gene used to normalise data in both kinase and inflammatory panel.

| 24 | Normalized Data Name | Treatment Group | Cartridge ID | Lane Nu... | Sample Name | Description | QC Flag | Normalization Flag | Positive ... | Content Normali... |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 20150107_fahad #1 huinflam_01 3v1_01.RCC | Pre | fahad #1 huinflam | 1 | 01 3v1 | | | | 1.14 | 0.39 |
| 2 | 20150107_fahad #1 huinflam_02 10v1_02.RCC | Pre | fahad #1 huinflam | 2 | 02 10v1 | | | | 1.18 | 0.68 |
| 3 | 20150107_fahad #1 huinflam_03 28v1_03.RCC | Pre | fahad #1 huinflam | 3 | 03 28v1 | | | | 1.04 | 0.68 |
| 4 | 20150107_fahad #1 huinflam_04 22v1_04.RCC | Pre | fahad #1 huinflam | 4 | 04 22v1 | | | | 1.08 | 1.31 |
| 5 | 20150107_fahad #1 huinflam_05 17v1_05.RCC | Pre | fahad #1 huinflam | 5 | 05 17v1 | | | | 1.11 | 0.95 |
| 6 | 20150107_fahad #1 huinflam_06 16v1_06.RCC | Pre | fahad #1 huinflam | 6 | 06 16v1 | | | | 1.16 | 2.04 |
| 7 | 20150107_fahad #1 huinflam_07 3v2_07.RCC | Post | fahad #1 huinflam | 7 | 07 3v2 | | | | 0.93 | 0.81 |
| 8 | 20150107_fahad #1 huinflam_08 10v2_08.RCC | Post | fahad #1 huinflam | 8 | 08 10v2 | | | | 1.08 | 0.93 |
| 9 | 20150107_fahad #1 huinflam_09 28v2_09.RCC | Post | fahad #1 huinflam | 9 | 09 28v2 | | | | 1.08 | 0.49 |
| 10 | 20150107_fahad #1 huinflam_10 22v2_10.RCC | Post | fahad #1 huinflam | 10 | 10 22v2 | | | | 1.05 | 0.7 |
| 11 | 20150107_fahad #1 huinflam_11 17v2_11.RCC | Post | fahad #1 huinflam | 11 | 11 17v2 | | | | 1.08 | 0.95 |
| 12 | 20150107_fahad #1 huinflam_12 16v2_12.RCC | Post | fahad #1 huinflam | 12 | 12 16v2 | | | | 1.01 | 5.07 |
| 13 | 20150120_fahad #1 huinflam_01 7v1_01.RCC | Pre | fahad #1 huinflam | 1 | 01 7v1 | | | | 0.74 | 7.71 |
| 14 | 20150120_fahad #1 huinflam_02 13v1_02.RCC | Pre | fahad #1 huinflam | 2 | 02 13v1 | | | | 0.8 | 19.17 |
| 15 | 20150120_fahad #1 huinflam_03 26v1_03.RCC | Pre | fahad #1 huinflam | 3 | 03 26v1 | | | | 0.8 | 3.57 |
| 16 | 20150120_fahad #1 huinflam_04 7v2_04.RCC | Post | fahad #1 huinflam | 4 | 04 7v2 | | | | 0.94 | 0.71 |
| 17 | 20150120_fahad #1 huinflam_05 13v2_05.RCC | Post | fahad #1 huinflam | 5 | 05 13v2 | | | | 0.8 | 2.6 |
| 18 | 20150120_fahad #1 huinflam_06 26v2_06.RCC | Post | fahad #1 huinflam | 6 | 06 26v2 | | | | 1.18 | 1.51 |
| 19 | 20150120_fahad #1 huinflam_07 NG_07.RCC | HV | fahad #1 huinflam | 7 | 07 NG | | | | 1.19 | 0.79 |
| 20 | 20150120_fahad #1 huinflam_08 PE_08.RCC | HV | fahad #1 huinflam | 8 | 08 PE | | | | 0.92 | 0.88 |
| 21 | 20150120_fahad #1 huinflam_09 006_09.RCC | HV | fahad #1 huinflam | 9 | 09 006 | | | | 0.9 | 1.72 |
| 22 | 20150120_fahad #1 huinflam_10 DR_10.RCC | HV | fahad #1 huinflam | 10 | 10 DR | | | | 0.97 | 1.08 |
| 23 | 20150120_fahad #1 huinflam_11 002_11.RCC | HV | fahad #1 huinflam | 11 | 11 002 | | | | 1.3 | 0.69 |
| 24 | 20150120_fahad #1 huinflam_12 JM_12.RCC | HV | fahad #1 huinflam | 12 | 12 JM | | | | 1 | 1.1 |

Figure 85: Inflammatory normalisation: 13 pre-treatment failed normalisation (high content normalisation)

| 24 | Normalized Data Name | Treatment Group | Cartridge ID | Lane Nu... | Sample Name | Description | QC Flag | Normalization Flag | Positive ... | Content Normali... |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 20150107_fahad #2 hukinase-v2_01 3v1_01.RCC | Pre | fahad #2 hukinase-v2 | 1 | 01 3v1 | | | | 1.18 | 0.43 |
| 2 | 20150107_fahad #2 hukinase-v2_02 10v1_02.RCC | Pre | fahad #2 hukinase-v2 | 2 | 02 10v1 | | | | 1.06 | 0.53 |
| 3 | 20150107_fahad #2 hukinase-v2_03 28v1_03.RCC | Pre | fahad #2 hukinase-v2 | 3 | 03 28v1 | | | | 0.95 | 0.7 |
| 4 | 20150107_fahad #2 hukinase-v2_04 22v1_04.RCC | Pre | fahad #2 hukinase-v2 | 4 | 04 22v1 | | | | 0.93 | 1.36 |
| 5 | 20150107_fahad #2 hukinase-v2_05 17v1_05.RCC | Pre | fahad #2 hukinase-v2 | 5 | 05 17v1 | | | | 0.88 | 0.84 |
| 6 | 20150107_fahad #2 hukinase-v2_06 16v1_06.RCC | Pre | fahad #2 hukinase-v2 | 6 | 06 16v1 | | | | 0.88 | 3.29 |
| 7 | 20150107_fahad #2 hukinase-v2_07 3v2_07.RCC | Post | fahad #2 hukinase-v2 | 7 | 07 3v2 | | | | 0.93 | 0.93 |
| 8 | 20150107_fahad #2 hukinase-v2_08 10v2_08.RCC | Post | fahad #2 hukinase-v2 | 8 | 08 10v2 | | | | 1.03 | 0.96 |
| 9 | 20150107_fahad #2 hukinase-v2_09 28v2_09.RCC | Post | fahad #2 hukinase-v2 | 9 | 09 28v2 | | | | 0.97 | 0.57 |
| 10 | 20150107_fahad #2 hukinase-v2_10 22v2_10.RCC | Post | fahad #2 hukinase-v2 | 10 | 10 22v2 | | | | 0.87 | 0.73 |
| 11 | 20150107_fahad #2 hukinase-v2_11 17v2_11.RCC | Post | fahad #2 hukinase-v2 | 11 | 11 17v2 | | | | 0.83 | 1.13 |
| 12 | 20150107_fahad #2 hukinase-v2_12 16v2_12.RCC | Post | fahad #2 hukinase-v2 | 12 | 12 16v2 | | | | 0.79 | 5.7 |
| 13 | 20150120_fahad #2 hukinase-v2_01 7v1_01.RCC | Pre | fahad #2 hukinase-v2 | 1 | 01 7v1 | | | | 0.93 | 5.4 |
| 14 | 20150120_fahad #2 hukinase-v2_02 13v1_02.RCC | Pre | fahad #2 hukinase-v2 | 2 | 02 13v1 | | | | 0.89 | 17.67 |
| 15 | 20150120_fahad #2 hukinase-v2_03 26v1_03.RCC | Pre | fahad #2 hukinase-v2 | 3 | 03 26v1 | | | | 0.98 | 2.51 |
| 16 | 20150120_fahad #2 hukinase-v2_04 7v2_04.RCC | Post | fahad #2 hukinase-v2 | 4 | 04 7v2 | | | | 1.13 | 0.68 |
| 17 | 20150120_fahad #2 hukinase-v2_05 13v2_05.RCC | Post | fahad #2 hukinase-v2 | 5 | 05 13v2 | | | | 1.09 | 2.37 |
| 18 | 20150120_fahad #2 hukinase-v2_06 26v2_06.RCC | Post | fahad #2 hukinase-v2 | 6 | 06 26v2 | | | | 1.27 | 1.07 |
| 19 | 20150120_fahad #2 hukinase-v2_07 NG_07.RCC | HV | fahad #2 hukinase-v2 | 7 | 07 NG | | | | 1.19 | 0.87 |
| 20 | 20150120_fahad #2 hukinase-v2_08 PE_08.RCC | HV | fahad #2 hukinase-v2 | 8 | 08 PE | | | | 1.19 | 0.94 |
| 21 | 20150120_fahad #2 hukinase-v2_09 006_09.RCC | HV | fahad #2 hukinase-v2 | 9 | 09 006 | | | | 1.05 | 1.43 |
| 22 | 20150120_fahad #2 hukinase-v2_10 DR_10.RCC | HV | fahad #2 hukinase-v2 | 10 | 10 DR | | | | 1.09 | 1.27 |
| 23 | 20150120_fahad #2 hukinase-v2_11 002_11.RCC | HV | fahad #2 hukinase-v2 | 11 | 11 002 | | | | 1.08 | 0.63 |
| 24 | 20150120_fahad #2 hukinase-v2_12 JM_12.RCC | HV | fahad #2 hukinase-v2 | 12 | 12 JM | | | | 1.19 | 0.96 |

Figure 86:Kinase normalisation: TG V1 13 pre-treatment failed normalisation (high content normalisation)

## 7.4 Statistical analysis

We took advice from the statistician. We were advised that data should be corrected for multiple corrections using Benjamini-Hochberg (BH), False discovery rate (FDR).

## 7.5 Results

This section discusses the results of the expression of 768 genes (519 kinase gene panel and 249 inflammatory gene panel). The results are presented in three main sections. The first section discusses the pre-treatment group (TG V1) compared to healthy control (HC). The second section discusses TG V1 compared to TG V2 (post treatment). The third section discussed TG V2 compared to HC.

### *7.5.1 Treatment group (pre-metformin) versus healthy control*

Without FDR, seventy-one genes are differentially expressed in TG V1 when compared to HC. Gene list of p values and fold changes are provided in Table 28. Figure 100 depicts a volcano plot. Figure 101 shows a heat map of significant genes. Except for nine genes, all the significant genes are overexpressed in TG V1 when compared to HC.

After analysing the data for multiple correction using FDR, none of the genes were differentially expressed.

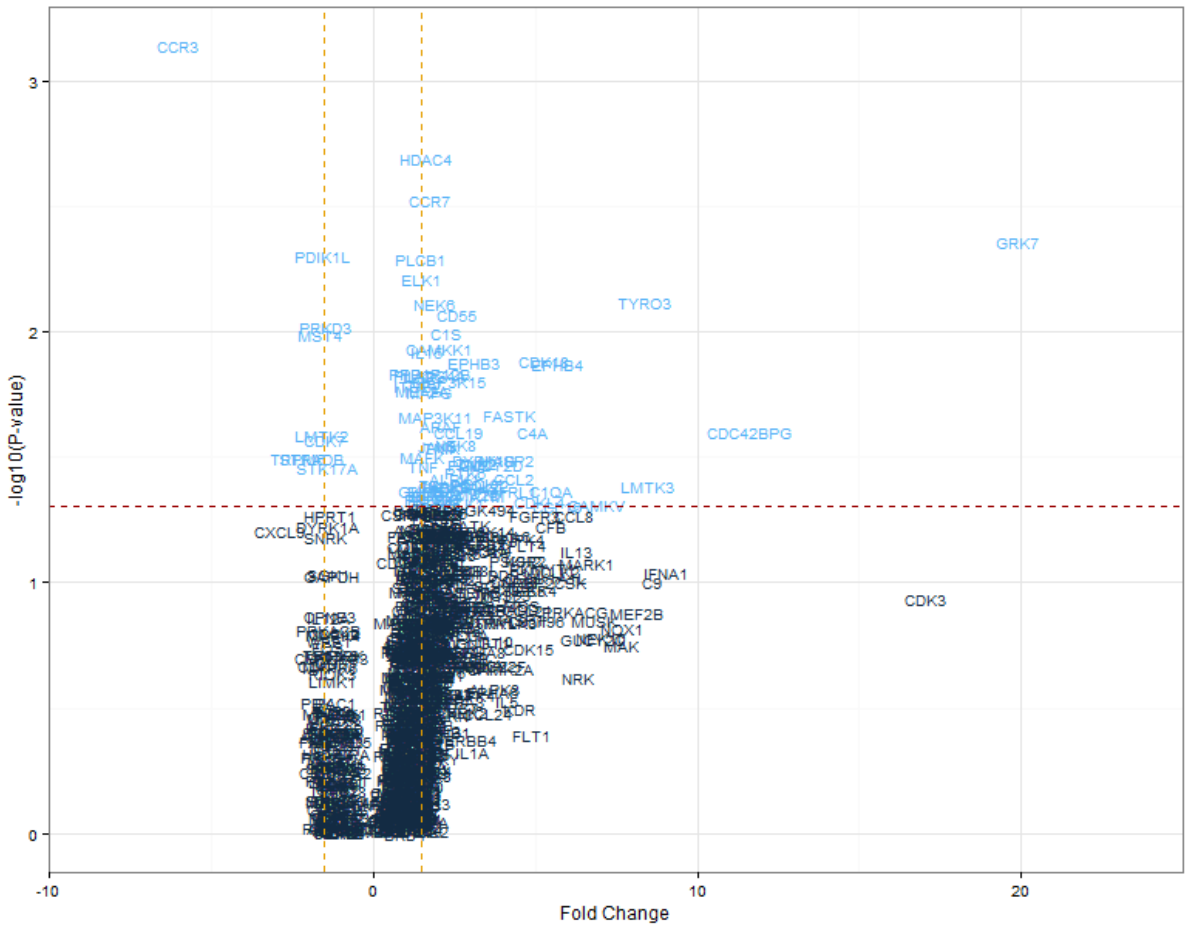
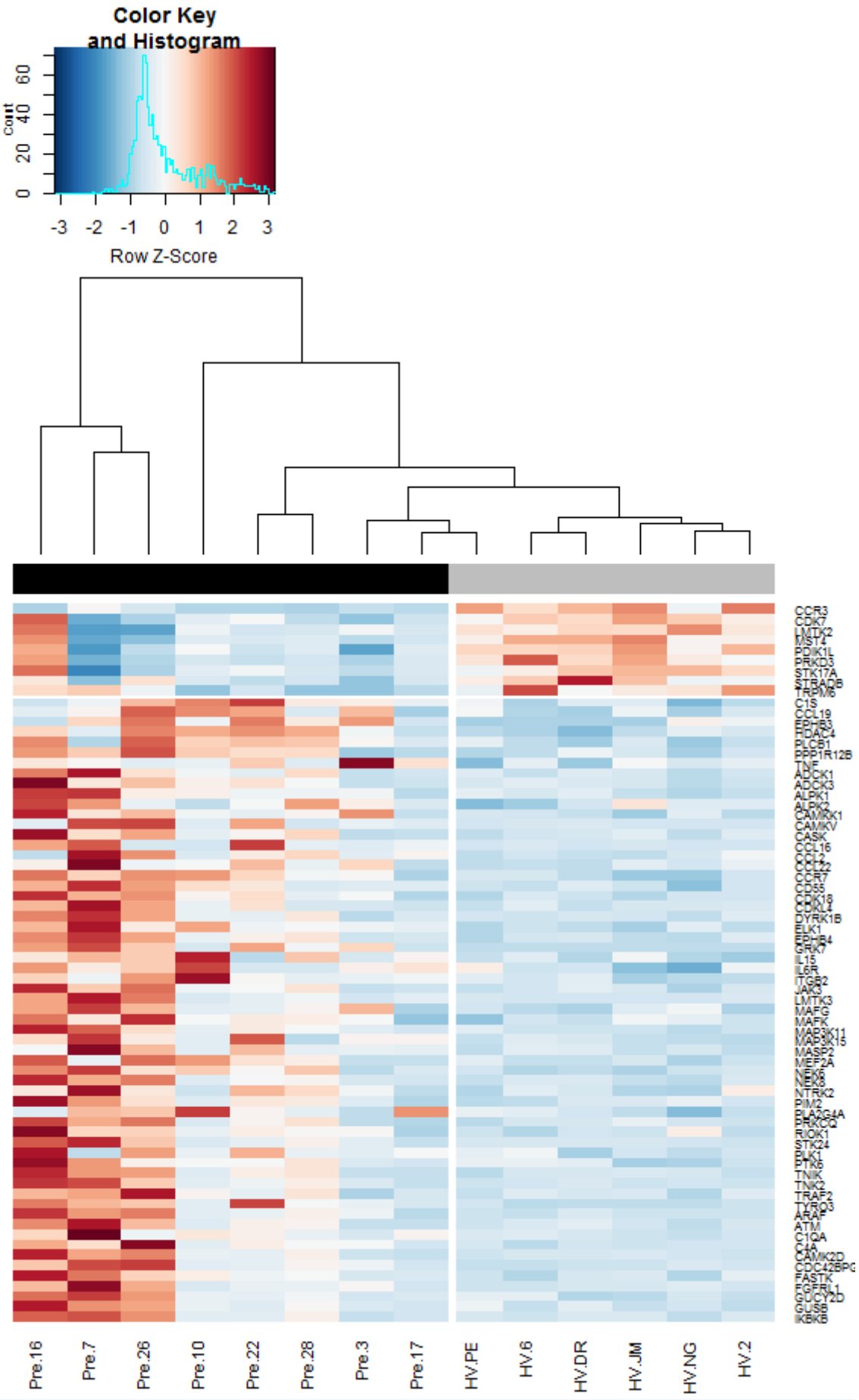



Figure 87: Volcano plot comparing pre-treatment (TG V1) with HC. Horizontal line depicts p-value of 0.05. Any gene above the line has a p value of <0.05 (non-corrected). Two vertical lines depicted fold change (-1.5 and +1.5). Significantly expressed genes are coloured in blue.


Color Key
and Histogram
Count
0
20
40
60
-3
-2
-1
0
1
2
3
Row Z-Score
CCR3
CDK7
LMTK2
MST4
PDIK1L
PRKD3
STK17A
STRADB
TRPM6
C1S
CCL19
EPHB3
HDAC4
PLCB1
PPP1R12B
TNF
ADCK1
ADCK3
ALPK1
ALPK2
CAMKK1
CAMKV
CASK
CCL16
CCL2
CCL22
CCR7
CD55
CDK19
CDKL1
DYRK1B
ELK1
EPHB4
GRK7
IL15
IL6R
ITGB2
JAK3
LMTK3
MAFG
MAFK
MAP3K11
MAP3K15
MASP2
MEF2A
NEK6
NEK8
NTRK2
PIM2
PLA2G4A
PRKCQ
RIOK1
STK24
BLK
PTK6
TNIK
TNK2
TRAF2
TYRO3
ARAF
ATM
C1QA
C1A
CAMK2D
CDC42BPG
FASTK
FGFR1
GUCY2D
GUSB
IKBKB
Pre.16
Pre.7
Pre.26
Pre.10
Pre.22
Pre.28
Pre.3
Pre.17
HV.PE
HV.6
HV.DR
HV.JM
HV.NG
HV.2

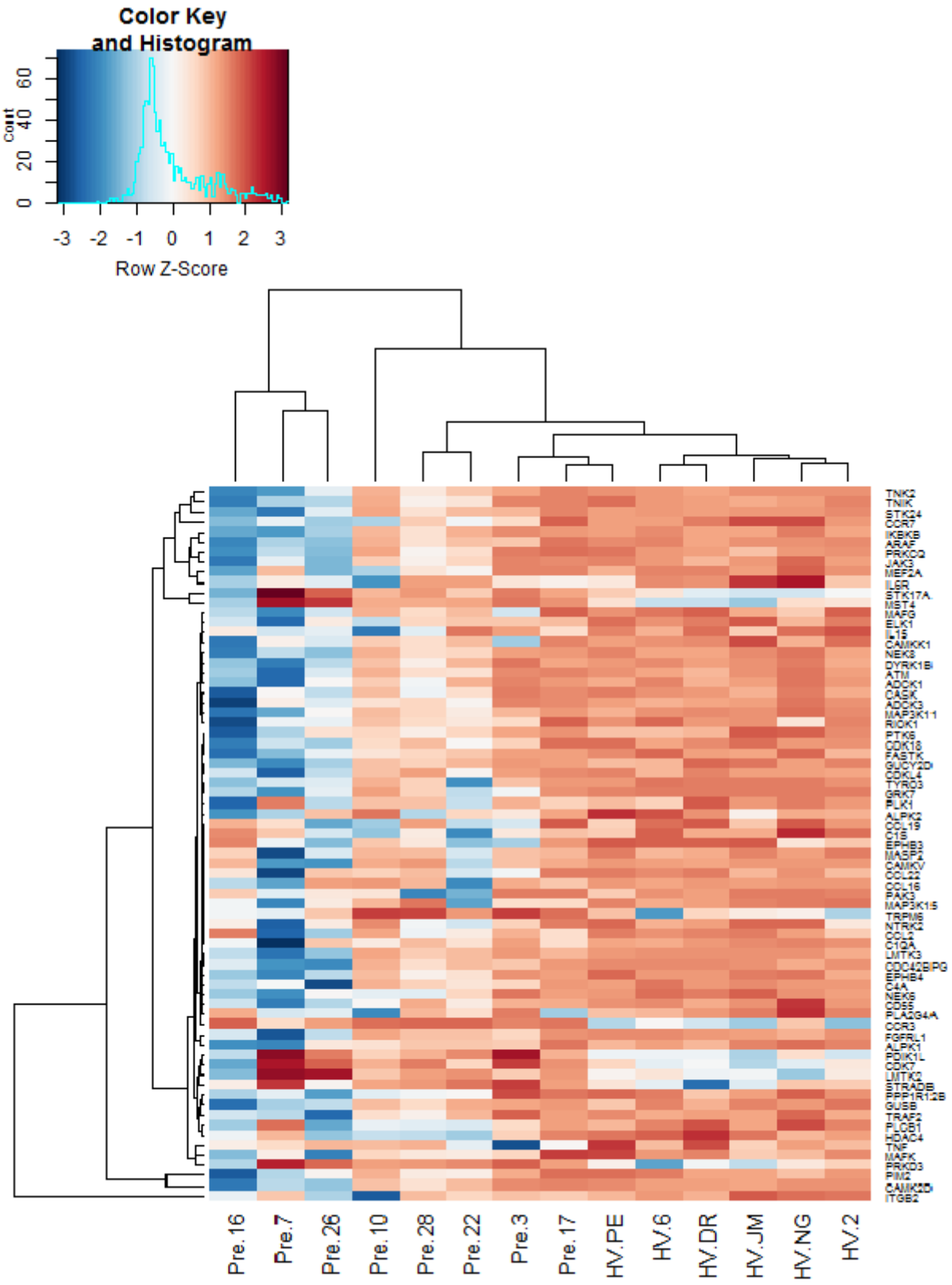



Figure 101: Heat Map showing up and downregulation of genes between TG V1 vs. HC

| Genes | FC | p value | q value | | Genes | FC | p value | q value |
|---|---|---|---|---|---|---|---|---|
| CCR3 | -5.99 | 0.0007 | 0.5059 | | IL6R | 1.43 | 0.0151 | 0.4996 |
| HDAC4 | 1.63 | 0.002 | 0.7108 | | MAP3K15 | 2.33 | 0.0158 | 0.498 |
| CCR7 | 1.79 | 0.003 | 0.6939 | | ITGB2 | 1.41 | 0.0164 | 0.4945 |
| GRK7 | 19.91 | 0.0044 | 0.7613 | | MEF2A | 1.56 | 0.0173 | 0.5001 |
| PDIK1L | -1.57 | 0.005 | 0.6932 | | GUCY2D | 3.62 | 0.0338 | 0.4857 |
| PLCB1 | 1.5 | 0.0052 | 0.5985 | | TNF | 1.53 | 0.0346 | 0.5774 |
| ELK1 | 1.49 | 0.0062 | 0.6157 | | STK17A | -1.4 | 0.0349 | 0.5652 |
| TYRO3 | 8.39 | 0.0077 | 0.6652 | | PTK6 | 2.88 | 0.0361 | 0.5889 |
| NEK6 | 1.91 | 0.0077 | 0.5957 | | ALPK1 | 2.56 | 0.0385 | 0.6003 |
| CD55 | 2.59 | 0.0086 | 0.5985 | | CCL2 | 4.34 | 0.0386 | 0.5812 |
| PRKD3 | -1.46 | 0.0096 | 0.6085 | | ADCK1 | 3.28 | 0.0404 | 0.563 |
| C1S | 2.28 | 0.0101 | 0.5868 | | CCL22 | 3.43 | 0.0405 | 0.5607 |
| MST4 | -1.6 | 0.0103 | 0.5511 | | TRAF2 | 2.23 | 0.0408 | 0.5711 |
| CAMKK1 | 2.07 | 0.0117 | 0.5823 | | LMTK3 | 8.52 | 0.0411 | 0.5768 |
| IL15 | 1.7 | 0.0121 | 0.5598 | | CASK | 2.07 | 0.0412 | 0.5678 |
| CDK18 | 5.31 | 0.0131 | 0.567 | | IKBKB | 2.32 | 0.0415 | 0.563 |
| EPHB3 | 3.12 | 0.0133 | 0.5434 | | NTRK2 | 3.06 | 0.042 | 0.5892 |
| EPHB4 | 5.7 | 0.0135 | 0.5198 | | PIM2 | 1.69 | 0.0429 | 0.5815 |
| PPP1R12B | 1.78 | 0.0147 | 0.5388 | | FGFRL1 | 4.16 | 0.043 | 0.5733 |
| PLA2G4A | 1.8 | 0.0148 | 0.5153 | | C1QA | 5.5 | 0.0433 | 0.5612 |

| Genes | FC | p value | q value |
|---|---|---|---|
| GUSB | 1.49 | 0.0434 | 0.5492 |
| CAMK2D | 2.9 | 0.0442 | 0.5506 |
| ALPK2 | 1.98 | 0.0443 | 0.5458 |
| ATM | 3.52 | 0.045 | 0.5343 |
| STK24 | 2.04 | 0.0452 | 0.534 |
| PRKCQ | 1.84 | 0.0459 | 0.5268 |
| RIOK1 | 1.85 | 0.0466 | 0.5339 |
| CDKL4 | 5.18 | 0.0475 | 0.5569 |
| MAFG | 1.72 | 0.0175 | 0.548 |
| FASTK | 4.2 | 0.0216 | 0.5611 |
| MAP3K11 | 1.9 | 0.022 | 0.5523 |
| ARAF | 2.09 | 0.0237 | 0.5448 |
| C4A | 4.93 | 0.025 | 0.5391 |
| CDC42BPG | 11.65 | 0.0251 | 0.5302 |
| CCL19 | 2.66 | 0.0251 | 0.5239 |
| LMTK2 | -1.54 | 0.0258 | 0.5214 |
| CDK7 | -1.45 | 0.0271 | 0.5229 |
| NEK8 | 2.57 | 0.0282 | 0.5155 |
| JAK3 | 2.01 | 0.0286 | 0.5106 |
| TNIK | 2.13 | 0.0292 | 0.5023 |
| MAFK | 1.56 | 0.0314 | 0.5033 |
| TRPM6 | -2.3 | 0.0318 | 0.4971 |
| STRADB | -1.88 | 0.0322 | 0.4969 |
| DYRK1B | 3.43 | 0.0323 | 0.4909 |
| MASP2 | 4.1 | 0.0324 | 0.4913 |
| TNK2 | 3.23 | 0.0333 | 0.4907 |
| PAK3 | 2.96 | 0.0338 | 0.4931 |
| PLK1 | 2.85 | 0.0482 | 0.4927 |
| CAMKV | 6.93 | 0.0493 | 0.4966 |
| CCL16 | 5.68 | 0.0494 | 0.4905 |
| ADCK3 | 1.93 | 0.0496 | 0.4857 |
| | | | |

Table 28: Significant gene expression TG V1 compared with HC. FC: Fold change; q value represents false discovery rate corrected values.

#### *7.5.1.1 Ingenuity Pathway Analysis*

The complement system, the role of macrophages, fibroblast and endothelial cell, NF-kB signalling complex and CCR3 signalling were upregulated significantly in TG V1 when compared with HC. The Z score was more than 2 and these were above the threshold. Figure 102 demonstrates top 20 canonical pathway.

ATP-γ-S, Lipopolysaccharide, interferon-α, ING-γ, BQ123 pathways were significantly upregulated ($P<0.05$). Lipopolysaccharide and Interferon-γ were predicted to be activated in TG V1 when compared with HC (Figure 103).

The analysis predicted that there would be a significant increase in levels of LDH, CRP, Creatinine and alkaline phosphatase in TG V1 compared with HC ($p<0.05$) (Figure104).

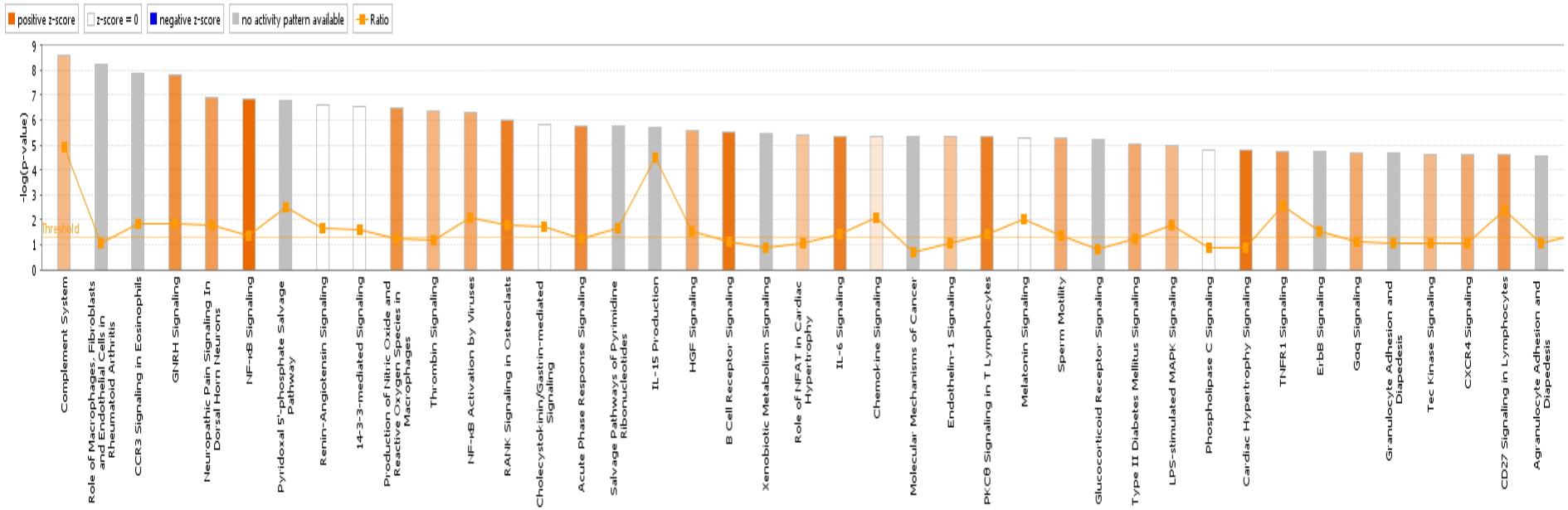



Figure 88: Top 20 Canonical pathway affected in TG V1 compared with HC.

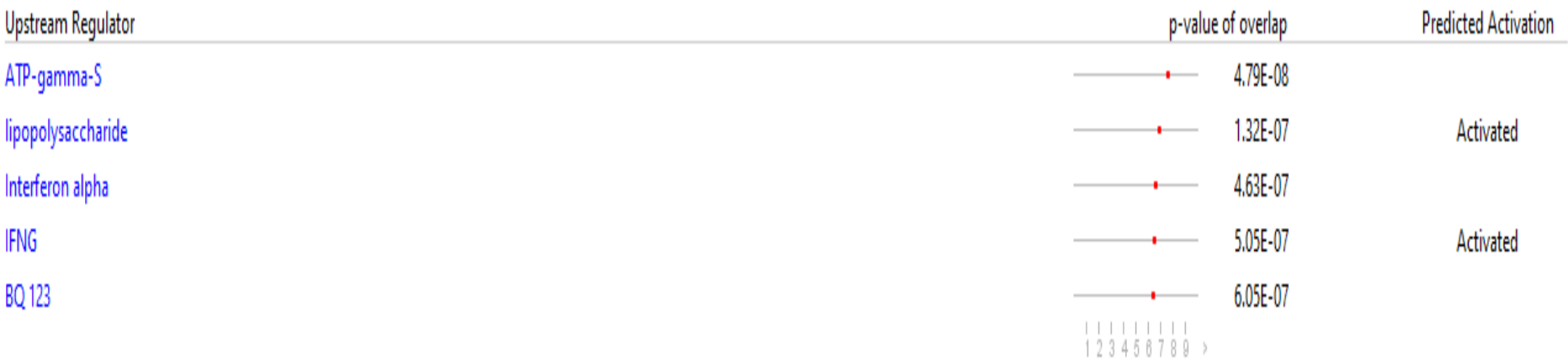



Figure 89: Top 5 upstream regulator affected in TG V1 when compared with HC.

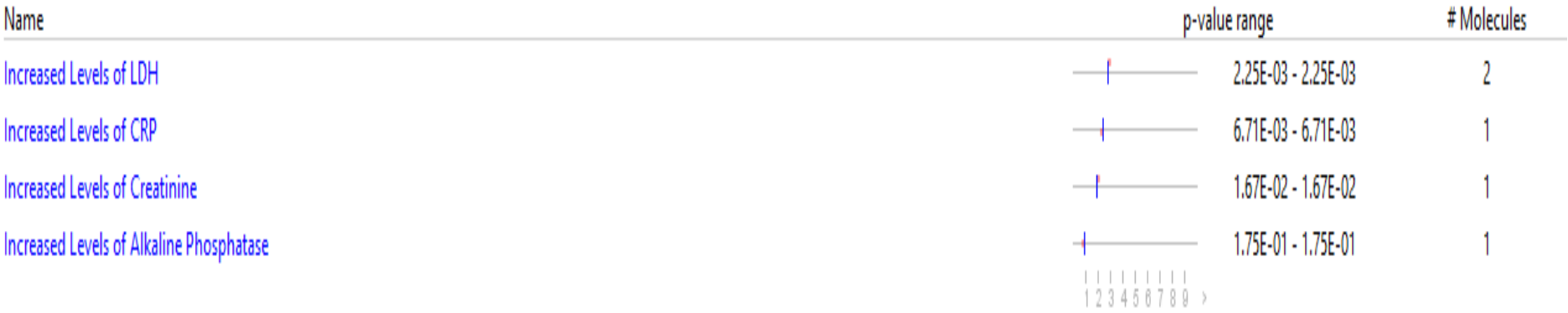



Figure 90:Top 5 biochemistry affected in TG V1 vs. HC.

The major regulatory networks affected in TG V1 were CD40LG and NF-κB complex when compared with HC. The affected networks increased activation of phagocytes. The analysis predicted that CD40LG, NF- κB, TNF and STAT-1 were activated (Figure 105). Analysis of these networks predicted activation of PBMC chemotaxis, homing and phagocytosis (Figure 106).

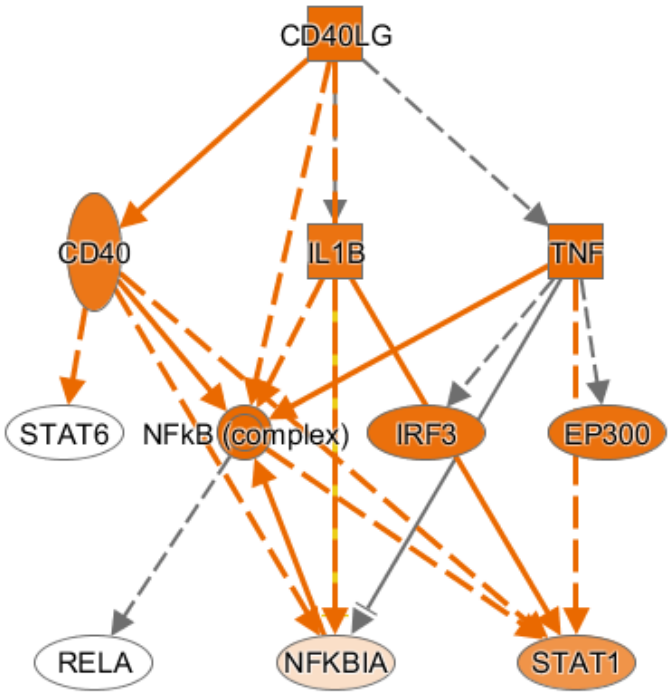



Figure 91: CD40LG, NF-kB, TNF and STAT1 was predicted to be upregulated in TG V1 vs. HC.

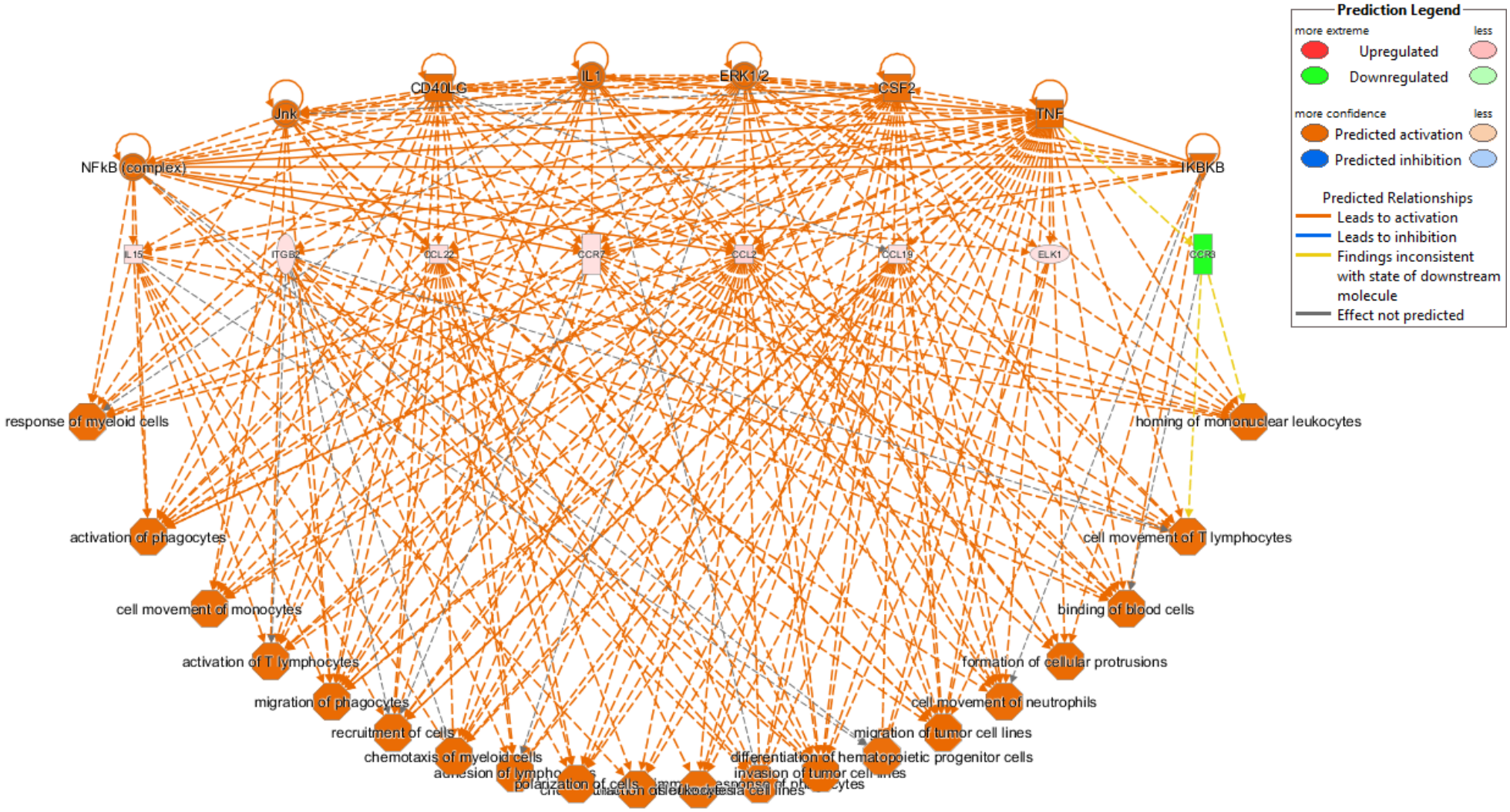



Figure 92: Predicted activation of PBMC functions in TG V1 vs. HC.

Analysis demonstrated that TNF, INF-γ, CD40L and CSF2 cytokines were significantly upregulated in TG V1 when compared to HC. The Inhibitor of Kappa Light Polypeptide Gene Enhancer In B-Cells (IKBKB) was also activated significantly in TG V1 compared with HC (Z-score ≥ 2) (Figure 107).

In TG V1, the inflammatory response, post-translational modification, cell death and survival, cellular movement and cell to cell signalling and interaction, immune cell trafficking, cell-mediated immune response, humoral immune response were significantly upregulated when compared with HC. It was predicted that TG V1 had increased adhesion of immune cell, inflammatory response, leukocytes chemotaxis and immune response of leukocytes. (Figure 108 to 111).

| Upstream Regulator | Exp Fold Change | Molecule Type | Predicted Activation State | Activation z-score | p-value of overlap | Target molecules in d... | Mechanistic Network |
|---|---|---|---|---|---|---|---|
| NFkB (complex) | | complex | Activated | 2.761 | 8.53E-06 | ↑CAMK2D, ↑CCL2, ↑... ...all 9 | 17 (10) |
| TNF | ↑1.530 | cytokine | Activated | 2.761 | 1.47E-05 | ↑CCL19, ↑CCL2, ↑... ...all 14 | 24 (15) |
| lipopolysaccharide | | chemical drug | Activated | 2.739 | 1.32E-07 | ↑CCL19, ↑CCL2, ↑... ...all 15 | 19 (13) |
| CD40LG | | cytokine | Activated | 2.383 | 1.31E-04 | ↑CCL19, ↑CCL2, ↑C... ...all 7 | 18 (11) |
| ERK1/2 | | group | Activated | 2.369 | 3.91E-05 | ↑CCL2, ↑CCL22, ↑C... ...all 6 | 17 (13) |
| IL1 | | group | Activated | 2.348 | 4.80E-04 | ↑CCL2, ↑CCR7, ↑CD55...all 6 | 24 (14) |
| IFNG | | cytokine | Activated | 2.218 | 5.05E-07 | ↑C1QA, ↑C4A/C4B, ↑... ...all 13 | 21 (12) |
| Jnk | | group | Activated | 2.191 | 8.11E-06 | ↑CCL2, ↑CCL22, ↑C... ...all 6 | 17 (13) |
| IKBKB | ↑2.320 | kinase | Activated | 2.183 | 3.75E-06 | ↑CCL2, ↑CCR7, ↑IK... ...all 5 | 15 (12) |
| CSF2 | | cytokine | Activated | 2.118 | 2.92E-04 | ↑ATM, ↑CCL2, ↑CCR7 ...all 6 | 16 (13) |

Figure 93: Predictor upstream regulator with Z-score ≥ 2.

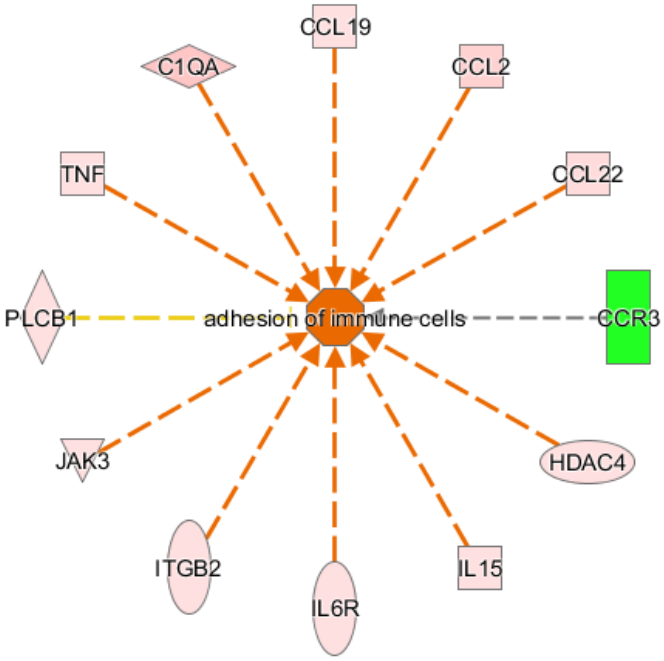



Figure 94: Adhesion of immune cells upregulation in TG V1 compared to HC.

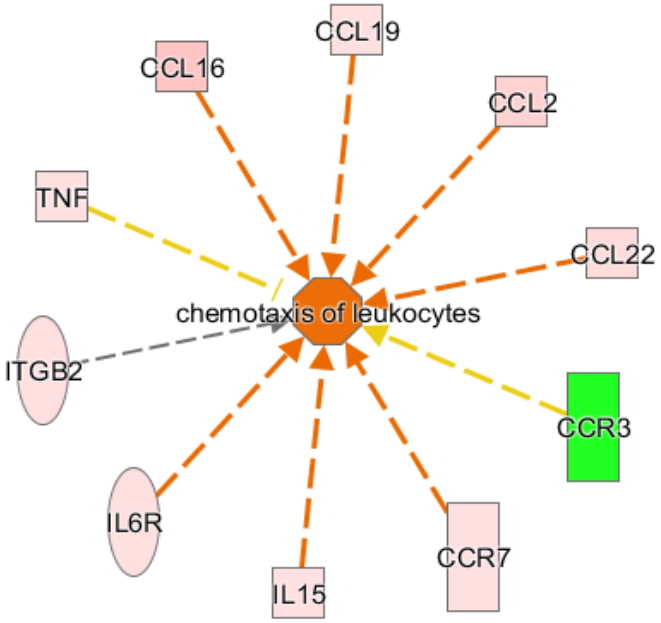



Figure 95: Chemotaxis of leukocytes upregulation in TG V1 compared to HC.

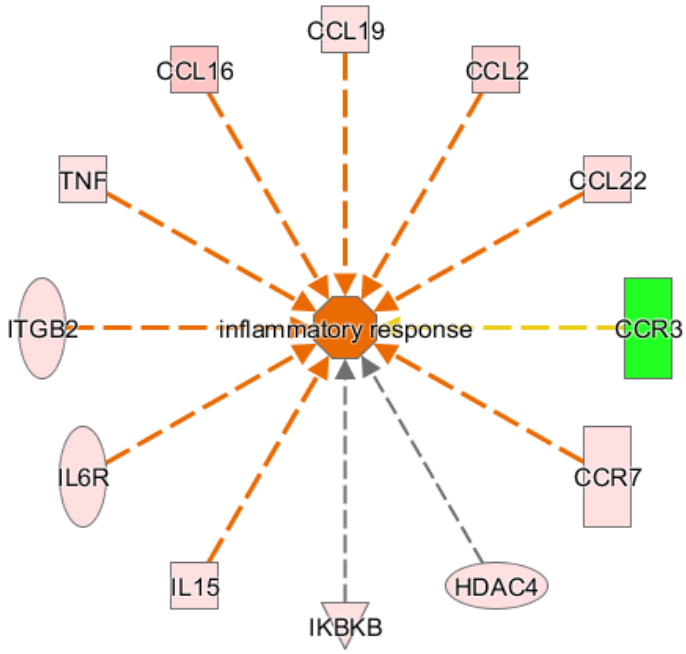



Figure 96: Inflammatory response upregulation in TG V1 compared to HC.

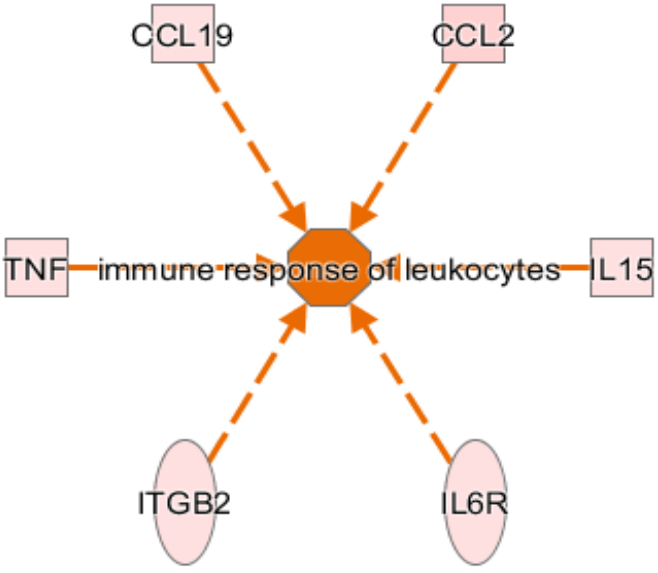



Figure 97: Immune response of leukocytes upregulation in TG V1 compared to HC.

Below figure 112 to 116 demonstrate heat maps of cellular function. Orange demonstrates upregulation. Blue demonstrates downregulation and grey demonstrates no pattern of predicted regulation.

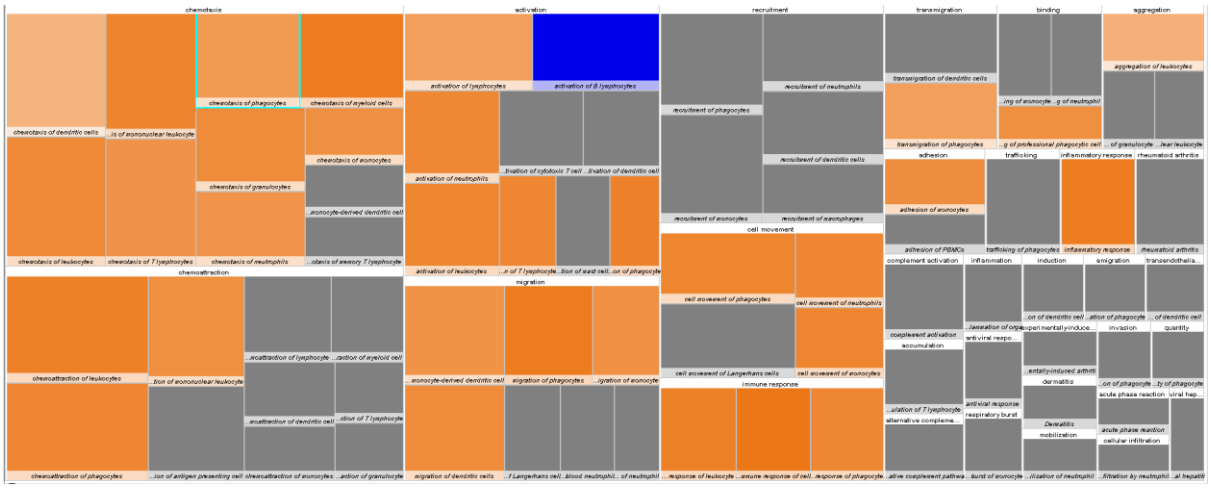



Figure 98: Inflammatory response heat map.

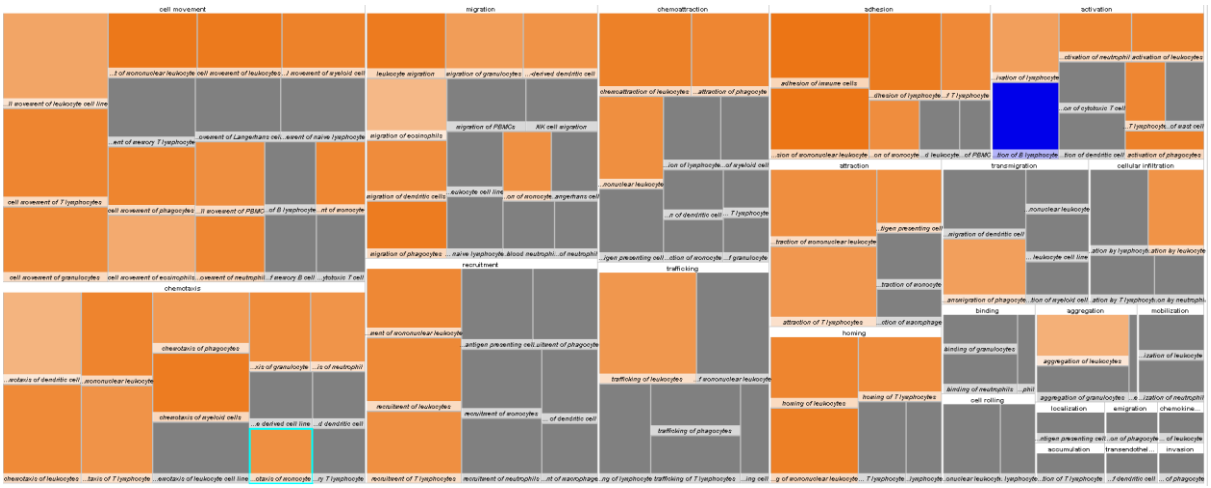



Figure 99: Immune cell trafficking heat map.

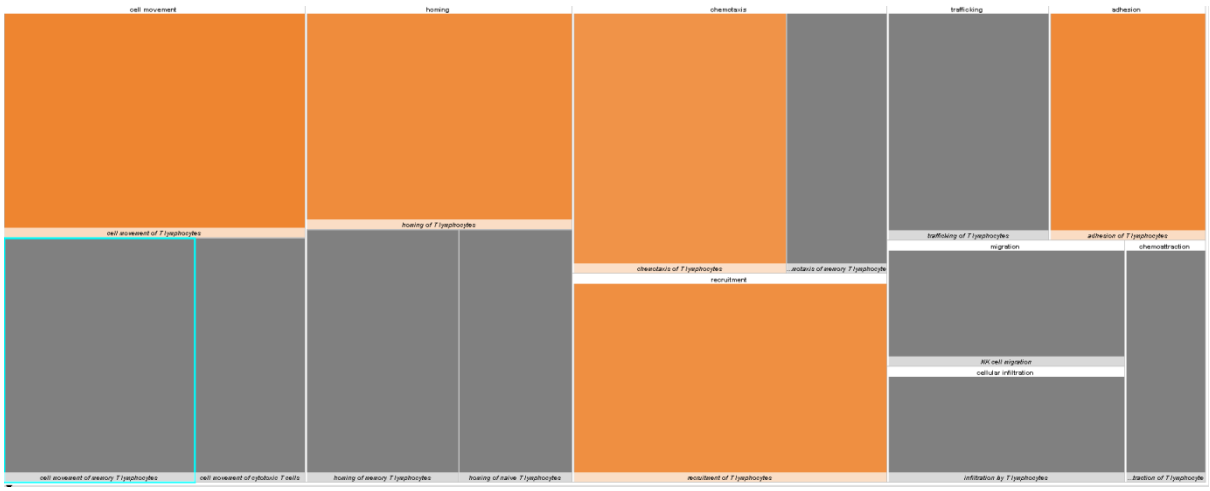



Figure 100: Cell mediated immune response.

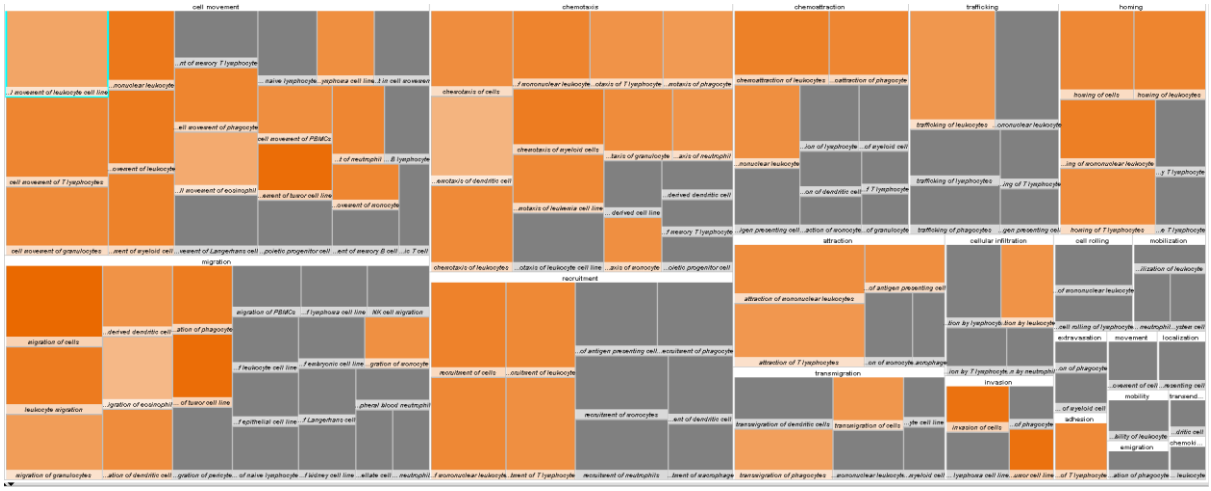



Figure 101: Cell movement heat map.

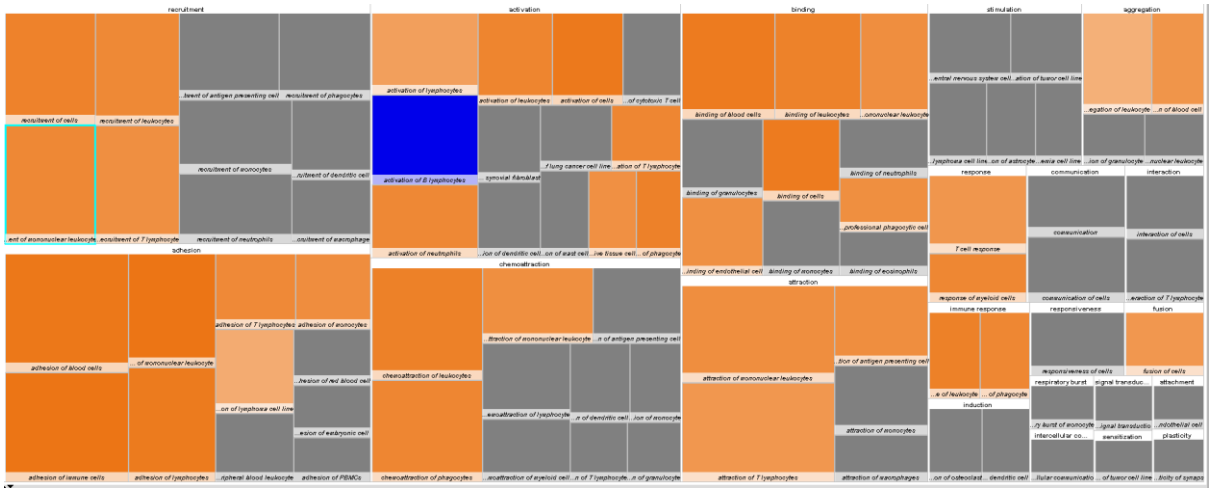



Figure 102: Cell to cell signalling heat map.

We performed analysis for links with disease markers and cellular function. Based on the z-score (≥2), TG V1 had increased chemotaxis, homing, movement, adhesion and activation of PBMC compared to HC (Figure 117).

| Categories | Diseases or Functions Annotation | p-Value | Predicted Activat | Activation z- | Notes | Molecules | # Molecules |
|---|---|---|---|---|---|---|---|
| Cell Death and S | cell viability of leukocytes | 3.82E-05 | Increased | 2.163 | bias | ↑CCR7, ↑IL15, ↑ITGB2, ↑JAK3, ↑PIM2, ↑TNF all 6 | 6 |
| Infectious Disea | Viral Infection | 6.75E-06 | Increased | 2.160 | bias | ↑ARAF, ↑ATM, ↑C1S, ↑C4A/C4B, ↑CCL2, ↑CCL22, ↑CCR7, ↑CD55, ↑IKBKB, ↑IL15, ↑IL6R all 21 | 21 |
| Cellular Movem | chemotaxis of leukemia cell lines | 4.99E-06 | Increased | 2.154 | bias | ↑CCL16, ↑CCL19, ↑CCL2, ↑CCL22, ↑TNF all 5 | 5 |
| Cellular Movem | homing of cells | 6.29E-09 | Increased | 2.147 | bias | ↑C4A/C4B, ↑CCL16, ↑CCL19, ↑CCL2, ↑CCL22, ↓CCR3, ↑CCR7, ↑IKBKB, ↑IL15, ↑IL6R, ↑I... all 14 | 14 |
| Cell-To-Cell Sig | activation of neutrophils | 1.24E-05 | Increased | 2.146 | bias | ↑CCL2, ↑CCL22, ↑IL15, ↑ITGB2, ↑TNF all 5 | 5 |
| Cell-To-Cell Sig | recruitment of mononuclear leuk | 2.09E-08 | Increased | 2.146 | bias | ↑CCL19, ↑CCL2, ↑CCL22, ↑IL15, ↑IL6R, ↑TNF all 6 | 6 |
| Gene Expression | transcription of RNA | 1.80E-04 | Increased | 2.136 | | ↑ATM, ↑CAMK2D, ↑CCL2, ↓CDK7, ↑DYRK1B, ↑ELK1, ↑HDAC4, ↑IKBKB, ↑IL6R, ↑MAFG, all 17 | 17 |
| Cell-To-Cell Sig | recruitment of leukocytes | 6.10E-08 | Increased | 2.128 | bias | ↑C4A/C4B, ↑CCL19, ↑CCL2, ↑CCL22, ↑IL15, ↑IL6R, ↑ITGB2, ↑TNF all 8 | 8 |
| Cell-To-Cell Sig | adhesion of T lymphocytes | 1.17E-05 | Increased | 2.117 | bias | ↑CCL19, ↑CCL22, ↑ITGB2, ↑JAK3, ↑TNF all 5 | 5 |
| Cellular Movem | chemotaxis of leukocytes | 9.61E-08 | Increased | 2.098 | bias | ↑CCL16, ↑CCL19, ↑CCL2, ↑CCL22, ↓CCR3, ↑CCR7, ↑IL15, ↑IL6R, ↑ITGB2, ↑TNF all 10 | 10 |
| Cellular Develop | differentiation of cells | 3.15E-05 | Increased | 2.085 | bias | ↑ATM, ↑CCL2, ↑ELK1, ↑EPHB3, ↑EPHB4, ↑HDAC4, ↑IKBKB, ↑IL15, ↑IL6R, ↑JAK3, ↑MA... all 17 | 17 |
| Cellular Movem | cell movement of granulocytes | 2.36E-07 | Increased | 2.083 | bias | ↑CCL16, ↑CCL19, ↑CCL2, ↓CCR3, ↑CCR7, ↑IKBKB, ↑IL15, ↑ITGB2, ↑TNF all 9 | 9 |
| Cellular Develop | differentiation of lymphatic syste | 1.86E-06 | Increased | 2.057 | bias | ↑ATM, ↑HDAC4, ↑IL15, ↑IL6R, ↑JAK3, ↑MAFG, ↑MAFK, ↑TNF, ↑TRAF2 all 9 | 9 |
| Cellular Movem | chemotaxis of granulocytes | 1.32E-05 | Increased | 2.041 | bias | ↑CCL19, ↑CCL2, ↑CCR7, ↑IL15, ↑ITGB2, ↑TNF all 6 | 6 |
| Cellular Develop | differentiation of hematopoietic c | 4.26E-06 | Increased | 2.028 | | ↑ELK1, ↑IL15, ↑IL6R, ↑JAK3, ↑MAFG, ↑MAFK, ↑TNF all 7 | 7 |
| Cellular Develop | differentiation of hematopoietic p | 3.38E-05 | Increased | 2.028 | | ↑ELK1, ↑IL15, ↑IL6R, ↑MAFG, ↑MAFK, ↑TNF all 6 | 6 |
| Cell-mediated Ir | homing of T lymphocytes | 4.76E-08 | Increased | 2.023 | bias | ↑CCL19, ↑CCL2, ↑CCL22, ↓CCR3, ↑CCR7, ↑IL15, ↑JAK3 all 7 | 7 |
| Cellular Movem | migration of dendritic cells | 8.28E-06 | Increased | 2.022 | bias | ↑CCL19, ↑CCL2, ↑CCL22, ↑CCR7, ↑TNF all 5 | 5 |
| Cellular Develop | development of epithelial cell line | 8.81E-06 | Increased | 2.000 | bias | ↑CDC42BPG, ↑CDK18, ↑IKBKB, ↑LMTK3 all 4 | 4 |
| Cell Morphology | formation of cilia | 1.17E-03 | Increased | 2.000 | bias | ↑CDC42BPG, ↑CDK18, ↑IKBKB, ↑LMTK3 all 4 | 4 |

Figure 103: Disease and function annotation. Only Z score of greater than 2 is included.

### *7.5.2 Treatment group (pre metformin) versus treatment group (post metformin)*

Eleven genes were differentially expressed in TG V2 when compared to TG V1 (Table 29). The list of genes with p values and fold changes are provided in Table 7. Figure 118 depicts volcano plot. Figure 119 shows a heat map of significant genes. Except for three genes, all the significant genes were overexpressed in TG V2 when compared to TG V1. After analysing the data for multiple correction using FDR, none of the genes were differentially expressed.

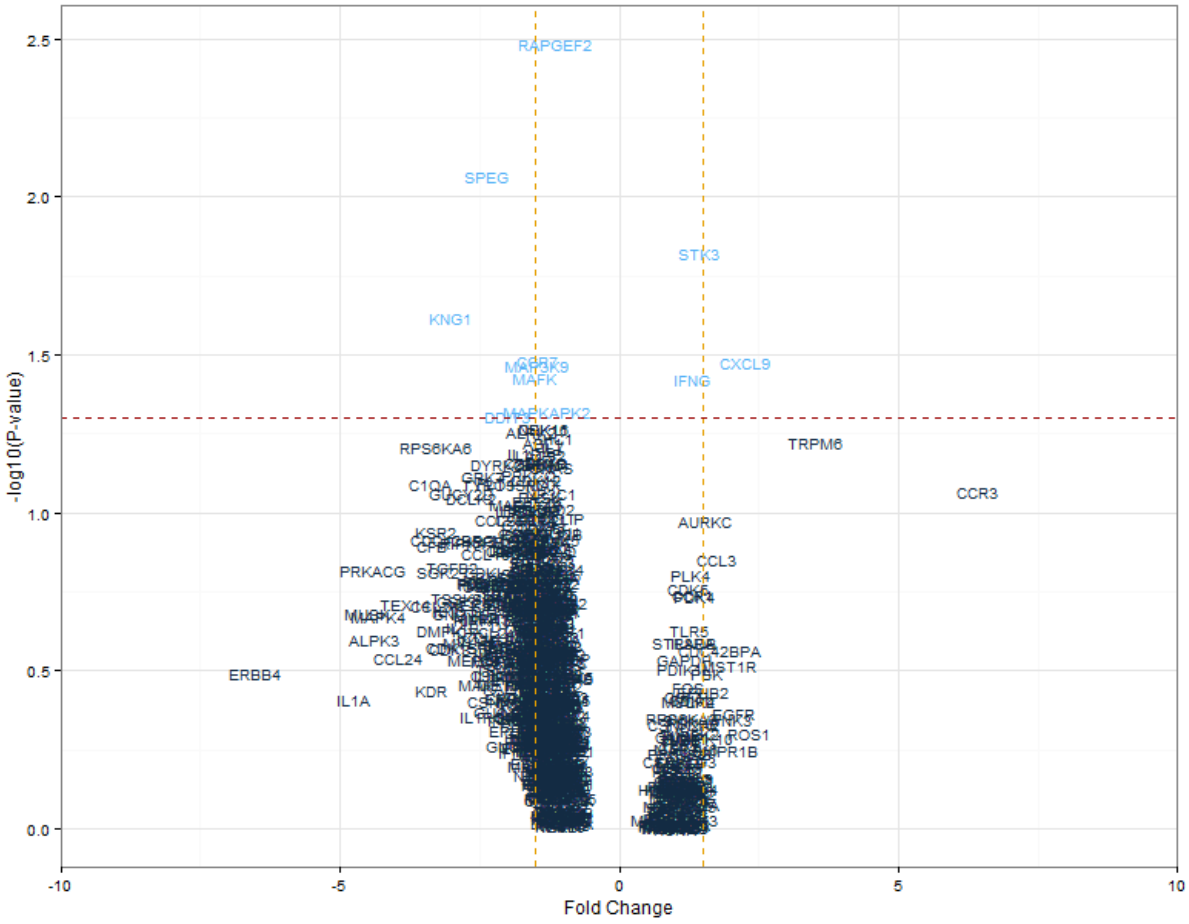



Figure 104: Volcano plot is comparing post-treatment (TG V2) with pre-treatment (TG V1). Horizontal line depicts p-value of 0.05. Any gene above the line has a p value of <0.05. Two vertical lines depicted fold change (-1.5 and +1.5). Significantly expressed genes are coloured in blue.

| Gene Name | Post vs. Pre FC | Post vs. Pre p-val | q value |
|---|---|---|---|
| RAPGEF2 | -1.15 | 0.003285 | 2.3 |
| SPEG | -2.35 | 0.008645 | 3 |
| STK3 | 1.45 | 0.015063 | 3.5 |
| KNG1 | -3.02 | 0.024251 | 4.2 |
| CCR7 | -1.46 | 0.032895 | 4.6 |
| CXCL9 | 2.26 | 0.033285 | 3.9 |
| MAP3K9 | -1.47 | 0.034378 | 3.4 |
| MAFK | -1.5 | 0.0373 | 3.2 |
| IFNG | 1.31 | 0.037935 | 2.9 |
| MAPKAPK2 | -1.27 | 0.047785 | 3.3 |
| DDIT3 | -1.99 | 0.049269 | 3.1 |

Table 29: Significant gene expression in TG V2 when compared with TG V1; q value represents false discovery rate corrected values.

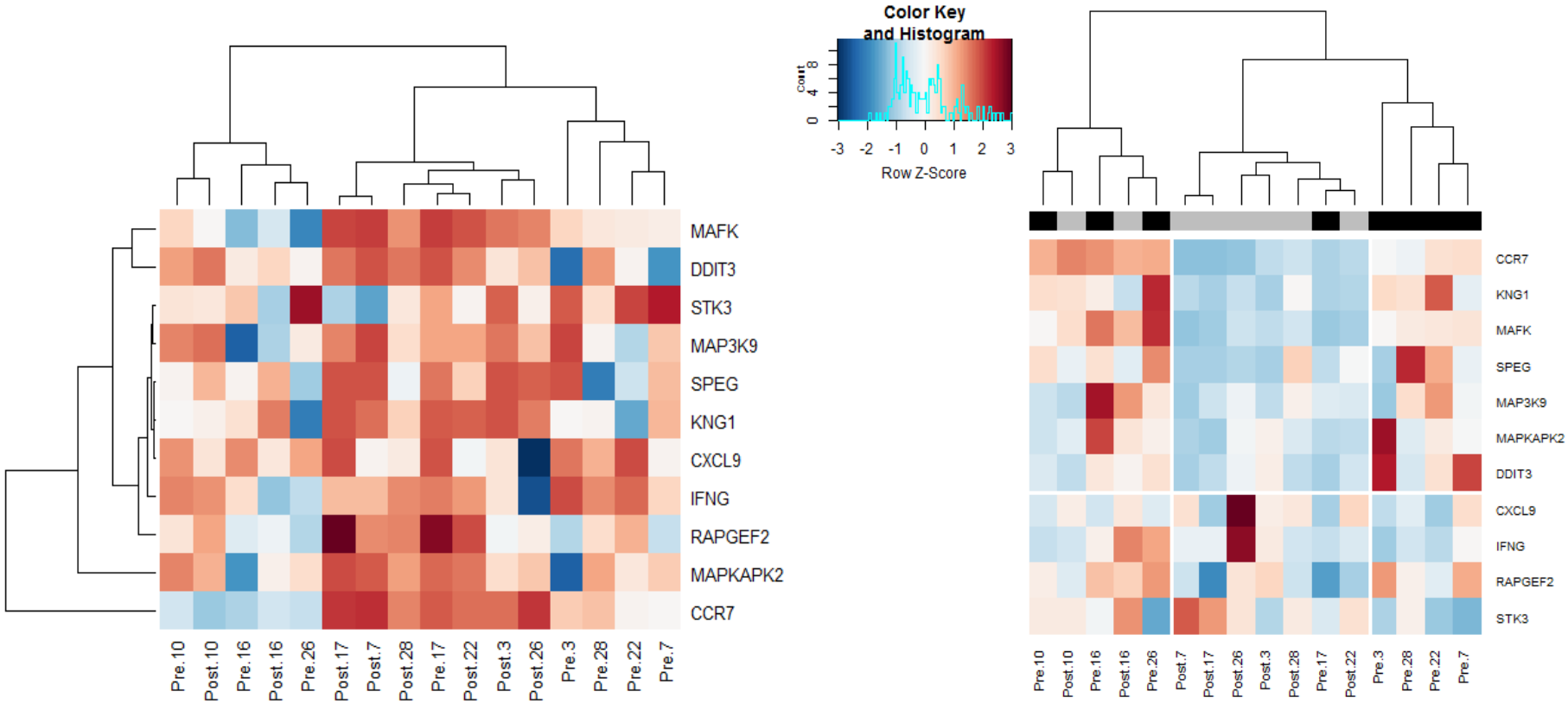



Figure 105: Heatmap is showing significant gene in TG V2 when compared with TG V1.

#### *7.5.2.1 Ingenuity Pathway Analysis*

The critical canonical pathways significantly affected by metformin in TG were communication between innate and adaptive immune cells, p38 Mitogen-activated protein kinase (MAPK) signalling, crosstalk between dendritic cells and NK cells (figure 120). However, the z-score did not point toward upregulation and downregulation (Figure 121).

Metformin treatment significantly affected INF-γ, p38 MAPK, RELA, SOD2 and TLR9. However, none of the upstream regulators could predict if there was activation or deactivation based on the Z-score. The maximum activation score was 0.468 (Figure 122) (Activation: Z score should be ≥ 2; Deactivation: Z score should be ≤ -2).

Metformin treatment significantly affected the inflammatory response, cellular movement, cell morphology, cell death and survival, cell to cell signalling, cell-mediated immune response and immune cell trafficking. However, the Z score was not greater or less than 2. The genes affected by metformin treatment could not predict activation of any specific network or pathway.

It was predicted that metformin treatment in TG reduced chemotaxis whereas phosphorylation of protein was increased (Figure 123 and 124 respectively).

| Name | p-value | Overlap |
|---|---|---|
| Hepatic Fibrosis / Hepatic Stellate Cell Activation | 1.29E-04 | 1.7 % 3/181 |
| Neuroprotective Role of THOP1 in Alzheimer's Disease | 2.30E-04 | 5.0 % 2/40 |
| Communication between Innate and Adaptive Immune Cells | 9.68E-04 | 2.4 % 2/82 |
| Crosstalk between Dendritic Cells and Natural Killer Cells | 1.14E-03 | 2.2 % 2/89 |
| p38 MAPK Signaling | 1.96E-03 | 1.7 % 2/117 |

1 2 3 4 5 6 7 8 9 ›

Figure 106: Top 5 canonical pathway affected in TG V2 compared with TG V1.

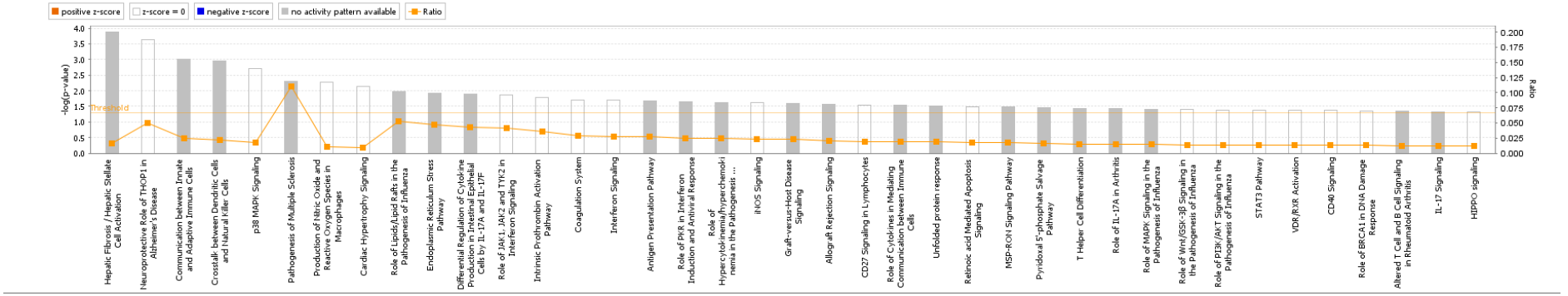



Figure 107: Canonical pathway depiction showing no pattern when TG V2 was compared to TG V1 (based on Z-score).

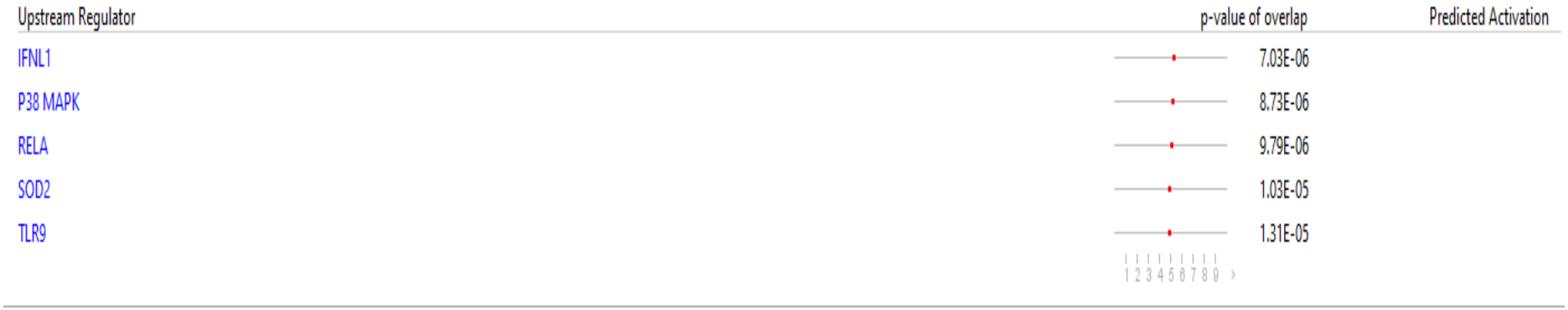



Figure 108: Upstream regulator significantly affected by metformin treatment.

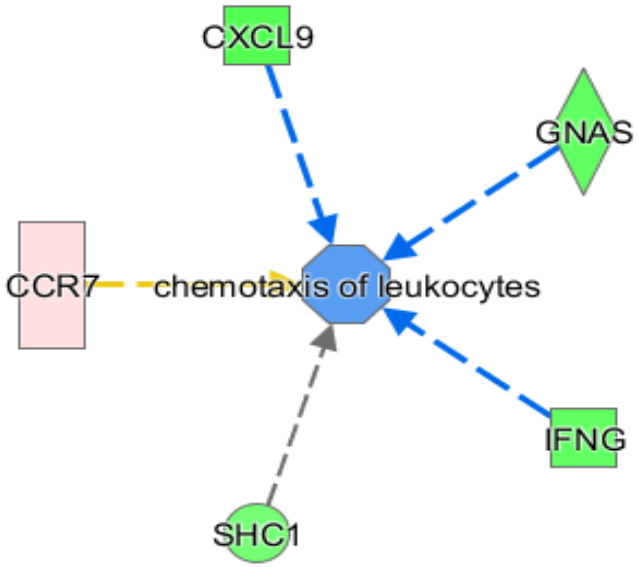



Figure 109: Predicted decreased chemotaxis of PBMC in TG V2 vs. TG V1.

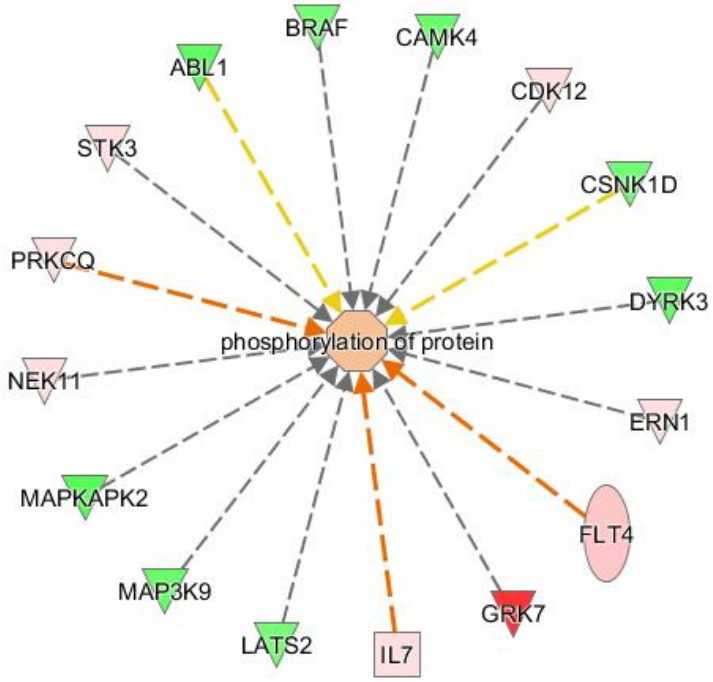



Figure 110: Predicted increased phosphorylation of protein in TG V2 vs. TG V1.

(Orange is upregulation and blue is down-regulation)

### *7.5.3 Treatment group (post metformin) versus healthy controls*

Thirty-one genes were differentially expressed in TG V2 when compared to healthy controls. The list of genes with p values and fold changes are provided in Table 30. Figure 125 depicts a volcano plot. Figure 126 shows a heat map of significant genes. Except for ten genes, all the significant genes were under expressed in TG V2 when compared to HC.

After analysing the data for multiple correction using FDR, none of the genes were differentially expressed.

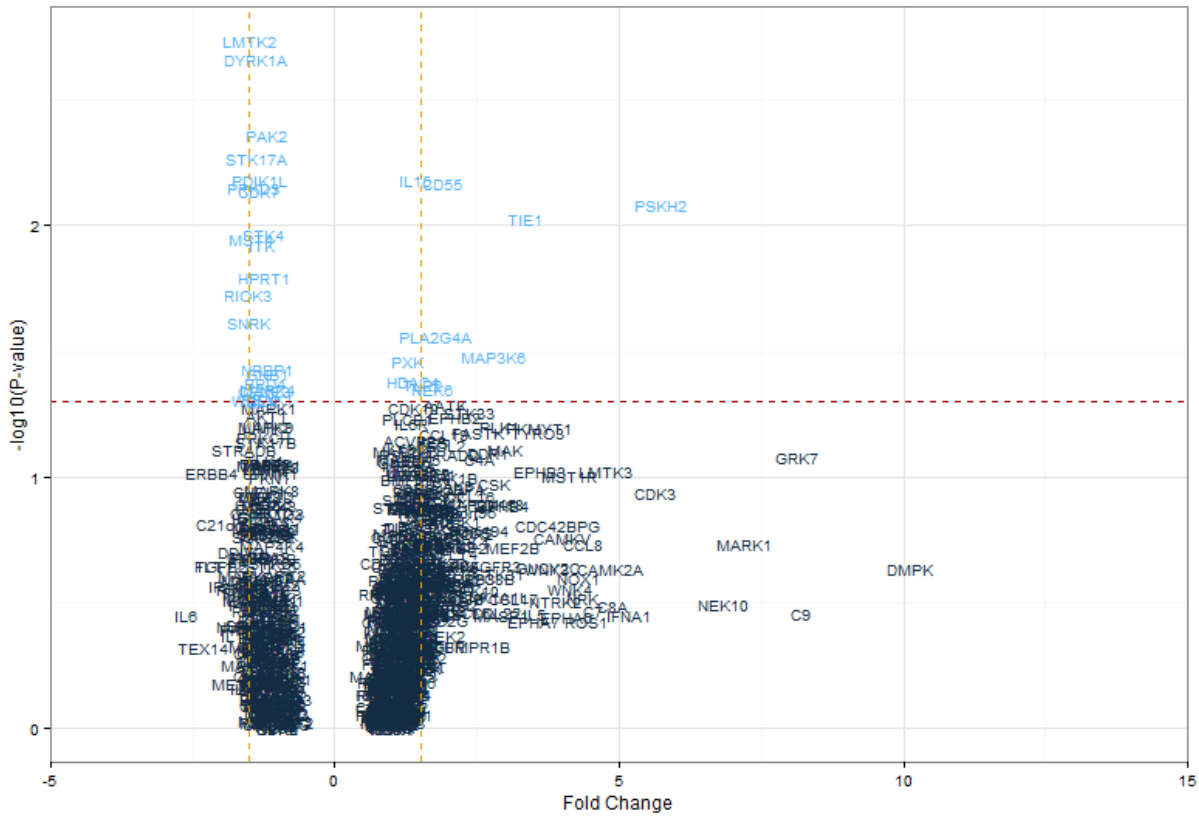



Figure 111: Volcano plot is comparing post-treatment (TG V2) with healthy control (HC). Horizontal line depicts p-value of 0.05. Any gene above the line has a p value of <0.05. Two vertical lines depicted fold change (-1.5 and +1.5). Significantly expressed genes are coloured in blue.

| Gene Name | Post vs. Ctl FC | Post vs. Ctl p-val | q value |
|---|---|---|---|
| LMTK2 | -1.49 | 0.001836 | 1.27581 |
| DYRK1A | -1.36 | 0.002171 | 0.75435 |
| PAK2 | -1.18 | 0.00439 | 1.01697 |
| STK17A | -1.36 | 0.005414 | 0.94076 |
| PDIK1L | -1.32 | 0.006547 | 0.9101 |
| IL15 | 1.44 | 0.006633 | 0.76838 |
| CD55 | 1.9 | 0.006789 | 0.67403 |
| PRKD3 | -1.44 | 0.007045 | 0.612 |
| CDK7 | -1.33 | 0.007262 | 0.56081 |
| PSKH2 | 5.74 | 0.008233 | 0.57218 |
| TIE1 | 3.36 | 0.009365 | 0.59172 |
| STK4 | -1.24 | 0.010795 | 0.6252 |
| MST4 | -1.46 | 0.01132 | 0.6052 |
| ITK | -1.26 | 0.01204 | 0.59768 |
| HPRT1 | -1.24 | 0.016025 | 0.7425 |
| RIOK3 | -1.52 | 0.018937 | 0.82256 |
| SNRK | -1.52 | 0.024433 | 0.99887 |
| PLA2G4A | 1.79 | 0.027556 | 1.06397 |
| MAP3K6 | 2.79 | 0.033321 | 1.21886 |
| PXK | 1.3 | 0.034712 | 1.20624 |
| NRBP1 | -1.17 | 0.037039 | 1.22581 |
| GNB1 | -1.18 | 0.038726 | 1.22339 |
| HDAC4 | 1.39 | 0.04169 | 1.25975 |
| BRD4 | -1.2 | 0.042494 | 1.23056 |
| TLR2 | 1.54 | 0.043047 | 1.19669 |
| MARK4 | -1.19 | 0.044631 | 1.19303 |
| NEK6 | 1.74 | 0.044851 | 1.1545 |
| CPNE3 | -1.24 | 0.045624 | 1.13246 |
| PEAK1 | -1.17 | 0.048384 | 1.15955 |
| WEE1 | -1.41 | 0.04917 | 1.13911 |
| RYK | -1.23 | 0.049959 | 1.12004 |

Table 30: Significant genes in TG V2 compared with HC; q value represents false discovery rate corrected values.

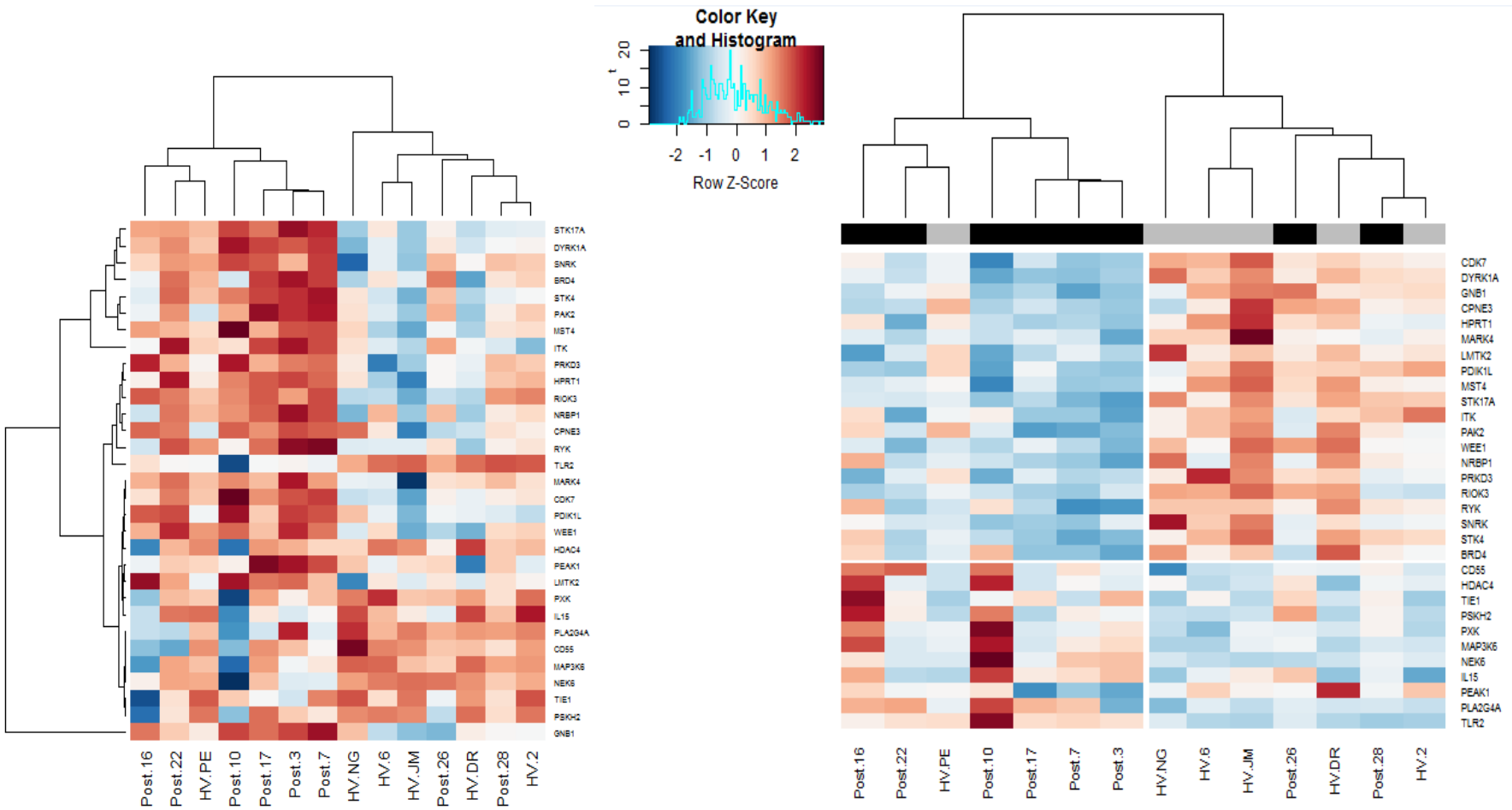



Figure 112: Heatmap is showing significant gene in TG V2 when compared with HC.

#### *7.5.3.1 Ingenuity Pathway Analysis*

eNOS and B cell receptor signalling were affected significantly in TG V2 when compared to HC ($p<0.05$) (Figure 127). There was a trend towards reduced B cell receptor signalling TG V2 when compared with HC. Even though eNOS activity was significantly affected, no activity pattern could be elucidated (Figure 128).

IL-10 was significantly up-regulator in TGV2 compared to HC. TNF, INF-γ and IL1B were significantly downregulated in TG V2 when compared with HC. However, none of the Z-score reached ≥ 2 (Figure 129).

The inflammatory response, post-translational modifications, cell death and survival, cellular assembly and organisation, cellular function and maintenance and cell to cell signalling and interaction were significantly different in TG V2 when compared with HC. There was a trend towards negative Z scores. However, none of the Z-scores for the relevant cellular functions reached ≥ 2or ≤ -2 (Figure 130).

The heat map demonstrated a decreased inflammatory response, immune cell trafficking, cell-mediated immune response, cell to cell signalling in TG V2 when compared with HC. Post-translational modification was upregulated in TG V2 when compared with HC. (Figure 131 to 135). Orange demonstrates upregulation, blue demonstrates downregulation, grey demonstrates no pattern of predicted regulation.

| Name | p-value | Overlap |
|---|---|---|
| Pyridoxal 5'-phosphate Salvage Pathway | 3.11E-06 | 6.3 % 4/63 |
| eNOS Signaling | 3.69E-06 | 3.4 % 5/148 |
| Hepatic Fibrosis / Hepatic Stellate Cell Activation | 9.86E-06 | 2.8 % 5/181 |
| B Cell Receptor Signaling | 1.01E-05 | 2.7 % 5/182 |
| Salvage Pathways of Pyrimidine Ribonucleotides | 1.41E-05 | 4.3 % 4/92 |

1 2 3 4 5 6 7 8 9 ›

Figure 113: Top 5 canonical pathway affected in TG V2 compared with HC.

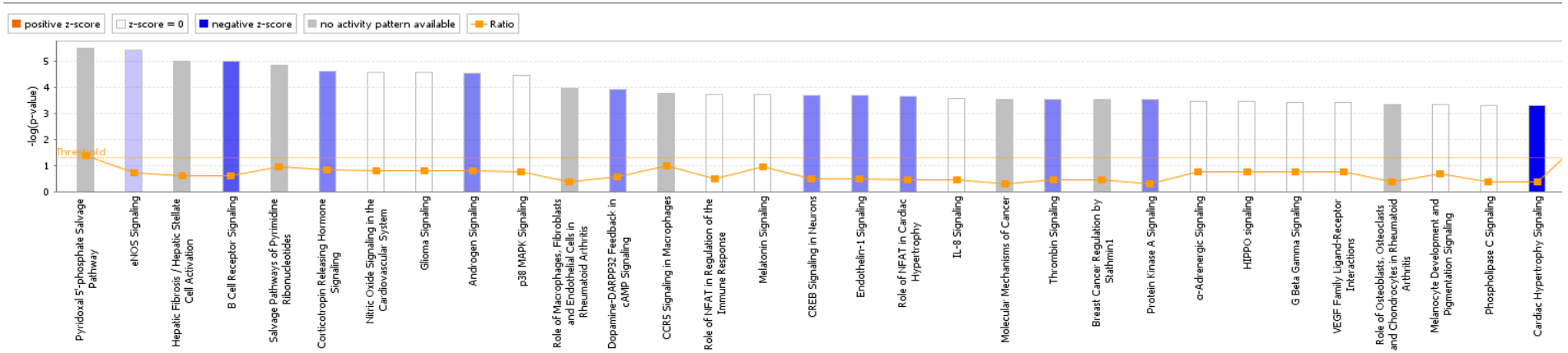



Figure 114: Canonical pathway depiction showing no pattern when TG V2 was compared to HC (based on Z-score).

| Upstream Regulator | Exp Fold Change | Molecule Type | Predicted Activation State | Activation z-score | p-value of overlap | Target molecules in d... | Mechanistic Network |
|---|---|---|---|---|---|---|---|
| IL10 | | cytokine | | 1.368 | 7.76E-04 | ↑CCR7, ↓CXCL9, ↓I... ...all 4 | 6 (5) |
| IRF4 | | transcription regulator | | 1.117 | 2.42E-05 | ↓CXCL9, ↓DDIT3, ↓I... ...all 4 | |
| sirolimus | | chemical drug | | 1.014 | 1.76E-03 | ↑CCR7, ↓DDIT3, ↓IF... ...all 4 | 5 (4) |
| nocodazole | | chemical reagent | | 0.765 | 5.95E-06 | ↓BRAF, ↓CSNK1D, ↓... ...all 4 | |
| IL1 | | group | | -0.072 | 9.44E-05 | ↓ABL1, ↑CCR7, ↓CX... ...all 5 | 7 (3) |
| lipopolysaccharide | | chemical drug | | -0.115 | 3.22E-04 | ↑CCR7, ↓CXCL9, ↓... ...all 7 | 8 (6) |
| TGFB1 | | growth factor | | -0.217 | 6.67E-03 | ↓ABL1, ↑CCR7, ↓G... ...all 6 | |
| Interferon alpha | | group | | -0.298 | 1.01E-03 | ↑CCR7, ↓CXCL9, ↓I... ...all 4 | 5 (4) |
| IL1B | | cytokine | | -0.533 | 8.72E-04 | ↑CCR7, ↓CXCL9, ↓I... ...all 5 | 7 (6) |
| TNF | | cytokine | | -0.943 | 6.86E-03 | ↑CCR7, ↓CXCL9, ↓... ...all 6 | 6 (7) |
| TP53 | | transcription regulator | | -1.172 | 1.13E-03 | ↓BRAF, ↓CSNK1D, ↓... ...all 6 | 6 (2) |
| IFNG | ↓-1.160 | cytokine | | -1.297 | 2.37E-02 | ↓CAMK4, ↓CXCL9, ↓.. ...all 4 | |
| cisplatin | | chemical drug | | -1.982 | 3.61E-04 | ↓ABL1, ↓CXCL9, ↓D... ...all 5 | |

Figure 115: Predictor upstream regulator

| Categories | Diseases or Functions Annotation | p-Value | Predicted Activatio | Activation z-sc | Molecules | # Molecules |
|---|---|---|---|---|---|---|
| Cell Death and Su | cell death of neuroblastoma cell line | 2.81E-07 | Decreased | -2.190 | ↓ABL1, ↓CAMK4, ↓DDIT3, ↓GNAS, ↓IFNG, ↓SHC1 all 6 | 6 |
| Cellular Assembly | organization of cytoskeleton | 3.63E-09 | | -1.967 | ↓ABL1, ↓BRAF, ↓CAMK4, ↑CCR7, ↓CSNK1D, ↓CXCL9, ↑ERN1, ↑FLT4, ↓GNAS, ↓IFNG, ↓KNG1 all 13 | 13 |
| Cell Morphology | orientation of cells | 5.50E-05 | | -1.934 | ↓CXCL9, ↓IFNG, ↓KNG1, ↓MAPKAPK2 all 4 | 4 |
| Cell Death and Su | necrosis | 2.05E-09 | | -1.772 | ↓ABL1, ↓BRAF, ↓CAMK4, ↑CCR7, ↓CXCL9, ↓DDIT3, ↑ERN1, ↑FLT4, ↓GNAS, ↓IFNG, ↑IL7, ↓... all 19 | 19 |
| Cellular Assembly | microtubule dynamics | 3.52E-08 | | -1.760 | ↓ABL1, ↓BRAF, ↓CAMK4, ↑CCR7, ↓CSNK1D, ↑ERN1, ↑FLT4, ↓GNAS, ↓IFNG, ↓KNG1, ↓RAP... all 11 | 11 |
| Cellular Assembly | growth of neurites | 6.03E-05 | | -1.731 | ↓ABL1, ↓CAMK4, ↓CSNK1D, ↓IFNG, ↓SHC1 all 5 | 5 |
| Cell Death and Su | apoptosis of leukocytes | 1.17E-04 | | -1.688 | ↑CCR7, ↓IFNG, ↑IL7, ↑PRKCQ, ↓SHC1 all 5 | 5 |
| Cell Death and Su | apoptosis of lymphatic system cells | 2.12E-05 | | -1.655 | ↑FLT4, ↓IFNG, ↑IL7, ↑PRKCQ, ↓SHC1 all 5 | 5 |
| Cell Signaling, Mo | quantity of Ca2+ | 1.79E-04 | | -1.509 | ↓CXCL9, ↓GNAS, ↓IFNG, ↓KNG1, ↓SHC1 all 5 | 5 |
| Cell Death and Su | cell death of tumor cell lines | 9.91E-07 | | -1.380 | ↓ABL1, ↓BRAF, ↓CAMK4, ↓DDIT3, ↑ERN1, ↓GNAS, ↓IFNG, ↑IL7, ↓LATS2, ↓MAPKAPK2, ↑N... all 14 | 14 |
| Cell Death and Su | cell death of immune cells | 6.08E-07 | | -1.345 | ↓CAMK4, ↑CCR7, ↓DDIT3, ↑ERN1, ↓IFNG, ↑IL7, ↑PRKCQ, ↓SHC1 all 8 | 8 |
| Cell-To-Cell Signa | response of mononuclear leukocytes | 5.09E-06 | | -1.236 | ↓ABL1, ↓CXCL9, ↓IFNG, ↑IL1RAP, ↑IL7 all 5 | 5 |
| Cell Death and Su | apoptosis of T lymphocytes | 9.59E-05 | | -1.228 | ↓IFNG, ↑IL7, ↑PRKCQ, ↓SHC1 all 4 | 4 |
| Cell Signaling, Mo | mobilization of Ca2+ | 1.46E-03 | | -1.213 | ↑CCR7, ↓CXCL9, ↓IFNG, ↓KNG1 all 4 | 4 |
| Cell Death and Su | apoptosis of cervical cancer cell lines | 2.08E-05 | | -1.210 | ↓ABL1, ↓BRAF, ↓DDIT3, ↓IFNG, ↓LATS2, ↑STK3 all 6 | 6 |
| Free Radical Scave | synthesis of reactive oxygen species | 8.96E-05 | | -1.206 | ↓ABL1, ↓GNAS, ↓IFNG, ↓KNG1, ↑PRKCQ, ↓SHC1 all 6 | 6 |
| Cellular Movemer | invasion of cells | 7.95E-07 | | -1.200 | ↓ABL1, ↓BRAF, ↑CCR7, ↑FLT4, ↓GNAS, ↓IFNG, ↓KNG1, ↓MAPKAPK2, ↑PRKCQ, ↑RPS6KA6, ↑... all 11 | 11 |
| Free Radical Scave | quantity of reactive oxygen species | 5.67E-05 | | -1.199 | ↓ABL1, ↓IFNG, ↓MAPKAPK2, ↓SHC1 all 4 | 4 |
| Cell Morphology, | formation of cellular protrusions | 2.02E-06 | | -1.180 | ↓ABL1, ↓BRAF, ↓CAMK4, ↑CCR7, ↓CSNK1D, ↓IFNG, ↓KNG1, ↓RAPGEF2 all 8 | 8 |
| Cell Death and Su | apoptosis | 7.98E-07 | | -1.178 | ↓ABL1, ↓BRAF, ↓CAMK4, ↑CCR7, ↓DDIT3, ↑ERN1, ↑FLT4, ↓GNAS, ↓IFNG, ↑IL7, ↓KNG1, ↓... all 16 | 16 |

Figure 116: Disease and function annotation.

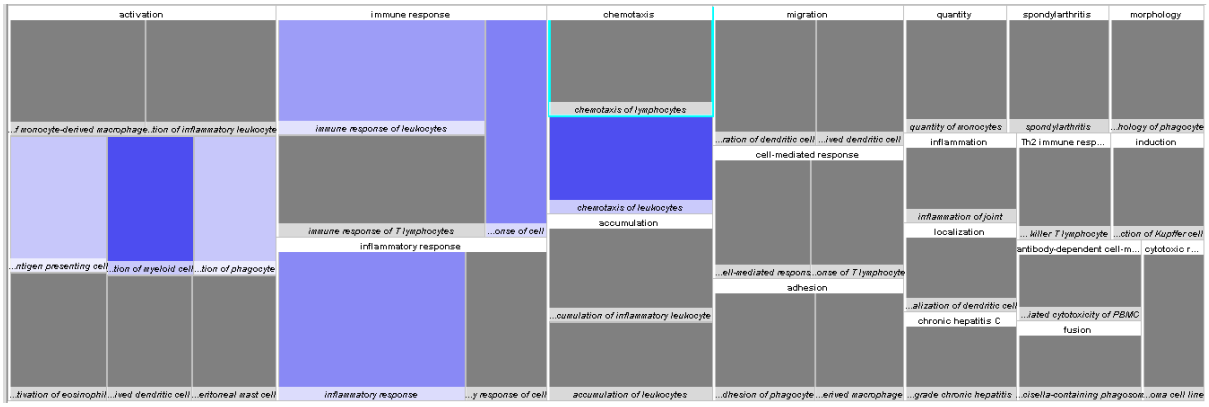



Figure 117: Inflammatory response heatmap.

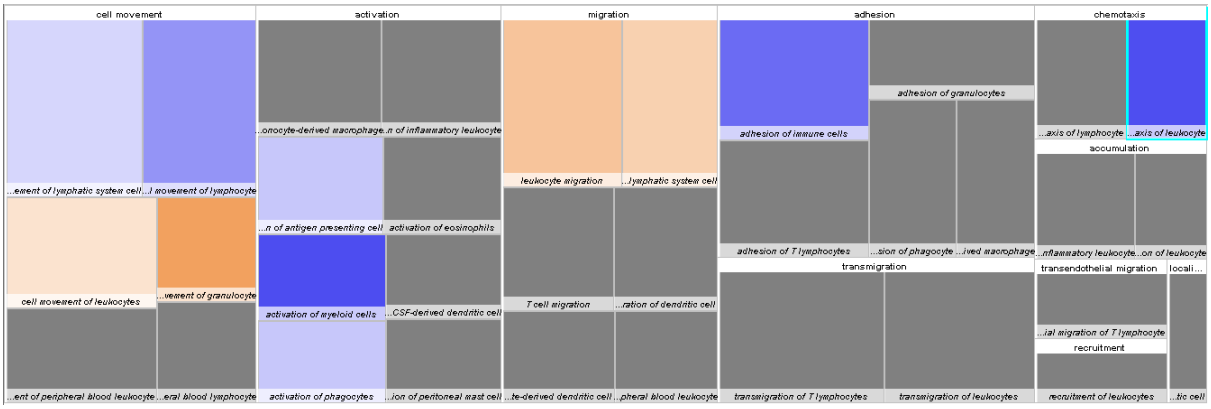



Figure 118: Immune cell trafficking heatmap.

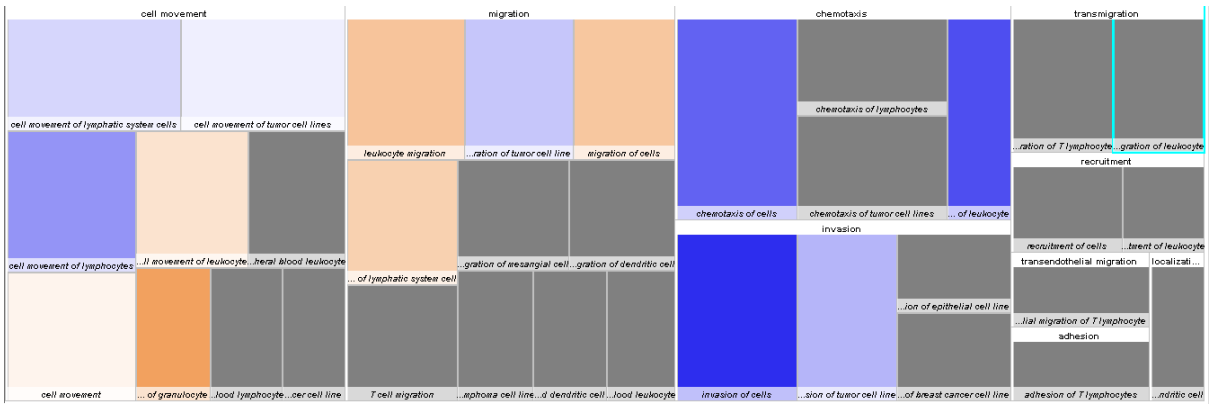



Figure 119: Cell-mediated immune response especially cellular movement heatmap.

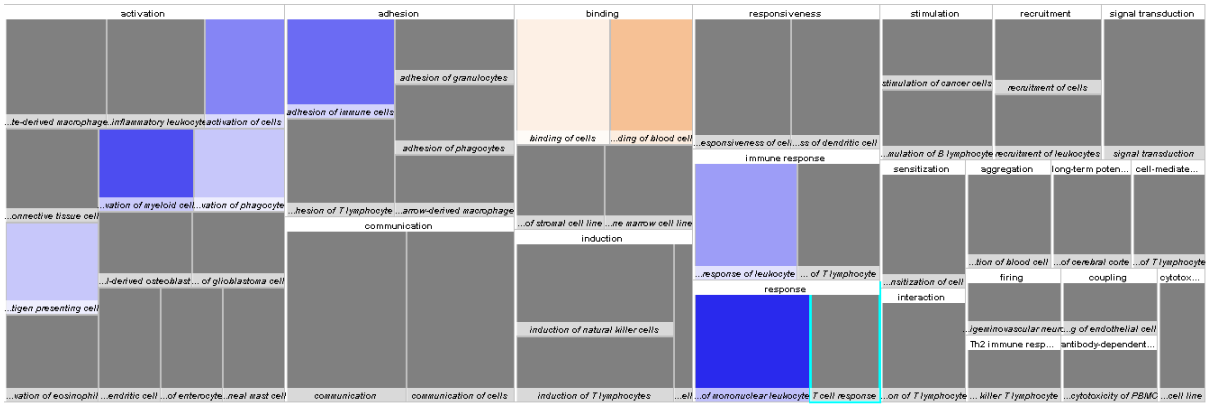



Figure 120: Cell to cell signalling heatmap.

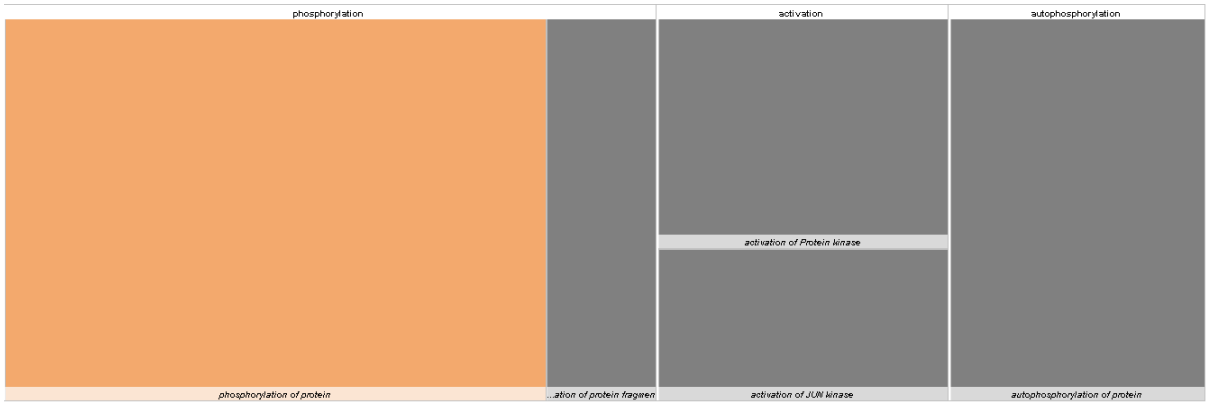



Figure 121: Post-translational modification heatmap.

It was predicted that EC death, immune cells adhesion, leukocytes chemotaxis, the inflammatory response and synthesis and quantity of ROS would be decreased and angiogenesis increased in TG V2 compared to HC. (Figure 136 to 143- Blue depicts downregulation and orange depicts upregulation).

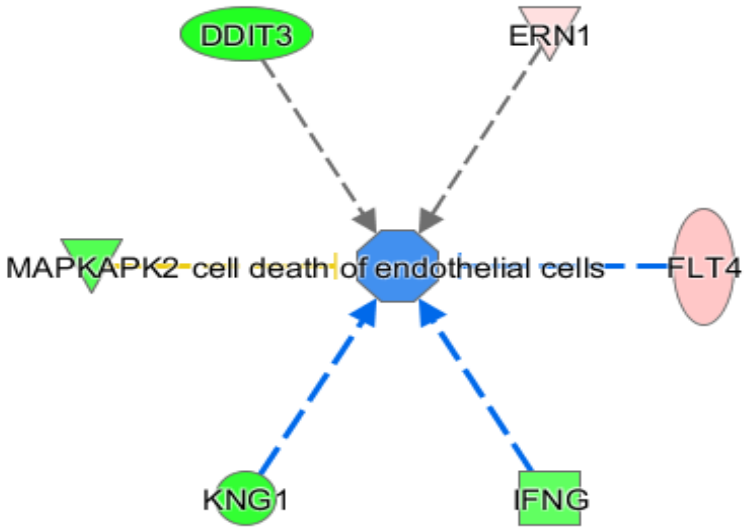



Figure 122: Predicted downregulation of cell death of EC.

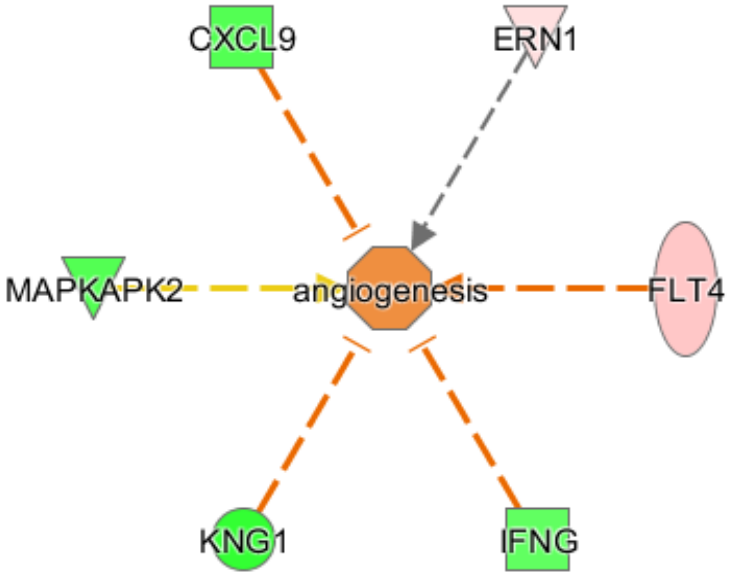



Figure 123: Predicted upregulation of angiogenesis in TG V2 when compared with HC.

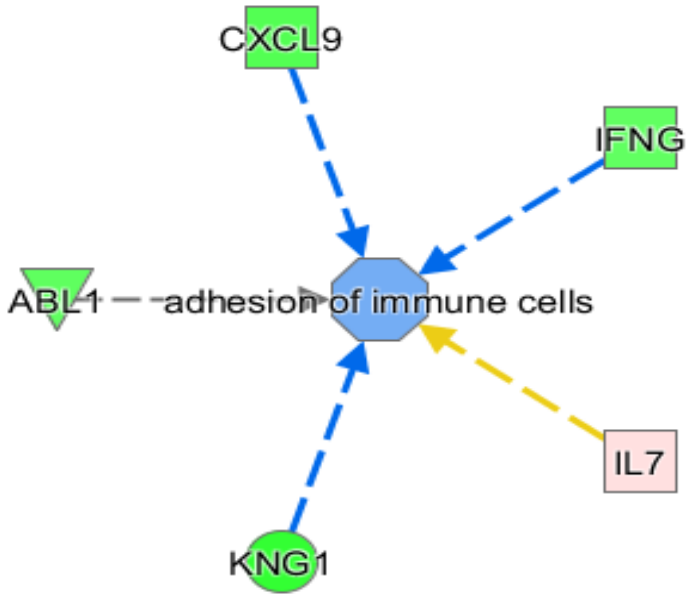



Figure 124: Predicted down-regulation of adhesion of immune cell in TG V2 compared to HC.

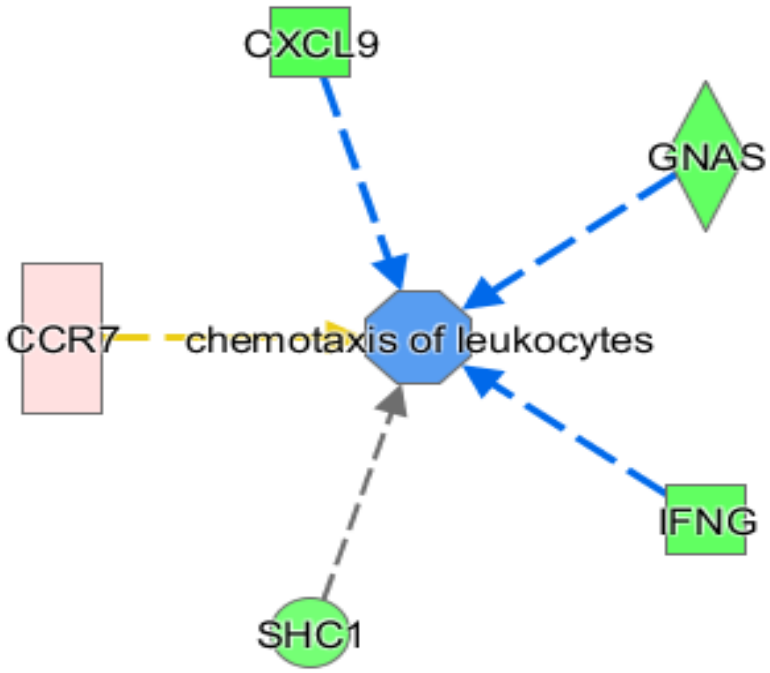



Figure 125: Predicted downregulation of chemotaxis of leukocytes in TG V2 when compared with HC.

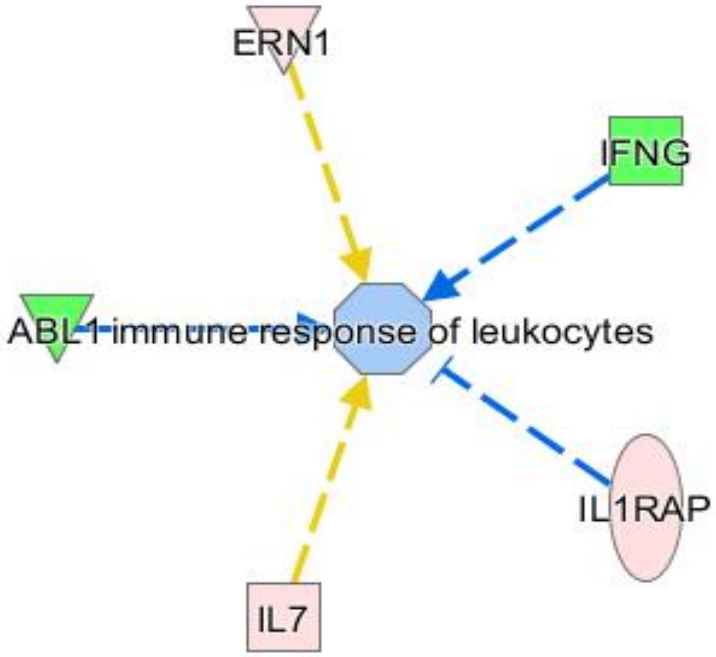



Figure 126: Predicted down-regulation of the immune response of leukocytes in TG V2 compared with HC.

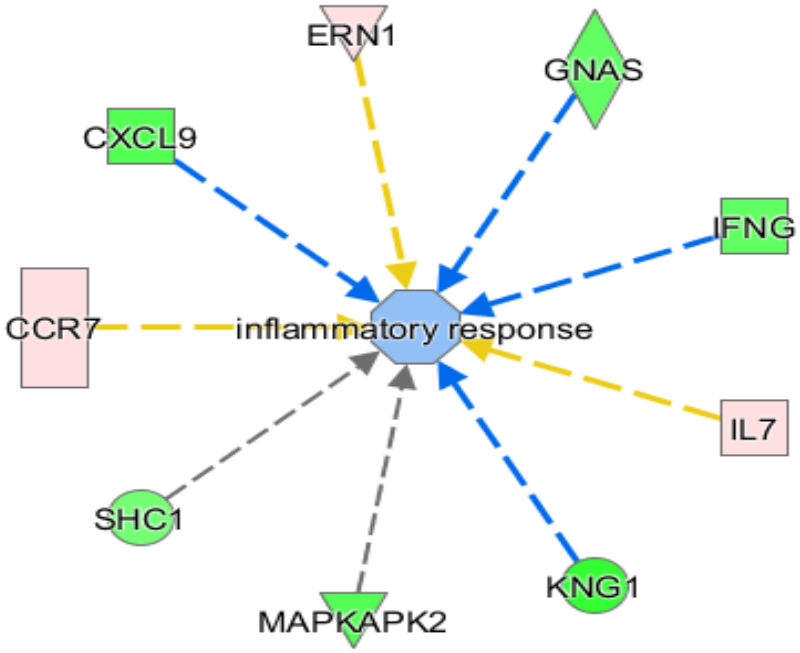



Figure 127: Predicted down-regulation of the inflammatory response in TG V2 compared with HC.

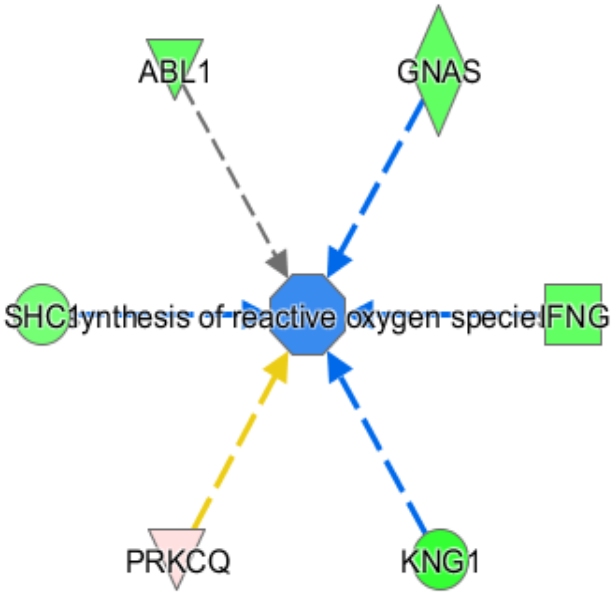



Figure 128: Predicted down-regulation of synthesis of reactive oxygen species in TG V2 compared with HC.

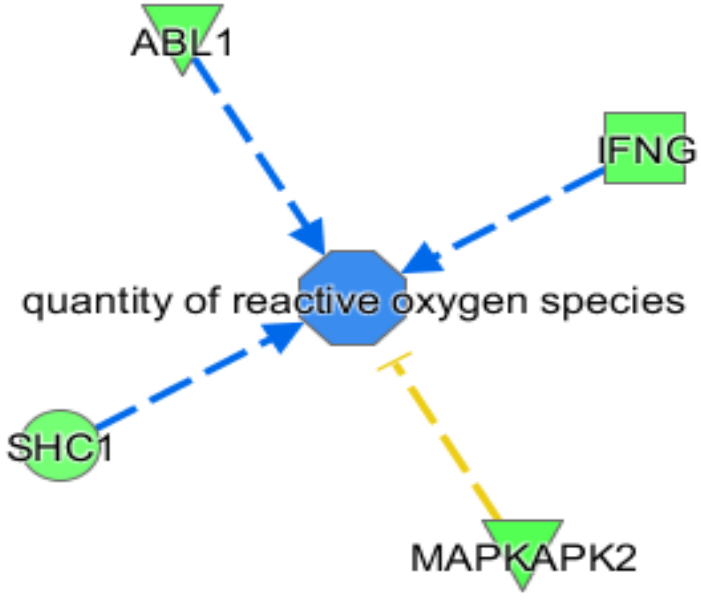



Figure 129: Predicted down-regulation of the quantity of reactive oxygen species in TG V2 compared with HC.

### *7.5.4 MAP kinase pathway*

Multiple pathways can be analysed to provide predicted up- or down-regulation of genes using KEGG pathway. R software (The statistical programming software) was used to provide prediction of activation or suppression of the pathway. MAP kinase was chosen as it was predicted to be affected. Furthermore, the MAP kinase pathway plays an important role atherosclerosis (Fisk *et al.*, 2014).

We analysed the effect of significantly expressed genes in group TG (TG V2 vs. TG V1) on MAP kinase pathway.

Metformin treatment was predicted to downregulate genes in both the classical and p38 MAP kinase pathways in TG (Figure 144).

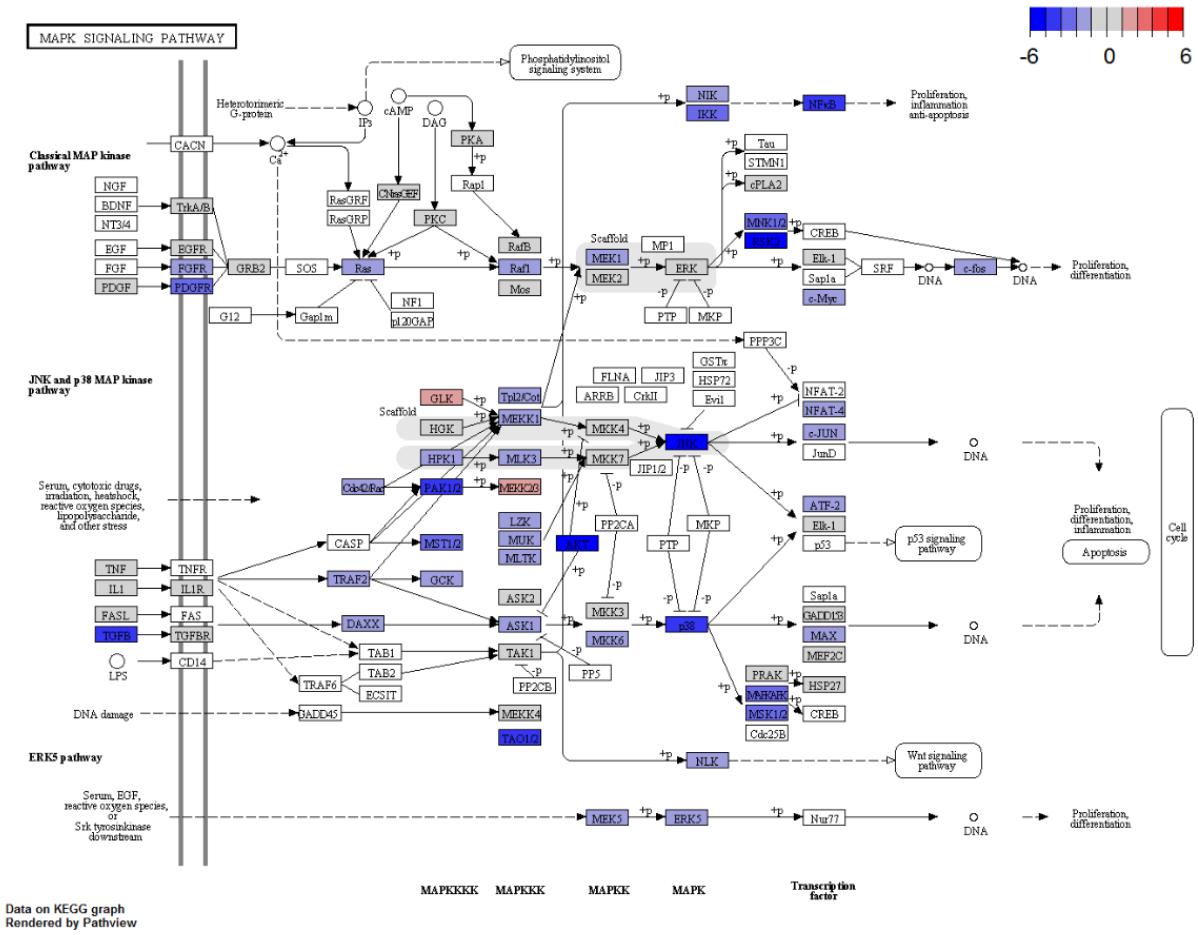



Figure 130: Predicted MAP kinase regulation comparing TG V2 vs. TG V1. Blue means down-regulation and red means upregulation.

## 7.6 Discussion

PBMCs from selected TG and matched HC were analysed using the nCounter technology to explore the differential gene expression profile. RNA from the treatment group PBMC were analysed before and after metformin treatment (TG V1 and TG V2) to study the effect of metformin on PBMC gene expression. After FDR, none of the genes were differentially expressed. Non-corrected analysis showed that seventy genes were differentially expressed in TG V1 compared to HC. All but nine genes were overexpressed. Metformin treatment altered gene expression of eleven genes in TG. Out of this, three were downregulated. After metformin treatment, thirty-one genes were differentially expressed when compared to HC. Out of these, ten genes were under-expressed.

After correcting for multiple testing (Benjamini-Hochberg -BH: FDR), none of the differential gene expression was significant. We did not set a limit on the fold changes (FC) greater than or equal to +/-1.5. Our TG group had good diabetes control as defined by HbA1c levels and these did not change with metformin treatment. Therefore, we needed to study PBMC gene expression in larger number of TG and HC.

It is of great interest to evaluate if metformin can alter gene expression in T1DM individuals with good diabetes control and to this end we used IPA to evaluate and analyse predicted targets and pathways. In TG at baseline, differentially expressed genes predicted the activation of INF-γ, NF-κB, TNF-α and IKBKB. All of these processes increased chemotaxis, adhesion, migration and the inflammatory response of the PBMC in T1DM compared to HC. Our cytokines data of increased CRP and TNF-α levels in TG V1 compared to HC complimented our gene expression analysis. Our gene expression data has demonstrated that in T1DM, the PBMC has an increased tendency towards inflammation. The increase in inflammation has been reported to play an important part in vascular damage and atherosclerosis (Libby *et al.*, 2002).

NF-κB was also predicted to be upregulated in TG V1. NF-kB plays a vital role in inflammation, angiogenesis and apoptosis (Ghosh and Hayden, 2008). We have shown that the CD40 ligand (CD40L) was predicted to be upregulated. CD40L is known to activate NF-kB pathway (Coope *et al.*, 2002). Activation of NF-κB pathway has been shown to increase expression of proinflammatory cytokines, such as IL-6, IL-8 and TNF-alpha and anti-inflammatory cytokines IL-10 (Monaco *et al.*, 2004; Pamukcu *et al.*, 2011). We have shown that all of these cytokines are increased in the plasma of our TG at baseline. NF-kB has also been shown to increase adhesion molecules in EC (Chen *et al.*, 1995). These are important in driving the inflammatory response and the development of atherosclerosis. *In vitro* studies have demonstrated that the NF-κB pathway is activated by hyperglycaemia (Yerneni *et al.*, 1999). NF-κB plays a role in diabetes-related complications such as retinopathy and nephropathy (Patel and Santani, 2009). The NF-κB pathway has been shown also to be activated in IHD patients (Liuzzo *et al.*, 2007). NF-κB pathway activation has been shown to be involved in the pathogenesis of atherosclerosis from a very early stage (de Winther *et al.*, 2005). Animal studies have shown that inhibition of the NF-κB pathway may reduce the inflammatory response and EC adhesion expression (Ferrante *et al.*, 2006). Inhibition of the NF-κB could provide a novel mechanism in preventing the development of atherosclerosis.

Evaluation of gene expression after metformin treatment predicted a decrease in leukocytes chemotaxis and increased protein phosphorylation. Phosphorylation of proteins in the AMPK pathway leads to activation of this pathway. This is one of the known effects of metformin. Metformin treatment in TG did demonstrate alteration in the MAP kinase pathway. However, IPA pathway analysis did not support either positive or negative regulation. MAP kinase is discussed in next section.

Comparison of gene expression between TG V2 and HC predicted reduced inflammatory and increased angiogenic responses, as demonstrated by increased IL-10 expression. IPA pathway analysis predicted reduced inflammatory response, reduced oxidative stress and increased angiogenesis.

If we compare TG V1 vs. HC and TG V2 vs. HC, it can be seen that individuals with T1DM have increased inflammatory signals when compared to HC. Metformin treatment shifts this focus towards reduced inflammation and improved angiogenesis.

We evaluated 14 subjects for gene analysis (8 from TG and 6 from HC). If we had included more subjects, than we could have increased the power of our analysis. Furthermore, we did not evaluate upstream and downstream markers effected by the genes. However, our data can be used to evaluate specific genes and their upstream and downstream effectors.

### *7.6.1 Predicted KEGG pathway affect in PBMCs by metformin treatment: MAP kinase*

KEGG pathway analysis demonstrated that metformin treatment reduces the MAP kinase pathway activity in PBMC in TG. This may have potential implications in the reduced inflammatory activity of PBMCs. MAP kinase pathway is known to increase pro-inflammatory cytokine activity by increasing production of IL-1 and TNF-α (Lee *et al.*, 1994). It may be that decreased activity of MAP kinase pathway reduced the PBMC mediated vascular damage. Also, the MAP kinase pathway has been implicated in the differentiation of monocytes into macrophages (Ayala *et al.*, 2000). Furthermore, it plays a critical role in the transformation of macrophages into foam cells (Zhao *et al.*, 2002). Furthermore, the fraction of PBMC constitutes potential EPCs. MAP kinase activity is implicated in the survival of EPC. Inhibition of MAP kinase has been shown to increase EPCs survival. Furthermore, inhibition of MAP kinase activity in EPCs has been shown to increase angiogenesis in an ex vivo ischaemic animal model (Seeger *et al.*, 2005).

### *7.6.2 Overall assessment*

We have shown that PBMCs in type 1 diabetes patients has the increased tendency towards inflammation compared to healthy control. NF-kB pathway likely plays an important role in increasing the inflammatory signals.

Metformin treatment in type 1 diabetes patients with good glycaemic control likely moves the focus towards reduced inflammation. In addition, inhibition of MAP kinase in PBMCs by metformin may play a significant role in decreased vascular damage by PBMC.

Our results need to be confirmed by exploring the effect of physiological concentration of metformin on MAP kinase activity in the PBMCs. This can be extended further by exploring the effect of metformin-treated PBMCs on EC.

# Chapter 8: General discussion and recommendation

## 8.1 General discussion

MERIT Study is an open-label observation study aiming to explore the effect of metformin on circulatory biomarkers in individuals with T1DM independent of the glycaemic control. Metformin is one of the first oral DM medications with cardio-protective properties. However, limitedresearch has been conducted to evaluate its cardio-protective effects in T1DM. Our study addressed an unmet area of research and evaluated early response to metformin on established biomarkers of CVD risk in patients with T1DM. So far, no study has addressed if metformin has clinical efficacy on biomarker established to prevent the development of early CVD in patients with T1DM. Recently, REMOVAL trial has demonstrated that metformin might reduce the progression of atherosclerosis in type 1 diabetes. Unfortunately, REMOVAL study was underpowered to demonstrate positive effect of metformin on CIMT in type 1 diabetes (Petrie *et al.*, 2017). However, REMOVAL study has not addressed if metformin can affect early biomarkers of vascular damage and repair. Our proof of concept study's primary outcome is an evaluation of the effect of metformin on cEPCs (biomarkers of vascular repair). The secondary outcome of MERIT study included exploring the influence of metformin on circulatory biomarkers representative of vascular repair and damage. Below, we summarise our major findings based on our hypothesis.

**Hypothesis 1:** Metformin therapy improves the number and function of EPCs in T1DM when controlled for euglycaemia.

We showed that cEPCs were lower in T1DM compared with HC. Metformin treatment significantly increased cEPCs.

**Hypothesis 2:** Metformin improves cECs in T1DM when controlled for euglycaemia.

We showed that cECs were higher in T1DM compared with HC. Metformin treatment significantly decreased cECs in TG.

**Hypothesis 3:** Metformin improves circulatory MPs in T1DM when controlled for euglycaemia.

We demonstrated that EMPs, PMPs, LMPs, CD34+ and cEPC MPs were higher in T1DM compared with HC. All the MPs remained unchanged after metformin treatment.

**Hypothesis 4:** Metformin increases angiogenic miRNA when controlled for euglycaemia.

We evaluated two angiogenic miRNAs: miR-126 and miR-210. miR-126 levels were similar in TG and HC. Plasma miR-126 levels remained unchanged after metformin treatment.

Plasma miR-210 levels were significantly higher in TG when compared to HC. Metformin treatment reduced plasma miR-210 levels in TG.

**Hypothesis 5:** Metformin decreases anti-angiogenic miRNA when controlled for euglycaemia.

We evaluated plasma miR-21a, miR-92a, miR-195, miR-222, miR-223 and miR-320 levels in our participants.

Plasma miR-222 levels were significantly higher in TG when compared to HC. Metformin treatment reduced plasma miR-222 levels in TG.

Plasma miR-195 levels were significantly higher in TG when compared to HC. Metformin treatment reduced plasma miR-195 levels in TG.

Plasma miR-223 levels were significantly higher in TG when compared to HC. Plasma miR-21a levels in TG remained unchanged after metformin treatment.

Plasma miR-320 levels were significantly higher in TG when compared to HC. Plasma miR-320 levels in TG remained unchanged after metformin treatment.

miR-92a levels were similar in TG and HC. Plasma miR-92a levels remained unchanged after metformin treatment.

**Hypothesis 6:** Metformin increases cytokines related to EPC mobilisation when controlled for euglycaemia

We evaluated plasma TIMP-1 levels in our participants. TIMP-1 levels were higher in TG when compared to HC. Metformin treatment significantly reduced TIMP-1 in TG.

**Hypothesis 7:** Metformin decreases cytokines representative of vascular injury markers when controlled for euglycaemia.

We measured E-Selectin, P-Selectin, sICAM-1, sVCAM-1, sICAM-3 and thrombomodulin in plasma of our participants. All the marker levels were similar in TG and HC. Only thrombomodulin reduced significantly in TG after metformin treatment.

**Hypothesis 8:** Metformin decreases inflammatory cytokines when controlled for euglycaemia.

We measured CRP, TNF-α, IL-6, IL-8, IP-10, INF-γ and IL-10 in plasma of our participants. Except for IP-10 and INF-γ, all other inflammatory markers were raised in TG when compared to HC. Metformin treatment reduced plasma IL-6 and IP-10 levels significantly while CRP, TNF-α, IL-8, INF-γ and IL-10 remained unchanged in TG.

**Hypothesis 9:** Metformin increases angiogenic cytokines when controlled for euglycaemia.

We measured bFGF, VEGF and VEGF-D in plasma of our participants. Plasma bFGF and VEGF levels were similar in TG and HC. VEGF-D levels were higher in TG when compared to HC. Plasma levels of these growth factors did not change after metformin treatment.

**Hypothesis 10:** Gene expression profile of PBMC from T1DM patient demonstrates increased inflammatory signals compared to healthy volunteer.

**Hypothesis 11:** Metformin will shift the gene expression profile of PBMC in T1DM towards decreased inflammation.

Nanostring gene expression analysis of PBMCs demonstrated that metformin treatment did not alter the gene expression of inflammatory and kinase gene in type 1 diabetes. Analysis of non-corrected data had shown that individuals with T1DM had increased tendency toward inflammation. Furthermore, metformin treatment demonstrated a tendency toward reduced inflammatory signal in PBMC in TG. This is likely to be mediated via MAP kinase.

Data generated from MERIT Study demonstrated that metformin may have positive clinical implication in preventing CVD in patients with T1DM by rebalancing the emphasis in their management from limiting damage alone to also improving vascular repair. cEPC, a marker of vascular repair increased significantly. In addition, there was a significant reduction in cEC numbers (vascular injury marker). Metformin treatment not only improved the level of cEPCs in T1DM but also brought PACs number/ adhesion and CFU-Hill colonies closer to the HC levels. We have been able to demonstrate this in T1DM individual with good overall diabetes control. Furthermore, this effect is generated without improvement in a variable of glycaemic control i.e. HbA1c and glucose variability.

Improvement in cEPC numbers could be mediated via increased survival, differentiation and/or mobilisation from bone marrow. It is likely all these processes contribute to this. We have shown that PAC adhesion function improved significantly. It is likely that similar mechanism is involved in increased survival. KEGG pathway analysis of gene expression data of PBMC demonstrated reduction in activity of MAP kinase pathway. PACs are a very small fraction of cells of PBMC. Similarly, cEPCs are

a cells derived from PBMCs. It is likely, reduced activity of MAP kinase by metformin treatment improved cEPC and PAC survival and function. Indeed, inhibition of MAP kinase activity has been shown to improve PAC survival and angiogenesis function (Seeger *et al.*, 2005). Our data provides an insight into the differentiation of CD34+ cells into cEPCs. 4 weeks of metformin treatment increased CD34+ cells, cEPC numbers did not increase. However, at 8 weeks CD34+ cell numbers reduced back to baseline whilst cEPC numbers increased. It may be that there was increased differentiation of CD34+ cells into cEPCs. Improvement in cEPC numbers is also likely due to increase in cEPC mobilisation. The possible mechanism may be mediated by reduction of TIMP-1 as seen in our study. TIMP-1 is known to inhibit matrix metalloproteinases in bone marrow, thereby limiting EPC mobilisation. Reduction in TIMP-1 would allow bone marrow matrix to allow for EPC mobilisation. However, this will need confirmation.

We have assessed EMPs, PMPs, LMPs, CD34+ MPs and cEPC MPs in our participants except for SG (due to change in assay). Metformin treatment did not result in change in MP concentration in TG. It might be that metformin did not have any effect on MP generation. It could be that metformin may shift the focus of MP function toward maintaining vascular homeostasis. There is extensive evidence that MPs play an important role in inflammation, coagulation and endothelial dysfunction. It is important to note that MPs can have both deleterious and beneficial effects on vascular haemostasis (Figure 145). MPs are a conduit for exchange of biological information. In addition, MPs can serve as protective mechanism whereby harmful products like oxidative stress markers produced by cells are released via generation of MPs. If these oxidative stress markers remained in the cells, it will cause damage. In vitro experiment had shown that ECs exposed to statin at physiological relevant concentration released more EMPs. However, more EC remained viable when exposed to statin. It was noted that oxidative stress marker (Caspase 3) laden MPs were increased (Diamant *et al.*, 2008). Thus, there is a need to explore if metformin changes the MPs content rather than numbers or move the balance toward vascular homeostasis.

We explored selected angiogenic and anti-angiogenic plasma miRNA levels. Major finding is significant reduction of anti-angiogenic plasma miRNA levels after metformin treatment. It may be likely those anti-angiogenic signals are reduced by metformin,

thereby shifting the focus towards angiogenesis. Metformin treatment did not affect plasma miR-126 levels. However, there was significant reduction in plasma miR-210 levels. Both, miR-126 and miR-210 have angiogenic properties. High miR-210 levels have been linked with development of atherosclerosis. Reduction in miR-210 levels after metformin treatment is suggestive of reduced signals for development of atherosclerosis. However, this hypothesis is speculative. Further work is needed to understand the effect on angiogenic and anti-angiogenic miRNAs and its upstream regulator and downstream regulators.

PBMC plays a critical role in development of atherosclerosis. This is mediated via multiple mechanisms including increased generation of inflammation. Metformin treatment shifted predicted gene expression profile towards reduced inflammatory tendency. In contrast, plasma inflammatory cytokines except for IL-6 did not decrease with 8 weeks metformin treatment. There is a need to explore the effect of metformin on inflammation and vascular damage over longer duration.

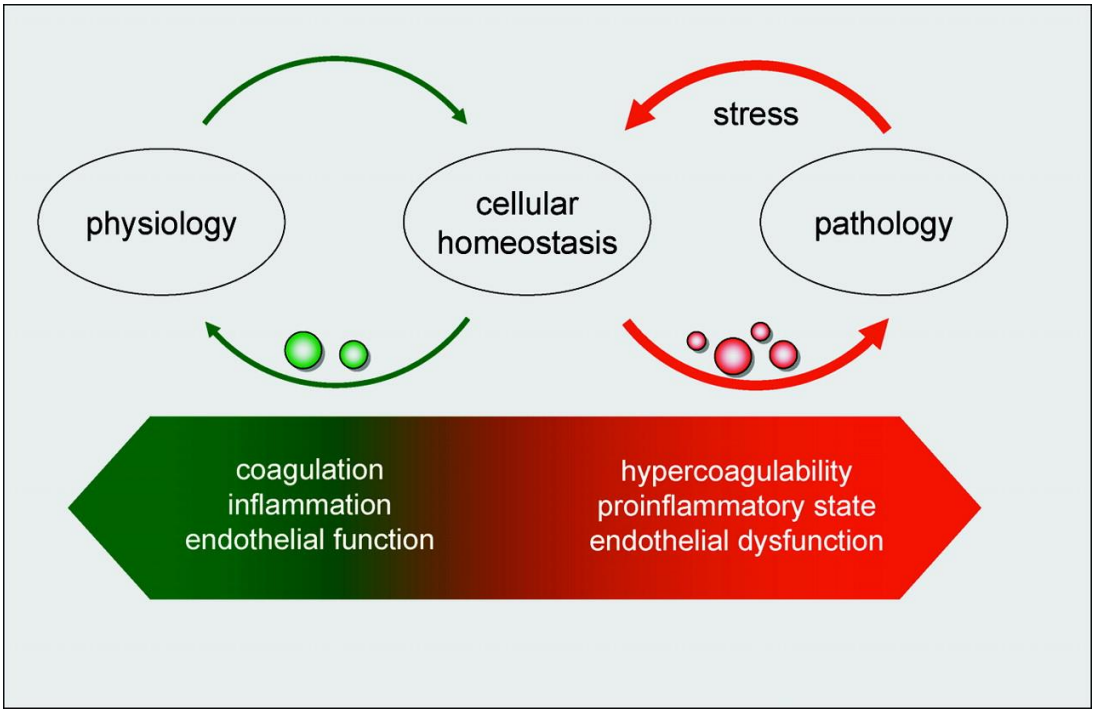



Figure 131: Proposed role of microparticle in vascular homeostasis.

Adapted from (Tushuizen *et al.*, 2011)

### *8.1.1 Limitations of our study*

In this section, we will discuss overall limitations of our work. Major limitation of MERIT study was that it was a non-randomised trial. MERIT study was designed as an open label proof of concept study. Therefore, there is a need to design a randomised controlled trial to explore the effect of metformin on primary CVD outcomes in type 1 diabetes. At this current moment, no randomised trial has been conducted in patients with type 1 diabetes exploring the effect of metformin on primary CV outcomes. Data generated from our work can be used to design randomised trials of longer duration in order to repurpose this widely used T2DM drug, for patients with T1DM (Bromage and Yellon, 2015). Our work can be supplemented by exploring the effect of metformin treatment on oxidative stress markers in T1DM.

MERIT study was limited by small sample size. However, it was adequately powered to evaluate the primary outcome. Our participants had heterogeneous features with a wide range of diabetes duration and age. This can also be seen as an advantage to improve generalisability of the results of our study. CGMS was not perfomed in SG. This can introduce bias as we could not access glucose variability. However, SG overall glycaemic control did not change as assessed by HbA1c. Furthermore, glucose variability should not be affected as SG was not given any treatment. Furthermore, home blood glucose readings were evaluated to make sure that overall profile remained unchanged. In TG, CGMS was performed at the beginning and middle of the study and not at the end of the study. However, CGMS in the middle of the treatment phase was done at the maximum dose of tolerated metformin. Therefore, it is representative of metformin effect on blood glucose levels.

MPs and gene expression was not undertaken in SG. MPs could not be analysed in SG due to changes made in the LSR machine. This meant that data from SG could not be compared reliably with TG and HC data. Lack of funding prevented us from analysing gene expression in SG group.

## 8.2 Future work

### *8.2.1 Animal study*

As discussed earlier that DM is associated with bone marrow dysfunction. It is speculated that it leads to impaired mobilisation of EPCs from bone marrow to vascular tissue. We propose to conduct an animal study to answer two important questions:

1. Is diabetes associated with impaired mobilisation of EPCs from bone marrow to vascular tissues?
2. Does metformin improve mobilisation of EPC from bone marrow to vascular tissue?

We propose to study type 1 diabetes mice model (C57BL/6), aged 8 to 9 weeks. There will be six different groups of mice with five animals in each group. The experiments will be performed in triplicates in order to validate the results. Therefore, the final number of animals studied in each group will be 15. Six groups (Table 6) are:

**1)** Control non-diabetic mice,

**2)** non-diabetic mice treated with metformin,

**3)** control mice treated with vehicle (citrate buffer control for STZ injection),

**4)** diabetic mice (streptozocin (STZ) induced),

**5)** STZ mice treated with insulin (insulin will be used to aim for euglycaemia state).

**6**) STZ mice treated with insulin + MF (we will use the same dose of MF of 40mg/kg/day and the dose of insulin will be adjusted so the level of glycaemia will be comparable with animal group

STZ will be injected intraperitoneally in a single dose of 187.5 mg/kg dissolved in sterile citrate buffer. All animals will undergo validated (Home Office) clinical scoring system with tail tip blood glucose testing. Weekly IPGTT (1.5g/kg) fasting and stimulated samples will be done for sensitive insulin (ELISA). Mice with a blood glucose level > 15 mmol/l will be considered diabetic. We will use glargine insulin (50u/kg) as previously described (Dong *et al.*, 2011).

The dose of metformin will reflect the dose used in patients (40mg/kg/day). The initial study will be set up to establish with few mice (n=6) that low dose of metformin does not lower significantly glucose levels in control mice and that comparable euglycaemia is achieved between the diabetic animals using insulin and insulin and metformin. We anticipate that that low dose of metformin will not alter significantly glucose levels in mice treated with metformin alone and that the dose of insulin required may be reduced in STZ mice treated with insulin and metformin.

EPCs defined as CD34/VEGFR+2, Lin – ( CD23, CD11b, Ter 119, and CD45) can be measured in bone marrow, peripheral circulation and aorta (by FACS) in all groups using protocols established by us and others (Sibal *et al.*, 2009b; Shakoor *et al.*, 2010) (Zengin *et al.*, 2006; Khoo *et al.*, 2009).

Angiogenesis assay can be performed using aortic ring culture assay (Zengin *et al.*, 2006). Fragments of the aorta will be cut into 1 mm thick rings and embedded between two layers of special collagen gel (Invitrogen) (Zengin *et al.*, 2006). Rings will be evaluated for angiogenesis every day using a phase contrast microscope. In 3 experiments, rings will be fixed and embedded in paraffin for immunohistochemistry. The immunohistochemical analysis will be performed before and after the ring assay using antibodies recognising endothelial and precursor cell markers CD34, CD31, CD45, CD105, CD133, CD68, von Willebrand Factor, TIE2 VEGFR2, VEcadherin, smooth-muscle-actin.

Cytokines like SDF-1, VEGF, VEGFR, leptin, adiponectin, MM9, TIMP-1 and inflammatory cytokines in blood at the end of the experiment and compare with EPC number and angiogenesis in all groups of animals (MSD multiplex assay/ELISA). We anticipate that MF will have additional effect on cytokine secretion to the effect of STZ and influence angiogenesis.

Plan mentioned above is summarised in Table 31.

| Animal group/assay | **Control** | **Control & MF** | **Mice & citrate** | **STZ DM mice** | **STZ DM mice & Insulin** | **STZ DM & insulin & MF** |
|---|---|---|---|---|---|---|
| EPCs in BM | x | x | x | x | x | x |
| EPCs in blood | x | x | x | x | x | x |
| EPCs in aorta | x | x | x | x | x | x |
| angiogenesis (aorta) | x | x | x | x | x | x |
| Insulin/glucose/cytokines | x | x | x | x | x | x |

Table 31: Plan of animal experiments; streptozocin-treated (STZ), metformin (MF), diabetic (DM) mice.

### *8.2.2 Clinical study*

In our proof of concept MERIT study, we have established important preliminary data.

Our work can meaningfully be extended to design a randomised controlled trial. Any work should include plans to explore mechanistic pathways. We propose a randomised controlled trial that will answer following questions:

1. Do cEPCs improve significantly with metformin treatment?
2. Does metformin improve endothelial function?
3. Does change of cEPC correlate with changes in endothelial function?
4. Do changes in cytokines determine the response of cEPCs?
5. Do changes in miRNAs determine the response of cEPCs?
6. Do cECs improve significantly with metformin treatment?
7. Does change of cECs correlate with changes in endothelial function?
8. Does metformin changes microparticles constituents?

We propose a multicentre, double-blind randomised placebo controlled trial using metformin versus placebo. Participants are to be randomised to receive either metformin or placebo for duration of 12 months. Inclusion and exclusion criteria are similar to MERIT Study (Table 1). The only difference will be the inclusion of T1DM patients with an HbA1c of ≤ 82 mmol/mol.

The study design is explained in Figure 146. Briefly, the participant will go through 8 weeks running in phase. Clinical, vascular and biomarkers will be assessed at 0, 6 and

12 months post randomisation. Further clinical assessment will be undertaken at 1, 4 and 7 months.

Protocol for enumeration of cEPC and cEC is well established by our group. Enumeration of microparticles will need further development. Cytokine array will be measured in plasma by MSD platform. miRNA will be assessed using NGS (Exiqon). We had a problem with the machine continuously. Endothelial function assessment can be conducted via pulse wave velocity (aortic stiffness).

This randomised controlled trial is aimed to confirm our proof of concept open label study and provide definitive data regarding the clinical efficacy of cEPCs in assessing early response to metformin treatment. This trial will also assess the size of effect whilst comparing with the functional improvement of endothelial function. Furthermore, we will be able to answer if metformin, an inexpensive drug can reduce early CVD in young T1DM individual.

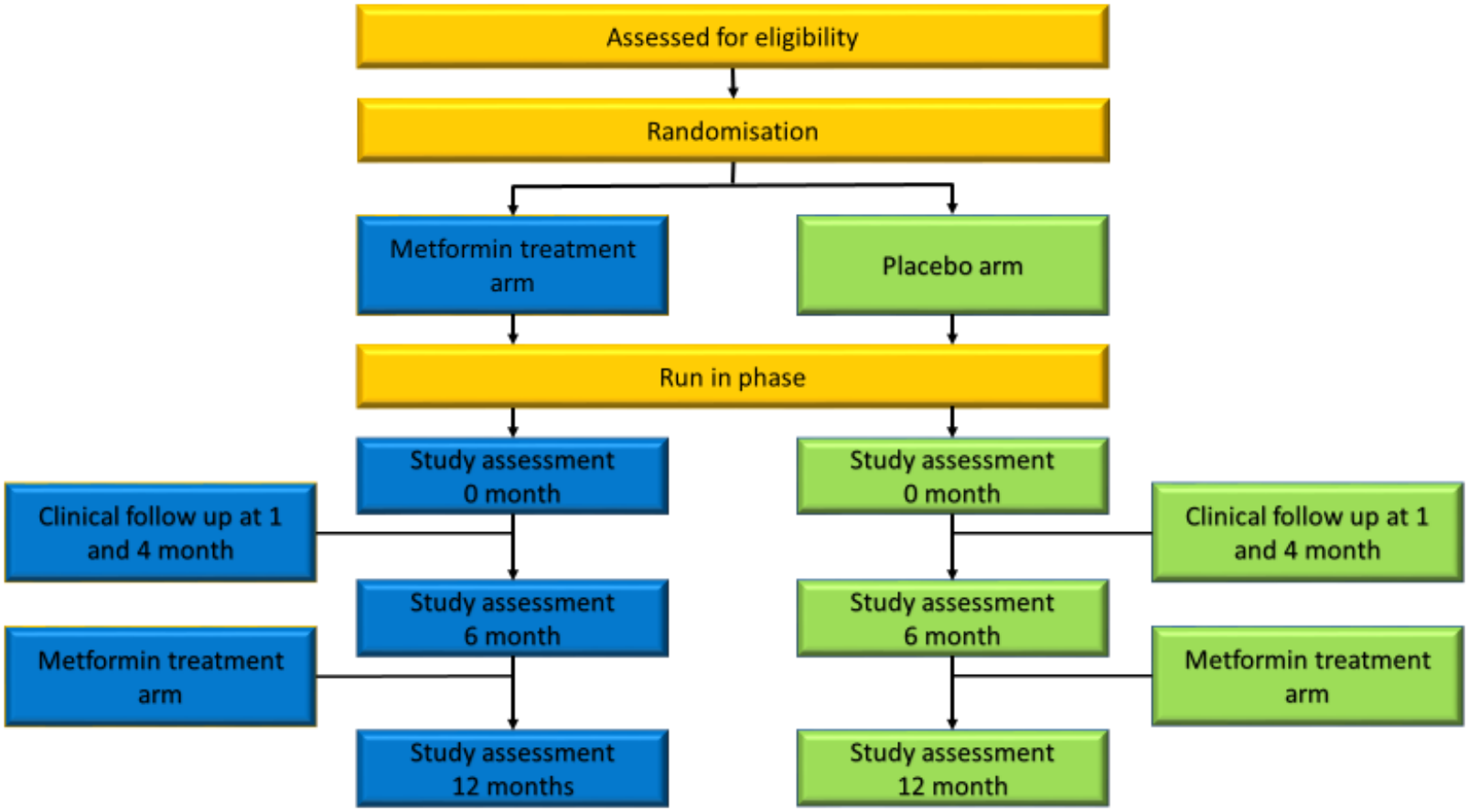



Figure 132: Proposed placebo controlled randomised controlled trial.

## 8.3 Conclusions

In summary, the data presented in this thesis has shown for the first time that metformin treatment may provide cardiovascular benefit by increasing markers of vascular repair (cEPCs, CFU-Hill Colonies, and PACs,) and reducing markers of vascular damage (cECs and anti-angiogenic miRNA). A pivotal study by Werner and colleagues (Werner *et al.*, 2005) demonstrated that CVD events are lowest in the group with higher levels of cEPCs. We believe that a 75% rise in cEPCs number in T1DM patients, as mentioned in chapter 4 of this thesis, might equate to the reclassification of our T1DM patients into a lower CVD risk group. This might equate to an approximate reduction of CVD risk by 23% as presented by Werner and collegues (Hazard Ratio of 0.77) (Werner *et al.*, 2005). If this decrease in CVD risk can be achieved in T1DM patients with good control, we believe that metformin may further reduce the CVD risk in T1DM patients with suboptimal control. However, this will need to be confirmed by large randomised a controlled trial examining cardiovascular events.

# Appendix A

| | cEPC | PAC | FAA | CFU | cEC | HDL Cholesterol | Insulin | TM | IL-6 | TIMP-1 | IP-10 | miR-210 | miR-222 | miR195 | miR-21 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| cEPC | 1.00 | | | | | | | | | | | | | | |
| PAC | .15 | 1.00 | | | | | | | | | | | | | |
| FAA | .05 | .46* | 1.00 | | | | | | | | | | | | |
| CFU | -.25 | .41 | .06 | 1.00 | | | | | | | | | | | |
| cEC | .13 | -.26 | .15 | -.60** | 1.00 | | | | | | | | | | |
| HDL Cholesterol | -.20 | .12 | -.30 | -.03 | -.18 | 1.00 | | | | | | | | | |
| Insulin | -.24 | .08 | .10 | -.01 | -.34 | .15 | 1.00 | | | | | | | | |
| TM | -.24 | -.07 | -.18 | .19 | -.06 | .26 | .14 | 1.00 | | | | | | | |
| IL-6 | .16 | -.06 | -.07 | -.20 | -.09 | -.04 | .31 | -.25 | 1.00 | | | | | | |
| TIMP-1 | -.23 | -.11 | -.54** | .12 | -.08 | .48* | -.20 | .26 | .11 | 1.00 | | | | | |
| IP-10 | .07 | .25 | .46* | .20 | -.19 | -.55** | .32 | -.14 | .09 | -.37 | 1.00 | | | | |
| miR-210 | -.08 | -.04 | .37 | .12 | .34 | .03 | -.29 | .20 | -.29 | -.05 | -.13 | 1.00 | | | |
| miR-222 | .03 | -.14 | .07 | -.21 | .42* | .04 | -.41* | .05 | -.28 | .01 | -.27 | .68** | 1.00 | | |
| miR195 | .05 | -.12 | -.11 | .04 | -.01 | .33 | -.08 | .10 | .25 | .22 | -.45* | .45* | .21 | 1.00 | |
| miR-21 | -.09 | -.16 | .08 | -.17 | .19 | .05 | -.13 | -.13 | -.07 | -.13 | -.23 | .40 | .41 | .42* | 1.00 |

Table 32: Correlation between significant changes in variable after metformin treatment. * denotes $p<0.05$ and ** denotes $p<0.01$.

| | cEPC | CD34CD133 | CD34 | cEC | PAC | FAA | CFU |
|---|---|---|---|---|---|---|---|
| cEPC | 1.00 | | | | | | |
| CD34CD133 | .91** | 1.00 | | | | | |
| CD34 | .28 | .19 | 1.00 | | | | |
| cEC | .11 | .12 | -.22 | 1.00 | | | |
| PAC | -.15 | -.09 | -.25 | -.48* | 1.00 | | |
| FAA | -.02 | .07 | -.14 | -.51* | .70** | 1.00 | |
| CFU | .20 | .21 | .12 | .10 | -.02 | .17 | 1.00 |

Table 33: Correlation matrix between cEPC, CPC, cECs, PACs, PAC adhesion function and CFU-Hill Colonies. * denotes $p<0.05$ and ** denotes $p<0.01$.

| | Age | DOD | HbA1c | Weight | BMI | Insulin | Creat | TC | Tg | LDL | HDL |
|---|---|---|---|---|---|---|---|---|---|---|---|
| cEPC | .14 | -.23 | .12 | .39 | .21 | .25 | .43* | .00 | .21 | .21 | -.36 |
| CD34CD133 | .01 | -.37 | .13 | .25 | .11 | .30 | .44* | .06 | .21 | .28 | -.30 |
| CD34 | -.29 | -.13 | .29 | -.17 | -.27 | -.19 | -.23 | -.08 | -.19 | -.15 | .17 |
| cEC | .03 | -.26 | -.23 | .37 | .41* | -.07 | -.02 | .36 | .40 | .43* | -.18 |
| PAC | .15 | .30 | -.34 | -.34 | -.26 | -.09 | -.08 | -.23 | -.22 | -.35 | .29 |
| FAA | .09 | .21 | -.03 | -.38 | -.42 | -.07 | .03 | -.23 | -.35 | -.29 | .26 |
| CFU | .00 | .17 | .27 | -.16 | -.08 | -.27 | .13 | .20 | .07 | .14 | .13 |
| Age | 1.00 | .51* | .19 | .14 | .28 | .14 | .32 | .20 | -.03 | -.11 | .09 |
| DOD | | 1.00 | .36 | .07 | .22 | -.01 | .08 | .05 | .06 | -.12 | .02 |
| HbA1c | | | 1.00 | -.21 | -.17 | -.05 | .15 | .21 | -.07 | .23 | -.09 |
| Weight | | | | 1.00 | .88** | .61** | .31 | -.05 | .53** | .21 | -.62** |
| BMI | | | | | 1.00 | .40 | .33 | .13 | .51* | .23 | -.37 |
| Insulin | | | | | | 1.00 | .10 | -.25 | .48* | -.07 | -.55** |
| Creat | | | | | | | 1.00 | .10 | .15 | .17 | -.31 |
| TC | | | | | | | | 1.00 | -.06 | .78** | .32 |
| Tg | | | | | | | | | 1.00 | .12 | -.50* |
| LDL | | | | | | | | | | 1.00 | |
| HDL | | | | | | | | | | | 1.00 |

Table 34: Correlation matrix between cEPC, CPC, cECs, PACs, PAC adhesion function and CFU-Hill Colonies and baseline metabolic variables. * denotes $p<0.05$ and ** denotes $p<0.01$.

| | MAGE | CONGA | AUC | MeanBG | StdevBG |
|---|---|---|---|---|---|
| cEPC | .10 | .21 | .37 | .24 | .10 |
| CD34CD133 | .07 | .05 | .25 | .10 | -.09 |
| CD34 | -.10 | .13 | .20 | .16 | .12 |
| cEC | -.11 | .37 | .30 | .34 | .19 |
| PAC | .18 | -.56** | -.49* | -.55** | -.34 |
| FAA | .26 | -.43* | -.38 | -.40 | -.28 |
| CFU | -.05 | .03 | .05 | .07 | .06 |

Table 35: Correlation matrix between cEPC, CPC, cECs, PACs, PAC adhesion function, CFU-Hill Colonies and glucose variability. * denotes $p<0.05$ and ** denotes $p<0.01$.

| | CD31MP | PMP | EMP | CD62eMP | CD144MP | LMP | CD34MP | EPC MP |
|---|---|---|---|---|---|---|---|---|
| cEPC | .33 | .35 | .04 | .18 | .07 | .40 | .47* | .20 |
| CD34CD133 | .24 | .26 | .11 | .11 | .02 | .38 | .45* | .29 |
| CD34 | .37 | .39 | -.17 | .37 | .40 | .38 | .42* | -.10 |
| cEC | .17 | .17 | -.03 | .13 | .03 | .31 | .32 | .30 |
| PAC | -.27 | -.27 | -.05 | -.23 | -.18 | -.11 | -.17 | -.04 |
| FAA | -.30 | -.33 | .17 | -.19 | -.08 | -.22 | -.24 | .15 |
| CFU | .08 | .08 | -.05 | .20 | .31 | .36 | .33 | .37 |
| CD31MP | 1.00 | 0.99** | .32 | .92** | .81** | .50* | .50* | .11 |
| PMP | | 1.00 | .25 | .89** | .78** | .54** | .54** | .12 |
| EMP | | | 1.00 | .36 | .33 | -.21 | -.26 | -.25 |
| CD62eMP | | | | 1.00 | .96** | .43* | .41 | .22 |
| CD144MP | | | | | 1.00 | .40 | .38 | .28 |
| LMP | | | | | | 1.00 | .98** | .41 |
| CD34MP | | | | | | | 1.00 | .48* |
| EPC MP | | | | | | | | 1.00 |

Table 36: Correlation matrix between cEPC, CPC, cECs, PACs, PAC adhesion function, CFU-Hill Colonies and microparticles. * denotes $p<0.05$ and ** denotes $p<0.01$.

| | miR-21 | miR-222 | miR-195 | mir-210 | miR-320 | miR-223 | miR-92a | miR-126 |
|---|---|---|---|---|---|---|---|---|
| cEPC | .01 | -.14 | -.20 | -.27 | .12 | .27 | .15 | -.17 |
| CD34CD133 | -.01 | -.18 | -.17 | -.16 | .19 | .38 | .21 | -.14 |
| CD34 | .26 | .42* | .34 | .10 | .23 | -.06 | .35 | .33 |
| cEC | .27 | .20 | .09 | .36 | .51* | -.01 | .39 | -.01 |
| PAC | -.08 | -.14 | -.07 | -.27 | -.31 | .19 | -.11 | .06 |
| FAA | -.31 | -.11 | .05 | -.12 | -.40 | .23 | -.23 | .18 |
| CFU | -.04 | .22 | .30 | -.04 | -.12 | -.24 | .19 | .31 |
| miR-21 | 1.00 | .51* | .51* | .18 | .41 | .21 | .56** | .37 |
| miR-222 | | 1.00 | .64* | .23 | .58** | -.09 | .66** | .88** |
| miR-195 | | | 1.00 | .13 | .13 | .08 | .33 | .62** |
| mir-210 | | | | 1.00 | .11 | -.13 | .09 | .20 |
| miR-320 | | | | | 1.00 | .11 | .81** | .42* |
| miR-223 | | | | | | 1.00 | -.03 | -.11 |
| miR-92a | | | | . | | | 1.00 | .58** |
| miR-126 | | | | | | | | 1.00 |

Table 37: Correlation matrix between cEPC, CPC, cECs, PACs, PAC adhesion function, CFU-Hill Colonies and miRNAs. * denotes p<0.05 and ** denotes p<0.01.

| | IL-6 | TNF-alpha | CRP | INF-gamma | IP-10 | P-Selectin | sVCAM-1 | sICAM-1 | E-Selectin | sICAM-3 | TMD | TIMP-1 | VEGF | bFGF | VEGF-D |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| cEPC | -.35 | .46* | .16 | -.14 | .32 | .18 | -.33 | -.06 | .25 | .11 | .70** | -.04 | -.40 | -.43* | .02 |
| CD34CD133 | -.36 | .31 | .07 | -.11 | .25 | .33 | -.44* | -.09 | .33 | .08 | .62** | .07 | -.44* | -.36 | .07 |
| CD34 | -.30 | .01 | -.04 | .02 | .09 | -.27 | -.18 | -.14 | -.19 | -.27 | -.01 | .13 | -.43* | .00 | .45* |
| cEC | .00 | -.07 | .34 | .08 | .17 | -.12 | .13 | .32 | -.31 | .13 | -.15 | -.59** | .15 | .05 | .00 |
| PAC | -.04 | -.08 | -.18 | -.37 | -.37 | .00 | -.12 | -.20 | .03 | -.19 | .17 | .34 | .12 | -.25 | .15 |
| FAA | -.33 | .06 | -.43* | -.13 | -.20 | .20 | -.33 | -.45* | .03 | -.01 | .32 | .52** | -.13 | -.47* | .24 |
| CFU | -.37 | .11 | -.05 | .15 | -.18 | .27 | -.19 | .00 | .16 | -.08 | .35 | .15 | -.06 | -.40 | .27 |
| IL-6 | 1.00 | -.35 | .13 | -.33 | .00 | .07 | .54** | .22 | .08 | -.15 | -.51* | -.41* | .53** | .47* | -.40 |
| TNF-alpha | | 1.00 | -.02 | .31 | .53** | .03 | -.04 | -.08 | .23 | .32 | .70** | .29 | -.23 | -.44* | -.15 |
| CRP | | | 1.00 | .14 | .33 | -.14 | .36 | .82** | .02 | .06 | .03 | -.23 | .13 | .04 | -.24 |
| INF-gamma | | | | 1.00 | .42* | -.14 | -.07 | .07 | -.04 | .46* | .23 | .14 | -.29 | .03 | -.21 |
| IP-10 | | | | | 1.00 | .05 | .25 | .14 | .11 | .27 | .21 | -.09 | -.09 | .02 | -.33 |
| P-Selectin | | | | | | 1.00 | .12 | .07 | .76** | -.04 | .18 | .27 | .38 | .06 | -.03 |
| sVCAM-1 | | | | | | | 1.00 | .63** | .17 | -.21 | -.27 | -.34 | .68** | .45* | -.25 |
| sICAM-1 | | | | | | | | 1.00 | .20 | -.16 | -.09 | -.27 | .48* | .21 | -.19 |
| E-Selectin | | | | | | | | | 1.00 | .10 | .39 | .25 | .22 | .13 | -.13 |
| sICAM-3 | | | | | | | | | | 1.00 | .44* | -.03 | -.09 | .08 | -.14 |
| TMD | - | | | | | | | | | | 1.00 | .29 | -.36 | -.48* | .04 |
| TIMP-1 | | | | | | | | | | | | 1.00 | -.16 | -.32 | .33 |
| VEGF | | - | | | - | | | | | | . | | 1.00 | .45* | .01 |
| bFGF | | | | | | | | | | | | | | 1.00 | -.03 |
| VEGF-D | | | | | | | | | | | | | | | 1.00 |

Table 38: Correlation matrix between cEPC, CPC, cECs, PACs, PAC adhesion function, CFU-Hill Colonies, cytokines. * denotes p<0.05 and ** denotes p<0.01.

| | Age | DOD | HbA1c | Weight | BMI | Insulin | Creat | TC | Tg | LDL | HDL |
|---|---|---|---|---|---|---|---|---|---|---|---|
| CD31MP | -.28 | -.35 | -.05 | -.05 | -.29 | -.28 | .15 | -.14 | -.20 | .00 | -.23 |
| PMP | -.26 | -.35 | -.04 | -.05 | -.28 | -.28 | .15 | -.12 | -.19 | .00 | -.21 |
| EMP | -.22 | -.13 | -.04 | .18 | -.08 | .26 | .01 | -.30 | -.13 | -.03 | -.41 |
| CD62eMP | -.35 | -.27 | -.08 | -.05 | -.25 | -.37 | .22 | -.17 | -.16 | -.07 | -.18 |
| CD144MP | -.42 | -.22 | -.07 | -.16 | -.32 | -.45* | .20 | -.16 | -.18 | -.11 | -.06 |
| LMP | -.07 | -.25 | -.13 | -.06 | -.13 | -.15 | .16 | -.01 | .20 | -.15 | .18 |
| CD34MP | -.08 | -.29 | -.09 | -.05 | -.12 | -.13 | .18 | .00 | .24 | -.12 | .13 |
| EPC MP | -.04 | -.39 | -.18 | -.21 | -.14 | -.33 | .42* | .15 | .08 | .05 | .26 |
| Age | 1.00 | .50* | .16 | .20 | .33 | .17 | .28 | .18 | .03 | -.10 | .04 |
| DOD | | 1.00 | .34 | .12 | .26 | .02 | .03 | .03 | .13 | -.12 | -.04 |
| HbA1c | | | 1.00 | -.15 | -.13 | .00 | .08 | .18 | .01 | .25 | -.18 |
| Weight | | | | 1.00 | .88** | .61** | .31 | .00 | .47* | .20 | -.62** |
| BMI | | | | | 1.00 | .38 | .33 | .17 | .47* | .23 | -.37 |
| Insulin | | | . | | | 1.00 | .19 | -.23 | .45* | -.08 | -.55** |
| Creat | | | | . | | | 1.00 | .05 | .34 | .21 | -.31 |
| TC | | | | | | | | 1.00 | -.01 | .79** | .29 |
| Tg | | | | | | | | | 1.00 | .11 | -.42* |
| LDL | | | | | | | | | | 1.00 | -.19 |
| HDL | | | | | | | | | | | 1.00 |

Table 39: Correlation matrix between microparticles and baseline metabolic variables. * denotes p<0.05 and ** denotes p<0.01.

| | MAGE | CONGA | AUC | MeanBG | StdevBG |
|---|---|---|---|---|---|
| CD31MP | -.36 | -.01 | .04 | .01 | .04 |
| PMP | -.36 | .00 | .08 | .03 | .04 |
| EMP | -.08 | -.30 | -.40 | -.27 | -.21 |
| CD62eMP | -.30 | .03 | -.01 | .06 | .11 |
| CD144MP | -.27 | -.01 | -.08 | .03 | .10 |
| LMP | -.36 | .04 | .18 | .06 | -.18 |
| CD34MP | -.34 | .09 | .26 | .12 | -.16 |
| EPC MP | -.23 | .00 | .05 | .04 | -.19 |
| MAGE | 1.00 | .45* | .40 | .43* | .66** |
| CONGA | | 1.00 | .93** | .99** | .78** |
| AUC | | | 1.00 | .94** | .67** |
| MeanBG | | | | 1.00 | .76** |
| StdevBG | | | | | 1.00 |

Table 40: Correlation matrix between microparticles and glucose variability. * denotes $p<0.05$ and ** denotes $p<0.01$.

| | miR-21 | miR-222 | miR-195 | mir-210 | miR-320 | miR-223 | miR-92a | miR-126 |
|---|---|---|---|---|---|---|---|---|
| CD31MP | -.02 | -.11 | .12 | .07 | -.19 | .00 | -.11 | -.31 |
| PMP | .03 | -.08 | .12 | .08 | -.15 | .00 | -.06 | -.29 |
| EMP | -.27 | -.38 | .12 | -.24 | -.45* | .19 | -.52* | -.37 |
| CD62eMP | -.23 | -.13 | .06 | .12 | -.26 | .00 | -.24 | -.31 |
| CD144MP | -.31 | -.02 | .12 | .14 | -.28 | -.05 | -.27 | -.16 |
| LMP | .15 | .16 | .10 | -.01 | .28 | -.18 | .42* | .03 |
| CD34MP | .17 | .16 | .09 | .04 | .33 | -.10 | .37 | .02 |
| EPC MP | -.20 | .01 | -.04 | .22 | .16 | .21 | .10 | .03 |
| miR-21 | 1.00 | .51* | .51* | .17 | .33 | .25 | .56** | .30 |
| miR-222 | | 1.00 | .61** | .23 | .58** | -.09 | .66* | .88** |
| miR-195 | | | 1.00 | .12 | .06 | .09 | .27 | .62** |
| mir-210 | | | | 1.00 | .09 | -.13 | .07 | .19 |
| miR-320 | | | | | 1.00 | .12 | .80** | .42* |
| miR-223 | | | | | | 1.00 | -.02 | -.11 |
| miR-92a | | | | | | | 1.00 | .58** |
| miR-126 | | | | | | | | 1.00 |

Table 41: Correlation matrix between microparticles and miRNAs. * denotes $p<0.05$ and ** denotes $p<0.01$.

| | IL-6 | TNF-alpha | CRP | INF-gamma | IP-10 | P-Selectin | sVCAM-1 | sICAM-1 | E-Selectin | sICAM-3 | TMD | TIMP-1 | VEGF | bFGF | VEGF-D |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CD31MP | .08 | -.14 | -.05 | -.28 | -.22 | -.41 | -.38 | -.23 | -.33 | -.23 | -.09 | -.36 | -.35 | -.08 | .13 |
| PMP | .05 | -.11 | -.04 | -.29 | -.22 | -.41 | -.40 | -.23 | -.31 | -.22 | -.06 | -.35 | -.35 | -.07 | .15 |
| EMP | .44* | -.38 | -.01 | -.05 | .04 | .06 | -.16 | -.14 | -.08 | -.03 | -.17 | -.17 | -.13 | -.06 | -.22 |
| CD62eMP | .00 | -.13 | -.10 | -.07 | -.15 | -.45* | -.34 | -.27 | -.39 | -.30 | -.10 | -.25 | -.40 | -.14 | .20 |
| CD144MP | -.12 | -.14 | -.16 | .07 | -.19 | -.42 | -.39 | -.32 | -.43* | -.33 | -.11 | -.10 | -.47* | -.20 | .24 |
| LMP | -.46* | .03 | .09 | -.06 | -.20 | -.30 | -.46 | .05 | -.30 | -.18 | .19 | -.03 | -.36 | -.23 | .27 |
| CD34MP | -.52* | .10 | .11 | -.05 | -.10 | -.29 | -.49 | .03 | -.27 | -.16 | .21 | -.01 | -.42 | -.24 | .27 |
| EPC MP | -.70** | .19 | .10 | .27 | -.07 | -.04 | -.53* | .04 | -.07 | .09 | .32 | .30 | -.34 | -.43* | .26 |
| IL-6 | 1.00 | -.34 | .11 | -.32 | .00 | .07 | .55** | .21 | .09 | -.14 | -.52 | -.49 | .53 | .47* | -.41 |
| TNF-alpha | | 1.00 | .10 | .30 | .53** | .02 | .05 | .05 | .22 | .29 | .69** | .21 | -.20 | -.44* | -.13 |
| CRP | | | 1.00 | .22 | .39 | -.12 | .24 | .85** | .08 | .18 | .11 | -.23 | .06 | .02 | -.32 |
| INF-gamma | | | | 1.00 | .42* | -.15 | -.03 | .15 | -.05 | .46* | .21 | .09 | -.27 | .04 | -.20 |
| IP-10 | | | | | 1.00 | .04 | .28 | .19 | .10 | .27 | .21 | -.12 | -.08 | .02 | -.33 |
| P-Selectin | | | | | | 1.00 | .15 | .12 | .75** | -.06 | .17 | .27 | .40 | .06 | -.02 |
| sVCAM-1 | | | | | | | 1.00 | .63** | .22 | -.15 | -.23 | -.22 | .68** | .46* | -.30 |
| sICAM-1 | | | | | | | | 1.00 | .31 | -.05 | -.01 | -.03 | .48* | .23 | -.28 |
| E-Selectin | | | | | | | | | 1.00 | .08 | .38 | .23 | .25 | .13 | -.12 |
| sICAM-3 | | | | | | | | | | 1.00 | .42 | -.14 | -.06 | .09 | -.13 |
| TMD | | | | | | | | | | | 1.00 | .25 | -.34 | -.48* | .06 |
| TIMP-1 | | | | | | | | | | | | 1.00 | -.09 | -.34 | .42 |
| VEGF | | | | | | | | | | | | | 1.00 | .51* | -.01 |
| bFGF | | | | | | | | | | | | | | 1.00 | -.04 |
| VEGF-D | | | | | | | | | | | | | | | 1.00 |

Table 42: Correlation matrix between microparticles, cytokines. * denotes p<0.05 and ** denotes p<0.01.

| | Age | DOD | HbA1c | Weight | BMI | Insulin | Creat | TC | Tg | LDL | HDL |
|---|---|---|---|---|---|---|---|---|---|---|---|
| miR-21 | .13 | .00 | -.01 | -.04 | .01 | .00 | -.51* | .15 | -.09 | .10 | .05 |
| miR-222 | -.32 | -.21 | -.17 | -.19 | -.10 | -.34 | -.61** | .16 | -.10 | .08 | .40 |
| miR-195 | -.34 | -.08 | .10 | -.43 | -.46* | -.6* | -.74** | .11 | -.29 | .09 | .36 |
| mir-210 | .09 | -.08 | .17 | -.34 | -.20 | -.40 | -.05 | .33 | -.03 | .13 | .23 |
| miR-320 | -.22 | -.51* | -.27 | .19 | .24 | .07 | -.29 | .28 | .25 | .28 | .12 |
| miR-223 | -.10 | -.33 | -.27 | .12 | .05 | .11 | .05 | -.18 | -.15 | .08 | -.18 |
| miR-92a | -.11 | -.29 | -.07 | -.01 | .08 | -.09 | -.36 | .35 | .00 | .28 | .28 |
| miR-126 | -.19 | -.03 | -.03 | -.31 | -.21 | -.31 | -.49* | .36 | -.18 | .21 | .53** |
| Age | 1.00 | .51* | .19 | .14 | .28 | .14 | .32 | .20 | -.03 | -.11 | .09 |
| DOD | | 1.00 | .36 | .07 | .22 | -.01 | .08 | .05 | .06 | -.12 | .02 |
| HbA1c | | | 1.00 | -.21 | -.17 | -.05 | .15 | .21 | -.07 | .23 | -.09 |
| Weight | | | | 1.00 | .88** | .61** | .31 | -.05 | .53** | .21 | -.62** |
| BMI | | | | | 1.00 | .40 | .33 | .13 | .51* | .23 | -.37 |
| Insulin | | | | | | 1.00 | .10 | -.25 | .48* | -.07 | -.55** |
| Creat | | | | | | | 1.00 | .10 | .15 | .17 | -.31 |
| TC | | | | | | | | 1.00 | -.06 | .78** | .32 |
| Tg | | | | | | | | | 1.00 | .12 | -.50* |
| LDL | | | | | | | | | | 1.00 | -.19 |
| HDL | | | | | | | | | | | 1.00 |

Table 43: Correlation matrix between miRNAs and baseline metabolic variables. * denotes p<0.05 and ** denotes p<0.01.

| | MAGE | CONGA | AUC | MeanBG | StdevBG |
|---|---|---|---|---|---|
| miR-21 | -.08 | -.13 | -.03 | -.14 | -.07 |
| miR-222 | .04 | .08 | .04 | .07 | .11 |
| miR-195 | -.41 | -.32 | -.31 | -.31 | -.36 |
| mir-210 | .03 | .14 | .19 | .19 | .09 |
| miR-320 | .10 | .32 | .30 | .29 | .16 |
| miR-223 | -.06 | -.28 | -.21 | -.27 | -.30 |
| miR-92a | .08 | .19 | .26 | .17 | .15 |
| miR-126 | .11 | -.09 | -.10 | -.11 | .00 |
| MAGE | 1.00 | .45* | .40 | .46* | .68** |
| CONGA | | 1.00 | .91** | .99** | .80** |
| AUC | | | 1.00 | .93** | .65** |
| MeanBG | | | | 1.00 | .78** |
| StdevBG | | | | | 1.00 |

Table 44: Correlation matrix between miRNAs and glucose variability. * denotes p<0.05 and ** denotes p<0.01.

| | IL-6 | TNF-alpha | CRP | INF-gamma | IP-10 | P-Selectin | sVCAM1 | sICAM-1 | E-Selectin | sICAM-3 | TMD | TIMP-1 | VEGF | bFGF | VEGF-D |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| miR-21 | .15 | -.14 | .45 | -.17 | .07 | -.12 | .09 | .33 | -.08 | .05 | -.25 | -.34 | .28 | .26 | .11 |
| miR-222 | -.30 | -.23 | .20 | .18 | -.19 | -.34 | -.07 | .07 | -.45* | .05 | -.19 | -.19 | -.15 | -.04 | .23 |
| miR-195 | .06 | -.45* | .17 | -.15 | -.27 | -.14 | -.17 | .04 | -.39 | -.12 | -.45* | .02 | .07 | -.15 | .31 |
| mir-210 | -.16 | -.05 | -.27 | .17 | .06 | -.10 | -.02 | -.19 | -.34 | .06 | -.33 | .00 | .20 | .34 | .33 |
| miR-320 | -.30 | -.15 | .34 | .12 | .12 | -.12 | .03 | .27 | -.17 | .02 | -.12 | -.38 | -.20 | .12 | .03 |
| miR-223 | -.02 | .15 | .42 | -.02 | .32 | -.04 | -.24 | .08 | .06 | .12 | .18 | .03 | -.31 | -.29 | -.04 |
| miR-92a | -.28 | -.21 | .20 | -.11 | -.17 | -.02 | .01 | .20 | -.03 | .01 | .01 | -.29 | -.02 | .11 | .34 |
| miR-126 | -.37 | -.14 | -.10 | .13 | -.31 | -.03 | -.20 | -.15 | -.27 | .05 | -.08 | .13 | -.08 | -.18 | .32 |

Table 45: Correlation matrix between miRNAs, cytokines. * denotes $p<0.05$ and ** denotes $p<0.01$.

| | Age | DOD | HbA1c | Weight | BMI | Insulin | Creat | TC | Tg | LDL | HDL |
|---|---|---|---|---|---|---|---|---|---|---|---|
| IL-6 | .08 | .25 | .07 | .21 | .15 | .29 | -.25 | -.13 | .01 | .05 | -.38 |
| TNF-alpha | .45* | .30 | .13 | .34 | .42 | .10 | .57** | .07 | .17 | .00 | -.12 |
| CRP | -.12 | -.21 | -.23 | .32 | .42* | .06 | -.21 | -.32 | .29 | -.03 | -.30 |
| INF-gamma | .04 | -.07 | .00 | .10 | .34 | -.16 | .29 | .11 | -.04 | -.05 | .23 |
| IP-10 | .36 | .13 | .26 | .55** | .57** | .30 | .22 | -.06 | .31 | -.02 | -.33 |
| P-Selectin | .15 | .20 | .64** | .00 | .01 | .33 | .28 | .34 | .20 | .51* | -.28 |
| sVCAM-1 | .16 | .37 | .15 | .29 | .40 | .20 | -.25 | -.21 | .37 | -.09 | -.33 |
| sICAM-1 | -.11 | -.08 | -.10 | .27 | .32 | .23 | -.21 | -.33 | .45* | -.09 | -.34 |
| E-Selectin | .16 | .13 | .49* | .10 | .18 | .37 | .36 | .14 | .04 | .28 | -.28 |
| sICAM-3 | .43* | .01 | .04 | .20 | .41 | -.08 | .27 | .26 | -.33 | .13 | .24 |
| TMD | .35 | .08 | .04 | .30 | .35 | .09 | .63** | -.04 | .01 | -.01 | -.05 |
| TIMP-1 | .01 | .20 | .31 | -.51 | -.46 | -.06 | .14 | -.07 | -.22 | -.27 | .41 |
| VEGF | .35 | .49* | .33 | -.11 | .08 | -.05 | -.23 | .17 | .14 | .15 | -.08 |
| bFGF | .08 | .05 | .28 | -.03 | .10 | .07 | -.13 | .03 | -.05 | -.02 | -.05 |
| VEGF-D | -.21 | .08 | .14 | -.26 | -.24 | -.37 | -.13 | .19 | -.18 | .07 | .35 |

Table 46: Correlation matrix between cytokines and baseline metabolic variables. * denotes $p<0.05$ and ** denotes $p<0.01$.

| | MAGE | CONGA | AUC | MeanBG | StdevBG |
|---|---|---|---|---|---|
| IL-6 | -.09 | -.09 | -.20 | -.13 | .01 |
| TNF-alpha | .05 | .21 | .33 | .19 | .12 |
| CRP | -.21 | .12 | .06 | .11 | -.01 |
| INF-gamma | -.07 | .11 | .06 | .12 | .04 |
| IP-10 | .18 | .40 | .41 | .43 | .17 |
| P-Selectin | .30 | .02 | .07 | .05 | .09 |
| sVCAM-1 | .17 | .43* | .23 | .37 | .41 |
| sICAM-1 | -.22 | .17 | .04 | .13 | .05 |
| E-Selectin | .16 | -.03 | .08 | -.01 | .08 |
| sICAM-3 | .07 | .09 | .25 | .11 | .00 |
| TMD | .21 | .11 | .27 | .13 | .09 |
| TIMP-1 | -.16 | -.39 | -.26 | -.35 | -.46* |
| VEGF | .07 | .05 | .00 | .04 | .13 |
| bFGF | .16 | .22 | .24 | .23 | .26 |
| VEGF-D | -.02 | .01 | .09 | .05 | .02 |

Table 47: Correlation matrix between cytokines and glucose variability. * denotes $p<0.05$ and ** denotes $p<0.01$.

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