Dissecting the role of apolipoprotein CIII in diabetic wound healing

Cho Mar Myint Wai

2019

Cho Mar Myint Wai. (2019). Dissecting the role of apolipoprotein CIII in diabetic wound healing. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/90276>

<https://doi.org/10.32657/10220/48515>

**DISSECTING THE ROLE OF APOLIPOPROTEIN CIII
IN DIABETIC WOUND HEALING**

CHO MAR MYINT WAI

**Interdisciplinary Graduate School
NTU Institute for Health Technologies**

A thesis submitted to the Nanyang Technological University in partial fulfillment
of the requirement for the degree of
Doctor of Philosophy

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research and has not been submitted for a higher degree to any other University or Institution.

25 April 2019

.....

Date

A handwritten signature in black ink, appearing to read 'Cho Mar Myint Wai', is displayed within a light gray rectangular box.

.....

Cho Mar Myint Wai

Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

25 April 2019

.....

Date

.....

Yusuf Ali

Authorship Attribution Statement

This thesis **does not** contain any materials from papers published in peer-reviewed journals or from papers accepted at conferences in which I am listed as an author.

25 April 2019

.....

Date

A handwritten signature in black ink, appearing to read 'Cho Mar Myint Wai', is displayed within a light gray rectangular box.

.....

Cho Mar Myint Wai

Table of Contents

ABBREVIATION

ABSTRACT

1. INTRODUCTION.....	17
1.1 Process of wound healing	17
1.1.1 Anatomy and physiology of the skin	17
1.1.2 Mechanisms of normal cutaneous wound healing	19
1.2 Dysregulated wound healing in diabetes	24
1.2.1 Diabetes mellitus and its complications	24
1.2.2 Pathology of chronic and diabetic wounds	25
1.2.3 Current treatments and management of chronic wounds	29
1.2.4 State of the art adjunctive therapies for managing chronic wounds	30
1.2.4.1 Use of skin substitutes in wound therapy	31
1.2.4.2 Hyperbaric oxygen therapy	32
1.2.4.3 Negative pressure wound therapy	32
1.2.4.4 Growth factors as wound therapy	33
1.2.4.5 Electrical therapy and shock wound therapy	34
1.2.4.6 Future wound therapies	34

1.3 Importance of Lipoproteins in physiology	36
1.3.1 Apolipoproteins	39
1.4 Pleiotropic effects of Apolipoproteins CIII (APOCIII) in health and disease	40
1.4.1. Regulation of ApoCIII expression	41
1.4.2. Role of ApoCIII in Lipoprotein metabolism	44
1.4.3 Role of ApoCIII in Atherosclerosis and Inflammation	45
1.4.4 Role of ApoCIII in Cardiovascular Diseases	45
1.4.5 Role of ApoCIII in diabetes	47
1.4.6 Current therapies modulating ApoCIII plasma level	50
1.5 Modulating levels of circulating ApoCIII	51
1.5.1 Antisense oligonucleotides to lower ApoCIII	51
1.5.2 ASO-mediated lowering of ApoCIII levels	52
1.6 Hypothesis and Aims	55
2. MATERIALS AND METHODS	56
2.1 Determining APOCIII levels in human serum.....	56
2.1.1 Information on human serum samples obtained	56
2.1.2 Measurements of human serum APOCIII.....	57
2.1.3 Data Analysis	58

2.2 Determining the effect of glucose on apolipoprotein expression in skin cell lines.....	59
2.2.1 Cell culture	59
2.2.2.Treatment of cells with differing concentration of D-glucose	60
2.2.3 Treatment of cells with human purified APOCIII	61
2.2.4 RNA isolation from cells	61
2.2.5 Reverse Transcription	62
2.2.6 Quantitative real-time PCR (qPCR)	62
2.3 Determining effect of human purified APOCIII on cell migration <i>in vitro</i>	66
2.3.1 <i>In vitro</i> scratch wound assay	66
2.3.2 Image acquisition and data analysis.....	67
2.3.3 Modified scratch assay for PCR array	68
2.3.4 RT ² Profiler PCR Array	69
2.4 Determining ApoCIII expression in murine skin.....	70
2.4.1 Animal work	70
2.4.2 Glucose tolerance test	70
2.4.3 Harvesting of skin tissue	71
2.4.4 RNA isolation from skin	71
2.4.5 qPCR analysis of skin tissue	72

2.4.6 Histological analysis	73
2.4.7 Deparaffinization and rehydration	73
2.4.8 Antigen Retrieval	74
2.4.9 Immunohistochemistry	75
2.5 Determining APOCIII expression in mouse wounds	76
2.5.1 Mouse excisional wound model	76
2.5.2 Collection of wound tissue	78
2.6 ApoCIII-ASO treatment and assessment of wound closure in mouse splinted excisional wound model	79
2.6.1 Mouse splinted excisional wound model	79
2.6.2 Pluronic F-127 gel preparation	80
2.6.3 Splinting rings preparation	80
2.6.4 Mouse specific ApoCIII-ASO	81
2.6.5 Topical ASO delivery	81
2.6.6 Monitoring wound closure	82
2.6.6 Wound tissue harvesting	82
2.6.8 Hematoxylin and eosin staining	83

2.6.9 Wound closure analysis	83
2.7 Determining ApoCIII and Toll-like receptor expression in islets.....	84
2.7.1 Pancreas tissue preparation	84
2.7.2 Immunohistochemistry of pancreas tissue	84
2.7.3 Expression of TLRs mRNA in mouse skin.....	86
2.8 Statistical analysis	86
3. RESULTS	88
3.1 Serum APOCIII concentration in non-diabetic and diabetic patients	88
3.1.1 Introduction.....	88
3.1.2 Results.....	91
3.1.3 Discussion.....	97
3.1.3 Limitations and Suggested Improvements	100
3.2 Effect of APOCIII on skin cells <i>in vitro</i>	102
3.2.1 Introduction.....	102
3.2.2 Effect of glucose on apolipoprotein gene expression	104
3.2.3 Effect of exogenous APOCIII on gene expression of inflammatory cytokines	107

3.2.4 Effect of APOCIII on keratinocyte migration <i>in vitro</i>	108
3.2.5 Analysis of wound healing-related genes	113
3.2.6 Discussion	118
3.2.6 Limitations and Suggested Improvements	121
3.3 Determining APOCIII expression and its role in skin and wounds of wild-type and diabetic mice	123
3.3.1 Introduction.....	123
3.3.2 APOCIII expression in intact skin of wild-type and diabetic mice	126
3.3.3 Expression of ApoCIII in wild-type mouse wounds	131
3.3.4 Expression of ApoCIII in diabetic mouse wounds	140
3.3.5 Accelerated wound healing in wild-type mouse with ApoCIII-ASO treatment	147
3.3.6 Effect of topical application of ApoCIII-ASO on wound healing in diabetic mice.....	154
3.3.7 Discussion	159
3.3.8 Limitations and Suggested Improvements	162
3.4 Expression pattern of TLRs and ApoCIII	163
3.4.1 Introduction.....	163
3.4.2 Expression of APOCIII in mouse pancreatic islets.....	167

3.4.3 Expression of TLRs in pancreatic islets of wild-type and diabetic mice	171
3.4.4 Expression of TLRs in the skin of wild-type and diabetic mice	183
3.4.5 Discussion	186
3.4.6 Limitations and Suggested Improvements	188
4. GENERAL DISCUSSION	190
5. CONCLUSIONS	194
6. FUTURE PERSPECTIVES	196
7. ACKNOWLEDGMENT	199
8. REFERENCES	200

ABBREVIATIONS

Apo	Apolipoprotein
ASO	Antisense Oligonucleotide
IPGTT	Intraperitoneal Glucose Tolerance Test
CAC	Coronary Artery Calcium
CCL	C-C motif Chemokine Ligand
CTGF	Connective Tissue Growth Factor
CTSK	Cathepsin K
COL4A3	Collagen Type IV Alpha 3
CXCL	C-X-C motif Ligand
ChREBP	Carbohydrate Response Element-Binding Protein
CVD	Cardiovascular Diseases
DM	Diabetes Mellitus
DFU	Diabetic foot ulcer
EC	Endothelial Cell
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
FCS	Familial Chylomicronemia Syndrome
FGF	Fibroblast Growth Factor

FoxO1	Forkhead box O1
FXR	Farnesoid X Receptor
GF	Growth Factor
GWAS	Genome-wide Association Study
HDL	High-Density Lipoprotein
HL	Hepatic Lipase
HTG	Hypertriglyceridemia
IDL	Intermediate-Density Lipoprotein
LOF	Loss-of-function
LPL	Low Density Lipoprotein
LDLR	Low-Density Lipoprotein Receptor
MMP	Matrix Metalloprotease
MOE	Methoxyethyl
mRNA	Messenger Ribonucleic Acid
NF- κ B	Nuclear Factor Kappa B
PPAR	Peroxisome Proliferator-Activated Receptor
PDGF	Platelet Derived Growth Factor
T1D	Type-1 Diabetes
T2D	Type-2 Diabetes

TLR	Toll-like Receptor
TG	Triglycerides
TGF- α	Transforming Growth Factor α
TNF- α	Tumor Necrosis Factor α
TRL	Triglyceride-rich lipoprotein
TLR	Toll-like Recptors
VCAM-1	Vasuclar Cell Adhesion Molecule-1
VEGF	Vascular Endothelial Growth Factor

ABSTRACT

The overarching aim of this thesis is to determine whether ApoCIII plays any role in diabetic wounds. We first determined ApoCIII levels in Asian diabetic serum and compared to our values to Caucasian counterparts. We next studied the role of ApoCIII in the skin and finally our mechanistic pursuit led us to study its putative binding partner (i.e. Toll-Like Receptors) in the pancreatic islet, the latter being an mini-organ that functionally responds to exogenously added ApoCIII. For our first aim, we determined APOCIII levels in a Singapore-based cohort of healthy, type-1 and type-2 diabetics (obtained through courtesy of local biobanks) and found no significant difference in serum APOCIII levels. Our ApoCIII serum values for healthy individuals were different from published values leading us to speculate that for APOCIII determination fresh serum samples are perhaps required. Nonetheless, as elevated APOCIII levels were reported in diabetics, and as the role of lipoproteins in the skin remains relatively unknown, we decided to determine the role of ApoCIII on wound healing. We sought to determine the role of ApoCIII during wound healing in healthy and diabetic states. We hypothesized that elevated levels of ApoCIII at the wound site delays wound healing and that this delay is exacerbated in diabetic wounds. In this study, we provided evidence that ApoCIII delays keratinocyte (HaCaT cells) migration *in vitro* through a yet undefined process that may involve changes in gene expression of pro-inflammatory cytokines and ECM depositing genes. ApoCIII was found to be expressed in the skin of healthy and diabetic mice. In addition, ApoCIII expression was observed to be elevated in the wounds and

its expression was highest around the wound edges. Given the increased levels of ApoCIII in wounds compared to intact skin and our *in vitro* observations that APOCIII hinders keratinocyte migration, we proceeded to knockdown ApoCIII at the wound site by topical application of ApoCIII-antisense oligonucleotide (ASO) at wound site. ApoCIII-ASO treatment significantly improved percent wound closure by 87.44% ($\pm 2.1\%$) compared to control wounds (74.13% $\pm 4.8\%$) in healthy mice at day 9 post-wounding. The observed higher rate of wound closure was corroborated with histology showing improved epithelialization and dermal collagen formation compared to control-treated wounds. The benefit of ApoCIII-ASO was also seen in the excisional wounds of type-2 diabetic mice (*db/db*). In diabetic mice, topical application of ApoCIII-ASO at the same dose and time as in healthy mice, significantly improved wound closure by 90.78 ($\pm 3.2\%$) at day 16 post wounding compared to adjacent control-treated wounds (70.54 $\pm 4.8\%$). Similar to healthy mice, histological examination also showed improved epithelialization and dermal collagen maturation in ApoCIII-ASO treated wounds. Taken together, the results here suggest that ApoCIII is expressed in the skin and that treatment with ApoCIII-ASO alters the wound healing process *in vivo*. This opens the possibility of targeting ApoCIII at the wound site to improve healing, with particular consideration for diabetic non-healing wounds.

1. Introduction

1.1 Process of wound healing

1.1.1 Anatomy and physiology of the skin

Skin is the largest organ of the human body and it forms a barrier that protects the internal organs from the external environment (Elias and Wakefield 2011). It has sensory functions related to touch, pressure, pain and temperature and regulates blood flow to maintain thermal homeostasis. The skin also forms part of the immune system containing components of innate as well as adaptive immunity. In addition, it can also be considered an endocrine organ because it is capable of generating essential hormones such as vitamin D and steroids (Zouboulis 2009).

The human skin consists of the epidermis (outermost), dermis and the innermost subcutaneous layer (Fig. 1.1). The epidermis is the outermost layer of the skin that is approximately 50-100µm thick and consists of 95% keratinocytes (Zaidi and Lanigan 2010). It also contains melanocytes, Langerhans cells (immune surveillance cells) and Merkel cells (mechanoreceptor cells). The epidermis has no blood vessels and therefore is entirely dependent on the underlying dermis for nutrients. The epidermis is made of several layers; from outermost stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum to the innermost stratum basale (Wickett and Visscher 2006). The epidermal cells are connected by different types of intercellular junctions

(tight junctions, desmosomes, hemi desmosomes and gap junctions) to form a barrier-like structure (Morita, Miyachi et al. 2011).

The dermis lies below the epidermis, is much thicker at around 1-2 mm with numerous capillaries. It is a fibrous layer that consists mostly of fibroblasts, macrophages and adipocytes. The dermis mostly consists of extracellular matrix (ECM) which is abundant in collagen, fibrin and elastin that are mainly secreted by fibroblasts in the dermis. They provide structure to the skin and are necessary for wound closure. The dermis also contains skin appendages such as hair follicles and various glands.

Figure 1.1 Illustration of the structure of skin tissue. (Retrieved from 'Human Anatomy Charts' <https://anatomybody101.com/>) (open source)

1.1.2. Mechanisms of normal cutaneous wound healing

Wound healing is a dynamic process and is highly regulated by cellular, humoral and molecular mechanisms. The process involves multiple cell types such as endothelial cells, blood platelets, immune cells, fibroblasts and keratinocytes. Changes in cell composition of the wound and its function during injury is coordinated by both autocrine and paracrine signaling of growth factors (GFs) and cytokines released by cells at and around the site of injury (Werner and Grose 2003,

Galkowska, Wojewodzka et al. 2006). Wound healing can be divided into four overlapping but distinct phases; (i) hemostasis, (ii) inflammation, (iii) new tissue formation and (iv) remodeling (Reinke and Sorg 2012) (Fig. 1.2).

The first stage of skin wound healing is hemostasis, which occurs immediately after injury and it begins with the aggregation of platelet and deposition of fibrin. Upon injury, platelets trigger the vasoconstriction to reduce blood loss and to fill the wound with blood clot. The blood clot contains fibrin molecules that physically crosslink to form a scaffold for infiltrating cells to the wound. Vasoconstriction is then followed by vasodilation which then allows the invasion of thrombocytes into the wound matrix (Robson, Steed et al. 2001). Aggregated platelets secrete cytokines, GFs such as transforming growth factor β (TGF- β), platelet derived growth factor (PDGF), epidermal growth factor (EGF), insulin-like growth factor (IGF) that are together responsible for activating fibroblasts, endothelial cells (ECs) and inflammatory cells required for wound healing (Broughton, Janis et al. 2006).

The inflammatory phase occurs simultaneously with hemostasis and can be further sub-divided into the 'early phase' with neutrophil recruitment and the 'late phase' with appearance of monocytes at wound site (Koh and DiPietro 2011). Neutrophils are first to infiltrate the wound site and they remove foreign material, bacteria and necrotic tissue by phagocytosis and protease secretion. They also release mediators such as tumor necrosis factor α (TNF- α) and interleukin 1 β (IL1 β) that amplify the inflammatory response (Gurtner, Werner et al. 2008). Approximately after

3 days, monocytes arrive at the site of injury, differentiate into macrophages and thereafter cleanse the wound by clearing it of biological debris. In addition, they secrete several GFs and mediators such as PDGF, TGF- β , transforming growth factor α (TGF- α), TNF- α , which orchestrate subsequent wound healing processes (Singer and Clark 1999).

The next phase of wound repair is the new tissue formation which involves re-epithelialization, angiogenesis and formation of granulation tissue. Initially, keratinocytes and fibroblasts at wound edges, activated by cytokines and GFs such as colony-stimulating factor 1 (CSF-1), PDGF and TNF- α , proliferate and migrate over the damaged region to cover the wound surface (Werner and Grose 2003). Cells become migratory by undergoing phenotypic changes by enzymatic loosening of epithelial junctions by collagenase and elastase and migrate along the chemotactic gradient of growth factors at wound site (Jacinto, Martinez-Arias et al. 2001). In addition, the ECM is broken down by matrix metalloproteases (MMPs) and plasminogen activators to pave way for the migrating cells. Finally, when the wound edges meet, the cell migration is halted by contact inhibition and by the formation of intercellular adhesion that closes the wound (Jacinto, Martinez-Arias et al. 2001). Angiogenesis occurs in concert with epidermal repair and is stimulated by multiple GFs including vascular endothelial growth factor (VEGF) fibroblast growth factor (FGF) and PDGF. Binding of GFs to ECs of existing vessels activates signaling cascade leading to ECs proliferation and migration into the wound (Reinke and Sorg 2012). The co-mix of newly formed blood vessels with cellular compounds consisting of a high density of fibroblast, granulocytes,

macrophages, capillaries and collagen bundles forms the granulation tissue (Gurtner, Werner et al. 2008). Fibroblasts produce collagen and ECM proteins such as fibronectin, glycosaminoglycans, proteoglycans and hyaluronic acid, forming the new ECM which act as scaffold in which cell migration and organization takes place (Jacinto, Martinez-Arias et al. 2001). Some fibroblasts are stimulated by macrophages to differentiate into myofibroblasts, contributing to wound contraction (Opalenik and Davidson 2005).

The final phase of wound healing process is remodeling or maturation phase, which may take weeks if not years, depending on the severity of the wound. The remodeling phase is characterized by the degradation of previously formed granulation tissues; ECs, macrophages and myofibroblasts (Greenhalgh 1998). Remodeling of acellular ECM is mediated by several MMPs, to replace the existing collagen III in the ECM with stronger collagen I, therefore strengthening the repaired tissue. By the end of this phase, the angiogenesis process diminishes and the acute wound metabolic activity stops. Although the wound is healed, its composition and properties will take time to fully regain the quality close to that of unwounded tissue (Enoch and Leaper 2008).

The process of wound healing are, by and large, well-conserved between species however the rate of healing may change depending on factors such as age, immunity levels and diseases, such as diabetes. Slow healing wounds in diabetes still remain a major clinical problem and this thesis is aimed at addressing that.

Figure 1.2. Phases of cutaneous wound healing: Hemostasis, inflammation, proliferation (granulation tissue formation) and remodeling. Image shows key molecular and cellular events involved in each phases. Abbreviation: PDGF, platelet-derived growth factor; TGF, transforming growth factor; FGFs, fibroblast growth factors; IL-1, interleukin-1; TNF, tumor necrosis factor; KGF, keratinocyte growth factor; IGF, insulin-like growth factor; IFN, interferon; VEGF, vascular endothelial growth factor; HGF, hepatocyte growth factor; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase. (Sun, Siprashvili et al. 2014)

1.2 Dysregulated wound healing in diabetes

1.2.1 Diabetes mellitus and its complications

Diabetes mellitus (DM) is a chronic metabolic disease characterized by hyperglycemia resulting from impairment in insulin action, insulin secretion or both. Diabetes significantly impacts the quality of life and its increasing prevalence, currently at 8.3% worldwide, makes it a burgeoning problem (Bommer, Heesemann et al. 2017). It is estimated to have a global cost of US\$ 1.3 trillion. In 2013, about 382 million people have diabetes and by 2035, this figure is expected to increase to 592 million (Guariguata, Whiting et al. 2014). In addition, according to international diabetes federation (IDF), this number is expected to rise above US\$ 640 million by 2040, which means that one in ten adults will have diabetes. According to IDF, the prevalence of diabetes in Singapore in 2017 was 13.7% and one in three adults, aged 65 and above, is diabetic (Phan, Alkema et al. 2014). In addition, Singapore spent over S\$ 1 billion in 2010 on managing diabetes and its associated complications and it is estimated that the cost could go up to \$2.5 billion annually by 2050 (Png, Yoong et al. 2016). Some factors that contribute to the rise in the number of diabetics in Singapore are an aging population and a generally more sedentary lifestyle.

There are a number of different types of diabetes but the two most common forms of DM are Type 1 diabetes (T1D), which is an autoimmune disease that stems from a lack of insulin production by the pancreas and Type 2 diabetes (T2D), which is a result of the body's ineffective use of insulin due to insulin resistance and/or insulin-producing β cell dysfunction. T2D is the most common

type which accounts for more than 90% of the diabetic population in both the world and in Singapore (Chatterjee, Khunti et al. 2017).

Poor control of diabetes results in various chronic complications including microvascular complications such as retinopathy, nephropathy and neuropathy and macrovascular complications such as heart diseases and stroke. One of the most serious, debilitating and common complications of DM is impaired wound healing (Brem and Tomic-Canic 2007). DM-related chronic wounds drastically affect quality of life and it imposes a significant medical and economic burden on both patients and the healthcare system.

1.2.2 Pathology of chronic and diabetic wounds

Chronic wounds are wounds that deviated from the natural physiology course of the healing process such that the wounds fail to resolve in a reasonable amount of time. The development of chronic wounds are influenced by intrinsic factors such as the immune cells, blood supply, metabolic status and previous injury. Extrinsic factors such as temperature, pressure and relative humidity can also influence the development of chronic wounds (Han and Ceilley 2017). It is estimated that almost 10% of world population will develop some form of a chronic wound over the course of a life time, with a wound-related mortality rate of 2.5% (Chatterjee 2012). Chronic wounds may often result in amputation after all available therapeutic interventions have been exhausted and it was reported that chronic wounds such as ulcers precede 85% of all amputations (Reiber, Bokyko et al. 1995).

Chronic wounds encompass more than just diabetic wounds. Other examples of chronic wounds besides diabetic wounds are bedsores. Bedsores are caused by intense or prolonged pressure on the skin whereas several factors (such as impaired GF production, collagen accumulation, quantity of granulation tissue, angiogenic response, macrophage function, keratinocyte and fibroblast migration, impairment in ECM components and MMPs, etc) contribute to wound healing deficiencies of diabetic wounds. In this thesis, we are interested specifically in diabetic wounds because ApoCIII has been reported to be significantly elevated in diabetics but its role during wound healing remains unknown. Diabetic wounds, are the most common and debilitating form of chronic wounds. In diabetic wounds, the healing process does not follow the timeline of classical wound healing and results in chronic non-healing wounds that are hindered in one or more of normal healing phases (Falanga 2005, Pradhan, Nabzdyk et al. 2009). Some of the factors affecting the diabetic wound healing are summarized below (Fig 1.3). Hitherto, out of all lipoproteins (introduced in the subsequent section) only ApoE has been studied in the wound healing context. ApoE deficient mice were shown to exhibit delayed wound healing (Hiebert, Wu et al. 2013, Gordts, Muthuramu et al. 2014). Apart from this report nothing much is known about the role of other apolipoproteins (e.g. ApoCIII) on wound healing.

In diabetic patients, a wide variety of factors such as hyperglycemia, chronic inflammation, autonomic and sensory neuropathy contribute to the impaired wound healing process, affecting several phases and nearly all the molecules involved in the process (Brem and Tomic-Canic 2007).

One of the main factors that leads to development of DFU is chronic diabetic neuropathy, which is characterized by progressive loss of peripheral nerve fibers due to hyperglycemia and decreased blood flow (Boulton, Malik et al. 2004, Vinik, Ullal et al. 2006). Approximately 50% of diabetic patients develop neuropathic symptoms within 10-20 years of diabetes diagnosis (M. and M. 2005).

Studies have reported that impaired healing associated with DFU is due to macrophage dysfunctions leading to prolonged inflammation and impaired expression of GFs and angiogenic factors resulting in deregulation of the neovascularization (Acosta, del Barco et al. 2008). In addition, aberrant proliferation and migration of epidermal cells (Bloomgarden 2008) as well as abnormal MMPs activities (Liu, Min et al. 2009) are associated with difficulties in diabetic wound healing. The systemic changes in characteristic of diabetes progression such as hyperglycemia, insulin resistance, obesity and subsequent microvascular or macrovascular complications have been associated with increased risk of non-healing wounds including DFU (Nunan, Harding et al. 2014). However, it is still not clear to what extent impaired healing is due to direct effects of these factors.

Regardless of etiology, one of the most debilitating complications of DM is impaired wound healing. DM-related chronic wounds impose a significant medical and economic burden on both patients and the healthcare system. DFU is the most common non-healing wound and is a leading cause of hospitalization in diabetic patients. DFU occurs in around 15% of all diabetic population

and therefore constitutes a major healthcare burden (Yazdanpanah, Nasiri et al. 2015). The cost of treating chronic wounds is estimated at up to \$25 billion annually in the US alone and treatment for a single diabetic ulcer cost nearly \$50,000 (Wellen and Hotamisligil 2005, Rice, Desai et al. 2014). Once DFU has developed, disease progression can ultimately lead to high risk of lower limb amputation and DFU precede 84% of all diabetes-related lower limb amputations (Reiber, Bokyko et al. 1995). In the US, there are more than 80,000 patients with DFU who undergo amputations each year (Zhang, Lu et al. 2017). In Singapore, there are there approximately 1500 lower limb amputations annually due to DM complications (Ang, Yap et al. 2016). In addition to high medical costs, non-healing wounds in diabetic patients significantly affect the patients' quality of life (Falanga 2005, Brem and Tomic-Canic 2007).

Figure 1.3. Summary of some factors that have been postulated to affect diabetic wound healing.

1.2.3 Current treatments and management of chronic wounds

Managing chronic wounds has always been a challenge in medical field. On top of standard wound care, several treatments that involve debridement, proper wound bed preparation with wound dressings, pressure off-loading, ensuring adequate revascularization and treatment of wound infection have been used for managing chronic wounds (Baltzis, Eleftheriadou et al. 2014). In addition general control of diabetic state such as good glycemic control, management of dyslipidemia and diet are also required in managing diabetic wounds such as DFUs (Steed, Attinger et al. 2006).

Wound care and dressings play important roles in chronic wound care. Debridement is the removal of necrotic tissue, hyperkeratotic epidermis (callus), foreign debris and bacteria from the wound bed using surgical, enzymatic or other methods to expose the healthy, well-perfused tissue (Gordon, Lebrun et al. 2012). The principle is to allow the proliferation and migration of healthy epithelial cells to wound bed in the place of necrotic tissue at wound site. In addition, debridement also allows the wound to better respond to topical treatments, thus facilitating healing (Tecilazich, Dinh et al. 2013). Many different types of wound dressings have been developed over past decades with ultimate aim to promote wound healing and to protect wound from infections (Moura, Dias et al. 2013). An ideal wound dressing should provide a moist environment, remove wound exudates, promote healing and protect wound from infection. Tegaderm, a semi-occlusive film, is a basic type of wound dressing that allows diffusion of air and water vapor while restricting

exudate and microbe penetration (Madeo, R. Martin et al. 1998). Hydrocolloids, hydrogels, alginate-based dressings and foams with different characteristics such as absorptive capability, durability, and permeability have been developed (Hilton, Williams et al. 2004, Jeffcoate, Price et al. 2004, Fonder, Lazarus et al. 2008). Currently, there is no research evidence of one type of dressing being the most effective in healing DFUs due to the different characteristics of wound at different stages of wound healing. Recent advances have focused on integrating therapeutic substances into the wound dressings (Moura, Dias et al. 2013). Another crucial factor for successful wound healing is pressure off-loading (Snyder, Frykberg et al. 2014). Several techniques are used to redistribute or reduce the pressure such as pressure-relieving braces and casts, modified surgical footwear and various foam dressings (Cavanagh and Bus 2010, Lewis and Lipp 2013). Another common treatment that involves surgery is revascularization since most chronic ulcers are associated with vascular insufficiency (Boulton, Armstrong et al. 2008).

1.2.4 State of the art adjunctive therapies for managing chronic wounds

Advanced wound therapies have been developed and can be used with the standard basic care as mentioned before. Options include skin substitutes, hyperbaric oxygen, negative pressure wound therapy, growth factors, electrical stimulation and shockwave therapy. However, there is contradicting or little published evidence and clinical trials to justify the use of these recent therapies.

1.2.4.1 Use of skin substitutes in wound therapy

To date, there have been two FDA approved bioengineered cellular skin substitutes; Dermagraft and Apligraf, and one acellular skin substitute; Omnigraft that has been approved by FDA for the treatment of DFUs. Dermagraft is an allogenic, human dermal fibroblast derived from neonatal foreskin tissue grown on bioabsorbable polyglactin mesh (Marston 2004). Fibroblasts in this scaffold generate ECM components such as glycosaminoglycans and collagen as well as growth factors and cytokines necessary for wound healing process. Apligraf is a bilayered culture skin equivalent composed of cultured dermis and sequentially cultured epidermis, derived from human neonatal foreskin (Newton, Khan et al. 2002). It is made up of viable dermal fibroblasts, epidermal keratinocytes, ECM and a stratum corneum mimicking similar environment of a normal healing wound. The keratinocytes form the epidermis and together with the underlying fibroblasts, produce growth factors to stimulate wound healing. ECM is made up of ECM components and bovine collagen. The stratum corneum protects against secondary infection or wound desiccation (Dinh and Veves 2006). Omnigraft is a bilayer acellular matrix consisting of collagen and chondroitin to assist in wound healing and silicone layer for protection and maintaining moist wound environment. It has been used for burn wounds since 20 years ago and it was recently approved for DFU treatment. Recent clinical trial showed that wound closure was greater (51%) in patients treated with Omnigraft compared to patients treated with standard care alone (32%) (Driver,

Lavery et al. 2015). Although widely used to promote wound healing, skin substitutes remain expensive and are thus not widely accessible to all segments of society.

1.2.4.2 Hyperbaric oxygen therapy

Hyperbaric oxygen therapy (FBOT) is exposure to pure oxygen at ambient pressure higher than atmospheric pressure in a chamber. It is based on the principle that it can stimulate angiogenesis, improve immune function enhance fibroblast proliferation and epithelialization, among other functions (Bhutani and Vishwanath 2012). However, the use of HBOT remain controversial due to conflicting reports of its efficacy in improving wound healing (Liu, Li et al. 2013, O'Reilly, Pasricha et al. 2013).

1.2.4.3 Negative pressure wound therapy

Negative pressure wound therapy (NPWT) or vacuum-assisted closures is the local application of subatmospheric pressure to the wound (Plikaitis and Molnar 2006). It is based on the principle that it helps remove exudates, reduce bacterial load, optimize blood flow whilst maintaining moist wound environment (Streubel, Stinner et al. 2012). Similar to HBOT, more research is required before the use of NPWT can become routine practice. To date there are studies with conflicting results on their wound healing efficacy and side effects (Isaac and Armstrong 2013).

1.2.4.4 Growth factors as wound therapy

Growth factors play a central role in many processes of wound healing and they have been of increased focus in the past few decades. Since chronic wounds contain many perturbations in growth factors and cytokines, addressing some of these molecules may be helpful in treatment of chronic wounds. It is known that chronic wounds have reduced levels of growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), transforming growth factor- β (TGF β) and vascular endothelial growth factor (VEGF). Chronic wounds also express lower levels of cytokines such as interleukin-1, IL-6, and tumor necrosis factor- α (TNF α) (Barrientos, Stojadinovic et al. 2008). Thus, over the past two decades, these molecules have been of target in wound healing research. Becaplermin, a gel based PDGF-BB, is the only FDA approved growth factor for the use of DFU treatment (Smiell, Wieman et al. 1999). Becaplermin has a biological activity similar to the endogenous PDGF-BB, the most common and potent PDGF isomer. PDGF is a chemoattractant and mitogen, exerting its actions on fibroblasts, smooth muscle cells and endothelial cells and has a pivotal role in all stages of wound healing (Nagai and Embil 2002, Papanas and Maltezos 2007). In conjunction with good ulcer care, daily treatment of becaplermin gel (100 $\mu\text{g/g}$) significantly increased the incidence of complete wound closure and significantly decreased the time to achieve complete closure of chronic diabetic neuropathic ulcers. It has been widely used for treatment of DFUs although prolonged use has increased risk of cancer mortality (Tecilazich, Dinh et al. 2013). Some of the

other growth factors mentioned before has also been used to stimulate wound healing process but no reported randomized clinical trials has confirmed the effectiveness of DFUs in wound healing (Galiano, Tepper et al. 2004, Tiaka, Papanas et al. 2011, Game, Hinchliffe et al. 2012).

1.2.4.5 Electrical therapy and shock therapy

Other therapies that have the potential to be used as adjunctive treatment for DFUs are pulsed electromagnetic field stimulation and shockwave therapy. Providing pulsed electrical stimulation to wounds seem to stimulate fibroblast and endothelial cells proliferation. It also reduced bacterial production and induce bactericidal effect by increasing neutrophils and macrophages migration (Thakral, LaFontaine et al. 2013). Shock therapy stimulate growth of new blood vessels by stimulating expression of angiogenesis-related growth factors, thus improved blood flow at wound site and increased cell proliferation and accelerate wound healing (Moretti, Notarnicola et al. 2009). However, more clinical evidence is needed to confirm the positive outcomes of these methods in DFU healing (Game, Hinchliffe et al. 2012).

1.2.4.6 Future therapies

Future therapies for DFUs, as well as other chronic wounds include stem cell therapies, gene therapies, angiotensin, substance P and cytokines. The use of bone marrow-derived stem cells have been examined for diabetic wound healing in animal and clinical studies with promising results (Humpert, Bartsch et al. 2005, Dubsky, Jirkovska et al. 2013, O'Loughlin, Kulkarni et al. 2013). Furthermore, delivery of growth factor encoding genes using adenoviral vectors has also been

proposed as potential therapy to wound treatment (Brem, Kodra et al. 2009). Studies have also shown that renin-angiotensin system which regulates blood pressure and fluid balance, plays an important role in the wound healing process (Steckelings, Henz et al. 2005). An angiotensin analog, NorLeu3-angiotensin (1-7) has been shown to be effective in treating DFUs and is now currently in phase III clinical trial (Balingit, Armstrong et al. 2012).

In addition, substance P, a neuropeptide which has a role in inflammatory disease, has been shown to improve wound closure in rats by increasing VEGF and TGFP expression (Kant, Gopal et al. 2013). Lastly, another approach is the activation and recruitment of anti-inflammatory macrophages to wound site to stall inflammatory phase and initiate cell proliferation and angiogenesis. Recently, it was shown that inhibiting a pro-inflammatory cytokine (IL-8) produced by activated macrophages promote the transition of macrophage from pro-inflammatory to anti-inflammatory phenotype, thus accelerates wound healing (Mirza, Fang et al. 2013). Despite emerging therapeutic targets and approaches for treatment of chronic and diabetic wounds, further studies are needed to evaluate the efficacy and safety and the translational relevance of these methods.

Treatment of chronic wounds has been a challenge due to the wounds' diverse aetiologies and pathological backgrounds, causing great difficulties in identifying the specific molecular mechanism behind the impaired wound healing. Multiple treatment modalities using cytokine replacement and transplantation of either keratinocytes or fibroblasts are effective in nondiabetic

populations, but their efficacy in patients with diabetes is diminished as a result of undetermined mechanisms (Laiva, O'Brien et al. 2018). There is a dire need to develop localized therapies for treating non-healing as well as slow healing wounds such as diabetic foot ulcers as the efficacies and/or application mode of the current approaches have not been sufficient to guarantee adequate DFU healing (Moura, Dias et al. 2013).

1.3 Importance of lipoproteins in physiology

As mentioned earlier, the role of lipoproteins in wound healing is not yet known except for delayed wound healing characteristic in ApoE^{-/-} mice (Gordts, Muthuramu et al. 2014). In this section, a general overview of lipoproteins and their importance in physiology will be given followed by a detailed introduction on its role in pathology especially on APOCIII and diabetes.

Lipids are naturally occurring hydrophobic biomolecules that store energy and provide fuel for metabolism (Rosen and Spiegelman 2006). They are structural components of cell membranes and play a vital role in cell signaling (Berg JM 2002, Wang 2004). Since lipids are insoluble in water or body fluids, lipids absorbed from the diet and synthesized by the liver and adipose tissues, are transported throughout the bloodstream by compartmentalizing them within lipid bilayer particles known as lipoproteins.

Lipoproteins are carrier proteins that transport insoluble hydrophobic lipids in blood or extracellular fluids (Fig. 1.4). The hydrophobic lipids are surrounded by a single layer of amphipathic phospholipids, cholesterol and apolipoproteins with the hydrophilic group facing

outward to the hydrophilic environment and hydrophobic portion facing inwards towards the hydrophobic core. Some integral apolipoproteins are embedded in the lipid layer and cannot be removed while peripheral apolipoproteins reside on the surface of lipoproteins and can be exchanged readily between different lipoproteins (Gaudet, Drouin-Chartier et al. 2017).

Plasma lipoproteins are classified into five major lipoproteins based on density, lipid content and size of particles: chylomicrons, very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), low density lipoprotein (LDL) and high density lipoprotein (HDL) (Semenkovich 2012). HDL or “good cholesterol” is formed in the periphery tissues and HDL aids in the reverse transport TG and cholesterol from peripheral tissues to the liver for removal (Feingold KR 2000). Lipoproteins are decorated on the exterior by apolipoproteins which play a role in regulating lipoprotein clearance.

Figure 1.4. Graphic representation of a generic lipoprotein. A nonpolar lipid core with cholesteryl ester and triglycerides sequestered by a monolayer of amphipathic phospholipids, free cholesterol and apolipoproteins. Integral apolipoprotein (e.g., ApoB) are embedded in the phospholipid layer and non-exchangeable. Peripheral apolipoprotein (e.g., ApoC) are exchangeable and can be freely transferred between different lipoproteins. All lipoproteins share these general features, but differ in size, lipid composition and the apolipoproteins bound. (Figure adapted from International Chair on Cardiometabolic Risk).

1.3.1 Apolipoprotein

Apolipoproteins are amphipathic proteins that interact with core lipids and the surrounding aqueous environment of the plasma (Mahley, Innerarity et al. 1984, Gangabadage, Zdunek et al. 2008, Semenkovich 2012). They generally serve as either structural component of lipoprotein particles, ligands for lipoprotein receptors, enzyme activators or enzyme inhibitors involved in lipoprotein metabolism. Apolipoproteins can be classified into 4 major groups: apolipoproteins (Apo-) A, B, C and E with their own sub-groups and 5 minor groups: Apo- D, F, G, H and J.

ApoAI, a predominant structural protein component of HDL, constitutes 70% of HDL protein (Murphy 2013). It is involved in reverse cholesterol transport (Bogan and Hennebold 2010) and ApoAI-HDL serum levels are inversely related to the predisposition of cardiovascular disease and atherosclerosis (Florvall, Basu et al. 2006, Camont, Chapman et al. 2011).

ApoB, a major structural component of VLDL, IDL and LDL, acts as a ligand for LDL receptor and is responsible for the uptake and clearance of lipoproteins from circulation (Twisk, Gillian-Daniel et al. 2000). ApoB-LDL plays a vital role in transporting cholesterol to peripheral tissues and the development of atherosclerosis (Boren and Williams 2016). The ratio of ApoA1/ApoB act as risk factors for cardiovascular events and coronary heart disease (Carnevale Schianca, Pedrazzoli et al. 2011, Lu, Lu et al. 2011).

ApoE is synthesized in multiple tissues including brain, adipose tissue, artery wall but mainly in liver and intestine (Mahley 2016). ApoE, a multifunctional protein, is associated with

neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and vascular dementia as well as multiple sclerosis, and ischemic stroke (Kim, Basak et al. 2009, Giau, Bagyinszky et al. 2015). ApoE mediates uptake of lipoprotein particles by binding to LDL receptor. Individuals with mutations in ApoE (homologous for ApoE2) develop familial dysbetalipoproteinemia, characterized by impaired lipid remnant clearance (Koopal, Marais et al. 2017). ApoE is the only apolipoprotein that has been linked to wound healing with a few studies showing delayed wound healing in ApoE^{-/-} mice (Hiebert, Wu et al. 2013, Gordts, Muthuramu et al. 2014). However, the direct effect of ApoE on wound healing is yet unknown. Among all the lipoproteins, an interesting lipoprotein that is gaining prominence especially in diabetes and cardiovascular disease is ApoCIII.

1.4 Pleiotropic effects of Apolipoprotein CIII in health and disease

Apolipoprotein CIII (ApoCIII) is first identified in 1969 as a regulator for triglyceride-rich lipoproteins (TRL) (such as LDL, VLDL and HDL) in the circulation (Brown, Levy et al. 1969). ApoCIII is a small 8.8 kDa O-linked glycoprotein composed of 6 amphipathic α -helices (Gangabadage, Zdunek et al. 2008). It is synthesized, mainly in the liver and to a lesser degree, in small intestine, as a 99-amino acid peptide that becomes a mature 79 amino-acid protein after removal of 20 amino-acid signal peptide in the endoplasmic reticulum (Ooi, Barrett et al. 2008). ApoCIII is mainly associated with chylomicrons, VLDL and to a lesser extent, LDL and HDL particles. ApoCIII has three isoforms: non-glycosylated isoform (ApoCIII₀) or glycosylated

isoforms containing one (ApoCIII₁) or two (ApoCIII₂) residues of sialic acid, contributing to approximately 10%, 55% and 35% respectively of total ApoCIII in circulation (Kashyap, Srivastava et al. 1981). All three isoforms have same plasma half-life and metabolism, suggesting similar functions (Mauger, Couture et al. 2006) and when β -cells were treated with ApoCIII, all three isoforms affected intracellular Ca^{2+} handling similarly (Juntti-Berggren, Refai et al. 2004). However it has been reported that the degree of sialylation of ApoCIII influences its function in LPL-mediated hydrolysis (Holdsworth, Stocks et al. 1982) although such studies have not been verified till date.

1.4.1 Regulation of ApoCIII expression

ApoCIII expression is downregulated by insulin, peroxisome proliferator-activated receptor α (PPAR α) and farnesoid X receptor (FXR) activation and nuclear factor- κ B (NF- κ B) (Fig 1.5) (Chen, Breslow et al. 1994, Staels, Vu-Dac et al. 1995, Claudel, Inoue et al. 2003). Nuclear transcription factor forkhead box O1 (FoxO1) induces ApoCIII expression by binding to its promotor region. Insulin, inactivates FoxO1 by exporting it out of the nucleus, thus repressing hepatic ApoCIII expression (Altomonte, Cong et al. 2004). Studies have shown that activating PPAR activity using fibrates represses ApoCIII expression *in vivo* (Peters, Hennuyer et al. 1997). In addition, repressing PGC-1 β , a coactivator of PPAR α , increases hepatic ApoCIII expression (Hernandez, Molusky et al. 2010). It was also shown that treatment with FXR agonist in mice reduced hepatic ApoCIII expression and decreased serum TG levels (Claudel, Inoue et al. 2003).

NF- κ B represses ApoCIII expression by attenuating HNF4a transactivation function through steric hindrance of HNF4a binding to the ApoCIII promoter (Nikolaidou-Neokosmidou, Zannis et al. 2006).

In contrast, ApoCIII expression is induced by insulin resistance, glucose and free fatty acids (Fig 1.5) (Gruber, Torres-Rosado et al. 1994, Altomonte, Cong et al. 2004, Pavlic, Valero et al. 2008, Caron, Verrijken et al. 2011). In the absence of insulin signaling, such as states of insulin resistance, FoxO1 nuclear localization increases and by interfering with the IRE1 element in the ApoCIII promoter, FoxO1 promotes ApoCIII expression. It was shown that in insulin-deficient and diabetic NOD and insulin-resistant *db/db* mice, FoxO1 expression was significantly increased compared to nondiabetic controls which contributes to increased liver and intestinal ApoCIII expression in these animals (Altomonte, Cong et al. 2004). Glucose activates transcription factors such as hepatocyte nuclear factor-4 α (HNF-4A) and carbohydrate response element-binding protein (ChREBP) which bind to promoter region of ApoCIII, thus inducing ApoCIII expression (Caron et al., 2011). In addition, increasing free fatty acids in plasma of healthy individuals resulted in increased triglyceride-rich ApoCIII production (Pavlic, Valero et al. 2008). This study used Intralipid/heparin (IH) infusion to raise plasma FFA concentration in human subjects and stable isotopes techniques to examine ApoCIII metabolism. ApoCIII production was significantly higher in the IH study compared with saline control study, indicating that acute elevation of plasma FFAs stimulated ApoCIII production. Furthermore,

high-fat diet with low saturated fatty acid and high monosaturated and omega-3 polyunsaturated fatty acids downregulates hepatic ApoCIII production by inhibiting FoxO1 expression (Chen, Chen et al. 2012, Kastelein, Maki et al. 2014).

Figure 1.5. Factors regulating ApoCIII expression. Glucose (Caron et al., 2011), fatty acid (Pavlic, Valero et al. 2008), insulin resistance (Altomonte, Cong et al. 2004) or activation of NF-κB (Gruber, Torres-Rosado et al. 1994) induces ApoCIII expression, whereas, insulin (Chen, Breslow et al. 1994) and activation of PPARα (Peters, Hennuyer et al. 1997) or FXR downregulate ApoCIII expression (Claudel, Inoue et al. 2003).

1.4.2 Role of ApoCIII in Lipoprotein Metabolism

ApoCIII regulates plasma TG levels through a variety of mechanisms. First, ApoCIII impairs the clearance of TRLs and their remnants by interfering with the binding of ApoB or ApoE to hepatic receptors (Jong, Hofker et al. 1999). Since ApoE mediates TRLs clearance from the circulation and ApoCIII inhibits the process by displacing ApoE from lipoprotein particles, the proportions of ApoCIII and ApoE regulate the metabolism of lipoproteins (Sacks 2015). In addition, human kinetic studies have provided evidence that ApoB-containing lipoproteins are removed from circulation significantly slower if they contain ApoCIII (Zheng, Khoo et al. 2010). Second, ApoCIII is a direct non-competitive inhibitor of lipoprotein lipase (LPL) which has an essential role in the lipolysis of TG in TRL in peripheral tissues (Ooi, Barrett et al. 2008). It was observed that ApoCIII null mice have increased LPL mediated TG hydrolysis and enhanced postprandial TG clearance (Jong, Rensen et al. 2001). A recent study found that ApoCIII inhibits LPL activity by displacing LPL from TRL, thus reducing lipolysis (Larsson, Vorrsjö et al. 2013). In addition, ApoCIII, at high concentrations inhibits hepatic lipase, a lipolytic enzyme secreted by the liver, thus further reducing TG hydrolysis (Ooi, Barrett et al. 2008). Lastly, ApoCIII affects lipoprotein production by directly promoting VLDL assembly and secretion in the liver (Sundaram, Zhong et al. 2010). Overexpression of ApoCIII increases TG-rich VLDL production (Yao 2012). The role of ApoCIII on TRL clearance and hypertriglyceridemia (HTG) is supported by the

hypertriglyceridemic state of ApoCIII transgenic mice (Qu, Perdomo et al. 2007) and by the hypotriglyceridemic state of mice deficient in ApoCIII (Hofker 2010).

1.4.3 Role of ApoCIII in Atherosclerosis and Inflammation

In addition to modulating lipid metabolism, ApoCIII induces vascular inflammation and may have a pro-atherogenic effect. It was seen in human population studies that patients with higher ApoCIII levels had a higher incident and severity of atherosclerosis than those with lower ApoCIII levels (Luc, Fievet et al. 1996, Sacks, Alaupovic et al. 2000). It was also supported by another study in ApoCIII transgenic and knockout mice (Li, Yu et al. 2015), demonstrating that ApoCIII could be a causative factor for atherogenesis. Studies have shown that ApoCIII exerts pro-atherogenic effects by increasing lipoprotein retention in the vessel wall (Hiukka, Stahlman et al. 2009) and by facilitating interaction between monocytes and vascular ECs (Kawakami, Aikawa et al. 2007, Zheng, Azcutia et al. 2013). Although these studies have shown the atherosclerotic effect of ApoCIII on vascular inflammation, the underlying molecular mechanism remains unclear.

1.4.4 Role of ApoCIII in Cardiovascular Diseases

As described previously, ApoCIII modulates plasma TG levels and it was identified that plasma TG, as well as plasma ApoCIII levels, are significant risk factors for cardiovascular diseases (CVD) (Sarwar, Danesh et al. 2007, Ooi, Barrett et al. 2008). Perhaps the best experiments elucidating the role of ApoCIII comes from nature where mutations within regulatory elements of ApoCIII leading to reduced expression were found to cause a phenotype.

The first to show the effect of reduced ApoCIII on human health arose from a genome-wide association study (GWAS) in the Lancaster Amish. A group of Amish individuals were shown to be carriers of a loss-of-function (LOF) mutation in an ApoCIII allele, resulting in these subjects having approximately half the normal circulating APOCIII levels as compared to non-carriers. Subjects with APOCIII mutation had 45% reduction in plasma TG and lower levels of LDL-cholesterol (LDL-C) and 22% increase in HDL-cholesterol levels (HDL-C) (Pollin, Damcott et al. 2008). In addition, coronary artery calcium (CAC) score, a measurement of subclinical atherosclerosis, was significantly lower in carriers of the ApoCIII-lowering mutation, which suggests that a lifelong reduction in ApoCIII probably confers some form of cardioprotection. Another natural occurring mutation is in a group of Ashkenazi Jewish centenarians and their offspring with a higher prevalence of one of ApoCIII alleles (-641C) (Atzmon, Rincon et al. 2006). Subjects with this genotype have lower serum ApoCIII levels, favorable lipoprotein profile, insulin sensitivity, lower risk of CVD and longevity compared to control.

Furthermore, the potential cardioprotective effect of lowering ApoCIII levels was supported by three larger population studies (Crawford, Dumitrescu et al. 2014, Crosby, Peloso et al. 2014, Jørgensen, Frikke-Schmidt et al. 2014). These studies showed that subjects with a LOF mutation in ApoCIII had lower TG levels, higher HDL-C levels and lower LDL-C levels in addition to lower risk of developing coronary heart disease.

In addition, studies have considered plasma content of ApoCIII and/or ApoCIII containing TRLs as a more reliable measure of CVD risk than conventional lipid markers (Chang, Lee et al. 2012, Jensen, Rimm et al. 2012, Sacks 2015). ApoCIII has been studied as a therapeutic target in treatment of CVD although the exact mechanism by which ApoCIII is associated to CVD remains unknown.

1.4.5 Role of ApoCIII in diabetes

Although ApoCIII had been extensively studied for its role in atherosclerosis and CVD, there have only been a handful of studies that provided evidence suggesting that ApoCIII may play a role in establishing insulin resistance and diabetes. A study conducted in Asian Indian men found that subjects with ApoCIII polymorphism exhibited 60% higher plasma APOCIII and TG levels, 46% lower plasma TG clearance and increased insulin resistance (Petersen, Dufour et al. 2010). In addition, ApoCIII may also play a direct role in pancreatic β -cell function. It was discovered that ApoCIII was responsible for calcium-dependent pancreatic β -cell apoptosis and the level of ApoCIII was increased in serum of T1D patient (Juntti-Berggren, Larsson et al. 1993, Juntti-Berggren, Refai et al. 2004). The increase in Ca^{2+} channel activity and β -cell apoptosis were ApoCIII-specific as neutralizing with anti-ApoCIII antibody abolished these effects. Although exact mechanisms are not known, it was demonstrated that ApoCIII activates Ca^{2+} channel through coactivation of protein kinase A and Src kinase pathways (Shi, Yang et al. 2014). In addition, it was shown that ApoCIII was directly involved in development of T1DM *in vivo* in diabetes-prone

bio-breeding (DPBB) rat model (Holmberg, Refai et al. 2011). The level of ApoCIII was higher in serum of DPBB compared to controls and lowering ApoCIII with antisense treatment significantly delayed the onset of T1D, thus providing a proof-of-concept for targeting ApoCIII in T1DM.

It is well-established that insulin resistance and β -cell failure occur in T2DM. It was recently shown that ApoCIII links insulin resistance and β -cell failure in T2DM (Avall, Ali et al. 2015). Our group and others found that insulin resistance in islet could promote local ApoCIII production which leads to local inflammation, irregular $[Ca^{2+}]$ handling and β -cell apoptosis. The study was conducted in ob/ob and high fat diet (HFD) -fed B57BL/6 (B6) mice, both characterized by high systemic serum APOCIII levels and impaired glucose tolerance. Avall and colleagues transplanted *ApoCIII* knockout islets to one eye of ob/ob and HFD-B6 mice and islets from ob/ob or B6 mice to the other eye respectively. This provided an environment where the *ApoCIII* knockout islets and normal islets coexisted in the same animal. All transplanted islets were exposed to high systemic level of APOCIII but the *ApoCIII* knockout islets could not produce APOCIII locally. Lack of local APOCIII production protected *ApoCIII* knockout islets from deranged inflammatory changes, increase in $[Ca^{2+}]$ and β -cell apoptosis. Thus, local APOCIII production in β -cell, induced by insulin resistance is an important diabetogenic factor for β -cell dysfunction (Avall, Ali et al. 2015). Furthermore, lowering systemic APOCIII with antisense oligonucleotide (ASO) treatment improved glucose tolerance. In a recent study with a small group of 15 T2D patients, treatment

with ApoCIII ASO reduced APOCIII and TG levels, as well as improved insulin sensitivity and glycemic control (Digenio, Dunbar et al. 2016). Recently, a prospective population based study in Rotterdam found that serum levels of ApoCIII, as well as ApoCIII-to-ApoA1 ratio are associated with a higher incidence of T2D, independent of known risk factors and are stronger biomarkers than HDL-C levels (Brahimaj, Ligthart et al. 2017). The involvements of ApoCIII in lipid metabolism, atherosclerosis, cardiovascular diseases and diabetes are summarized in Fig 1.6.

Figure 1.6. Pleiotropic effects of Apolipoprotein CIII (ApoCIII). Abbreviations: TRL, triglyceride-rich lipoprotein; LPL, lipoprotein lipase; HL, hepatic lipase; TG, triglyceride; VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; SMase, sphingomyelinase; EC, endothelial cells; CVD, cardiovascular disease. (Avall et al., 2015; Crawford et al., 2014; E. M. Ooi et al., 2008; Fogelstrand & Boren, 2012; Hiukka et al., 2009; Holmberg et al., 2011; Kawakami et al., 2007; Pollin et al., 2008; Sacks, 2015; Shi et al., 2014; Sundaram et al., 2010; Zheng et al., 2013)

1.4.6 Current therapies modulating ApoCIII plasma levels

Based on the apparent beneficial effects of lowering APOCIII, attempts are being made through pharmacological means to reduce APOCIII levels for improving HTG and CVD. Due to its association with HTG and potential development of CVD, ApoCIII has been a target of drugs to reduce plasma TG levels. There has been a number of lipid-lowering drugs that modulate ApoCIII levels. PPAR agonists such as fibrates (Hertz, Bishara-Shieban et al. 1995), pioglitazone (Nagashima, Lopez et al. 2005) and tesaglitazar (Fagerberg, Edwards et al. 2005), statins such as atorvastatin (Dallinga-Thie, Berk-Planken et al. 2004) and rosuvastatin (Ooi, Watts et al. 2008), ezetimibe (Kosoglou, Statkevich et al. 2004), niacin (Song and FitzGerald 2013), omega-3 polysaturated fatty acids ethyl esters (Kastelein, Maki et al. 2014) decrease plasma APOCIII and TG levels. However, all these pharmacological approaches decrease APOCIII plasma level by 10% to 30% (Norata, Tsimikas et al. 2015). A more robust reduction in APOCIII is necessary to achieve any clinical benefit as it was observed that a genetic mutation resulting in 50% plasma APOCIII reduction has cardioprotective effects (Crosby, Peloso et al. 2014). The high concentration of circulation APOCIII (10-20 mg/dl) and the intracellular action of APOCIII on VLDL secretion limits the use of antibody-based therapy against ApoCIII. A promising new approach is to use mRNA-silencing antisense strategies to lower ApoCIII synthesis.

1.5 Modulating levels of circulating ApoCIII

1.5.1 Antisense oligonucleotides to lower ApoCIII

Antisense oligonucleotides (ASOs) are short (typically 16-20 bases) chemically modified single-stranded DNA molecules that bind to their complementary mRNA sequence encoding specific proteins (Fig 1.7). Upon binding the target mRNA, the RNA/ASO duplex is recognized and degraded by endogenous nuclease RNaseH1, thus inhibiting target protein translation (Gouni-Berthold and Berthold 2011). Over the past 25 years, considerable progress has been made in the evolution of antisense technology to enhance stability, specificity and tolerability of these therapeutic agents (Lee, Crosby et al. 2013). The most advanced ASO currently in clinical trials is the second generation ASO with 2' methoxyethyl (MOE) modifications. The next generation ASO with increased potency, improved safety and efficacy is currently under development (Gaudet and Brisson 2015). It is based on ligand conjugation antisense technologies where ASO is conjugated to a high-affinity ligand for a hepatocyte-specific receptor thereby enhancing its potency in the liver. ASO agents are water-soluble and can be easily administered in saline via subcutaneous (s.c) injection and other routes of delivery, including intravenous, aerosol, oral and topical. While these drugs are widely distributed throughout the body, liver is the primary site of ASO uptake, thus making it an attractive choice as liver-targeting lipid lowering therapeutic. While there is only one FDA approved ASO that target ApoB (mipomersen) for the treatment of lipid disorder (Wong and Goldberg 2014), there is currently no available FDA approved ASO for lowering APOCIII.

Figure 1.7. Mechanism of action of ASO. Adapted from (Gouni-Berthold 2017)

1.5.2 ASO as a therapy to lower ApoCIII levels

Species-specific antisense ASO targeting ApoCIII has been evaluated in several animal strains, diets and disease models (Graham, Lee et al. 2013). This study demonstrated that antisense reduction of ApoCIII produced a robust reduction in serum ApoCIII and TG levels with a good tolerance towards the drug. In a phase I double-blind, placebo-controlled, dose-escalation clinical

study, volanesorsen (ISIS 304801 or ISIS-APOCIII_{Rx}), a second generation 2'MOE modified ASO targeting human ApoCIII, was given to healthy volunteers. It was shown to have dose-dependent and prolonged reduction in plasma concentrations of APOCIII and TG without serious adverse effects (Graham, Lee et al. 2013). There was also a trend for an increase in HDL-C levels, although this effect was not dose-dependent.

Phase II study of volanesorsen evaluated its pharmacodynamics effects on patients with familial chylomicronemia syndrome (FCS) (Gaudet, Brisson et al. 2014) and HTG (Gaudet, Alexander et al. 2015). FCS is a rare autosomal recessive disease caused by a mutation in gene encoding LPL or protein involved in LPL function. Patients with FCS have very high plasma chylomicrons, severe HTG, resulting in increased risk of pancreatitis and other complications (Brahm and Hegele 2015). Current treatment for these patients is very strict diet with TG-lowering medications, which are not very effective. LPL gene-replacement therapy, approved in Europe, has limited use due to transient TG-lowering effects and extremely high cost (Gaudet, Méthot et al. 2013). Gaudet and colleagues showed that in the three patients with FCS and high TG levels, administration of volanesorsen significantly reduced baseline plasma APOCIII levels by 71% - 90% and TG levels by 56% - 86%. This data suggested that inhibition of ApoCIII had a profound and clinically relevant effect on TG levels, through LPL-independent mechanism. Furthermore, in patients with HTG, treatment with volanesorsen resulted in dose-dependent reduction of plasma APOCIII and

TG concentrations, as well as increased in HDL-C levels (Gaudet, Alexander et al. 2015). In addition, no serious ASO treatment-associated adverse events were identified in both studies.

Currently, there are three ongoing Phase III trials of volanesorsen; the APPROACH (the APPROACH study: A study of ISIS 304801 in Patients with Familial Chylomicronemia Syndrome, NCT02211209), the COMPASS (the COMPASS study: A study of Volanesorsen in Patients with Hypertriglyceridemia, NCT02300233), and the BROADEN (the BROADEN study: A study of Volanesorsen in Patients with Partial Lipodystrophy, NCT02527343). The primary objective of these studies is to evaluate the efficacy of volanesorsen as compared to placebo on the percent change in fasting TG from baseline to 13 weeks. Although the results of these phase III studies have yet to be finalized, it does point to high possibility that ApoCIII ASO could be used as an officiating therapy to lower human ApoCIII.

1.6 Hypothesis and Aims

There is a need for expanding therapeutic approaches to effectively improve the treatment of chronic diabetic wounds especially with the increasing incidence of diabetes. Serum APOCIII is increased in diabetes and increased ApoCIII has been shown to provoke inflammatory responses in vascular ECs, monocytes and islets. It was recently reported that ApoCIII is present in the wound fluids collected after surgical trauma (Bekeschus, Lackmann et al. 2018). It is well established that wound healing is delayed in diabetes and excess and prolonged inflammation impairs diabetic wound healing (Guo and DiPietro, 2010). However, the role of ApoCIII in diabetic wound healing remains hitherto, unknown. The overarching hypothesis was that elevated levels of ApoCIII in the diabetic skin impedes wound healing and therefore lowering the levels of ApoCIII at wound site could improve diabetic wound healing.

The principal aim of this thesis was to explore the role of ApoCIII, a diabetogenic and pro-inflammatory serum protein as a therapeutic target for non-healing diabetic wounds.

The specific aims of this thesis were:

1. To determine if serum APOCIII is increased in diabetic Asian patients in Singapore, similar to what was reported in Caucasian patients
2. To investigate whether ApoCIII is expressed in skin and if there is a link between its expression and the delayed wound healing
3. To evaluate the therapeutic utility of lowering ApoCIII at the wound site
4. To determine putative receptors for ApoCIII

2. MATERIALS AND METHODS

2.1. Determining APOCIII levels in human serum

2.1.1 Information on human serum samples obtained

Anonymized serum samples were obtained from Singapore Bio-bank and Germany were obtained through Prof Boehm Bernhard Otto. Non-diabetic (control) (n=25), T1D (n=38) and T2D (n=47) serum samples were obtained from Singapore bio-bank. Non-diabetic controls (n=27), T1D (n=20) and T2D (n=10) were obtained from Germany. In addition, anonymized serum samples were obtained from KTPH (Study on profiles of metabolites in diabetes mellitus, hypertension and hypercholesterolemia). Non-diabetic (control) serum samples (n=33) and T2D serum samples (n=56) were obtained through Associate Professor Tavintharan Subramaniam as part of an earlier study (DSRB Ref: 2008/00431). Details of serum samples obtained is listed in Table 2.1.

Table 2.1. Classifications of serum samples. T1D, Type-1 diabetes; T2D, Type-2 diabetes; M, Male; F, Female; NA, not available; KTPH, Khoo Teck Puat Hospital.

Source	Category	Number	Sex	Age (Median)	BMI
Singapore Bio-bank	Non-diabetic	25	M	48.8 ± 1.0 (47)	NA
	T1D	38	M	50.6 ± 1.6 (50.5)	
	T2D	47	M	49.6 ± 0.9 (50)	
Germany	Non-diabetic	27	F	52.3 ± 1.2 (51)	NA
	T1D	20	F	55.9 ± 1.0 (54)	
	T2D	10	F	52.9 ± 0.8 (53)	
KTPH	Non-diabetic	33	M/F	37.3 ± 2.4 (35)	29.4 ± 1.6
	T2D	56	M/F	48.3 ± 1.7 (50.5)	29.0 ± 1.1

2.1.2 Measurements human serum APOCIII

APOCIII levels in the serum samples were determined using APOCIII human enzyme-linked immunosorbent assay (ELISA) kit (Assaypro, St Charles, MO, USA) or (Abnova Corp., Taipei, Taiwan) according to manufacturers' instructions. Briefly, the serum samples were first diluted 4000-fold in EIA® diluent buffer. Diluted serum samples along with human APOCIII standards (1.95 ng/ml to 500 ng/ml) were added to the 96-well plates pre-coated with polyclonal antibody specific for human APOCIII and incubated for 2 hours. The plate was washed five times with the Wash Buffer® and tapped dry. Biotinylated polyclonal antibody specific for human APOCIII was

then added to each well and incubated for 1 hour. After washing for three times with Wash Buffer, the APOCIII-antibody complex was recognized by adding streptavidin-peroxidase (SP) conjugate® solution to each well. After washing away all unbound materials, chromogen peroxidase enzyme substrate was added. The reaction was stopped after 15 minutes incubation in the dark. The absorbance was measured at 450 nm and 570 nm on a Synergy HTX microplate reader (BioTek, Winooski, VT, U.S.A) immediately. The absorbance readings at 570 nm were subtracted from those at 450 nm to correct for optical imperfections.

2.1.3 Data Analysis

The APOCIII serum concentration was determined from the four-parameter sigmoidal standard curve. Data was presented as median with 10th to 90th percentile range (10-90 percentiles box-whisker plot). The whiskers were drawn down to the 10th percentile and up to 90th percentile. Values below and above the whiskers (10% of the sample size) were drawn as individual points (black dots). Statistical analysis was determined using a two-tailed unpaired student's t-test for comparison between two groups. Comparison between more than two groups was performed using one-way analysis of variance (ANOVA) with Bonferroni's multiple comparisons test, where appropriate. To evaluate the associations between variables, correlation analysis was computed using Pearson correlation coefficient. All statistical analysis were done using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, U.S.A). Statistical significance is displayed as * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$ and the number of experiments (N) are stated in each figure legend.

2.2. Determining the effect of glucose on apolipoprotein expression in skin cell lines

2.2.1 Cell Culture

The mouse fibroblast cell line NIH/3T3 (3T3 cells) was obtained as a kind gift from Professor David Becker (LKCMedicine, NTU Singapore). 3T3 cells were maintained at 37 °C, 5% CO₂ and humidified environment and cultured in high glucose Dulbecoo's modified Eagle medium (DMEM; GIBCO, Dublin, Ireland) (4.5 g/L glucose) with sodium pyruvate (110 mg/L) and L-glutamine (584 mg/L), supplemented with 10% Fetal Bovine Serum (FBS) (GIBCO, Dublin, Ireland), 100 µg/ml streptomycin and 100 units/ml penicillin (pen/strep) (GIBCO, Dublin, Ireland). Cell cultures were passaged every three to four days, before reaching 90% confluency. Cells were rinsed with Phosphate Buffered Saline (PBS) and detached using a solution containing 0.05% trypsin and 0.02% ethylene diamine tetra-acetic acid (EDTA) (GIBCO, Dublin, Ireland). After 5 mins incubation at room temperature, the trypsin solution was neutralized by adding 10 ml of culture media. The cell suspension was collected in a 10 ml tube (Greiner bio-one, Kremsmünster, Austria) and subjected to a 1000 x g centrifugation for 3 minutes. The supernatant was carefully removed and cells were re-suspended in fresh pre-warmed cell culture media.

The human keratinocyte cell line HaCaT was obtained as a kind gift from Professor David Becker (LKCMedicine, NTU Singapore) and was maintained in 5% CO₂ chamber at 37°C in high glucose DMEM (Lonza, Basel, Switzerland) (4.5 g/L glucose) with L-glutamine (584 mg/L), supplemented with 10% FBS and pen/strep. It is important to note that HaCaT keratinocytes,

similar to many other cell lines, are maintained in media that contains a high concentration of glucose, higher than what is typically found in diabetic individuals, so that these cells proliferate optimally. Cells were trypsinized using 0.25% trypsin-EDTA (GIBCO, Dublin, Ireland) and sub-cultured every two to three days at 90% confluency. All reagents, unless otherwise stated, were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.).

2.2.2 Treatment of cells with differing concentrations of D-glucose

3T3 cells, and separately HaCaT cells, were seeded into 12-well plates (Corning Inc, Corning, NY, U.S.A.) at the density of 300,000 cells per well, two days before the start of treatment. Prior to treatment, cells were moved from the high glucose containing cell culture media (25 mmol/ L; 4.5g/ L D-glucose) to a culture media containing 1.5mmol/ L (0.27 g/ L D-glucose) for 8 hours. After incubation in low glucose media cells were then treated by adding the respective culture media containing either low glucose (1.5 mmol/ L), normal glucose (6 mmol/ L) or high glucose (25 mmol/ L) for 24 hours. Cells were washed twice in ice-cold PBS and cell lysate was collected in RLTplus® lysis buffer (Qiagen, Hilden, Germany) with β -mercaptoethanol (GIBCO, Dublin, Ireland) before scraping and transferring to an 1.5 mL tube (Eppendorf, Hamburg, Germany) for storage at -80°C. When ready, tubes were removed from freezer and thawed on ice for RNA extraction and immediate cDNA conversion.

2.2.3 Treatment of cells with human purified APOCIII

3T3 or HaCaT cells were treated with human APOCIII purified from plasma (Athens Research & Technology, Athens, GA). Human ApoCIII was dissolved in 10 mmol/ L ammonium bicarbonate, pH 7.4 (Athens Research & Technology, Athens, GA). Cells were treated with either 0 µg/ mL (diluent control), 30 µg/ mL, 50 µg/ mL or 100 µg/ mL of APOCIII for 24 hours prior to cell lysis. Cells were washed twice in ice-cold PBS and cell lysate was collected in RLTplus® lysis buffer (Qiagen, Hilden, Germany) with β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, U.S.A.) before scraping and transferring to an 1.5 mL microtube (Corning Inc., NY, USA) for storage at -80°C (Thermo Fisher Scientific, Waltham, MA, U.S.A.). When ready, tubes were removed from freezer and thawed on ice for RNA extraction and immediate cDNA conversion.

2.2.4 RNA isolation from cells

Cell lysates in RLTplus® lysis buffer were allowed to thaw on ice. Instruments and surfaces were swabbed with RNase Away (Molecular BioProducts, Inc., San Diego, CA, USA) before commencement of RNA isolation. Total RNA was purified from cell lysates using the RNeasy Plus Mini Kit® (Qiagen, Hilden, Germany), according to the manufacturers' protocol. Briefly, cell lysates were disrupted by vortexing for 30 seconds. The lysates were then homogenized using QIAshredder® homogenizers (Qiagen, Hilden, Germany) for 2 minutes at 10,000 x g. The homogenized lysate was then passed through gDNA eliminator spin columns® (Qiagen, Hilden, Germany) to eliminate the contaminating genomic DNA. The collected flow-through was then

mixed with an equal volume of 70% ethanol (vol/ vol in RNase-free water) and transferred to RNeasy spin columns for RNA purification. After repeating washing, all column-bound RNA was eluted with RNase-free water in sterile 1.5ml tubes (Corning Inc., NY, USA) and the quantity and purity of RNA was assessed using a NanoDrop 2000 Spectrophotometer (Thermo Fisher, Waltham, MA, U.S.A.).

2.2.5 Reverse Transcription

Single-stranded cDNA was synthesized from 1 µg of total RNA purified from cell lysates using reverse transcription using High Capacity cDNA Reverse Transcription Kit® (Applied Biosystems, Foster City, CA, U.S.A.). Reaction mixtures of 20 µL were incubated for 10 minutes at 25 °C followed by 120 minutes at 37 °C. Reaction was terminated by heating at 85 °C for 5 minutes. cDNA was stored in 4 °C and subsequently used for real-time quantitative PCR (qPCR).

2.2.6 Quantitative real-time PCR (qPCR)

qPCR was performed on cDNA samples thawed at room temperature according to recommended instructions (Applied Biosystems, Foster City, CA, U.S.A.). Gene expression levels were measured by qPCR using Fast SYBR Green Master Mix (2X)® (Applied Biosystems, Foster City, CA, U.S.A.), with 1 µmol/ L forward primer and 1 µmol/ L reverse primer, on a QuantStudio 6 Flex thermocycler instrument (Applied Biosystems, Foster City, CA, U.S.A.). Gene-specific intron-spanning primers were designed for the RT-PCR using either Primer3Plus (NCBI, Bethesda, MD, U.S.A.) or the universal primer library (Roche Diagnostics, Risch-Rotkreuz,

Switzerland) and synthesized in HyPure™ Molecular Biology Grade, nuclease-free water (Thermo Fisher Scientific, Waltham, MA, U.S.A.). Only primers that produced single peak on PCR melt curve were used. Genes analyzed by RT-PCR and their respective primer sequences used are listed in Table 2.2 and 2.3. Thermocycler was set up for initial denaturation at 94°C for 2 minutes, followed by 40 cycles of 95°C for 5 seconds, 59 °C for 15 seconds and 72 °C for 10 minutes. Relative gene expressions were obtained by first normalizing each gene Ct to that of the housekeeping gene Ct, β -actin, to obtain Δ Ct, followed by a normalization to that of control (i.e. 6 mmol/ L glucose or 0 μ g/ mL APOCIII) to obtain $\Delta\Delta$ Ct which equates to the relative gene expression change following the different treatments.

Table 2.2. Primer sequences of mouse genes used to measure gene expression in mouse 3T3 fibroblast.

Gene	Accession No.	Mouse Sequences (5' → 3')
<i>ApoA1</i>	NM_009692.4	Forward: CGGCAGAGACTATGTGTCC Reverse: CAGTTTTCCAGGAGATTCAGGT
<i>ApoA2</i>	NM_001305549.1	Forward: CACAGAATCGCAGCACTGTT Reverse: CGTCTGCCTGTCTCTTAACCA
<i>ApoB</i>	NM_009693.2	Forward: TTGAAGTGTCCAGCCCCATC Reverse R: CAGCCACTGGAGGATGTGAG
<i>ApoC1</i>	NM_001110009.2	Forward: AGAGATCCTTAGATCCAGGGTG Reverse: TGGCTACGACCACAATCAGG
<i>ApoC2</i>	NM_001277944.1	Forward: TCAGCATGGATGAGAACTCAG Reverse: CAGCTACTCTCCCCTCAGGA
<i>ApoC3</i>	NM_001289755.1	Forward: CTTGGCTCTCCTGGCATCT Reverse: GAGGCTTGTTCCATGTAGCC
<i>ApoE</i>	NM_001305819.1	Forward: AGACCCTGGAGGCTAAGGAC Reverse: AGAGCCTTCATCTTCGCAAT
<i>β-actin</i>	NM_007393.5	Forward: TGTGATGGTGGGAATGGGTCAG Reverse: TTTGATGTCACGCACGATTTCC
<i>Actb</i>		
<i>Ccl-2/</i>	NM_011333.3	Forward: CATCCACGTGTTGGCTCA Reverse: GATCATCTTGCTGGTGAATGAGT
<i>MCP-1</i>		
<i>IL-1β</i>	NM_008361.4	Forward: CAGGCAGGCAGTATCACTCA Reverse: GTCACAGAGGATGGGCTCTT
<i>IL-6</i>	NM_001314054.1	Forward: GCTACCAAAGTGGATATAATCAGGA Reverse: CCAGGTAGCTATGGTACTCCAGAA
<i>IL-18</i>	NM_001357221.1	Forward: CATGTACAAAGACAGTGAAGTAAGAGG Reverse: TTTCAGGTGGATCCATTTCC
<i>TNF-α</i>	NM_001278601.1	Forward: GGTCCCCAAAGGGATGAG Reverse: TGGGCTACAGGCTTGTCACT

Table 2.3. Primer sequences of human genes used to measure gene expression in human HaCaT keratinocytes.

Gene	Accession No.	Human Sequences (5' → 3')
<i>APOA1</i>	NM_000039.2	Forward: CTTGGGAAAACAGCTAAACC Reverse: CCAGAACTCCTGGGTCACA
<i>APOA2</i>	NM_001643.1	Forward: GAGAAGGTCAAGAGCCCAGA Reverse: CCTTCTTGATCAGGGGTGTC
<i>APOB</i>	NM_000384.2	Forward: GACGACTTTTCTAAATGGAAGTTCTAC Reverse: CTCAGTTTTGAATATGGTGAGTTTTT
<i>APOC1</i>	NM_001321065.1	Forward: GCCTTGGATAAGCTGAAGGA Reverse: GAAATGTCTCTGAAAACCACTCC
<i>APOC2</i>	NM_000483.4	Forward: GGGACTTGTACAGCAAAAGCA Reverse: TGGTCAGTAAAAATGCCTGTGT
<i>APOC3</i>	NM_000040.2	Forward: GCCAAGGATGCACTGAGC Reverse: GAACTGAAGCCATCGGTCAC
<i>APOE</i>	NM_000041.3	Forward: GGTCGCTTTTGGGATTACCT Reverse: CATGGTCTCGTCCATCAGC
<i>β-actin</i>	NM_001101.4	Forward: CCAACCGCGAGAAGATGA Reverse: CCAGAGGCGTACAGGGATAG
<i>ACTB</i>		
<i>CCL-2/</i>	NM_002982.3	Forward: AGTCTCTGCCGCCCTTCT Reverse: GTGACTGGGGCATTGATTG
<i>MCP-1</i>		
<i>IL-1β</i>	NM_000576.2	Forward: AAATACCTGTGGCCTTGGGC Reverse: TTTGGGATCTACACTCTCCAGCT
<i>IL-6</i>	NM_000600.4	Forward: GTAGCCGCCCCACACAGA Reverse: CATGTCTCCTTTCTCAGGGCTG
<i>IL-18</i>	NM_001243211.1	Forward: CAACAAACTATTTGTCGCAGGA Reverse: CAAAGTAATCTGATTCCAGGTTTTTC
<i>TNF-α</i>	NM_000594.3	Forward: CCCAGGGACCTCTCTCTAATCA Reverse: GCTTGAGGGTTTGCTACAACATG

2.3. Determining effect of human purified APOCIII on cell migration *in vitro*

2.3.1 *In vitro* scratch wound assay

HaCaT keratinocyte cells were seeded onto 24-well plates at a density of 300,000 cells per well in culture media. Cells were allowed to attach, spread and form a confluent monolayer for 48 hours. For the 24-hour pre-treatment with APOCIII, culture media was replaced with media containing varying concentrations of APOCIII just 24 hours after cells were seeded. A scratch wound assay was performed by using a sterile 200 μ l pipette tip to scrape the cells in a straight line at the center from the top to the bottom edge, creating a cell-free zone or 'scratch wound' into which cells at the wound edge can migrate into (Fig. 2.1A). Extra caution was taken to create scratches with similar size (and width) to minimize variation caused by the difference in the size of the wound. Following the scratch, the wells were washed twice with pre-warmed culture media to remove all cell debris before incubation with medium containing either ammonium bicarbonate (for control wells) or varying concentrations of purified human APOCIII. The cell migration was monitored and images captured at regular time intervals as described hereinafter.

Exogenous human APOCIII was prepared in culture media at concentrations of 0 μ g/ mL, 30 μ g/ mL, 50 μ g/ mL or 100 μ g/ mL and added to cells following scratching and washing. Culture media containing APOCIII was either added after the scratch or 24 hours prior to the scratch (pre-treatment).

Figure 2.1. Schematic of wound healing scratch assay. A) A pipette tip was used to physically scrap an intact monolayer of cells to introduce a wound model. The denuded area was imaged at regular time interval to measure the wound margin as the cells migrate to fill the gap. Dotted boxes showing regions where images were captured. B) Six edge-to-edge scratches were introduced to the confluent cell monolayer, creating multiple wound gaps which maximum number of migrating cells in the well. Illustrations adapted from (Hulkower and Herber 2011).

2.3.2 Image acquisition and data analysis

Images of closure of the scratch were captured using a 10x objective, under 100x magnification, every 15 minutes over the next 27 hours using temperature- and humidity-controlled, fully automated DMI 6000 inverted microscope (Leica Biosystems, Wetzlar, Germany). Images were acquired at three different regions of similar positions from each well for consistency. The degree of wound closure was quantified using Image J (NIH, Bethesda, ML, U.S.A) by tracing the wound margin or ‘wound edge’ and measuring the area between the two wound margins (gap) over time.

Data is expressed as a percentage of the area of the gap filled by the migrating keratinocytes at a particular measured timepoint.

$$\text{Percentage of wound closure} = \frac{(\text{area of initial wound} - \text{area of wound measured at time } t)}{\text{area of initial wound}} \times 100\%$$

2.3.3 Modified scratch assay for PCR array

HaCaT keratinocytes were seeded onto 12-well plates at a density of 500,000 cells per well in culture media. Cells were allowed to grow into a confluent monolayer for 48 hours before scratch wound induction. To maximize number of cells along a wound edge in any given well, a sterile 200µl pipette tip was used to scrape the cells in three straight lines each, from left to right and from top to bottom. In doing so, multiple wound gaps are created thus maximizing the number of migrating keratinocytes (Fig. 2.1B). Cells were washed twice with culture media and treated with human APOCIII or ammonium bicarbonate (control-treated) for 6 or 12 hours before cell lysis. HaCaT keratinocytes treated with APOCIII in this experiment were divided into 4 groups: A6 (APOCIII-treated cells collected 6 hours after scratch induction), A12 (APOCIII-treated cells collected 12 hours after scratch induction), C6 (control-treated cells collected 6 hours after scratch induction) and C12 (control-treated cells collected 12 hours after scratch induction). The experiment was run in duplicates and repeated three independent times. After 6 or 12 hours treatment with human APOCIII or diluent, the media was removed and cells were washed twice

with ice-cold PBS. RLTplus® lysis buffer was added to the wells. Cells were scraped, collected in a 1.5 mL Eppendorf tubes, and total cellular RNA was purified as described previously.

2.3.4 RT² Profiler PCR Array

The RT² Profiler PCR array for human wound healing (PAHS-121Z) was purchased from SABiosciences (Qiagen, Hilden, Germany) and performed according to the manufacturer's instructions. A total of 84 key genes involved in tissue remodeling during the repair and healing of wounds and 5 housekeeping genes were included in the array. cDNA synthesized from 400ng of total RNA, obtained from each group (A6, A12, C6 and C12) was used for the reaction. Fast SYBR Green 2x Master Mix (Applied Biosystems, Foster City, CA, U.S.A.) was used for the preparation of reaction mixture. PCR array in 384-well plate was used for the analysis of four biological samples. PCR cycling program was set up according to manufacturer's instructions for the QuantStudio 6 Flex (Initial Taq Polymerase activation at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1min). Among the five housekeeping genes included in the array, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was selected as the housekeeping control (reference) as it had the least varying gene expression across all the analyzed samples. Gene expression analysis using PCR array was performed for group comparisons (1) A6 versus C6; (2) A12 versus C12; (3) C12 versus C6; (4) A12 versus A6. Data analysis was performed according to literature (Chomiski, Gragnani et al. 2016). Ct value of GAPDH was used to standardize gene expression and determine between-group differences. Values obtained from

different groups were used as a reference for between-group comparisons. A 1.5-fold regulation was used as cut-off to identify differentially expressed genes. Data was analyzed by Excel-based data analysis template provided by Qiagen's data analysis webpage (<https://www.qiagen.com/sg/shop/genes-and-pathways/data-analysis-center-overview-page/>).

2.4. Determining ApoCIII expression in murine skin

2.4.1 Animal work

All mice used in this study were kept in standard temperature-controlled animal facility with 12-hour light-dark cycle and adequate ventilation and received water and food ad libitum. All animal experimentations were conducted in accordance with the National Advisory Committee for Laboratory Animal Research guidelines approved by Institutional Animal Care and Use Committee (IACUC; 140905, A0373) of Nanyang Technological University, Singapore. The animals utilized in all the experiments received humane care. Skin samples were collected from the dorsal region of 12 week-old healthy wild type (C57Bl/6N) and C57Bl/6J Lepr^{db} (*db/db*) mice. N=3 or as stated in individual experiments.

2.4.2 Glucose tolerance test

Intraperitoneal glucose tolerance tests (IPGTT) were performed in 12-week old wild-type and *db/db* mice. Following a 12 hour fasting period, glucose was administered via intraperitoneal injection (2g/kg body weight) and blood glucose was measured at time 0 and every 15 minutes

over a span of 2 hours using blood glucose meter (Accu-Check) (Roche Holding AG; Basel, Switzerland).

2.4.3 Harvesting of skin tissue

Animals were anaesthetized using isoflurane (2% in air) prior to being euthanized using carbon dioxide chamber. After depilation with hair removal cream (Nair, USA), a full thickness excision was made from the dorsal region of the mouse. For collection of samples for RNA purification, the samples were placed in cryotube (Nunc, Roskilde, Denmark) and snap frozen in liquid nitrogen immediately. The samples were subsequently stored in -80°C until RNA extraction. For collection of samples for histological analysis, skin tissues were placed on a piece of filter paper to avoid tissue folding during the fixation process and placed in freshly prepared 4% (*wt/vol*) paraformaldehyde (PFA) in PBS. Samples were kept at 4°C for up to two days until used for histological analysis. Extra care was taken to ensure that samples remained fully submerged in 4% PFA during the fixation period.

2.4.4. RNA isolation from cells and tissues

Tissues were removed from -80°C storage and chopped into small pieces using sterile blade on ice. Tissues were thoroughly disrupted and homogenized in RLT Plus lysis buffer (Qiagen), by handheld rotor-stator homogenizer TissueRuptor (Qiagen, Hilden, Germany). Disposable probes were used for each sample to prevent cross-contamination. In addition, QIAshredder homogenizers (Qiagen, Hilden, Germany) was used to ensure complete homogenization of the tissue samples.

As described previously in section 2.2.4, total RNA was purified from tissue homogenate using RNeasy Plus Mini Kit with gDNA eliminator spin columns to remove contaminating genomic DNA. The quantity and purity of RNA was assessed using a NanoDrop (Thermo Fisher, Waltham, MA, U.S.A.).

2.4.5 qPCR analysis of skin tissue

Single strand cDNA was synthesized from total RNA isolated from wound tissues as described previously in section 2.2.5. Gene expression levels were determined using qPCR. Expression of each gene was normalized to the level of housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to obtain ΔC_t . The $2^{-\Delta\Delta C_t}$ method was used to calculate gene expression difference in the wounded skin of diabetic and control mice at respective days (3, 5, 10 and 14). Gene specific intron-spanning primers were either designed as described previously. Only the primers that produced single peak on PCR melt curve were used. Genes analyzed and their respective primer sequences used are listed in Table 2.4.

Table 2.4 Genes and their primer sequences used. *ApoCIII*: Apolipoprotein CIII; *Gapdh*: glyceraldehyde 3-phosphate dehydrogenase.

Genes	Accession No.	Sequences (5' → 3')
<i>ApoCIII</i>	NM_001289755.1	Forward: AGGAGTCCGATATAGCTGTGGT Reverse: CCAGTAGCCTTTCAGGGATCT
<i>Gapdh</i>	NM_001289726.1	Forward: TGTGTCCGTCGTGGATCTGA Reverse: CCTGCTTCACCACCTTCTTGA

2.4.6 Histological analysis

Sample tissues were removed from the fixative and wrapped in filter paper to prevent wrinkling before placing them in cassettes. Cassettes containing formalin fixed tissues were placed in HistoCore Pearl tissue processor (Leica Biosystems, Wetzlar, Germany) for paraffin infiltration and processed according to Table 2.5. Samples were then embedded in paraffin using heated paraffin embedding station, HistoCore Arcadia H (Leica Biosystems, Wetzlar, Germany). Formalin-fixed paraffin-embedded (FFPE) tissues were sectioned using RM2245 Semi-Automated Rotary Microtome (Leica Biosystems, Wetzlar, Germany) at 5 μ m thickness and tissue sections were collected on positively charged Superfrost Plus slides (Thermo Fisher Scientific, Waltham, MA, U.S.A.). Tissues were analyzed using hematoxylin and eosin (H&E) and immunofluorescence staining.

2.4.7 Deparaffinization and rehydration

Slides holding the paraffin sections were placed in 65°C oven for 30 minutes to melt the paraffin. Slides were deparaffinized in 2 changes of xylene for 4 minutes each followed by rehydration through decreasing alcohol concentration. They were placed in 100% ethanol for 2 changes, 95% ethanol, 70% ethanol and 50% ethanol for 4 minutes each. The slides were kept in the tap water until H&E staining or antigen retrieval for immunostaining.

Table 2.5. Paraffin Tissue Processing Program for formalin fixed tissue samples.

Steps	Reagent	Duration	Temperature (°C)
1	Ethanol (70%)	45 min	45
2	Ethanol (80%)	45 min	45
3	Ethanol (95%)	45 min	45
4	Ethanol (100%)	45 min	45
5	Ethanol (100%)	45 min	45
6	Ethanol (100%)	45 min	45
7	Xylene	45 min	45
8	Xylene	45 min	45
9	Xylene	45 min	45
10	Paraffin	45 min	62
11	Paraffin	45 min	62
12	Paraffin	45 min	62

2.4.8 Antigen Retrieval

After optimization with different buffers, Tris/EDTA buffer (10mM Tris Base, 1mM EDTA Solution, 0.05% Tween 20, pH 9.0) was chosen as antigen retrieval buffer to be used for heat-induced epitope retrieval of paraffin samples. Briefly, the water bath was pre-heated with the staining dish containing antigen retrieval buffer until the temperature reaches 95°C - 98°C. Once the desired temperature was reached, the slides were immersed in the staining dish containing the buffer and boiled for 20 minutes. The staining dish together with the slides was removed from the

water bath and allowed to cool down at room temperature for 20 minutes before proceeding with immunohistochemical staining.

2.4.9 Immunohistochemistry

After deparaffinization and antigen retrieval steps, the tissue sections were blocked with 10% FBS in 0.1% (v/v) Triton X-100 in PBS for 1 hour at room temperature. The sections were stained with rabbit polyclonal IgG against mouse ApoCIII (M-75) (200 μ g/ml) (Santa Cruz, SC-50378) at a 1:50 dilution overnight at 4°C. For no primary antibody control, tissues were incubated with antibody diluent alone with no primary antibody. For isotype control, sections were incubated with rabbit polyclonal IgG (1mg/ml) at the dilution of 1:250. The slides were kept at room temperature for 1 hour before washing 3 times in PBS with 0.05% (v/v) Tween-20 (PBS-T) for 5 minutes each. The sections were incubated in Alexa Fluor® 488 anti-rabbit IgG (H+L) (ThermoFisher, A-21206) at a 1:200 dilution for 1 hour at room temperature. All sections were counterstained with DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) nuclei staining (5mg/ml) (ThermoFisher, D1306) for cell identification reference at 1:3000 dilution during secondary antibody incubation. After three washes with PBS, coverslips were mounted on the sections with Vectashield mounting medium for fluorescence imaging (Vector Laboratories, Burlingame, CA). Images were captured at 400 X magnification using LSM800 Inverted Confocal Laser Scanning Microscope (ZEISS, München-Hallbergmoos, Germany). Zen 2 Core (ZEISS, München-Hallbergmoos, Germany) was used for image acquisition and processing.

2.5. Determining ApoCIII expression in mouse wounds

2.5.1 Mouse excisional wound model

Diabetic C57Bl/6J Lepr^{db} (*db/db*) mice and non-diabetic (C57Bl/6N) (control) littermates at 12-week of age were used for determining the role of APOCIII in a mouse excisional wound model. Excisional wound models were performed on 12 non-diabetic and 12 diabetic mice for the collection for wound tissues on days 3, 5, 10 and 14 post wounding (n=3 for each group). The mouse excisional wound model was carried out as recommended with a few modifications (S. and Walter 2013) (Fig. 2.2A). Each mouse was anesthetized individually just before the wounding procedure. The animal was anesthetized using isoflurane in air with an induction chamber fitted to a precision vaporizer. Once the animal was placed into the chamber, oxygen flow was started and the animal was exposed to isoflurane at induction level of 3% isoflurane (v/v). Once the animal was fully anesthetized, it was removed from the chamber and switched to nose cone where the isoflurane delivery was maintained at 1.5% - 2% isoflurane (v/v). Dorsal hair was shaved off using an electric clipper and a thin layer of hair removal cream (Nair, Ewing, NJ, USA.), was applied for 2 to 3 minutes. Extra precaution was taken to ensure that the cream was not applied for more than 5 minutes to minimize skin damage. The cream and the hair were scraped off using plastic spatula tool and the exposed skin was cleaned with a gauze pad dampened with warm water. The depth of anesthesia was routinely assessed by monitoring the toe pinch withdrawal reflex at regular intervals before and during the wounding procedure.

The dorsal skin was pulled up and folded along the dorsal midline using index fingers and thumbs. With the folded skin in place, the animal was placed on its side on a sterile sheet. Two symmetrical full-thickness excisional wounds were created on each side of the dorsal skin along the midline by punching through both layers of the folded skin using a disposable 5-mm diameter sterile biopsy punch (Miltex Inc., Mettawa, IL, U.S.A.). Excisional wounds were covered with semi occlusive Tegaderm dressing (3M, Maplewood, Minnesota, U.S.A.). Mice were given subcutaneous an injection of Buprenorphine (0.1 mg/kg) for post-operative pain relief. Mice were individually caged after the surgery, placed under heat lamp and monitored continuously until full recovery from anesthesia. For post-operative care, Buprenorphine (0.1 mg/kg) was given once daily for up to two days post- surgery. Animals were monitored for the food and water consumption, appearance (grooming), weight loss and other pain indicators (e.g. spine curvature and running gait) every two days until the terminal end of the experiment.

Figure 2.2. Illustration of the mouse excisional wound model used. Two 5-mm full thickness excisional wounds were created on the depilated dorsal skin of each 12 week-old C57Bl/6N or C57Bl/6J *Lepr^{db} (db/db)* mouse with a biopsy punch. A) In conventional non-splinting wound model, no splinting ring was applied. B) In splinted excisional wound model, a silicon ring (grey area) was placed around each wound and secured using superglue and fastened using interrupted sutures (red crosses). All topical treatments were applied onto the wound bed at the center of the splint (pink area).

2.5.2 Collection of wound tissue

Wounds were harvested on post-operative days 3, 5, 10 and 14 for downstream processing and histological analysis. On the day of the tissue harvest, animals were anaesthetized (isoflurane) and killed using carbon dioxide chamber. The Tegaderm® dressings were carefully removed and a full

thickness excision was made around the entire wound tissue, along with some surrounding skin tissue. One wound from each mouse was used for RNA isolation and the other wound was used for histological analysis. For collection of wound tissue for RNA purification, wound bed along with the surrounding 1-2mm of skin was placed in a cryotube (Nunc, Roskilde, Denmark) and snap frozen in liquid nitrogen. The samples were subsequently stored in -80°C until RNA extraction. For collection of wounds for histological analysis, to avoid tissue folding during the fixation process, the wound tissue along with the surrounding 5mm of skin tissue was carefully placed on a piece of sterile cardboard paper and placed in freshly prepared 4% (v/v) PFA. The samples were kept at 4°C until they were processed for histology and immunocytochemistry. Tissues were processed and immunohistochemistry performed as described previously in section 2.4.4 to 2.4.9.

2.6 ApoCIII-ASO treatment and assessment of wound closure in mouse splinted excisional wound model

2.6.1 Mouse splinted excisional wound model

Diabetic C57Bl/6J *Lepr^{db}* (*db/db*) mice and non-diabetic (C57Bl/6N) (control) littermates were used for determining the role of ApoCIII in a mouse excisional wound model. Splinted excisional wound models were performed on 3 diabetic mice and 5 non-diabetic littermates to determine the effect of ApoCIII-ASO in wound healing. The mouse excisional wound model was carried out as described previously in section 2.5.1. Animals were anesthetized with isoflurane, shaved and

depilated. Withdrawal reflex was monitored regularly to make sure the animals were fully anesthetized before and during the surgery. As described earlier, two full thickness excisional wounds of 5mm diameters were created on the each side of the dorsal region along the midline of each animals. It was ensured that wounds were approximately 1 cm apart to give ample space for the splints. Splints were removed from 70% (v/v) ethanol and air-dried preceding the surgery. Super glue was applied to one side of a splint and the splint was carefully placed around the wound with the adhesive side facing the skin. The splint was affixed to the skin with four interrupted 6-0 nylon sutures. ApoCIII-ASO was applied to the wounds at this point, described in details hereinafter.

2.6.2 Pluronic F-127 gel preparation

Thirty percentage of pluronic gel (w/ v) was prepared by mixing 30 g of pluronic F-127 powder (Sigma-Aldrich. St. Louis, MO, U.S.A.) in 100 ml sterile autoclaved Milli-Q water. The powder was mixed vigorously and allowed to dissolve overnight at 4 °C on a tube roller. After complete dissolution, the solution was stored at 4°C to prevent gelation.

2.6.3 Splinting rings preparation

Circles of 15-mm diameter were cut from 0.5-mm-thick culture well silicone sheet (Grace bio-labs, Bend, OR, USA) and a 5-mm diameter biopsy punch was used to create a small hole in the middle of the disc to form donut-like splints (Fig. 2.2, area shaded grey). Splints were incubated in 70 % (v/v) ethanol until use.

2.6.4 Mouse-specific ApoCIII-ASO

ApoCIII antisense oligonucleotides (ASO) were obtained through a collaborator (Professor Phan Anh Tuan) who synthesized the ASO based on published literature (Graham, Lee et al. 2013). A series of chimeric 20-mer phosphorothioate antisense oligonucleotides (ASOs) containing 2'-O-methoxyethyl groups at positions 1 to 5 and 16 to 20 targeted to murine apoCIII mRNA was synthesized and purified with an automated DNA synthesizer as described in aforementioned literature. The mouse-specific apoCIII sequence synthesized was (5'-CCAGCTTTATTAGGGACAGC-3'), with underlined sequences indicating 2' MOE modified bases. In addition, a scrambled mismatch control 20-mer containing a random mix of all four bases was used as a control (in the treatment of two diabetic animals). ASO was received in lyophilized aliquots of (approx. 0.72 mg) at room temperature.

2.6.5 Topical ASO delivery

Prior to treatment, ASO was reconstituted by gentle mixing with freshly prepared 30 % (w/v) pluronic gel. Briefly, 2 µl of ASO (1 mmol/ L) was complexed with 18 µl of 30 % (w/v) pluronic to make a final concentration of 100 µmol/ L ApoCIII-ASO. The solution was kept on ice, until application to the mouse wound, to avoid gel formation. As soon as the splints were affixed with nylon sutures, 20 µl of pluronic solution with ASO was applied topically onto one of the wounds. After gel formation occurred (in less than a minute), Tegaderm® dressing was applied. On the adjacent wound of the same mouse, 20 µl of 30 % (v/v) pluronic solution with water (vehicle

control) or scrambled oligonucleotide (ASO control) was applied as control treatment. The treatments were re-applied, with the same preparation steps, topically every two days after the surgery until the wound was visually observed to be healed. These mice received the same post-operative care as described in section 2.5.1.

2.6.6 Monitoring wound closure

Animals were anesthetized for the documentation of wound closure and reapplication of treatment compounds every other day post-surgery until the day of wound harvest. General anesthesia was induced using 2% isoflurane as described hereinbefore and the Tegaderm dressing was gently removed using forceps. Digital photographs of individual wounds were taken on the day of surgery and every two days after the surgery. Re-application of the treatment gel and the vehicle control was performed before fresh Tegaderm dressing was applied. Splint securement was also checked and suture was replaced when required. Animals were monitored individually until fully recovered from anesthesia.

2.6.7 Wound tissue harvesting

Wound tissues from non-diabetic control mice were collected on day 9 post-surgery and wound tissues from diabetic littermates were collected 16 days after the surgery for histological analysis. On the day of tissue harvest, animals were euthanized using carbon dioxide chamber. The Tegaderm dressings along with splint and sutures were carefully removed. A full thickness

excision was made around the entire wound tissue, along with some surrounding 5mm of skin tissue. Wound tissues were processed as described previously in section 2.5.2.

2.6.8 Hematoxylin and eosin staining

After the sections were de-paraffinized and rehydrated, they underwent routine hematoxylin and eosin (H&E) processing. Briefly, the slides were stained with hematoxylin for 1 min before washing with water for 3 minutes. The slides were then quickly dipped in acid alcohol (0.3% HCL in ethanol) for 3 seconds before washing in water for 2 minutes. Next, the slides were briefly submerged in Scott's tap water (2 g of sodium bicarbonate and 20g of magnesium sulfate in 1 L of distilled water) for 5 seconds, washed with water for 2 minutes and placed in 70% ethanol for 2 minutes. They were stained in eosin for 30 seconds and dehydrated through 70% ethanol, 2 changes of 95% ethanol and 2 changes of 100% ethanol. Finally, the samples were put in xylene before mounting with Surgipath Micromount (Leica Biosystems, Wetzlar, Germany). Images were captured with Axioscan.Z1 Slide Scanner (ZEISS, München-Hallbergmoos, Germany).

2.6.9 Wound closure analysis

For images of wound tissues captured using a digital camera, the wound sizes were measured by tracing the wound margin and calculating the pixel area using Java's Image J software (NIH, Bethesda, ML, U.S.A. Data presented as percentage of wound closure as below. Since the splint has a constant area, it was used to normalize the wound sizes.

$$\text{wound closure (\%)} = \frac{(\text{wound area on day 0} - \text{wound area measured on indicated day})}{\text{wound area on day 0}} \times 100\%$$

2.7. Determining ApoCIII and Toll-like receptors expression in islets

2.7.1 Pancreas tissue preparation

Total pancreata from 12 week old non-diabetic mice and diabetic (*db/db*) littermates were excised and fixed in fresh 4% PFA in PBS at 4°C overnight. The tissues were transferred to 30% sucrose in PBS for another 24-48 hours at 4°C. Tissues were placed in Surgipath FSC 22 Clear Frozen Section Compound (Leica Biosystems, Wetzlar, Germany) and frozen at -80°C.

2.7.2 Immunohistochemistry of pancreas tissue

Frozen pancreas blocks obtained above were cut at 20-30 mm thickness using CM1950 cryostat (Leica Biosystems, Wetzlar, Germany), collected on SuperFrost® microslides (Thermo Fisher Scientific, Waltham, MA, U.S.A.) and stored at -80°C. The frozen slides were thawed at room temperature for 1 hour and washed with TBS three times for 5 minutes each. Sections were blocked with 10% FBS in TBS-T or M.O.M Mouse Ig Blocking Reagent from the Vector M.O.M Immunodetection kit (Vector Laboratories, Burlingame, CA) for 1 hour at room temperature. Primary antibodies were diluted in 10% FBS in TBS-T or M.O.M Diluent. The sections were preincubated in respective antibody diluent before primary antibody staining. The sections were

stained with primary antibodies at 4°C overnight and at room temperature for 1 hour the next day. The slides were washed three times in TBS-T before incubating in secondary antibodies for 1 hour at room temperature. Antibodies used in this experiment and their dilutions are listed in Table 2.6 and 2.7. The sections were counter-stained with DAPI nuclear staining during the secondary antibody incubation. Finally, the sections were mounted with Vectashield® (Vector Laboratories, Burlingame, CA). The pancreas samples were visualized on a ZEISS LSM800 Inverted confocal microscope (ZEISS, München-Hallbergmoos, Germany). Negative control assays were performed without primary antibodies.

Table 2.6. List of primary antibodies and dilutions used for immunohistochemistry.

Primary antibodies (Origin)	Company	Catalogue No.	Dilution
<i>ApoCIII (Rabbit)</i>	Santa Cruz Biotechnology	SC-50378	1:50
<i>CD31/PECAM-1(Goat)</i>	R&D Systems	AF3628	1:100
<i>CD68 (Rat)</i>	Bio-Rad Laboratories	MCA1957T	1:100
<i>Insulin (Guinea pig)</i>	Dako	A056401	1:400
<i>TLR-1(Rabbit)</i>	Novus Biologicals	BNBP1-77244	1:50
<i>TLR-2 (T2.5) (Mouse)</i>	Novus Biologicals	NBP2-31222	1:50
<i>TLR-3 (Mouse)</i>	Novus Biologicals	NBP2-24875	1:50
<i>TLR-4 (Mouse)</i>	Santa Cruz	SC-293072	1:50
<i>TLR-5 (Rabbit)</i>	Novus Biologicals	NBP1-76662	1:50
<i>TLR-9 (Mouse)</i>	Novus Biologicals	NBP2-24729	1:50

Table 2.7. List of secondary antibodies and dilutions used for immunohistochemistry.

Secondary antibodies (Origin)	Company	Catalogue No.	Dilutions
Alexa 488 anti-mouse (Goat)	Thermo Fisher	A11029	1:200
Alexa 488 anti-mouse (Donkey)	Thermo Fisher	A-21202	1:200
Alexa 488 anti-rabbit (Donkey)	Thermo Fisher	A-21206	1:200
Alexa 568 anti-rat (Rabbit)	Abcam	ab175719	1:200
Alexa 568 anti-goat (Donkey)	Thermo Fisher	A-11057	1:200
Alexa 647 anti-guinea pig (Donkey)	Jackson Immuno	706-605-148	1:400

2.7.3 Expression of TLRs mRNA in mouse skin

Skin tissues were collected from non-diabetic (n=4) and *db/db* (n=4) mice were used for total RNA isolation and single stranded cDNA synthesis as described previously. Gene expression levels were determined using qPCR. Expression of each gene was normalized to the level of housekeeping gene, GAPDH. Expression of each gene was normalized to the level of the house keeping gene, GAPDH. Gene specific intron-spanning primers were designed and only the primers with single peak on PCR melt curve were used for the RT-PCR. Genes analyzed and their respective primer sequences used in the RT-PCR are listed in Table 2.8.

2.8 Statistical analysis

All data was assumed to be normally distributed and presented as mean $\pm$ s.e.m. A two-tailed Student's t-test (unpaired) was used for comparison between two groups. A two-tailed paired t-test was used for comparison between wound closures of individual mice. Comparison between

more than two groups was performed using one way or two-way analysis of variance (ANOVA) with Tukey's or Bonferroni's multiple comparisons test post-hoc test, when applicable. All statistical analysis were performed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA). Statistical analysis was displayed as *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, when applicable. A post-hoc analysis was performed using G*Power for computation of achieved power of the *in vivo* studies (Faul, Erdfelder et al. 2007). The number of experiments (n) are stated in each corresponding figure legend.

Table 2.8 Genes and their primer sequences used. *Tlr*: Toll-like receptor.

Genes	Accession No.	Sequences (5' → 3')
<i>Tlr-1</i>	NM_001276445.1	Forward: GGGTAAGGTTGTCTTGACGGAAC Reverse: GGTATCCAACACGTGGGCTC
<i>Tlr-2</i>	NM_011905.3	Forward: GGGGCTTCACTTCTCTGCTT Reverse: AGCATCCTCTGAGATTTGACG
<i>Tlr-3</i>	NM_001357316.1	Forward: GATACAGGGATTGCACCCATA Reverse: TCCCCCAAAGGAGTACATTAGA
<i>Tlr-4</i>	NM_021297.3	Forward: GGACTCTGATCATGGCACTG Reverse: CTGATCCATGCATTGGTAGGT
<i>Tlr-5</i>	NM_016928.3	Forward: CTGGAGCCGAGTGAGGTC Reverse: CGGCAAGCATTGTTCTCC
<i>Tlr-9</i>	NM_031178.2	Forward: GAGAGACCCTGGTGTGGAAC Reverse: CCTTCGACGGAGAACCATGT

3. RESULTS

3.1 Serum APOCIII concentration in non-diabetic and diabetic patients

3.1.1 Introduction

Various studies have reported increased level of serum APOCIII in type-1 and type-2 diabetic patients compared to healthy individuals (Juntti-Berggren, Refai et al. 2004, Sundsten, Ostenson et al. 2008). However, the exact serum [APOCIII] reported varies between the studies. One study reported average APOCIII concentrations in human serum as 120 µg/ml (Atzmon, Rincon et al. 2006) where [APOCIII] was measured using ELISA (Wako Chemicals USA, Richmond, Virginia, USA). However in another study, APOCIII serum concentration, measured by conventional immunoturbidimetric method, varied between 28.1 µg/ml and 225.8 µg/ml in healthy individuals (Tilly, Sass et al. 2003). In some studies that compared the serum APOCIII level in control and T1D patients using radial immunodiffusion method, the concentration of serum APOCIII varied between 60 µg/ml to 140 µg/ml in control individuals and 90 µg/ml to 270 µg/ml in T1D patients (Joven, Vilella et al. 1989, al Muhtaseb, al Yousuf et al. 1992, Reverter, Senti et al. 1993). Another study reports serum APOCIII levels in T2D between 36 µg/ml and 125 µg/ml (Brahimaj, Ligthart et al. 2017) where the measurements were performed by the immunoassay testing company Myriad RBM (Austin, TX; www.myriadrbm.com).

All these studies involve an analysis of Caucasian/ Scandinavian datasets with no reference to Asian counterparts. To determine whether APOCIII is elevated in Asian diabetic serum, we measured serum APOCIII concentrations in serum from Singapore samples obtained through the LKCMedicine bio-bank and Khoo Teck Puat Hospital (KTPH) bio-bank. Serum samples from German patient samples were also included in the studies as a control and comparator for published Caucasian datasets. Serum samples were LKCMedicine bio-bank and German patients were a kind gift from Professor Bernhard Boehm (LKCMedicine). APOCIII concentration in the serum was determined using commercial ELISA kits as described in method section.

Non-fasted serum samples of Singapore male patients (n=113) from diverse ethnicity (Chinese, Malay and Indians) were obtained from LKCMedicine Bio-bank as part of the samples collected from other study (Ong, Koh et al. 2017). The samples were characterized into non-diabetic (n=25), T1D (n=38) and T2D (n=47). Serum [APOCIII] levels were measured using ELISA kit (AssayPro, St Charles, MO, USA) according to manufacturer's instructions. All samples were diluted 4000X so that the absorbance values obtained will fall within the linear range of the detection curve.

Next, we measured the APOCIII serum concentration in non-fasted German female serum samples obtained from LKCMedicine Bio-bank as part of the samples collected from other study (Ong, Koh et al. 2017). The samples were characterized into non-diabetic (n=27), T1D (n=20) and T2D (n=10). Serum [APOCIII] was determined using ELISA kit (AssayPro, St Charles, MO, USA) according to manufacturer's instruction.

We then proceeded to determine serum APOCIII levels in a separate cohort of fasted serum in Singapore patients, obtained as a kind gift from Associate Professor Tavintharan Subramaniam (KTPH). Fasted serum samples from non-diabetic (n=33) and T2D (n=56) were obtained from KTPH as part of the samples collected from earlier study (DSRB Reference Number: 2008/00431). Serum APOCIII level was determined using different ELISA kits (AssayPro, St Charles, MO, USA) and (Abnova Corp., Taipei, Taiwan) according to manufacturer's instruction.

In healthy Caucasian individuals, APOCIII levels were reported to be influenced by factors such as age, BMI and gender (Tilly, Sass et al. 2003). The study reported higher serum APOCIII concentration in males compared to females and higher in adults compared to children younger than 18 years old. In addition, another study reported that polymorphism in ApoCIII is associated with aging and longevity in Ashkenazi Jews centenarians (Atzmon, Rincon et al. 2006). Therefore, we proceeded to determine the association between APOCIII and a few factors. Additional patient information was not available at that time for non-fasted Singapore and German samples (LKC Medicine bio-bank). However, additional patient information such as age, body mass index (BMI), fasting blood glucose, total cholesterol (TC), triglycerides (TG), high density lipoprotein (HDL), low density lipoprotein (LDL) and serum creatinine were available for fasted KTPH samples. Thus, we proceeded to determine if there was a correlation between and these factors and serum APOCIII level in these patients.

3.1.2 Results

APOCIII concentration in human serum was measured using ELISA kit (AssayPro, St Charles, MO, USA). We found that in non-fasted serum samples from Singapore male patients (LKC Medicine bio-bank) (Fig. 3.1A), APOCIII levels were significantly higher in non-diabetic patients ($309.4 \pm 21.02 \mu\text{g/ml}$) compared to those in T1D ($172.2 \pm 9.50 \mu\text{g/ml}$) and T2D ($202.9 \pm 10.04 \mu\text{g/ml}$). There was no significant difference in serum APOCIII concentrations between T1D and T2D.

In the non-fasted serum samples from German female patients, the serum levels of APOCIII in non-diabetic patients ($253.2 \pm 21.23 \mu\text{g/ml}$) were significantly higher compared to that of T1D patients ($159.4 \pm 21.42 \mu\text{g/ml}$) and T2D ($160.3 \pm 41.45 \mu\text{g/ml}$) (Fig. 3.1B). The serum samples from the German cohort therefore does not corroborate with prevailing literature that show increased serum APOCIII concentrations in both T1D and T2D. Noteworthy, is the large variation in serum [APOCIII] especially in T2D and this perhaps calls into question the conditions of serum collection. There was no significant difference in the serum APOCIII concentrations between the serum samples from Singapore and Germany (Fig. 3.1C). It was observed that in both Singapore and Germany non-fasted serum samples, APOCIII serum levels were higher than what is reported in literature. Thus, a separate cohort of fasted serum samples of Singapore patients were obtained from KTPH. In the fasted serum, there was no significant difference in APOCIII concentration between non-diabetic patients ($268.8 \pm 25.13 \mu\text{g/ml}$) and T2D patients ($263.6 \pm 20.84 \mu\text{g/ml}$) (Fig.

3.1D). Since all values of serum APOCIII concentrations measured using AssayPro ELISA kit were higher, especially in non-diabetic patient samples, compared to literature reports, we then tested another APOCIII ELISA kit from that was recently released.

APOCIII concentration in fasted serum samples from KTPH were measured using ELISA kit (Abnova Corp., Taipei, Taiwan) according to manufacturer's instruction. There was no significant difference in serum [APOCIII] between non-diabetic patients ($179.6 \pm 13.72 \mu\text{g/ml}$) and T2D patients ($182.9 \pm 14.20 \mu\text{g/ml}$) (Fig. 3.2A). To determine whether there is a difference in APOCIII levels based on gender, serum [APOCIII] of male and female patients were compared within non-diabetic and T2D groups (Fig. 3.2B). Serum [APOCIII] were slighter lower in male non-diabetic ($174.7 \pm 18.13 \mu\text{g/ml}$; n=24) compared to that of female non-diabetic patients ($192.5 \pm 14.49 \mu\text{g/ml}$; n=9). APOCIII concentration in male T2D serum was higher ($195.7 \pm 17.33 \mu\text{g/ml}$) compared to that of female T2D serum ($146.3 \pm 21.41 \mu\text{g/ml}$). However, the findings were not statistically significant, likely due to the difference in sample size between the two groups. In addition, there was no significant difference between the serum APOCIII concentrations of male and female patients when a Mann-Whitney test (two-tailed) assuming that the data did not follow a normal distribution. Notably, APOCIII serum concentrations were significantly lower in non-diabetic (Fig. 3.2C) and T2D (Fig. 3.2D) samples measured using ELISA kit from Abnova compared to ELISA kit from AssayPro.

Subsequently, to determine if there is a possible correlation, serum APOCIII concentration of KTPH fasted samples was plotted against age, body mass index (BMI), fasting blood glucose, total cholesterol (TC), triglycerides (TG), high density lipoprotein (HDL), low density lipoprotein (LDL) and serum creatinine (Fig. 3.3). Among the groups compared, we found that TG level was strongly and positively correlated with serum APOCIII concentration ($r = 0.63$) which was expected as APOCIII is a major component of circulating TG-rich lipoproteins. It was found that serum APOCIII concentration was moderately associated with TC levels ($r = 0.33$) and weakly associated with LDL ($r = 0.26$) and serum creatinine ($r = 0.28$) although caution should be taken when interpreting r-squared data. However, there was no association between serum APOCIII levels and age, BMI, fasting glucose or HDL.

Figure 3.1. Serum APOCIII concentrations in non-diabetic and diabetic patients. Boxplots showing serum ApoCIII concentrations (μg/ml) in non-diabetic (red), type 1 diabetic (T1D; white), and type 2 diabetics (T2D; blue) in non-fasted serum samples from (A) Singapore male patients (n=25-47) and (B) German female patients (n=10-27). (C) represents a comparison between (A) and (B) (n=52-58). Serum APOCIII concentrations (μg/ml) in fasted samples of non-diabetic and T2D Singapore patients (n=33-54) from KTPH (D). Data represented as box-plot (10-90th percentile), unpaired student's t-test or one way ANOVA with Tukey's multiple comparison test was used whenever applicable. **P < 0.001, ****P < 0.0001. ND, Non-diabetic; SG, Singapore; DE, Germany.

Figure 3.2. APOCIII concentrations in fasted serum samples from non-diabetic and type-2 diabetic patients. Boxplots showing serum APOCIII concentrations (µg/ml) in non-diabetic (red) and type 2 diabetics (T2D; blue) in fasted serum samples from Singapore patients from KTPH (n=33-54) (A). [APOCIII] in serum samples from KTPH sub-categorized based on gender (n=10-40) (B). Comparison between serum APOCIII concentrations in non-diabetic patients (C) and T2D patients (D) measured by AssayPro and Abnova ELISA kits. Data represented as box-plot (10-90th percentile) and statistical analysis performed using unpaired student's t-test. **P < 0.001.

Figure 3.3. Associations between serum APOCIII concentrations and age, BMI, FBG, lipid profile and creatinine in non-diabetic and T2D patients. Data expressing the correlation between [APOCIII] and (A) age, (B) body mass index (kg/m^2), (C) fasting blood glucose (mmol/l), (D) total cholesterol (mmol/l), (E) triglycerides (mmol/l), (F) high density lipoprotein (mmol/l), (G) low density lipoprotein (mmol/l) and (H)serum creatinine ($\mu\text{mol/l}$). r: Pearson correlation coefficients. $P^{***}<0.0001$, $P^{**}<0.01$, $P^*<0.05$.

3.1.3 Discussion

Studies have reported increased serum APOCIII concentrations in T1D and T2D patients compared to healthy individuals. To determine if APOCIII concentrations are similarly elevated in Singapore patients, we measured the [APOCIII] in non-fasted serum samples from the LKCmedicine bio-bank. In addition, we included German non-fasted patient serum samples in this study as a control and reference for published Caucasian datasets. In our study, the serum samples from the German cohort does not corroborate with prevailing literature that show increased serum APOCIII concentrations in both T1D and T2D. However, there was also a concern that the non-diabetic serum [APOCIII] of LKCmedicine Bio-banked samples ($309.4 \pm 21.02 \mu\text{g/ml}$) and German samples ($253.2 \pm 21.23 \mu\text{g/ml}$) were higher compared to published literature that ranges from $28.1 \mu\text{g/ml}$ to $225.8 \mu\text{g/ml}$ in one study (Tilly, Sass et al. 2003) and from $60 \mu\text{g/ml}$ to $140 \mu\text{g/ml}$ in others (Joven, Vilella et al. 1989, al Muhtaseb, al Yousuf et al. 1992). Serum [APOCIII] levels can fluctuate following a meal and postprandial [APOCIII] can remain altered for several hours. Therefore, non-fasted blood samples may not be reliable for measurement of [APOCIII] when comparing between diabetic and non-diabetic individuals. For consistency and accessibility, we obtained a separate cohort of fasted serum sample from KTPH and measured

[APOCIII] in these samples. However, the serum [APOCIII] measured in non-diabetic patients of fasted samples ($268.8 \pm 25.13 \mu\text{g/ml}$) were still higher compared to that of published data. In addition, we did not observe significant difference in serum [APOCIII] between T2D and non-diabetic patients.

As mentioned, there is a wide range of values of serum APOCIII concentrations reported in literature. In these reports, different methods such as electroimmunoassay, RP-HPLC, radial immunodiffusion assays or ELSIA were used to determine APOCIII concentration with each methodology producing a different value. Therefore, the difference could be due to different methodologies used.

To clarify if different methods used in determining APOCIII concentrations make a difference, we re-measured the fasted serum samples (KTPH) using an ELISA kit from a different manufacturer. We observed that the serum APOCIII concentrations were significantly lower in both non-diabetic and T2D patients in the Abnova ELISA kit compared to that of the earlier used AssayPro ELISA kit. Re-measured [APOCIII] in non-diabetic patients ($179.6 \pm 13.72 \mu\text{g/ml}$) was close to that of published data. Thus, different methods and ELISA kits used in determining the serum APOCIII levels could result in the different APOCIII concentrations measured. Nonetheless, we did not observe any significant difference in serum APOCIII concentration between T2D and non-diabetic patients in Singapore (KTPH).

Irrespective of methodologies, APOCIII concentration in diabetic serum had either no difference or was lower compared to the non-diabetic serum. This could be due to different handling of samples during collection and serum preparation since these samples were derived from different clinics and institutes, each having a particular technique for sampling and storage. The lack of difference between diabetic and non-diabetic serum APOCIII is corroborated by a recent report where there was no significant difference in APOCIII level was observed between diabetic and non-diabetic patients in China (Luo, Liu et al. 2017). If so, this data is intriguing as it suggests another fundamental difference between Singaporean diabetics and Caucasian diabetics over and above the Asian lean diabetic phenotype. Further investigations are required to determine if this data is truly representative Singapore/Asian population. We certainly did not expect the variation in data obtained, especially for the non-diabetic samples. Our current N is 89, and based on the variation in data obtained, and the stipulated degrees of freedom (=4) the N needs to be 200 before any statistical comparison can be made.

Similar to what was reported in literature (Qamar, Khetarpal et al. 2015), we observed positive correlation of APOCIII concentration in fasted serum samples of Singapore patients (KTPH) with TG and TC. It was expected as APOCIII, is major component of VLDL and LDL, both triglyceride-rich lipoproteins, suggesting that these patients have high levels of VLDL and LDL to begin with. APOCIII may play a direct role of TG and TC as it inhibits lipoprotein lipase

resulting in less TG hydrolysis, leading to less cellular uptake thus increasing circulatory levels of TG and TC.

However in contrast to the reported literature, we did not find any association between APOCIII serum concentration and age, gender, BMI and fasting glucose (Tilly, Sass et al. 2003, Brahimaj, Ligthart et al. 2017). Another study of Ashkenazi centenarian families, reported that reduction in APOCIII due to genetic mutation, is associated with aging and longevity (Atzmon, Rincon et al. 2006). However, we did not observe an association between APOCIII and age of participants in our study but it is worth pointing out that our study was not specifically set up to test this association in the first place.

One study reported that plasma APOCIII concentrations were higher in individuals with chronic kidney disease (Ooi, Chan et al. 2011). In accordance with the previous report, we observed positive correlation between APOCIII concentration and serum creatinine despite the latter being in the normal range found in human serum. Correlation studies are usually conducted in studies with large sample size, thus our study was unfortunately underpowered.

3.1.4 Limitations and Suggested Improvements

Some limitations of the current study need to be mentioned. First, we have chosen ELISA as a method to measure APOCIII concentrations in the serum. The specific ELISA kits were chosen using published papers as reference however in-house validations were not performed we assumed minimal batch to batch variations. For future studies, different methods such as

immunoturbidimetric assay will need to be used for confirming the APOCIII.ELISA values. Second, serum samples were collected from a variety of sources with different sample handling, processing and storage. Moving forward, sample handling and processing should be more tightly controlled with SOPs and relevant information on obtained samples should be made available for future studies. Finally, while reports of APOCIII values were widely reported in Caucasians, there were very few studies showing deranged values of APOCIII in diabetic Asians. However, using available samples, we could not replicate many of these published reports showing increased APOCIII levels in diabetic patients. For future studies, prospective experiments should be conducted on a well stratified cohort with numbers large enough to attain sufficient statistical power.

3.2 Effect of ApoCIII on skin cells *in vitro*

3.2.1 Introduction

APOCIII is produced mainly in the liver and to a small extent, within the small intestine. Recently, diabetic islets have been found to produce APOCIII as well (Åvall, Ali et al. 2015). However, there has been no report on APOCIII production in skin. However, there has been no studies of APOCIII in skin. We embarked on a high risk project to study APOCIII in skin. The purpose of this project was to first determine whether APOCIII is produced in skin cells. In this thesis, we first sought to uncover if APOCIII is expressed in keratinocytes and fibroblast and whether hyperglycemia has any effect on APOCIII expression. We used 3T3 and HaCaT cell lines which are fibroblast and keratinocytes, respectively as they were previously shown to be functionally altered in the presence of high glucose (hyperglycemic conditions) (Lan, Liu et al. 2008, Lamers, Almeida et al. 2011). Mouse 3T3 fibroblast cell line was used in our initial experiments because our *in vivo* validation will be carried out in wounded control and diabetic mice.

ApoCIII has been shown to mediate pro-inflammatory effects in ECs and monocytes (Kawakami, Aikawa et al. 2006, Kawakami, Aikawa et al. 2007). Kawakami and colleagues showed that ApoCIII induces vascular inflammation, enhances monocyte adhesion to vascular endothelial cells and contributes to the formation of atherogenic plaques. Therefore, we also measured changes in inflammatory cytokine expression in fibroblast and keratinocytes exposed to purified human APOCIII.

Next, in order to study the effect of APOCIII on wound healing, we determined whether the addition of exogenous APOCIII affects the migratory function of HaCaT keratinocyte cells. Keratinocytes are the major component of the epidermis, responsible for re-epithelialization during wound healing. HaCaT cells are an immortal cell line that have been widely used in keratinocyte monolayer culture models (Seo, Kang et al. 2012). In this work, we performed an *in vitro* scratch assay on HaCaT cells treated with different concentration of purified human APOCIII. Scratch assay is a conventional *in vitro* technique that is convenient, inexpensive and is widely used for the analysis of cell migration in two dimensional monolayer manner (Liang, Park et al. 2007). It involves three basic steps: wounding, monitoring of the cell migration process with data acquisition and finally, data analysis.

Following the scratch assay, we determined the effect of exogenous APOCIII on the gene expression of HaCaT cells using a commercially available human RT² Profiler PCR array (Qiagen, Hilden, Germany). This qPCR array represents the top 84 genes that are known, through the Ingenuity Pathway Analysis (IPA, Qiagen, Hilden, Germany), to be involved in the human wound healing process. PCR array results were analyzed by comparing differentially expressed genes in the vehicle and APOCIII treatment groups as well as by comparing the dynamic changes in gene expression of wound healing related genes across the first twelve hours after ‘wounding’.

3.2.2 Effect of glucose on apolipoprotein gene expression

Besides liver and intestine, it was recently found that APOCIII is synthesized in pancreatic islets. However, there has not been no report of APOCIII production in skin cells. In order to study ApoCIII in wound healing, we first determined if APOCIII is produced in skin cells and if high glucose can stimulate *ApoCIII* expression in these cells.

The normal culture media for both 3T3 mouse fibroblast cells and HaCaT human keratinocyte cells contain 25mmol/L glucose. Since the cells were already grown in high glucose environment, 3T3 and HaCaT cells were cultured in media containing low glucose (1.5mmol/L) for 8 hours prior to glucose treatment to condition the cells to a low glucose environment. A low-glucose treatment time of longer than 8 hours will impact cellular growth and division. Subsequently, cells were exposed to different glucose concentrations (1.5mmol/L (hypoglycemia), 6mmol/L (euglycemia) or 25mmol/L (hyperglycemia)) for 24 hours prior to lysis and the relative abundance of *ApoCIII* measured by real-time PCR. For analysis, in addition to *ApoCIII*, we measured the expression of a few other apolipoproteins that were linked to pathological conditions.

We observed that most measured apolipoproteins were expressed at relatively low levels in the 3T3 and HaCaT cell lines (delta Ct values in the range of 18-22 when compared to actin). ApoA1 was reduced in 3T3 cells treated with 1.5 mmol/L compared to cells in normal glucose condition (6mmol/L). Apart from *ApoA1* in 3T3 fibroblasts, there was no significant difference observed

between expression levels of the apolipoproteins measured under conditions of increasing glucose concentrations in both 3T3 and HaCaT cells (Fig. 3.4A and B).

Taken together, the data suggests that 3T3 and HaCaT cells express very low levels of apolipoproteins and furthermore, the addition of high glucose failed to induce apolipoprotein (including *ApoCIII*) expression. Glucose treatment was further extended to 48 hours to determine if a prolonged incubation will result in an induction of *ApoCIII* expression. However even with 48-hour incubation in high glucose, there were no significant changes in *ApoCIII* or any of the other apolipoprotein transcript levels in 3T3 and HaCaT cells (results not shown). This data suggests that cell lines of keratinocytes and fibroblasts (unlike hepatocytes) do not produce APOCIII and that high glucose does not induce the expression of *ApoCIII*. The lack of *ApoCIII* expression suggests that *ApoCIII* is silenced in keratinocytes and fibroblasts and that high glucose levels are not able to relieve the repression of this gene. Although these cells do not produce APOCIII or induce *ApoCIII* expression (unlike observations on hepatocytes), it will be premature to conclude that these cells will not be functionally affected by extracellular APOCIII.

Figure 3.4. Expression of different apolipoproteins when exposed to increasing glucose concentration. 3T3 cells (A) and HaCaT cells (B) were exposed to different concentration of glucose (1.5 mmol/L, 6 mmol/L and 25 mmol/L) for 24hours prior to lysis. Ct values for each gene were first normalized to actin and subsequently to normal glucose (6 mmol/L) controls to determine the relative gene expression. Transcript expression was determined by gene-specific intron-spanning primers and quantitative-PCR. Data represents means $\pm$ s.e.m. n=3, *P < 0.05.

3.2.3 Effect of APOCIII on gene expression of inflammatory cytokines

To determine the effect of ApoCIII on cellular inflammation it is important to use endotoxin-free apoCIII that has been purified from human plasma. We treated 3T3 and HaCaT cells with increasing concentrations of purified human APOCIII (30 µg/ml, 50 µg/ml and 100 µg/ml) for 24 hours prior to lysis for gene expression determination by RT-PCR. These concentrations were determined by referencing previous studies of purified APOCIII in other cells and systems (Juntti-Berggren, Refai et al. 2004, Kawakami, Aikawa et al. 2006). Changes in expression of inflammatory cytokines known to be perturbed by ApoCIII such as IL-1 β , IL 18, TNF- α , IL-6 and MCP-1 were examined. No significant changes in *IL-18*, *IL-6* and *MCP-1* expression were observed in 3T3 cells exposed to human recombinant APOCIII (Fig. 3.5A). The expression of a few other inflammatory cytokines, *IL-1 β* and *TNF- α* was barely detectable in 3T3 fibroblast. In HaCaT keratinocytes, there was a significant reduction in the expression of *TNF- α* and *MCP-1* upon treatment with 100 µg/ml APOCIII compared to untreated control (Fig. 3.5B). There was no significant change in the expression of *IL-1 β* , *IL18* or *IL-6* in HaCaT cells exposed to APOCIII. Our data suggests that APOCIII reduces the expression of inflammatory cytokines *TNF- α* and *MCP-1* and contrary to what was published, our data suggests that ApoCIII could possibly exert weak anti-inflammatory effects on HaCaT cells. These observations have also been corroborated by a recent publication that suggests that ApoCIII could exert both pro- and anti-inflammatory effects depending on the cell type and exact biological context (Storling et al., 2011).

Figure 3.5. Expression of inflammatory cytokines exposed to increasing concentration of human purified APOCIII. 3T3 cells (A) and HaCaT cells (B) were exposed to different doses of purified human APOCIII for 24hrs prior to lysis. Transcript expression was determined by gene-specific intron-spanning primers and quantitative-PCR. Ct values for each gene were first normalized to actin and subsequently normalized to control untreated cells. Data represents means $\pm$ s.e.m. $n=3$, * $P < 0.05$, ** $P < 0.01$.

3.2.4 Effect of APOCIII on keratinocyte migration *in vitro*

The effect of APOCIII on HaCaT keratinocyte migration in an *in vitro* scratch wound assay has not been reported before. To confirm if the effect of migration is not limited to fibroblast migration, we tested similar concentrations of APOCIII on human keratinocyte migration. The *in vitro* scratch assay was performed on HaCaT keratinocytes in the presence of exogenously added purified APOCIII. Cells were grown to confluency for 48 hours before a physical scratch wound was introduced to the monolayer cells with sterile pipette. Cells were grown in culture media containing

30 µg/ml, 50 µg/ml or 100 µg/ml of exogenous purified human APOCIII and cell migration was monitored at regular intervals for the next 27 hours. A time-lapse study upon scratch induction, showed that the percentage of wound closure was significantly reduced in HaCaT keratinocytes treated with 50 µg/ml APOCIII compared to cells treated with APOCIII diluent, ammonium bicarbonate (control) (Fig. 3.6A). When the cells were treated with 50 µg/ml APOCIII, a significant reduction in the percentage wound closure was observed from 52.21 ± 3.3 to 42.02 ± 2.5 at 9 hours, from 72.06 ± 3.7 to 58.52 ± 3.6 at 12 hours, from 85.28 ± 3.0 to 72.29 ± 4.3 at 15 hours and from 92.90 ± 1.8 to 81.81 ± 4.8 at 18 hours after scratch induction, compared to cells treated with control at respective time points. Wound closure was significantly hindered (10-14%) in the cells treated with 50 µg/ml APOCIII compared to that of control and the effect was observed first observed at 9 hours after wound introduction. It was also found that at 30 µg/ml and 100 µg/ml concentrations, APOCIII had no significant effect on HaCaT cell migration suggesting that APOCIII may have a biphasic effect on the migration of HaCaT keratinocyte upon wound induction *in vitro* (Fig. 3.6A and B).

In order to determine whether exposure to APOCIII affected migratory regulation globally or if the effect was confined to when the cells were migrating, APOCIII (50 µg/ml or 100 µg/ml) was added for 24 hours before the *in vitro* scratch assay. After wound induction, cells were replenished with fresh media containing APOCIII and cell migration was monitored for 27 hours thereafter with image acquisition at regular time intervals. It was found that the additional APOCIII

pretreatment exacerbated the effect of APOCIII on HaCaT migration (Fig. 3.7). Treatment with 50 µg/ml APOCIII significantly delayed wound closure from 62.69 ± 2.5 % to 47.92 ± 5.1 % at 15 hours, from 75.32 ± 2.7 % to 57.66 ± 5.9 % at 18 hours, from 83.56 ± 2.6 % to 64.47 ± 6.4 % at 21 hours, from 89.54 ± 2.1 % to 69.42 ± 6.6 % at 24 hours and from 93.33 ± 1.6 % to 73.13 ± 6.5 % at 27 hours post wound induction, compared to the control cells treated with APOCIII diluent at respective time points. Wound closure was significantly attenuated (14-20%) in the cells pre-treated with 50 µg/ml APOCIII compared to that of control and the effect was observed first observed at 15 hours post wounding. However, pre-treatment with higher APOCIII concentration at 100 µg/ml had no significant effect on the migration of HaCaT keratinocytes. We have demonstrated that, at 50 µg/ml APOCIII delayed wound closure of HaCaT cells *in vitro* and pretreatment exacerbated the effect of APOCIII on HaCaT keratinocyte migration. This data suggests that APOCIII negatively affects HaCaT cell migration *in vitro*.

Figure 3.6. APOCIII impedes human keratinocytes (HaCaT) migration in an *in vitro* wound model. Representative images of *in vitro* wound healing assay performed in HaCaT keratinocytes, obtained at 100x magnification at indicated time points after wound introduction (A and B, left panels). Cells were grown to confluence and scratch wound was introduced by mechanically introducing a gap on the cell monolayer by scraping with a sterile yellow pipette tip. Cells were treated with diluent ammonium bicarbonate (control) or at either 30 µg/ml, 50 µg/ml human APOCIII (n=6) (A) or 100 µg/ml human APOCIII (n=5) (B) for 27 hours. Cell migration from the wound margin into the gap area was monitored and images of the same fields were taken immediately (0 hour) or at indicated time points, after wound induction. The original wound edge, at t=0 is depicted as white dotted lines. Graphs represent quantitative analysis of percent

wound closure and expressed by the scratch area filled by the migrating keratinocytes (right panel). Data was analyzed by two way ANOVA with Bonferroni's multiple comparisons test and represented as means $\pm$ s.e.m., * $P < 0.05$ compared with control. Scale bar: 100 μ m.

Figure 3.7. Pretreatment with APOCIII delayed HaCaT keratinocytes migration. Representative images of *in vitro* wound healing assay performed in HaCaT keratinocytes, obtained at 100x magnification at indicated time points after wound introduction (left panel). HaCaT keratinocytes were treated with control, 30 μ g/ml or 50 μ g/ml human APOCIII for 24 hours before the scratch wound assay. After scratch

introduction, cells were incubated in fresh media with APOCIII. Cell migration was monitored and images of the same fields were taken at regular time intervals as indicated. The original wound edge at t=0 is depicted as white dotted lines. Graphs represent quantitative analysis of percent wound closure and expressed by the scratch area filled by the migrating keratinocytes (right panel). Data was analyzed by two way ANOVA with Bonferroni's multiple comparisons test and represented as means $\pm$ s.e.m., *P<0.05 compared with control, n = 8. Scale bar: 100 μ m.

3.2.5 Analysis of wound healing-related genes

In order to determine whether ApoCIII alters the expression of several genes involved in the wound healing process, through a yet undefined mechanism, the expression of wound-healing-related genes in 'wounded' HaCaT cells, treated or untreated with APOCIII were compared. An *in vitro* scratch assay, with slight variation from the conventional assay (described in section 2.3.3) was performed. Six edge-to-edge scratches were introduced to the confluent cell monolayer, to create multiple wound gaps in order to maximize the number of migrating cells in the well. After scratch induction, HaCaT cells were treated with APOCIII diluent (control) or 50 μ g/ml APOCIII for either 6 or 12 hours and cell lysis were collected for RNA extraction (Fig. 3.8). In our *in vitro* data, APOCIII-mediated delayed migration was observed by as early as 9 hours after APOCIII treatment. To perhaps understand the mechanisms, if the effect is driven by changes in gene expression in particular, we decided to look at two timepoints around the 9 hour mark, at 6 hour (3 hours before the delayed effect was observed) and at 12 hour (where migration was significantly hindered) times. Our rationale is that gene expression effects may take hours and so looking at the 6 hour and 12 hour timepoints will maximize the chance that we catch differences in ApoCIII-

driven gene expression. Gene expression was analyzed by PCR array and comparisons were performed among comparisons (1) A6 versus C6; (2) A12 versus C12; (3) C12 versus C6; (4) A12 versus A6 (Table 3.4). Comparison 1 looks at the effect of APOCIII treatment on gene expression 6 hours after ‘injury’. Comparison 2 examines the effect of APOCIII treatment on gene expression 12 hours after ‘injury’. Comparison 3 shows the difference in gene expression in normal ‘wounded’ cells at 12 hours compared to at 6 hour post injury. Comparison 4 shows the difference in gene expression in ‘wounded’ cells with APOCIII treatment at 12 hours compared to 6 hours post injury.

Figure 3.8. Experimental design to determine gene expression of ‘wounded’ cells at different time point with or without APOCIII treatment. Wounds were introduced to cell monolayer and immediately treated with control or APOCIII. Cell lysis was collected at 6 hours post wounding (A6 and C6) and 12 hours post wounding (C12 and A12) for control cells and ApoCIII-treated cells, respectively.

Table 3.4. Number of differentially expressed genes in relative gene expression among groups. A6 = APOCIII-treated cells collected at 6 hour post scratch; A12 = APOCIII-treated cells collected at 12 hour post scratch; C6 = control-treated cells collected at 6 hour post scratch; C12 = control-treated cells collected at 12 hour post scratch. ^The second group in every comparison was always used as reference.

	Comparison	Up-regulated genes	Down-regulated genes	Total
1	A6 versus C6 ^	1	0	1
2	A12 versus C12 ^	0	3	1
3	C12 versus C6 ^	6	9	15
4	A12 versus A6 ^	7	10	17

A threshold of 2-fold difference is commonly used in microarray analysis. However, when applying a threshold of 2, only six genes are altered. As the RT2 Profiler PCR array is essentially a targeted qPCR analysis, we applied a threshold of 1.5 (fold difference) to increase the number of genes altered from 6 to 19 genes. Differentially expressed genes at a cut-off of 1.5 fold (up- or down-regulated) compared to the reference group are then listed in Table 3.5 along with respective fold change values. The relative expression level of a few genes were low ($C_t > 30$) in both groups, requiring more biological replicates to validate the result for these genes (indicated by ¥). One gene was up-regulated in Comparison 1 and one gene was down-regulated in Comparison 2. Thirteen differentially expressed genes were common to Comparison 3 and Comparison 4, of which 5 were up-regulated and 8 were down-regulated (Table 3.4 and 3.5).

A component of collagen family, collagen type 1 alpha 1 (*COL1A1*) was significantly up-regulated in comparison 3. This upregulation was not observed in APOCIII-treated cells in comparison 4.

Cathepsin K (*CTSK*), a protease involved in intracellular collagen degradation, was significantly upregulated in comparison 3 and 4. Matrix metalloproteinase 9 (*MMP9*), a type IV collagenase, was upregulated although not significant, in comparison 4. This upregulation was not seen in control cells of comparison 3. Connective tissue growth factor (*CTGF*) which plays a role in stimulating fibroblast proliferation and differentiation, was downregulated in APOCIII-treated cells at 12 hours post-injury (comparison 2). *CTGF* was downregulated to a greater extent in APOCIII-treated cells of comparison 4 compared to control-treated cells of comparison 3. Other growth factors such as *FGF2* and *HBEGF*, which are essential for proliferation and migration is downregulated, although not significant, in comparison 4. Transforming growth factor beta receptor 3 (*TGFBR3*), which involves in keratinocyte proliferation is significantly upregulated in comparison 3. The robust upregulation of *TGFBR3* was dampened in the APOCIII-treated cells at 12 hour compared to 6 hour (comparison 4).

Table 3.5. Genes differentially expressed in comparisons groups 1, 2, 3 and 4.

Genes	(1) A6 vs C6	(2) A12 vs C12	(3) C12 vs C6	(4) A12 vs A6
<u>ECM Components</u>				
<i>COL1A1</i>	-	-	*1.57 ↑	-
<i>COL5A2</i>	-	-	-	1.59 ↑*
<u>Remodeling Enzymes</u>				
<i>CTSK</i>	1.69 ↑*	-	*3.66 ↑	*2.14 ↑
<i>F3</i>	-	-	3.37 ↓	3.25 ↓
<i>MMP1</i>	-	-	2.18 ↑	2.31 ↑
<i>MMP9</i>	-	-	-	1.55 ↑
<i>PLAT</i>	-	-	1.53 ↑	1.64 ↑
<i>PLAUR</i>	-	-	1.76 ↓	1.85 ↓
<u>Cytoskeleton</u>				
<i>ACTA2</i>	-	-	1.89 ↑	1.78 ↑
<u>Inflammatory Cytokines & Chemokines</u>				
<i>CXCL1</i>	-	-	1.88 ↓	2.09 ↓
<i>CXCL2</i>	-	-	1.99 ↓	2.00 ↓
<i>IL1B</i>	-	-	1.53 ↓	1.58 ↓
<u>Growth Factors</u>				
<i>CTGF</i>	-	1.65 ↓*	2.47 ↓	3.70 ↓
<i>FGF2</i>	-	-	-	1.61 ↓
<i>HBEGF</i>	-	-	-	1.80 ↓
<i>TGFA</i>	-	-	*2.34 ↓	2.47 ↓
<i>TNF</i>	-	-	1.56 ↓	-
<i>VEGFA</i>	-	-	1.81 ↓	1.83 ↓
<u>Signal Transduction</u>				
<i>TGFBR3</i>	-	-	*3.44 ↑	2.18 ↑

↑ = up-regulated gene; ↓ = down-regulated gene; '-' = no change or less than 1.5 fold change; *P<0.05; *

Low relative gene expression.

3.2.6 Discussion

To study the function of ApoCIII in wound repair, we first sought to determine whether *ApoCIII* is expressed in fibroblasts (3T3) and keratinocytes (HaCaT) skin cell lines. Our results suggest that *ApoCIII*, along with other apolipoproteins, are expressed at relatively low levels in 3T3 and HaCaT cells (delta Ct values in the range of 18-22 when compared to actin). The earlier reported stimulatory effect of glucose on hepatocyte *ApoCIII* was also not observed in the cell lines tested (Caron, Verrijken et al. 2011). In addition, our data suggests that high glucose does not induce *ApoCIII* expression in 3T3 and HaCaT cells. It was observed that at high concentration (100 µg/ml), APOCIII represses inflammatory cytokines, TNF- α and MCP-1. This suggests APOCIII may exert dual function effects based on cell types and exact biological contexts as previously shown (Storling, Juntti-Berggren et al. 2011).

In this chapter, we showed for the first time that human purified APOCIII impeded keratinocyte migration *in vitro*. Using scratch assay, we showed that keratinocytes treated with 50 µg/ml APOCIII had a significantly reduced wound closure percentage when compared to the control treated cells. APOCIII had no significant effect on wound closure at a lower dose of 30 µg/ml. When the concentration of APOCIII was increased to 100 µg/ml the reduced migration effect seen at 50 µg/ml was lost. These results do not show a dose-dependent effect but rather a possible biphasic effect indicating that there is a physiological window within which APOCIII levels should be kept. Pre-treatment of HaCaT cells with APOCIII prior to wound induction, did not exacerbate

the effect of APOCIII on wound closure. This suggests that APOCIII-mediated effect on cell migration is observed only when the cells are driven to migrate upon ‘injury’. Whether or not APOCIII affects migratory pathway warrants further investigation.

In order to determine the underlying mechanism by which APOCIII delays wound closure in HaCaT cells *in vitro*, the expression of genes involved in wound healing were compared in ‘wounded’ keratinocytes, with or without APOCIII treatment. In our *in vitro* studies, we have observed APOCIII-mediated delay of HaCaT cells at 9 hour after APOCIII treatment and the effect was more significant at 12 hours and 15 hours. Therefore, we decided to look at two timepoints, at 6 hour mark before the effect was observed and at 12 hour mark when the effect was at most significance. It was observed that in control ‘wounded’ cells at 12 hour post injury there was increased expression of *COL1A1* compared to at 6 hour post injury. This upregulation was not seen in APOCIII-treated ‘wounded’ cells comparison at similar time points, suggesting that ApoCIII could interfere with collagen deposition therefore, delaying the wound healing process. In addition, *MMP-9* which encodes a type IV collagenase, was found to be slightly elevated albeit not significant in APOCIII-treated ‘wounded’ cells at 12 hour post injury compared to at 6 hour post injury. *MMP-9* is transiently expressed in normal acute wounds but *MMP-9* levels are elevated in chronic wounds such as diabetic wounds and venous ulcers (Reiss, Han et al. 2010). It was suggested that *MMP-9* delays wound healing by interfering with re-epithelialization and more replicates are required to determine if *MMP-9* expression was indeed affected in the presence of

exogenous APOCIII. In addition, *CTSK*, a potent collagen protease, was up-regulated, although again not significantly, in cells treated with APOCIII compared to control at 6 hour post injury (comparison 1). Inhibition of *CTSK* accelerates migration of fibroblasts *in vitro* and *CTSK* levels were elevated in wound tissues of *db/db* mice (Nair, Guo et al. 2017). The upregulation of *CTSK* by APOCIII at 6 hours post-wounding suggests that the observed wound closure delay is possibly due to less collagen available in the ECM to facilitate the migration of keratinocytes. Further work is required to determine if the changes in *CTSK* are significant and if so, it will be useful to measure the amount of collagen in the ECM of APOCIII-treated and control-treated keratinocytes in order to establish a mechanism for ApoCIII-mediated keratinocyte migration delay.

Our data also showed that *CTGF* is downregulated after 12 hour of injury in normal ‘wounded’ cells compared to 6 hour post injury. A study has shown that CTGF is present in early phase of wounds and has a role in early tissue repair (Alfaro, Deskins et al. 2013). It was also shown that *CTGF* is required for efficient re-epithelialization of corneal wounds (Gibson, Pi et al. 2014). This suggests that *CTGF* is required only in the early phase of ‘wounding’ for keratinocyte migration and its downregulation is necessary. This downregulation is also seen in APOCIII-treated ‘wounded’ cells but to a lesser extent and the change observed was not significant. Thus, effect of APOCIII on impaired wound closure could also be associated with the CTGF pathway. Growth factors are required in wound healing process for proliferation and migration of keratinocytes and fibroblasts. In our data, there was a trend of reduction in expression of growth factors such as

FGF2 and *HBEGF* in APOCIII-treated ‘wounded’ cells at 12 hours compared to at 6 hours after injury. A significant upregulation of *TGFBR3*, which was observed in normal ‘wounded’ cells (comparison 3) was also dampened in the APOCIII-treated ‘wounded’ cells (comparison 4). Collectively, our results allude to the possibility that ApoCIII could delay wound closure by interfering with a subset of growth factors that are essential for wound healing process.

Taken together, our results suggest that APOCIII although not expressed in our fibroblast and keratinocyte cell lines tested, responded to exogenous APOCIII negatively by delaying cell migration. Using the scratch wound assay, we showed that ApoCIII delays the migration of HaCaT keratinocytes and that this delay could possibly be associated with reduced collagen deposition and growth factors both of which has been shown to implicate wound repair *in vitro*.

3.2.7 Limitations and Suggested Improvements

A few limitations of the current study deserve mention. We used HaCaT cell line in our studies as it is one of the most established human keratinocyte cell lines. However, HaCaT cells require high glucose (25mM glucose) DMEM as their basal media. In order to study the effect of ApoCIII on keratinocyte migration under diabetic state *in vitro*, it would also be important to validate our results using a different keratinocyte cell line that does not require high glucose basal medium. In addition, for future studies, human fibroblast cells could be used to study the effect ApoCIII on fibroblast migration *in vitro*. Lastly, we have used PCR array as a first step towards identifying wound-healing related genes that could be affected by APOCIII in HaCaT keratinocytes *in vitro*.

For further studies, it would be also be interesting to study the changes in gene expression in wounds *in vivo*. In addition, although the PCR array was a quick and relatively cheaper, a more global expression analysis (such as microarray or more current sequencing methods) may reveal several other pathways that are altered in the presence of ApoCIII. If funds are available, measuring global changes to the transcriptome, especially at the single cell level would be of great value.

3.3 Determining APOCIII expression and its role in skin and wounds of wild-type and diabetic mice

3.3.1 Introduction

ApoCIII has been studied mainly in the field of cardiovascular diseases with emphasis on inflammation and atherosclerosis (Kawakami, Aikawa et al. 2006). A few studies have shown that ApoCIII is linked to diabetes, in relation to β -cell function in T1D and T2D (Holmberg, Refai et al. 2011, Avall, Ali et al. 2015).). Serum APOCIII is increased in diabetes and this increase provokes inflammatory responses in vascular ECs, monocytes and pancreatic islets. It is well established that wound healing is delayed in diabetes and excessive prolonged inflammation impairs diabetic wound healing (Guo and DiPietro, 2010). However, the involvement of ApoCIII in normal and diabetic wound healing has not yet been studied till date. Based on published literature that APOCIII is higher in serum of T2D, we sought to uncover whether increased APOCIII hinders the wound healing process.

Wound healing is a complex, sequential process which requires the many tissues and cell types. The process involves inflammation, cell proliferation and migration of keratinocytes at the wound edges, as well as the formation of granulation tissue and matrix remodeling (Werner and Grose 2003). The wound healing process is impeded in diabetic patients and this results in chronic wounds. In this chapter, to study wound repair, we introduced two full-thickness splinted excisional wounds in 12 weeks-old control and diabetic mice as previously described (Tan and

Wahli, 2011). In mice, wound healing primarily occurs via contraction, which is fundamentally different to that of humans. To closely mirror the wound healing process in humans, we used the splinted excisional mouse model that adds intricate steps to remove wound contractile properties upon wounding thereby allowing the wound healing process to occur via epithelialization and cell proliferation. In our splinted model, silicon splints are attached on the mouse skin and secured with sutures to limit skin contraction throughout the course of our study. In this chapter, we aim to study the role of APOCIII, a diabetogenic and pro-inflammatory serum protein, in the healing process of normal and diabetic mouse splintered wounds. We aim to determine the expression of APOCIII at the wound sites and to evaluate whether APOCIII ablation specifically at wound sites could alter the wound healing process in normal and diabetic mice.

First, we determined the presence of APOCIII on the normal ‘unwounded’ skin of healthy wild-type and diabetic mice. Next, we compared and contrasted the levels and localization of ApoCIII at wound site of healthy and diabetic mice by immunohistochemistry (immunofluorescence) analysis of full-thickness excisional wounds at day 3, 5, 10 and 14 post-wounding. Next, we sought to uncover if the increased presence of APOCIII in diabetic skin is due to increased *ApoCIII* expression (mRNA) in the cells of the skin at wound site, or if the APOCIII is an extrusion from serum, by measuring transcript levels of *ApoCIII* from the different wound samples. Finally, we ablated APOCIII at wound sites by ASO treatment and measured the rate of wound closures to determine the effect of APOCIII on wound repair in both healthy and diabetic mice. Each mouse

acts as its own control as treatments were applied on the two adjacent wounds of the same animal, thus allowing us to accurately compare the effect of APOCIII ablation at wound site.

To determine whether the ApoCIII-ASO treatment at wound site has beneficial effect on wound healing in normal and diabetic mice, we used an ASO specific for mouse ApoCIII that is currently in Phase III human trials for the treatment of hyperglyceridemia. The ASO sequence and method is published and was reproduced kindly by Professor Phan Anh Tuan (Graham, Lee et al. 2013). This second generation 2'-O-methoxyethyl (2'-MOE)-modified ASO has increased binding affinity for target mRNA and enhanced resistance against degradation (Graham, Lee et al. 2013). This aforementioned study showed a dose-dependent reduction in hepatic *ApoCIII* mRNA (66% - 98%) and plasma proteins in different mouse strains treated with ApoCIII-ASO. These mice also showed significant reduction in fasting triglyceride levels (19% - 79%) and this reduction in fasting plasma triglyceride was not seen in ApoCIII^{-/-} mice with same ApoCIII-ASO treatment, suggesting that the changes observed are attributable to reduction in APOCIII protein. In ApoCIII-ASO studies that showed robust decrease in APOCIII production and triglyceride concentrations, treatments of ApoCIII-ASO were given via systemic administration (Visser, Witztum et al. 2012, Burdett 2016). However there is no study available to date that administers ApoCIII-ASO locally or topically (i.e. at wound sites to study its effect on wound healing). To facilitate the topical application, a pluronic F127 (F127) gel was used as a medium for ASO delivery due to its low toxicity, reverse thermal gelation, high drug loading capabilities and ability to gel in physiological

conditions at relatively low concentrations (Khattak, Bhatia et al. 2005, Brunet-Maheu, Fernandes et al. 2009, Gioffredi, Boffito et al. 2016).

3.3.2 APOCIII expression in intact skin of wild-type and diabetic (T2D) mice

Prior reports on the expression of APOCIII has thus far been confined to the liver and to a lesser extent the small intestine and the diabetic islet. Other tissues, such as the skin, have yet to be looked at and for this reason, skin APOCIII expression had to be determined *in vivo*. In this context, we checked the expression of APOCIII in the skin in a genetic mouse model of Type-2 diabetes with wild-type (non-diabetic) age-matched mice as controls. To confirm that 12-week old *db/db* mice in our hands displayed all signs of diabetes, we conducted the intraperitoneal glucose tolerance test (IPGTT), which measures the rate of blood glucose clearance over two hours following a glucose load (Fig. 3.9).

The expression and localization of APOCIII in the skin of wild-type (healthy) and *db/db* (diabetic) mice was determined using immunofluorescence (IF) staining with a rabbit polyclonal antibody specific for mouse APOCIII. A no-primary-antibody control (without the addition of primary antibody) and isotype controls were also performed to eliminate non-specific background staining and detection of auto-fluorescence from the skin. There was no background signal detected in both control staining in healthy (Fig. 3.10) and diabetic mice (data not shown). Immunostaining of 12-week healthy and diabetic mouse skin confirmed the presence of APOCIII protein at the epidermal and, to a reduced extent, the dermal regions of the skin (Fig. 3.11). The presence of APOCIII in

the epidermis of healthy skin suggests that APOCIII is expressed in keratinocytes *in vivo*. This runs contrary to our earlier observation of HaCaT cells not expressing APOCIII. It is worth noting that as an immortal cell line, genetic drifts through long periods of culture could occur, which could account for the absence of APOCIII in HaCaT cells.

To corroborate the above IF observations, we examined *ApoCIII* mRNA expression in intact skin of the same 12-week wild-type and *db/db* mice. The ability to detect *ApoCIII* transcripts also suggests that APOCIII can be produced locally within the skin of mice. Full thickness skin punches from both group of mice were collected, homogenized, lysed and subjected to mRNA extraction and cDNA conversion. qPCR results, normalized to *Gapdh*, show that the level of *ApoCIII* expression in *db/db* mice were almost 16-fold higher as compared to levels found in the intact skin of wild-type mice (Fig. 3.12). Taken together, the results obtained suggest that *ApoCIII* is expressed in the mouse skin and that its transcript and protein levels are elevated in diabetic conditions.

Figure 3.9. Assessment of glucose homeostasis of *db/db* mice. Glucose homeostasis was assessed by intraperitoneal injection glucose tolerance test (IPGTT) in 12 week old wild-type and diabetic mice. Data represents mean $\pm$ s.e.m. $n=3$ mice per group. Statistical analysis performed using two-way ANOVA with Bonferroni's multiple comparison test.

Figure 3.10. Determining GFP autofluorescence in wild-type mouse skin. A no-primary-antibody staining of mouse skin as control (top). Rabbit antibody at a matched concentration was also used as isotype control (bottom). GFP signal (left panel) was countered by a blue DAPI stain of cell nuclei (mid panel). Merged channels as shown on the right panel. Images were acquired at 400x magnification and figures shown at best fit. Images acquired and processed using Zen 2 Core. n=3. Scale bar: 50 μ m.

Figure 3.11. Representative images of APOCIII staining in wild-type and diabetic mouse skin. Immunofluorescence staining of paraffin sections from intact skin of 12-week old wildtype (C57BL/6N) and diabetic mice using anti-APOCIII antibody. APOCIII immunofluorescence signal (green) (left panel) was counterstained by a blue DAPI stain of cell nuclei (mid panel). Merged channels as shown on the right panel. Images were acquired at 400x magnification and figures shown at best fit. Images acquired and processed using Zen 2 Core. n=3. Scale bar: 50 μ m.

Figure 3.12. *ApoCIII* expression in intact skin of healthy and diabetic mice. Relative mRNA expression of ApoCIII in intact skin of 12-week *db/db* mice and control litter mates. Statistical analysis performed using unpaired student's t-test. Data represented as mean $\pm$ s.e.m. n=3-4, **P < 0.01.

3.3.3 ApoCIII expression in wild-type mouse wounds

To the best of our knowledge, this is the first report of ApoCIII presence in mouse intact skin. In addition to its presence, we also found that the ApoCIII expression was significantly elevated in the diabetic intact skin. We next sought to uncover whether the ApoCIII levels in the skin will be

perturbed during wounding. Therefore, to determine the presence of APOCIII at wound sites, two 5mm-diameter full-thickness excisional wounds were created on the dorsal skin of wild-type mouse using a sterile biopsy punch as described previously. In order to assess the presence of APOCIII at wound sites during different phases of wound healing (inflammation, epithelialization/proliferation, remodeling), wound tissues were collected on day3, 5, 10 and 14 post-wounding. This post-wounding timeline has been well-established by various groups (Lin, Fang et al. 2011, Takemoto, Li et al. 2012, Dunn, Prosser et al. 2013, Katagiri, Park et al. 2016). The harvested samples were fixed in 4% PFA and embedded in paraffin. FFPE wound sections (5µm) were stained with ApoCIII-specific antibody followed by Alexa Fluor conjugated secondary antibodies. Immunoreactivity was visualized using a confocal microscope under both 50x and 400x magnifications to determine the presence, localization and relative signal of APOCIII protein the wound site.

3 days after wound induction, there was positive staining of APOCIII observed in cells of the epidermis and dermis layers (Fig. 3.13). APOCIII was expressed mainly in the keratinocytes of epidermal regions. In addition, APOCIII expression was observed to be cytoplasmic and was higher at cells surrounding the ‘wound edges’ (dotted line) and ‘migrating wound tip’ (enlarged boxes) compared to unwounded region of the epidermis. Stronger APOCIII expression was observed in wound tissues collected on day 5 post wounding compared to that of day 3 wounds (Fig. 3.14). Similarly, APOCIII was mostly present at ‘wound edges’ and ‘wound tip’ of day 5

wounds. In addition APOCIII was observed to be strongly expressed at the plasma membrane of migrating keratinocytes at wound margin towards the migrating tip (arrow head). In the wound tissues collected on day 10 post-wounding, APOCIII expression was present along the newly formed epidermis. We observed that at day 10, wounds were partially healed with hyperplastic epidermis at the wound edges and center of the wound (Fig. 3.15). APOCIII expression at these areas were similar to that of the unwounded epidermis. Similarly, at day 14 post-wounding, when the wounds were healed, APOCIII expression was present along the epidermis of wound edge and hyper thickened keratinocytes of newly formed epidermis (Fig. 3.16). The results obtained show that following *in vivo* wounding, the expression of APOCIII is not only significantly increased in the cells around the wound edge, but it also appears that the APOCIII subcellular localization is dynamically altered during the healing process (i.e. from a diffused cytoplasmic pattern within keratinocytes to a plasma membrane confined cell halo).

Next, to examine whether the increased APOCIII presence at wound sites is due to increased expression in the skin cells at wound site or extrusion from serum, we determined the *ApoCIII* mRNA expression in wounds on day 3, 5, 10 and 14 after surgery in healthy (wild-type) mice (Fig. 8). The *ApoCIII* mRNA expression in the wounds were comparatively higher than that of unwounded intact skin (Fig. 3.17). There was increased, though not statistically significant, expression of *ApoCIII* in day 5 and day 10 wounds compared to day 3 wounds. The relative expression level of *ApoCIII* was reduced at day 14 post-wound. However, a limitation of the

current *ApoCIII* mRNA expression study lies in the variations arising from the harvesting as wounds (i.e. differences in wound margin collected even though cuts were made as precise as possible). To overcome this limitation, a laser capture dissection microscope will add greater resolution and precision to wound harvesting and the subsequent mRNA transcript determination.

Figure 3.13. Representative image of APOCIII staining in wild-type mouse day 3 post wounding. Immunofluorescence staining of APOCIII expression in FFPE sections from excisional wounds of 12-week old wild-type mouse, 3 days after wound induction. APOCIII immunofluorescence signal (green) was countered by a blue DAPI stain of cell nuclei. Arrow indicate the direction of migration of wound tip and dotted line indicates wound margin. Images are acquired at 50x magnification (top panel) and boxed areas are acquired at 400x magnification (bottom panel). Figures are shown at best fit. Scale bar as indicated in individual images. n=3.

Figure 3.14. Representative image of APOCIII staining in wild-type mouse day 5 post wounding. Immunofluorescence staining of paraffin sections from excisional wounds of 12-week wild-type mouse 5 days after wounding. APOCIII immunofluorescence signal (green) was counterstained by a blue DAPI stain of cell nuclei. Arrow indicates the direction of migration of wound tip and dotted line indicates wound margin. Arrowhead indicates APOCIII expression at plasma membranes of keratinocytes. Images are acquired at 50x magnification (top panel) and boxed areas are acquired at 400x magnification (bottom panel). Figures are shown at best fit. Scale bar as indicated in individual images. n=3.

Figure 3.15. Representative image of APOCIII staining in wild-type mouse day 10 post wounding. Immunofluorescence staining of paraffin sections from excisional wounds of 12-week wild-type mouse 10 days after wounding. APOCIII immunofluorescence signal (green) was counterstained by a blue DAPI stain of cell nuclei. Arrows indicate the direction of migration of wound tip and dotted line indicates wound margin. Images are acquired at 50x magnification (top panel) and boxed areas are acquired at 400x magnification (bottom panel). Figures are shown at best fit. Scale bar as indicated in individual images. n=3.

Figure 3.16. Representative image of APOCIII in wild-type mouse day 14 post wounding. Immunofluorescence staining of paraffin sections from excisional wounds of 12-week wild-type mouse 14 days after wounding. APOCIII immunofluorescence signal (green) was counterstained by a blue DAPI stain of cell nuclei. Arrows indicate the direction of migration of wound tip and dotted line indicates wound margin. Images are acquired at 50x magnification (top panel) and boxed areas are acquired at 400x magnification (bottom panel). Figures are shown at best fit. Scale bar as indicated in individual images. n=3.

Figure 3.17. Relative *ApoCIII* expression in wild-type mouse wounds. Relative mRNA expression of *ApoCIII* in non-diabetic mouse wounds on day 3, 5, 10 and 14 post surgery measured by qPCR. Relative gene expression normalized to housekeeping gene, GAPDH. Statistical analysis performed using unpaired student's t-test. Data represented as mean $\pm$ s.e.m. n=3 for each group, **P < 0.01.

3.3.4 ApoCIII expression in diabetic mouse wounds

Given our recent finding that APOCIII was present at skin wounds at day 3, 5, 10 and 14 post wounding in normal mice, we proceeded to look into wounds of diabetic mouse. To determine the presence of ApoCIII in diabetic mouse wounds, two 5mm-diameter full-thickness excisional wounds were created on the dorsal skin of 12-week-old C57Bl/6J *Lepr^{db}* (*db/db*) mouse as described in methods section. Similarly, wound tissues were collected on day 3, 5, 10 and 14 post wounding for immunohistochemistry.

In the wound tissue collected on day 3 post surgery, APOCIII expression was present in the epidermis around wound edge and in the migrating wound tip (Fig. 3.18). There were traces of APOCIII staining at plasma membrane of keratinocytes at wound margin. In the wound tissue collected on day 5 post-wounding, APOCIII was strongly expressed in the epidermis at wound margin and migrating tip (Fig. 3.19). Around the wound margin, where migrating keratinocytes are found, APOCIII expression was mainly in the plasma membranes whereas its expression was cytoplasmic at other epidermal regions. In the wounds at day 10 post surgery, APOCIII expression was still strongly present in the epidermal region along the wound margin and at the wound tip (Fig. 3.20). Similarly in wounds at day 14 post-wounding, strong membrane-bound expression of APOCIII was still observed in the epidermis along the migrating keratinocytes from the wound margin towards the wound tip (Fig. 3.21).

Next, we determined the mRNA expression of *ApoCIII* in wounds on day 3, 5, 10 and 14 after surgery in diabetic mice (Fig. 3.22). The *ApoCIII* mRNA expression was highest in the day 3 wounds compared to the subsequent days, although this increase was not statistically significant. There were wide variations in the data obtained perhaps due to small sample size (n=3) and difference in wound sizes on different days of harvest. Also, similar to what was observed above, there were variations in the ratio of the actual wound tissue and surrounding unwounded skin collected in the wound sample.

Figure 3.18. Representative image of APOCIII staining in diabetic mouse day 3 post wounding. Immunofluorescence staining of paraffin sections from excisional wounds of 12-week *db/db* mouse 3 days after wounding. APOCIII immunofluorescence signal (green) was counterstained by a blue DAPI stain of cell nuclei. Arrow indicates the direction of migration of wound tip and dotted line indicates wound margin. Arrow head indicates APOCIII expression at plasma membranes of keratinocytes. Image shown at 50x magnification (top panel) and boxed areas are enlarged to 400x magnification (bottom panel). Scale bar as indicated in individual images. n=3.

Figure 3.19. Representative image of APOCIII staining in diabetic mouse day 5 post wounding. Immunofluorescence staining of paraffin sections from excisional wounds of 12-week *db/db* mouse 5 days after wounding. APOCIII immunofluorescence signal (green) was counterstained by a blue DAPI stain of cell nuclei. Arrow indicates the direction of migration of wound tip and dotted line indicates wound margin. Arrow head indicates APOCIII expression at plasma membranes of keratinocytes. Image shown at 50x magnification (top panel) and boxed areas are enlarged to 400x magnification (bottom panel). Scale bar as indicated in individual images.

Figure 3.20. Representative image of APOCIII staining in diabetic mouse day 10 post wounding. Immunofluorescence staining of paraffin sections from excisional wounds of 12-week *db/db* mouse 10 days after wounding. APOCIII immunofluorescence signal (green) was counterstained by a blue DAPI stain of cell nuclei. The arrows indicate the direction of migration of wound tip and dotted line indicates wound margin. Arrow head indicates APOCIII expression at plasma membranes of keratinocytes. Image shown at 50x magnification (top panel) and boxed areas are enlarged to 400x magnification (bottom panel). Scale bar as indicated in individual images.

Figure 3.21. Representative image of APOCIII staining in diabetic mouse day 14 post wounding. Immunofluorescence staining of paraffin sections from excisional wounds of 12-week *db/db* mouse 14 days after wounding. ApoCIII immunofluorescence signal (green) was countered by a blue DAPI stain of cell nuclei. The arrow indicate the direction of migration of wound tip and dotted line indicates wound margin. Arrow heads indicate APOCIII expression at plasma membranes of keratinocytes. Image shown at 50x magnification (top panel) and boxed areas are enlarged to 400x magnification (bottom panel). Scale bar as indicated in individual images.

Figure 3.22. Relative *ApoCIII* expression in diabetic mouse wounds. Relative mRNA expression of *ApoCIII* in *db/db* mouse wounds on day 3, 5, 10 and 14 post surgery measured by qPCR. Relative gene expression normalized to housekeeping gene, GAPDH. Statistical analysis performed using unpaired student's t-test. Data represented as mean $\pm$ s.e.m. $n=3$ for each group, $**P < 0.01$.

3.3.5 Accelerated wound healing in wild-type mouse with ApoCIII-ASO treatment

It was previously shown that APOCIII is present in intact (unwounded) skin and in wounds of normal mice. To determine if the expression of APOCIII, especially its increased expression in wounded skin, plays a role in skin wound healing we downregulated the expression of APOCIII at the wound site by topically applying antisense oligonucleotide (ASO) against the mouse *ApoCIII* mRNA. As mentioned, the ASO used is of the same sequence and chemistry as the ASO therapeutic agent (Ionis Pharma, Carlsbad, CA, USA) that is currently in Phase II Clinical Trials for the treatment of hypertriglyceridemia (Graham, Lee et al. 2013). This sequence has been validated to knockdown APOCIII *in vitro* and *in vivo* (Holmberg, Refai et al. 2011). To determine the effect of APOCIII knockdown at the wound site, two full-thickness excisional splinted wounds were created on the dorsal region of wild-type mice (n=5) (Fig. 3.23A). Control wounds were treated with vehicle (pluronic gel with nuclease-free water) while the adjacent wound of the same mouse was treated with 100 μ mol/ L ApoCIII-ASO. Wound closure was monitored for 9 days and images were taken at specific time points (Fig. 3.24). Topical application of 100 μ mol/ L ApoCIII-ASO every second day significantly accelerated wound closure compared to vehicle-treated control wounds of the same mouse. The acceleration of wound closure in ApoCIII-ASO treated wounds, was observed within 4 days after injury. In 4 out of 5 animals, ApoCIII-ASO-treated wounds were observed to be healed by day 9 whereas all the vehicle-treated control wounds remained open with visible epithelial gap, typical of the time taken to healing (S. and Walter 2013).

Measurements of the wound area on the digital images show that ApoCIII-ASO treatment significantly accelerated wound closure in comparison with controls on days 4, 6 and 8 after wounding in healthy mice (Fig. 3.25). The treatment of ApoCIII-ASO on the wound site caused a significant increase in the wound closure percentage ($41.12 \pm 2.7\%$) compared to vehicle treated wound ($17.60 \pm 6.5\%$) just 4 days (day 4) after 2 treatments. Wound closure was significantly accelerated in ApoCIII-ASO-treated wounds in comparison with vehicle-treated wounds on day 6 ($68.91 \pm 5.8\%$ vs $47.48 \pm 8.4\%$) and day 8 ($83.73 \pm 2.1\%$ vs $67.70 \pm 6.4\%$) post injury. By day 9 post wounding, wounds received ApoCIII ASO treatment were 87.44% ($\pm 2.1\%$) closed compared to control wounds that were 74.13% ($\pm 4.8\%$) closed. A post-hoc analysis computed using G*Power showed the achieved powers of the in vivo study on day 4, 6, 8 and 10 were 95.6%, 76.2%, 75.3% and 83.6% respectively. Our data suggests that treatment with ApoCIII ASO accelerated wound healing in wild-type mice. Again, to the best of our knowledge, this is the first time ApoCIII ASO agent has been delivered topically (usually delivered systemically) in a living animal.

IF analysis of wound tissue at the end of the experiment on day 9, when APOCIII levels were observed to be lower, showed that there was no significant difference in the APOCIII expression between the ASO-treated and control wounds (data not shown). However, in our preliminary experiment, there was a reduction in APOCIII expression in ASO-treated wounds on day 6 (Future perspective). Further histological examinations from cross-sections of healthy excisional wounds

at day 9 after surgery (Fig. 3.26) showed overt morphological differences between the ASO-treated and control wounds with regard to wound size, re-epithelialization and dermal collagen maturation. It was observed that most of the control wounds were not closed with a clear gap between the wound edges at both epidermis and dermis levels. We observed granulation tissue with fibrin and neutrophil infiltration was observed at the wound center. In addition, hyperplastic epidermis, fibroblast proliferation and lymphocyte infiltrations were observed around the wound edges. On the other hand, most ASO-treated wounds were completely closed and fully epithelialized with dermal collagen formation. In addition, the re-epithelialized epidermis had reduced to normal thickness. Some of the ASO-treated wounds were healed to a lesser extent with hyperplastic epidermis around the wound margin and some defects in dermis with incomplete dermal collagen maturation (not shown).

Figure 3.23. Treatment of ApoCIII ASO in full thickness excisional mouse model. Two full-thickness excisional splinted wounds were created on the dorsal skin of (A) wild-type (C57BL/6N) mouse and (B) diabetic (C57Bl/6J Lepr^{db}) (*db/db*) mice. Wounds were treated with 30% (w/ v) pluronic gel mixed with water for control or with 100 μ mol/ L ApoCIII ASO.

Figure 3.24. Topical application of ApoCIII-ASO enhanced wound healing in wild-type mice. Images from five different animals showing the macroscopic appearance of the control and ASO-treated wounds on post-surgery days 4, 6, 8 and 9. 5mm silicon splint ring for scale.

Figure 3.25. Topical application of ApoCIII-ASO enhanced wound healing in wild-type mice. Measurements of wound images at indicated time points were performed using image analysis to determine wound area and data expressed as percentage of wound closure (%). Statistical significance was evaluated using paired student's t-test. Data are expressed as mean $\pm$ s.e.m., *P<0.05, **P<0.01, n=5 for each group.

Figure 3.26. Histological examination of control and ASO-treated wounds in wild-type mice. Hematoxylin and eosin (H&E) staining of the cross-section of (A) control wound and (B) ASO-treated wound samples from wild-type mouse on post-surgery day 9. Boxed areas are enlarged to (a) and (b), respectively. Arrowheads indicate the wound edges. Arrows: 1 – granulation tissues; 2 – normal epidermis; 3 – hyperplastic epidermis; 4 – fibroblasts; 5 – hair follicles. Scale bar: 200µm.

3.3.6 Effect of topical application of ApoCIII-ASO on wound healing in diabetic mice

We have demonstrated that treatment with ApoCIII-ASO accelerated wound healing in healthy wild-type mice. To study the effect of APOCIII on diabetic wound healing, ApoCIII-ASO was applied onto wounds of diabetic (*db/db*) mice. Two full-thickness excisional splinted wounds were created on the dorsal region of diabetic mice as described previously (n=5) (Fig. 3.23B). Wounds were treated with either control or 100 $\mu\text{mol/L}$ ApoCIII-ASO. Control wounds were treated with vehicle control (nuclease-free water in pluronic gel) (n=3) or with ASO control (scrambled ASO in pluronic gel) (n=2). Wound closure was monitored for 16 days and images were taken at indicated time points (Fig. 3.27). The topical application of 100 $\mu\text{mol/L}$ ApoCIII-ASO every second day significantly increased wound closure compared to the adjacent control wounds of the same mouse. The acceleration of wound closure in ApoCIII-ASO treated diabetic wounds was observed within 4 days post wounding, after 2 treatments. Measurement of the wound area on the digital images indicated that wound closure was significantly increased in ApoCIII ASO-treated wounds compared to control wounds on day 4 ($14.99 \pm 2.8\%$ vs $7.34 \pm 2.5\%$), day 6 ($28.48 \pm 5.6\%$ vs $16.65 \pm 3.6\%$), day 8 ($46.59 \pm 7.2\%$ vs $30.07 \pm 6.7\%$), day 10 ($66.32 \pm 6.1\%$ vs $38.95 \pm 6.8\%$) and day 12 ($66.32 \pm 6.1\%$ vs $43.50 \pm 8.3\%$) (Fig. 3.28). At the end of wounding experiment on day 16, wounds that received ASO treatment were $90.78 \pm 3.2\%$ healed compared to control wounds that were $70.54 \pm 4.8\%$ healed. A post-hoc analysis of the achieved powers in the *in vivo* studies on day 4, 6, 8, 10, 12 and 16 were 74.1%, 62.5%, 62.0%, 80.5%, 80.2% and 98.5% respectively. Our data suggests that treatment with ApoCIII-ASO accelerated wound healing in

diabetic mice. Confocal IF image analysis of wound tissue at the end of the experiment on day 16, when APOCIII levels were observed to be lower, showed that there was no significant difference in the APOCIII expression between ASO-treated and control wounds in diabetic mice (data not shown).

Further histological analysis from cross-sections of the excisional wounds of diabetic mice at day 16 after surgery (Fig 3.29) showed morphological differences between the ASO-treated and control wounds with respect to wound size, re-epithelialization and dermal collagen maturation. Most of the control-treated diabetic wounds were not completely closed, as there was an observable gap between the wound edges. Granulation tissues with fibrin and lymphocytes were visually observed at wound center of H&E wound sections. Hyperplastic epidermis and fibroblastic proliferation were observed at wound edges. In two out of five mice where control wounds were 75-80% healed, complete re-epithelialization with normal epidermal thickness and partial dermal collagen formation were observed (figure not shown). On the other hand, ASO-treated diabetic wounds in all five mice were completely re-epithelialized with normal epidermal thickness and dermal collagen maturation (Fig.3.29, bottom panel).

Figure 3.27. Effect of topical application of ApoCIII-ASO on wound closure in diabetic (*db/db*) mice.

Images from five different animals showing the macroscopic appearance of the control and ASO-treated wounds at indicated days after the surgery. 5mm (mouse 1-3) and 6mm (mouse 4 and 5) silicon splint rings for scale.

Figure 3.28. Effect of topical application of ApoCIII ASO on wound closure (%) in diabetic (*db/db*) mice.

Measurements of wound images at indicated time points were performed using image analysis to determine wound area and data expressed as percentage of wound closure (%). Statistical significance was evaluated using paired student's t-test. Data are expressed as mean $\pm$ s.e.m., n=3 wounds for each group.

Figure 3.29. Histological examination of control and ASO-treated wounds in diabetic mice. Hematoxylin and eosin (H&E) staining of the cross-section of (A) control wound and (B) ASO-treated wound samples of *db/db* mouse on post-surgery day 16. Boxed areas are enlarged to (a) and (b), respectively. Arrowheads indicate the wound edges. Arrows: 1 – granulation tissues; 2 – hyperplastic epidermis; 3 – fibroblasts; 4 – normal epidermis; 5 – hair follicles. Scale bar: 200µm.

3.3.7 Discussion

In this chapter, we have shown for the first time, the presence of ApoCIII protein and mRNA expression in the skin of *db/db* and wild-type mice, apart from earlier reported liver, small intestine and pancreatic β -cell ApoCIII (Avall, Ali et al. 2015). Our IF analysis showed that APOCIII was present mainly in the keratinocytes of epidermis and to a much lesser extent, fibroblasts of dermis of the skin. In parallel to the increased serum APOCIII levels in diabetes, *ApoCIII* mRNA expression was also higher in *db/db* mouse skin compared to that of control and there was a greater presence of APOCIII protein in IF analysis of *db/db* mouse skin..

We also showed that APOCIII was present in the wounded skin and that the expression is increased in the wounds compared to unwounded skin in both wild-type and diabetic mouse. In the mouse wounds, we found that APOCIII was highly expressed in the keratinocytes at the epidermal region along the wound margin and the migrating wound tips. Interestingly, when the keratinocytes are actively proliferating at the wound margins on day 3 and 5 post-surgery of wild-type mouse and on day 3, 5, 10 and 14 of diabetic mouse, APOCIII expression was strongly present on the plasma membrane of the keratinocytes. Whereas, in the other regions, APOCIII expression was found in the cytoplasmic region of the cell. This difference in spatial expression of APOCIII at different regions of the wounds could perhaps suggest mechanistic dynamism of APOCIII in normal intact versus proliferating or migrating keratinocytes.

We observed that the relative mRNA levels of *ApoCIII* were higher in wounds compared to that of unwounded skin in both wild-type and diabetic mice. However, relative mRNA levels in the wounds were not directly relatable to the IF data, the latter being a semi-quantitative technique. This lack of changes observed at the mRNA level could be due to: 1) the actual size of the wound at different time points and 2) the ratio of wound edge to surrounding normal skin in the harvested wound tissue, which was difficult to control. This limitation can be mitigated using a laser capture microdissection technique for precise wound harvesting and subsequent mRNA transcript level determination.

There was no available literature regarding the dosage and formulation of ApoCIII-ASO for topical wound application. In our preliminary studies, ApoCIII-ASO was applied only once at three different doses (10, 30 or 100 $\mu\text{mol/L}$) immediately after surgery and wound closure was monitored for 3 to 7 days. There was no difference in wound sizes between ASO treated and untreated wounds observed perhaps due to the relatively short half-life of the ApoCIII-ASO (data not shown). We proceeded with our current study using the highest dose of 100 $\mu\text{mol/L}$ treatment given every two days throughout the experiment until day of wound collection.

We have demonstrated, for the first time that local ApoCIII-ASO treatment to reduce APOCIII at wound area in vivo, significantly improved wound healing in splinted excisional wound model of healthy wild-type mice. The improvement was seen in as early as day 4 post-wounding and by the end of the study at day 9, wounds with ASO treatment were almost 90% closed compared to control

wounds without ASO treatment that were 74% closed. Histological examination of wound section at the end of ASO treatment study (day 9) also showed that ASO-treated wounds have improved epithelialization and dermal collagen formation compared to control wounds.

Similarly, we have also demonstrated that local topical application of ApoCIII-ASO at wound area *in vivo* significantly accelerated wound closure in splinted excisional wound model of diabetic (*db/db*) mice. The improvement in wound closure (%) was seen in as early as 4 days after wounding and by the end of the study at day 16, ASO-treated wounds were approximately 90% closed compared to control wounds that were only approximately 70% closed. Histological examination of wound sections (H&E staining) also showed improved re-epithelialization and dermal collagen maturation in ASO-treated wounds compared to control wounds.

From our IF analysis, we observed that the APOCIII expression was low at the end of our study, at day 9 and day 16 in wild-type and diabetic mice when the wounds were almost healed. This could be one possible reason why we did not observe any significant difference in the APOCIII expression between ASO-treated and control-treated wounds at the end of experiment (day 9 and 16). Repeated experiments with wounds harvested at earlier time points could prove to be better in validating the knockdown efficiency of APOCIII expression at wound site. Given the scarcity of diabetic mice, we have repeated the topical application of ApoCIII-ASO experiment in 12-week wild-type mouse and harvested wounds on day 6 post surgery as a preliminary study (Section 6). IF analysis of wound tissues on day 6 post wound showed that there was lower APOCIII expression

in the ASO-treated wound compared to control wound (Fig. 3.39). In the control wound, APOCIII was strongly expressed in the plasma membranes of migrating keratinocytes at the wound edge (Fig 3.39A). This membranous expression of APOCIII is absent in ASO-treated wound (Fig 3.39B). Therefore, repeating experiments using more mice could better validate the efficiency of topical application of ApoCIII-ASO in reducing ApoCIII levels at wound site.

Our data alludes to APOCIII involvement in the earlier stages of wound healing phases (inflammation and proliferation). ApoCIII-ASO treatment significantly improved wound healing in both healthy and diabetic mice by day 4 post wounding.

3.3.8 Limitations and Suggested Improvements

One of the limitations is in the method used to harvest wound tissue for *ApoCIII* mRNA expression. Manual dissection of wound tissues arises to variation in the ratio of the amount of wound to surrounding skin tissue collected, which could be responsible for the variation in the data. This could be improved using a more precise technique such as a laser capture microdissection for precise wound harvesting. Another way is to use RNAscope®, an in-situ mRNA detection method, to stain for *ApoCIII* expression in wounds instead of quantifying mRNA levels. Another limitation is that we tested only one dose of ApoCIII-ASO in both healthy and diabetic mice in our in vivo studies due to lack of information with regards to the dosage and formulation of ApoCIII-ASO for topical application. For future studies, optimization of the ApoCIII-ASO treatment in terms of dosage and formulation is required to achieve maximum

efficiency. Another limitation is the sample size in the in vivo studies, which could be increased in future studies to increase statistical power. Finally, further mechanistic studies are required to prove direct effects of ApoCIII on wound healing and to evaluate the underlying mechanisms by which ApoCIII delays wound healing

3.4 Expression pattern of TLRs and ApoCIII

3.4.1 Introduction

In parallel with investigations of ApoCIII in intact skin, wounded skin, in diabetic intact skin and diabetic wounded skin, we sought to determine downstream mediators of ApoCIII signaling. Paucity of APOCIII receptors and or binding partners known to date make this task even more challenging. Thus far, there only 2 putative molecular players involved with ApoCIII signaling. The term putative is used here as none of these binding partners have been shown to physically interact with APOCIII. The above statement is true at the time of writing because the one paper that showed binding between APOCIII and TLR2 was retracted in 2014 due to improper data documentation. To capture current knowledge, a brief summary of the three possible pathways of ApoCIII signaling will be provided. Additionally, an explanation for why APOCIII-TLR association was looked at in this chapter will also be given.

One group has shown that APOCIII interacts with the scavenger receptor class B type I (SR-BI) and upregulate β_1 -integrin expression in PKA and Src kinase pathway-dependent fashion (Shi,

Yang et al. 2014). This mechanism leads to hyperactivation of voltage-gated Ca^{2+} channels in the pancreatic β -cell, leading to apoptosis. Whether or not this pathway is unique to pancreatic β -cells remains to be established. SR-B1 is known to be largely expressed in the liver but global knockout of SR-B1 results in a relatively weak phenotype of hypercholesterolemia and does not recapitulate with the *ApoCIII* knockout mice (Huby, Doucet et al. 2006). This suggests that ApoCIII may work through other receptors in addition to SR-B1.

Another study demonstrated that ApoCIII could activate the pertussis toxin (PTX)-sensitive G protein pathway, which in turn induces phosphatidylcholine-specific phospholipase (PC-PLC) activation (Kawakami, Aikawa et al. 2007). This leads to activation of the diacylglycerol (DAG), which activates with $\text{PKC}\alpha$. $\text{PKC}\alpha$ activation in turn induces NF- κ B translocation into nucleus thereby triggering β 1-integrin expression in monocytes, resulting in enhanced adhesion to ECs. In addition, ApoCIII also increases $\text{PKC}\beta$ and NF- κ B in ECs, which induces expression of VCAM-1 and intercellular adhesion molecules (ICAM-1) in ECs (Kawakami, Aikawa et al. 2006). These studies allude to a possible interaction between ApoCIII although the lack of binding studies leaves the possibility that ApoCIII works through a different receptor that in turn activates the G protein-coupled receptor remains a possibility.

In addition, ApoCIII is a potent inhibitor of LPL binding to its receptor but ApoCIII does not bind to LPLR per se. It inhibits the LPL-LPLR interaction by replacing the strong LPLR binding proteins such as ApoE from the lipoprotein, thereby interfering with LPL clearance.

Given the rising importance of ApoCIII especially for the treatment of hypertriglyceridemia, it is important to understand the mechanism by which ApoCIII exerts its function. As shown above, it seems that ApoCIII interacts with a myriad of receptors and that this interaction may differ from cell to cell. ApoCIII disrupts LPL-LPLR interactions by displacing other LPLR-binding proteins from the lipoprotein. ApoCIII through an unknown GPCR also exerts effects through G-protein signaling in ECs. ApoCIII in pancreatic beta-cells is suggested to signal through β 1 integrin or SR-B1 receptors based on knockdown studies but the lack of rescue through upregulation of either receptor calls in question the association between ApoCIII and these receptors. Noteworthy, there were only two papers that showed a direct binding between ApoCIII and a receptor. However both papers have been retracted due to questionable documentation on effect of ApoCIII on ECs. Retracted papers normally do not deserve mention but in this instance a single figure showing APOCIII with TLR2 association through direct binding studies (BiaCore) led us first to suspect that TLR2 may indeed be a relevant binding partner of ApoCIII (Apolipoprotein CIII Induces Monocyte Chemoattractant Protein-1 and Interleukin 6 Expression Via Toll-Like Receptor 2 Pathway in Mouse Adipocytes). The paper was retracted because some of the western blot results were not reproducible but the direct binding studies (BiaCore figure) was not called into question. The downstream effect of ApoCIII differs from cell to cell. Studies demonstrate that ApoCIII directly activates the pro-inflammatory signal transduction in vascular cells, thus contributing the development of atherosclerosis (Hiukka, Stahlman et al. 2009). ApoCIII-rich lipoprotein as well

as ApoCIII itself enhances the adhesion of human monocytes to ECs *in vitro* (Kawakami, Aikawa et al. 2006, Tao, Xiong et al. 2016). As mentioned before, the role of ApoCIII in diabetes and its effects on pancreatic β -cells has been explored, especially in the context of T1D. Briefly, ApoCIII promote hyperactivation of voltage-gated Ca^{2+} channel, leading to β -cell apoptosis. Recently, it was also shown that *ApoCIII* mRNA was expressed in T2D pancreatic islets and that the locally produced APOCIII contributes to β -cell dysfunction (Avall, Ali et al. 2015).

To determine whether subtypes of TLRs are indeed endogenous receptors for mediating ApoCIII signaling, we asked whether the different TLRs are expressed in cell types that have been shown to respond to ApoCIII. TLRs were shown to be involved in the inflammatory process in atherosclerosis, similar to ApoCIII. Vascular cells, which ApoCIII acts on, responds to a wide range of TLR ligands (TLR 2, TLR4, TLR 5 and TLR 9) (Erridge, Burdess et al. 2008). Multiple reports suggest the involvement of TLR2, TLR4 and TLR9 in atherogenesis although the exact mechanisms by which TLRs play in atherosclerosis remain uncertain (Roshan, Tambo et al. 2016)

In this chapter, we decided to probe the possible interaction between APOCIII and TLR using an established system of ApoCIII effect on pancreatic β -cells. We first sought to determine APOCIII surface expression on pancreatic islets using immunofluorescence staining, a study that has not been conducted to date. Next, we verified TLR expression in pancreatic islets by qPCR and further stained islets with TLR specific antibodies to both verify the expression and to identify islet cells that express the different TLRs. Using immunofluorescence staining, we determined whether

TLRs are only expressed on β -cells or if other cell types within islets such as macrophages and ECs, also express TLRs.

3.4.2 Expression of APOCIII in the mouse pancreatic islets

Overnight incubation of added APOCIII to pancreatic β -cells results in Ca^{2+} -dependent apoptosis (Juntti-Berggren, Refai et al. 2004). Antisense (ASO) mediated knockdown of APOCIII in isolated islets improves Ca^{2+} signaling and insulin secretion. Furthermore, FACS-sorted β -cells were shown to be enriched for *ApoCIII* mRNA (Åvall, Ali et al. 2015) suggesting that islets and specifically β -cells do express *ApoCIII*. However, the presence of APOCIII within pancreatic islets has not been unequivocally shown till date because of the paucity of reliable immunofluorescence antibodies against APOCIII. It is widely accepted that the presence of mRNA does not necessarily correlate with the protein presence. Buoyed by a new reliable antibody against APOCIII, similar to that used for mouse APOCIII ELISA, we proceeded to verify whether APOCIII is indeed present in β -cells.

The presence of APOCIII in pancreatic islets of C57BL/6N (wild-type) and C57Bl/6J *Lepr^{db}* (*db/db*) was determined by co-immunostaining various antibodies with ApoCIII antibody on cryo-sections of 12-week wild-type and *db/db* mice (Fig. 3.30). Immunostaining of mouse pancreas showed positive staining of APOCIII in the islets of both wild-type (Fig. 3.30A) and *db/db* mice (Fig. 3.30B). However, in our hands, the APOCIII –positive cells did not co-localize with insulin-positive cells, suggesting that APOCIII was expressed in other islets cell types but not in β -cells.

Noteworthy, APOCIII immunostaining produced a high background signal especially in islets of *db/db* mice and this signal: noise ratio did not improve with altered antibody concentrations and different incubation times. Despite the higher background staining in diabetic islets, APOCIII still does not co-localize with insulin, suggesting that they are not present in β -cells. The pattern of APOCIII staining in the islets resembled that of islet capillaries and till date, there has been no reports on APOCIII being expressed in ECs. To pursue the identity of APOCIII-expressing cells in pancreatic islets, wild-type pancreas sections were further stained with antibodies specific for APOCIII and CD31, the latter being a cell surface marker for vascular endothelial cells (ECs). APOCIII was indeed co-localized with CD31-positive cells suggesting that ECs and not β -cells express and produce the apolipoprotein APOCIII within the mouse islets (Fig. 3.31A). A no-primary-antibody control was also performed as negative control (Fig. 3.31B). *ApoCIII* mRNA detection in islets from previous published studies may have come from ECs and the higher expression of ApoCIII noted in obese diabetic mice (T2D model) is perhaps a reflection of the higher islet vascular density and ECs present in the diabetic islets (Ilegems, Dicker et al. 2013).

Figure 3.30. Representative images of APOCIII staining in wild-type and diabetic mouse islets. Immunofluorescence staining of APOCIII in islets of (A) wild-type and (B) *db/db* mouse pancreas cryosections. APOCIII immunofluorescence signal (green) was co-stained with insulin (red) and DAPI stain of cell nuclei (blue) Images shown at Maximum Intensity Projection under 400x magnification. Scale bar = 20 μ m. n=3.

Figure 3.31. Representative images of APOCIII staining with CD31 and no primary control in mouse islets. Immunofluorescence staining of wild-type mouse pancreatic islets with (A) APOCIII (green) and insulin (purple) (left) and APOCIII (green) and CD31 (red) (middle), merged image with DAPI nuclei stain (blue) (right) (B) No primary antibody was added in negative control stain. Images shown at Maximum Intensity Projection under 400x magnification. Scale bar = 20 μ m. n=3.

3.4.3 Expression of TLRs in pancreatic islets of wild-type and diabetic mice

Upon determining that the localization of APOCIII is in endothelial cells and not beta cells, we then proceeded to determine the expression of TLRs (a putative APOCIII receptor) in the islets of both healthy and diabetic mice. The localization of TLR expression relative to APOCIII may suggest cell autocrine or paracrine action of APOCIII within the islet. Various TLRs have been shown expressed in β -cells (Vives-Pi, Somoza et al. 2003, Wen, Peng et al. 2004, Nackiewicz, Dan et al. 2014). To determine presence, localization and changes in expression of different TLRs (TRL1, 2, 3, 4, 5 and 9) in pancreatic islets (healthy and diabetic), 20-30 μ m cross-sections of mouse pancreas were obtained from male diabetic C57Bl/6J *Lepr^{db}* (*db/db*) and C57BL/6N wild-type (control) mice. Pancreas cryosections were stained with varied antibodies specific for TLR-1 (Wen, Peng et al. 2004), -2 (Shahrara, Pickens et al. 2009), -3 (Helminen, Huhta et al. 2017), -4 (Shen, Qin et al. 2016), -5 (Kauppila, Mattila et al. 2013), and -9 (Veselits, Tanaka et al. 2014). All the sections were co-stained with CD31 for ECs, CD68 for macrophages, insulin for β -cells and DAPI for nuclei.

Immunostaining of mouse pancreas revealed cells that were positive for TLR1 in the islets of both healthy and diabetic mice, with no observed change in TLR1 signal between healthy and diabetic mice (Fig. 3.32). A majority of TLR1 positive cells were found at the islet periphery with a few cells in the islet mantle of healthy islets, and this staining pattern resembles that of pancreatic α -cells (glucagon-expressing cells). Indeed, co-staining with the insulin antibody showed that TLR1

was not expressed in the insulin-positive β -cells, strongly suggesting that β -cells are not the source of TLR1 expression (especially for cells in the mantle) in islets (Steiner, Kim et al. 2010). In both wild-type and diabetic islets, TLR1 did not co-localize with CD31-positive cells, suggesting TLR1 was not expressed by ECs in the islets (Fig. 3.32A). Similarly, a paucity of cells that are double-stained for TLR1 and CD-68 suggests that TLR1 within islets was not expressed on macrophages in islets of healthy and diabetic mice (Fig. 3.32B). Together, TLR1 is not expressed in islet β -cells, ECs and macrophages and the staining pattern of TLR1, suggests that TLR1 expression may be confined to pancreatic α -cells. Further experiments are required to show this.

A

B

Figure 3.32. TLR1 expression in healthy and diabetic pancreatic islets. Representative immunofluorescence images of TLR1 (green) and insulin (purple) along with CD31 (red) and DAPI (blue) staining in pancreatic islets from healthy mice (top) or *db/db* mice (bottom) (A). Representative immunofluorescence staining of TLR1 (green) and insulin (purple) along with CD68 (red) and DAPI (blue) in pancreatic islets from healthy mice (top) or *db/db* mice (bottom) (B). Images shown at Maximum Intensity Projection under 400x magnification. Scale bar = 10 μ m. n=3.

Pancreatic islets of healthy and diabetic mice showed higher positivity for TLR2 compared to TLR1 (Fig. 3.33). In our immunofluorescence results, TLR2 signal was higher in diabetic islets compared to healthy islets. For localization of the TLR2 signal, we found that TLR2 did not co-localize with insulin, indicating that cell types other than β -cells within the islet express TLR2, contrary to what was reported before (Li, Song et al. 2012). Immunostaining of TLR2 with CD31 showed that TLR2 co-localizes with CD31-positive cells (yellow stained cells), albeit not all CD31-positive cells express TLR2 and not all TLR2 expressing cells are CD31 positive (Fig. 3.33A). In both control and diabetic islets, a subset of CD68-positive macrophages were also positive for TLR2, and may account for the remainder of TLR2-expressing cells that were not CD31 positive (Fig. 3.33B). These expression of TLR2 on islet macrophages corroborate a previous finding that suggests that palmitate-mediated β -cell dysfunction is exacerbated in the presence of TLR2 expressing macrophages.

Figure 3.33. TLR2 expression in healthy and diabetic pancreatic islets. Representative immunofluorescence staining of TLR2 (green) and insulin (purple) along with CD31 (red) and DAPI (blue) in pancreatic islets from healthy mice (top) or *db/db* mice (bottom) (A). Representative immunofluorescence staining of TLR2 (green) and insulin (purple) along with CD68 (red) and DAPI (blue) in pancreatic islets from healthy mice (top) or *db/db* mice (bottom) (B). Images shown at Maximum Intensity Projection under 400x magnification. Scale bar = 10 μ m. n=3.

Immunostaining of mouse pancreas showed that TLR3 was positively stained in pancreatic islets of both healthy and diabetic mice, albeit at lower levels compared to TLR1 and TLR2 (Fig. 3.34). In our immunofluorescence results, we did not observe a difference in TLR3 signal between healthy and diabetic islets. TLR 3 did not co-localize with insulin, indicating that TLR3 was not expressed by the pancreatic β -cells. We found that 100% of TLR3-positive cells co-localized with CD31 in these islets (Fig. 3.34A), which strongly indicates that ECs are the main source of TLR3 in healthy and *db/db* mouse islets. In addition, a paucity of CD68-positive macrophages were also positive for TLR3 in both control and diabetic islets (Fig. 3.34B). The results obtained here suggest that TLR3 expression in healthy and diabetic islets are confined to islet ECs, contrary to what was reported before (Giarratana, Penna et al. 2004).

A

B

Figure 3.34. TLR3 expression in healthy and diabetic pancreatic islets. Representative immunofluorescence staining of TLR3 (green) and insulin (purple) along with CD31 (red) and DAPI (blue) in pancreatic islets from healthy mice (top) or *db/db* mice (bottom) (A). Representative immunofluorescence staining of TLR3 (green) and insulin (purple) along with CD68 (red) and DAPI (blue) in pancreatic islets from healthy mice (top) or *db/db* mice (bottom) (B). Images shown at Maximum Intensity Projection under 400x magnification. Scale bar = 10 μ m. n=3.

Pancreatic islets of healthy and diabetic mice showed that TLR4 was positively stained, albeit at lower levels compared to TLR1 and TLR2 (Fig. 3.35). From our immunofluorescence results, there was no observed difference in TLR4 signal between healthy and diabetic mouse islets. Co-staining with insulin antibody showed that TLR4 was not expressed in the insulin-positive β -cells, strongly suggesting that β -cells are not the source of expression in islets. Immunostaining of TLR4 with CD31 showed that TLR4 co-localizes with CD31-positive cells, albeit all not TLR4 positive cells express CD31 and not all CD31 expressing cells are TLR4 positive (Fig. 3.35A). In addition, a subset of CD68-positive cells were also positive for TLR4 and may constitute other TLR4-positive cells that are not CD31 positive (Fig. 3.35B). This data suggest that TLR4 is not expressed in islet β -cells of healthy and diabetic mice, in contrary to what was reported before (Giarratana, Penna et al. 2004) and the expression of TLR4 is confined to ECs and macrophages.

A

B

Figure 3.35. TLR4 expression in healthy and diabetic pancreatic islets. Representative immunofluorescence staining of TLR4 (green) and insulin (purple) along with CD31 (red) and DAPI (blue) in pancreatic islets from healthy mice (top) or *db/db* mice (bottom) (A). Representative immunofluorescence staining of TLR4 (green) and insulin (purple) along with CD68 (red) and DAPI (blue) in pancreatic islets from healthy mice (top) or *db/db* mice (bottom) (B). Images shown at Maximum Intensity Projection under 400x magnification. Scale bar = 10 μ m. n=3.

From our immunofluorescence results, we did not detect any presence of TLR5 in the islets of healthy and diabetic mice (Fig. 3.36). For the validity of this TLR5 antibody, in addition to the published literature (Kauppila, Mattila et al. 2013), we have verified this antibody using peritoneal exudate cells, which show a positive staining for TLR5 (data not shown). Therefore, murine pancreatic islets do not express TLR5.

Immunostaining of mouse pancreas showed cells that were positive for TLR9 in the islets of both healthy and diabetic mice, albeit weaker signal compared to other TLRs (Fig. 3.37). In our immunofluorescence results, TLR9 signal was higher in diabetic islets compared to healthy islets. Co-staining with insulin antibody showed that TLR9 did not co-localize with insulin-positive cells, suggesting that TLR9 was not expressed in the β -cells. Immunostaining of TLR9 with CD31 showed that 100% of TLR9-positive cells co-localized with CD31 in these islets, which strongly indicates that ECs are the main source of TLR9 in healthy and *db/db* mouse islets. The results suggest that TLR9 expression is higher in islets of diabetic mice compared to healthy mice and that the expression of TLR9 in pancreatic islets was solely confined to islet ECs.

Figure 3.36. TLR5 expression in healthy and diabetic pancreatic islets. Representative immunofluorescence staining of TLR5 (green) and insulin (purple) along with CD31 (red) and DAPI (blue) in pancreatic islets from healthy mice (top) or *db/db* mice (bottom). Images shown at Maximum Intensity Projection under 400x magnification. Scale bar = 10 μ m. n=3.

Figure 3.37. TLR9 expression in healthy and diabetic pancreatic islets. Representative immunofluorescence staining of TLR9 (green) and insulin (purple) along with CD31 (red) and DAPI (blue) in pancreatic islets from healthy mice (top) or *db/db* mice (bottom). Images shown at Maximum Intensity Projection under 400x magnification. Scale bar = 10 μ m. n=3.

3.4.4 Expression of TLRs in the skin of wild-type and diabetic mice

TLRs have been reported to be expressed in healthy skin and elevated in healthy wounds (Chen, Guo et al. 2013, Chen and DiPietro 2017). However, to date, only two TLRs; TLR2 (Dasu, Thangappan et al. 2010) and TLR4 (Dasu and Jialal 2013) have been studied in diabetic mouse wounds. In addition, the same group showed that TLR1, 2, 4 and 6 are elevated in diabetic foot ulcers (Dasu and Martin 2014). We sought to determine the gene expression of selected TLRs, namely *TLR2*, 3, 4 and 9, as they have been shown to be involved in acute cutaneous wounds of healthy mouse. Here, we determined the relative gene expression of *TLR 1*, 2, 3, 4, 5 and 9 in intact skin of healthy mice C57BL/6N (control) and diabetic C57Bl/6J *Lepr^{db}* (*db/db*) mice using qPCR (Fig.3.38).

We found that in the healthy (wild-type) mouse skin, the expression level of *TLR1* was the lowest compared to all other TLRs measured (Fig 3.38G). *TLR3* mRNA expression was the highest among the TLRs measured and the expression of *TLR2*, 4 and 5 were also relatively high in the normal skin. In *db/db* skin, *TLR1* expression was again the lowest and *TLR4* was the highest among the TLRs measured, as opposed to TLR3 in the healthy mouse skin (Fig 3.38H). Despite the differences in the relative TLR expression in healthy and diabetic mouse skin, all the TLRs measured except *TLR-3* were significantly elevated in the skin of diabetic mice compared to non-diabetic controls (Fig 3.38A-F). The relative expression of *TLR1*, 4 and 9 were at least 4-fold higher in diabetic skin than non-diabetic skin. *TLR2* and *TLR5* mRNA expression levels were also

increased by 2-fold in diabetic skin compared to that of healthy skin. Taken together these results suggest that within intact skin, the expression of TLRs is increased in diabetic skin and this perhaps suggests a response by skin cells (e.g. keratinocytes, fibroblast and ECs) to the low grade inflammation often characterized in the diabetic animals and patients alike (Pereira and Alvarez-Leite 2014). Our results are in line with the earlier studies showing increased TLR2, 4 and MyD88 expression in the STZ-induced diabetic mouse skin (Dasu, Thangappan et al. 2010). Increased TLR expression in the skin of the *db/db* mouse model corroborates with the increased level of TLRs such as TLR1, 2 and 4 in T2D diabetic skins ulcers (Dasu and Martin 2014).

Figure 3.38. Toll-like receptor expression in intact skin of healthy (control) and diabetic mice. Relative mRNA expression of TLR 1, 2, 3, 4, 5 and 9 (A-F) in intact skin of 12-week control and *db/db* mice. TLR mRNA expressions data in control (G) and *db/db* (H) skin were compiled for a better comparison of the different TLR present in skin. Gene expressions normalized to GAPDH levels and data represented as means $\pm$ s.e.m. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. $n=4$.

3.4.5 Discussion

In this chapter, we have shown that APOCIII is expressed in pancreatic islets of control and *db/db* mice. Using IF staining, we observed that vascular ECs as the main source of APOCIII expression in these islets and this had not been reported before. The increased vascularization seen in diabetic islets may be a possible reason why, APOCIII expression was also increased in diabetic islets compared to healthy islets. There has been emerging studies of the effects of metabolic aberrations of TLRs and their role in diabetes and its complications. TLR2 and TLR4 has been studied extensively for its role in the pathogenesis of insulin resistance, inflammation and diabetes (Song, Kim et al. 2006, Kim, Han et al. 2007, Dasu, Devaraj et al. 2010). qPCR studies on pancreatic islets show that TLRs are expressed in both mouse and human islets (Vives-Pi, Somoza et al. 2003, Giarratana, Penna et al. 2004, Wen, Peng et al. 2004). However, it is puzzling that none of these studies show surface staining of TLRs on the islets to identify if all islet cells or only a subset of islet cells express the different TLRs. ApoE has been shown to suppress TLR3- and TLR4-mediated inflammatory responses (Ali, Middleton et al. 2005) through Heparan sulfate proteoglycan (HSPG) binding related mechanism (Zhu, Kodvawala et al. 2010). ApoA1 was shown to interact with TLR4 receptor in vitro, in primary chondrocytes and fibroblast-like synoviocytes (de Seny, Cobraiville et al. 2015). However, ApoCIII-TLR association remains uncertain.

To determine if TLRs are possible receptors for APOCIII, we sought to determine the expression pattern of TLRs in pancreas in the Islet of Langerhans, where APOCIII was consistently shown to exert a pro-apoptotic effect. We determine the repertoire of TLRs (TLR 1, 2, 3, 4, 5 and 9) that are expressed in the pancreatic islets of healthy and *db/db* mice and identified the source of TLR expressing cells in pancreatic islets. We have shown that β -cells were not the source of TLRs expression in pancreatic islets, but in fact, TLRs were mainly expressed in CD31-positive vascular ECs and a few TLRs (2, 3 and 4) were expressed in CD68-positive macrophages of pancreatic islets. None of the TLRs studied are expressed on β -cells, and single-cell exome studies seem to corroborate this finding (Dorajoo & Ali et al 2017). TLRs have been known to be expressed in ECs (Salvador, Arranz et al. 2016), and thus it is perhaps unsurprising that we observed TLRs expressed on microvascular ECs of pancreatic islets. Beside co-localization with ECs, TLR1 and TLR2 were mostly expressed in periphery of pancreatic islets, which is similar to localization of pancreatic α -cells. This needs to further confirm by immunostaining with antibody specific for glucagon.

A few studies corroborate with our data showing that macrophages are the major contributors of islet inflammation in response to TLR2, 4 and 6 in T2D (Eguchi, Manabe et al. 2012, Westwell-Roper, Ehses et al. 2014). It was shown that TLR-mediated islet inflammation and impair β -cell insulin gene expressions are mediated by macrophages (Nackiewicz, Dan et al. 2014). Another study also suggests that TLR4 mediated macrophage recruitment leads to β -cell dysfunction in HF diet-induced mice (Li, Chen et al. 2013). It is tempting to speculate that perhaps ApoCIII, similar

to the palmitate, works in a paracrine manner to affect β -cell function. While palmitate is suggested to act on β -cells via macrophages, it is tempting to speculate that ApoCIII could act on β -cells via ECs based on our findings (through activation of ECs, TLR2, 3 or 4). Further experiments are required to definitively show this.

We also sought to understand if TLR expression in the skin is altered in diabetic states using the *db/db*, T2D mice. We found that with the exception of TLR3, the relative expression of all other TLRs studied (TLR1, 2 and 4) were 2-4 fold higher in *db/db* mice skin compared to that of non-diabetic control skin. It is likely that keratinocytes and fibroblasts are the main source of TLR expression as they are the main constituent of epidermis and dermis and TLR1-5 and 9 are known to be present in skin keratinocytes and fibroblasts (Curry, Qin et al. 2003, Köllisch, Kalali et al. 2005). TLR2, 3 and 4 are increased in mouse skin wounds and colocalize with keratinocytes (Lin, Wang et al. 2012, Chen, Guo et al. 2013). There has been no report of ApoCIII expression in skin cell lines and we have found that *ApoCIII* is not expressed in HaCaT cell lines *in vitro* but in the skin, ApoCIII appears to be expressed in keratinocytes. Further work, using skin specific knockouts of the various TLRs will help to elucidate if indeed ApoCIII works through TLRs in the skin to retard keratinocyte migration during wound healing.

3.4.6 Limitations and Suggested Improvements

A few limitations of the current study should be acknowledged. It is noted that similar expression pattern of ApoCIII and TLRs in the pancreatic islets does not qualify TLRs as cognate receptors

of ApoCIII. To study the interaction of TLRs and ApoCIII, we could use a BiaCore system which is a widely used platform to study ligand-receptor interaction in vitro. Fractions of human recombinant TLR receptors can be prepared using an overexpression construct in HEK293T cells and human recombinant ApoCIII can be commercially purchased. TLRs will be immobilized to the sensorchip while ApoCIII is used as the analyte. In addition, LigandTracer technology can be used to measure radioactive or fluorescence labelled ligand and targets that can be immobilized to the plastic surface of a petri dish. In our case, β -cells can be grown on the petri dish followed by addition of labeled ligand, ApoCIII to measure their interaction. Lastly, since TLRs are known to be expressed in keratinocytes, we can also study TLR-ApoCIII interaction in keratinocytes as well. Knowing now that exogenously added ApoCIII can retard keratinocyte migration (measurable outcome), it is worth pursuing studies on ApoCIII effects on TLR knockdown and/or overexpressing keratinocytes.

4. GENERAL DISCUSSION

Diabetes is a multifactorial, metabolic and lifelong disease associated with various debilitating chronic complications including diabetic foot ulcers. In diabetic wounds, healing is known to be impaired by several factors, especially excess and prolonged inflammation (Guo and DiPietro 2010). ApoCIII is a diabetogenic and pro-inflammatory molecule that is reported to increase in Caucasian Type-2 diabetic and has been shown to have negative effect on insulin resistance and β -cell function thereby exacerbating diabetes progression (Holmberg, Refai et al. 2011, Avall, Ali et al. 2015). To the best of our knowledge, ApoCIII has not been studied in skin and wound healing. The main focus of this thesis was to determine the role of ApoCIII during both normal (healthy) wound healing and wound healing during diabetes.

In this study, we measured APOCIII levels in Singapore patients to determine whether there was significant increase in the serum concentration of APOCIII in diabetic patients compared to non-diabetic patients. Given the dearth of information on levels of APCIII in Asians, it was important to validate if the Caucasian values of APOCIII in T2D could be extended to include Asians as well. Our results (which include internal controls from Caucasian samples) suggest that there is no difference in circulating [APOCIII] between T2D and age-matched healthy patients. In our study, we observed that the measured value of [APOCIII] varied between ELISA kits used from different manufacturers. There is lack of consistency in the serum [APOCIII] concentrations reported in different studies. Therefore, to ensure reliability in measurements of serum [APOCIII] across

cohorts, it is necessary to standardize a protocol that encompasses blood sampling, blood handling/storage and measuring/ analyzing of serum [APOCIII]. Standardization will allow us to accurately compare and contrast values between the different studies.

To first understand the role of ApoCIII in cells of the skin that are involved in healing, namely fibroblasts and keratinocytes, we determined the expression of *ApoCIII* in established cell lines *in vitro*. Our data suggests that *ApoCIII* is not expressed in the fibroblast and keratinocytes cell lines tested but that the addition of exogenous purified APOCIII delay wound closure *in vitro*. In an *in vitro* wound model, APOCIII impedes wound closure of HaCaT keratinocytes and this APOCIII-mediated effect on cell migration is observed only upon injury. In addition, we have observed that a subset of genes are marginally altered in APOCIII-treated cells *in vitro*. Our results allude to the possibility that APOCIII treatment affected the expression of genes involved in collagen and ECM deposition as well as inflammation. Our data suggests that the negative impact of APOCIII on wound healing could possibly be associated with pro-inflammation and reduced collagen deposition, both of which have been shown to reduce the rate of wound repair *in vitro* (Anderson and Hamm 2014).

The results of ApoCIII expression on cell lines *in vitro* do not reflect that of cells *in vivo*. *In vivo*, we found ApoCIII protein (by immunofluorescence) and transcript (by qPCR) expression in intact skin and wounds of wild-type (healthy) and *db/db* (diabetic) mice. The expression of APOCIII was found to be mainly in the epidermis and to a lesser extent, the dermis. ApoCIII expression is higher

in diabetic mouse skin compared to that of control as determined using mRNA and protein measurements. Furthermore, ApoCIII expression is significantly higher in the wounds compared to unwounded skin in both wild-type and diabetic mice, suggesting that ApoCIII could have a role in wound healing.

Our splinted excisional wound model with topical application of ApoCIII-ASO *in vivo* corroborated the *in vitro* data showing that ApoCIII indeed exerts a negative impact on splinted wound healing. The splinted wound model was used as it better approximates human wound healing as compared to the conventional wound model as it inhibits contraction that predominates in mouse wound healing. In this study, we show for the first time that lowering of APOCIII at the wound site with ApoCIII-ASO treatment, significantly improves wound healing in both wild-type and diabetic mice *in vivo*. Topical application of ApoCIII-ASO at 100 μ mol/L every two days significantly accelerated wound closure compared to control-treated wounds with improvements in re-epithelialization and dermal collagen maturation in ASO-treated wounds. In wild-type mice at day 9 post surgery, wounds with ASO treatment were 90% healed compared to control wounds that were 74% healed. In diabetic mice, at day 16 post wounding, ASO-treated wounds were 90% healed compared to control wounds that were 70% healed.

The data obtained on expression patterns of APOCIII and TLRs in pancreatic islets suggest that ApoCIII could possibly act on β -cell via paracrine interactions of EC, TLR2, or TLR4. TLR2 has been suggested to directly bind to APOCIII and TLR4 has been shown to bind to saturated fatty

acids. Further investigations are required to determine whether TLR2 and/or TLR4 are *bona fide* binding partners of APOCIII in skin. Further studies on skin-specific knockouts various TLRs may help to elucidate if indeed ApoCIII works through TLRs in the skin during wound healing.

Nonetheless, the experiments herewith show a significant advance by highlighting for the first time the utility in reducing APOCIII levels in skin wounds and, by showing that beyond the cardiovascular system, APOCIII plays a role in skin wound healing. Further experiments are required to show the molecular mechanism by which APOCIII inhibits wound healing. The results contained in this thesis will be part of a patent application.

5. CONCLUSIONS

1. There is no significant difference in the serum ApoCIII concentrations between diabetic and non-diabetic patients in Singapore. There is a possible fundamental difference between Asian/Singapore diabetic and Caucasian diabetics, besides Asian diabetic having lean phenotype. And this may represent a lack of difference in serum APOCIII concentrations in Asians versus Caucasians. Also, the Asian diet, being rich in carbohydrates as opposed to fat may have implications on the serum APOCIII measured.
2. Addition of purified APOCIII delays HaCaT keratinocytes migration in vitro. There is no dose-dependent effect and the ApoCIII-mediated effect on keratinocyte migration is only observed upon injury (no added effect on pre-treated cells). This suggests that ApoCIII affects the cell migration and specifically a few wound-healing related genes were found to be altered when treated with ApoCIII in 'wounded' keratinocytes. Our data suggests APOCIII delay wound healing by modulating inflammation, ECM formation and thus keratinocyte migration.
3. ApoCIII is expressed in skin and its expression is higher in *db/db* intact skin and is further increased in the wounds of wild-type and *db/db* mice. APOCIII is mainly expressed in the epidermis and to a lower extent, dermis. In the wounds, APOCIII is strongly expressed in keratinocytes around the wound margin. There is a difference in spatial expression of APOCIII at different regions of the wounds suggesting different roles of APOCIII in physiological stage or upon injury (when cells are proliferating or migrating).

4. Topical application of ApoCIII-ASO in pluronic gel significantly improved wound healing compared to control treatment in wild-type mice. Histological examination of ASO-treated wounds revealed improved epithelialization and dermal collagen formation compared to control-treated wounds.
5. In the splinted excisional wound model of *db/db* mice, topical treatment with ApoCIII- ASO also significantly increased wound closure as well as improved re-epithelialization and dermal collagen maturation compared to controls.
6. APOCIII is expressed in ECs of pancreatic islets and its expression is increased in *db/db* islets. We speculate that ApoCIII exerts its effect on β -cells by acting through EC, TLR2 or TLR4.

6. FUTURE PERSPECTIVES

Further investigation should be carried out to validate if serum concentrations of APOCIII are truly different between Asian or at least Singapore diabetics and Caucasian diabetics. It is also of importance to standardize a protocol that allows for accurate measurements of serum APOCIII concentrations.

We have identified a few wound-healing-related genes that are differentially expressed in HaCaT keratinocytes upon ‘injury’ with purified human APOCIII treatment. To tease out the underlying molecular mechanisms by which ApoCIII affects wound healing, it would be important to validate the genes (by either knockdown or overexpression studies) that were identified to be altered upon APOCIII treatment. Also, a detailed time course experiment on gene expression changes along with protein measurements may shed light on the effect of APOCIII on delayed wound healing. A proteomic analysis of ECM proteins may also shed more light on the effect of APOCIII-mediated retardation of keratinocyte migration. Future analysis could also be performed, with the use of PCR array, in mouse wounds treated with purified mouse APOCIII protein.

Having shown that local ApoCIII-ASO treatment significantly improves wound healing in splintered wounds of both healthy and diabetic mice, it would be interesting to investigate whether wound healing is delayed in *ApoCIII*^{-/-} mouse and if APOCIII ASO has no effect on wound healing in the *ApoCIII*^{-/-} mouse. As part of the future studies, we have found that topical application of ApoCIII-ASO could knockdown APOCIII expression at wound site in wild-type mouse wounds

harvested on day 6 post surgery (n=1) (Fig 3.39). Experiments need to be repeated to better validate the knockdown efficiency of APOCIII expression at wound sites. In future studies, the ASO chemistry could be optimized for skin delivery of ApoCIII-ASO. We will, in collaboration with SPMS, test various chemical backbones of ASOs to achieve the optimum knockdown of ApoCIII at wound site. In addition, APOCIII ASO needs to be designed in the context of a wound dressing that is safe, convenient and that allows for spatial and temporal release of ApoCIII-ASO at wound site (especially since our data indicates that APOCIII knockdown is beneficial in early stage diabetic wounds).

To ensure that this work is relevant, it is of importance to determine if ApoCIII is expressed in human skin and whether ApoCIII is similarly increased in human wounds, especially in diabetic chronic wounds. We will, in conjunction with KTPH, determine APOCIII levels in diabetic foot ulcer exudates.

Figure 3.39. Topical application of ApoCIII-ASO reduces APOCIII expression at wound site in a wild-type mouse day 6 post wounding. Immunofluorescence staining of paraffin sections from excisional wounds of a 12-week wild-type mouse treated with (A) vehicle or (B) ApoCIII-ASO (100 μmol/ L). Boxed areas are enlarged to adjacent corresponding boxes. APOCIII immunofluorescence signal (green) was countered by a blue DAPI stain of cell nuclei. Arrows indicate the direction of migration of wound tip. Scale bar as indicated in individual images, n=1

7. ACKNOWLEDGMENT

The work as presented in this thesis was carried out at the Lee Kong Chain School of Medicine-Nanyang Technological University in Singapore. I would like to thank everyone who has helped me along the way to complete this thesis and in particular:

I would like to express my deepest gratitude to my main supervisor Asst. Prof. Yusuf Ali, without whom I will not be writing this acknowledgement. Thank you for giving me this opportunity. Your patience, guidance and encouragements are the most crucial factors for me in achieving this goal. You have the rare gift of putting things in perspective when all hope seems lost. I could not have imagined having a better supervisor.

I would also like to heartily thank my co-supervisors Prof. David Becker, for your expertise and guidance and Asst. Prof Dang Thuy Tram, for your insightful comments and encouragements. I would like to thank my mentor, Dr.Jingxiang Huang, for your clinical expertise and insightful comments on H&E analysis. I would like to thank Prof. Phan Anh Tuan for providing us with ASO for the experiments.

I thank Vanessa Tay, Dr.Manesh Chittezhath and Minni Chua for lending me a hand whenever I need help with my experiments. I also wish to express my gratitude to all my fellow lab mates who have provided a positive atmosphere to work with.

I thank my friends Alaric, Alex and Jia Wang for being there for me in my darkest times.

Finally, I would like to thank my parents for their love and unconditional trust in me.

8. REFERENCES

- Acosta, J. B., D. G. del Barco, D. C. Vera, W. Savigne, P. Lopez-Saura, G. Guillen Nieto and G. S. Schultz (2008). "The pro-inflammatory environment in recalcitrant diabetic foot wounds." Int Wound J **5**(4): 530-539.
- al Muhtaseb, N., A. al Yousuf and J. S. Bajaj (1992). "Apolipoprotein A-I, A-II, B, C-II, and C-III in children with insulin-dependent diabetes mellitus." Pediatrics **89**(5 Pt 1): 936-941.
- Alfaro, M. P., D. L. Deskins, M. Wallus, J. DasGupta, J. M. Davidson, L. B. Nanne, A. G. M., M. Gannon and P. P. Young (2013). "A physiological role for connective tissue growth factor in early wound healing." Lab Invest **93**(1): 81-95.
- Ali, K., M. Middleton, E. Pure and D. J. Rader (2005). "Apolipoprotein E suppresses the type I inflammatory response in vivo." Circ Res **97**(9): 922-927.
- Altomonte, J., L. Cong, S. Harbaran, A. Richter, J. Xu, M. Meseck and H. H. Dong (2004). "Foxo1 mediates insulin action on apoC-III and triglyceride metabolism." J Clin Invest **114**(10): 1493-1503.
- Anderson, K. and R. L. Hamm (2014). "Factors That Impair Wound Healing." The journal of the American College of Clinical Wound Specialists **4**(4): 84-91.
- Ang, Y., C. W. Yap, N. Saxena, L.-K. Lin and B. H. Heng (2016). "Diabetes-related lower extremity amputations in Singapore." Proceedings of Singapore Healthcare **26**(2): 76-80.
- Atzmon, G., M. Rincon, C. B. Schechter, A. R. Shuldiner, R. B. Lipton, A. Bergman and N. Barzilai (2006). "Lipoprotein genotype and conserved pathway for exceptional longevity in humans." PLoS Biol **4**(4): e113.
- Avall, K., Y. Ali, I. B. Leibiger, B. Leibiger, T. Moede, M. Paschen, A. Dicker, E. Dare, M. Kohler, E. Ilegems, M. H. Abdulreda, M. Graham, R. M. Crooke, V. S. Y. Tay, E. Refai, S. K. Nilsson, S. Jacob, L. Selander, P. O. Berggren and L. Juntti-Berggren (2015). "Apolipoprotein CIII links islet insulin resistance to beta-cell failure in diabetes." Proc Natl Acad Sci U S A **112**(20): E2611-2619.
- Avall, K., Y. Ali, I. B. Leibiger, B. Leibiger, T. Moede, M. Paschen, A. Dicker, E. Daré, M. Köhler, E. Ilegems, M. H. Abdulreda, M. Graham, R. M. Crooke, V. S. Y. Tay, E. Refai, S. K. Nilsson, S. Jacob, L. Selander, P.-O. Berggren and L. Juntti-Berggren (2015). "Apolipoprotein CIII links islet insulin resistance to β -cell failure in diabetes." Proceedings of the National Academy of Sciences **112**(20): E2611-E2619.
- Balingit, P. P., D. G. Armstrong, A. M. Reyzelman, L. Bolton, S. J. Verco, K. E. Rodgers, K. A. Nigh and G. S. diZerega (2012). "NorLeu3-A(1-7) stimulation of diabetic foot ulcer healing: results of a randomized, parallel-group, double-blind, placebo-controlled phase 2 clinical trial." Wound Repair Regen **20**(4): 482-490.
- Baltzis, D., I. Eleftheriadou and A. Veves (2014). "Pathogenesis and treatment of impaired wound healing in diabetes mellitus: new insights." Adv Ther **31**(8): 817-836.
- Barrientos, S., O. Stojadinovic, M. S. Golinko, H. Brem and M. Tomic-Canic (2008). "Growth factors and cytokines in wound healing." Wound Repair Regen **16**(5): 585-601.
- Bekeschus, S., J. W. Lackmann, D. Gumbel, M. Napp, A. Schmidt and K. Wende (2018). "A Neutrophil Proteomic Signature in Surgical Trauma Wounds." Int J Mol Sci **19**(3).
- Berg JM, T. J., Stryer L (2002). Biochemistry. 5th edition
- Bhutani, S. and G. Vishwanath (2012). "Hyperbaric oxygen and wound healing." Indian Journal of Plastic Surgery : Official Publication of the Association of Plastic Surgeons of India **45**(2): 316-324.
- Bloomgarden, Z. T. (2008). "The Diabetic Foot." Diabetes Care **31**(2): 372-376.
- Bogan, R. L. and J. D. Hennebold (2010). "The Reverse Cholesterol Transport System as a Potential Mediator of Luteolysis in the Primate Corpus Luteum." Reproduction (Cambridge, England) **139**(1): 163-176.

Bommer, C., E. Heesemann, V. Sagalova, J. Manne-Goehler, R. Atun, T. Barnighausen and S. Vollmer (2017). "The global economic burden of diabetes in adults aged 20-79 years: a cost-of-illness study." Lancet Diabetes Endocrinol **5**(6): 423-430.

Boren, J. and K. J. Williams (2016). "The central role of arterial retention of cholesterol-rich apolipoprotein-B-containing lipoproteins in the pathogenesis of atherosclerosis: a triumph of simplicity." Curr Opin Lipidol **27**(5): 473-483.

Boulton, A. J. M., D. G. Armstrong, S. F. Albert, R. G. Frykberg, R. Hellman, M. S. Kirkman, L. A. Lavery, J. W. LeMaster, J. L. Mills, M. J. Mueller, P. Sheehan and D. K. Wukich (2008). "Comprehensive Foot Examination and Risk Assessment: A report of the Task Force of the Foot Care Interest Group of the American Diabetes Association, with endorsement by the American Association of Clinical Endocrinologists." Diabetes Care **31**(8): 1679-1685.

Boulton, A. J. M., R. A. Malik, J. C. Arezzo and J. M. Sosenko (2004). "Diabetic Somatic Neuropathies." Diabetes Care **27**(6): 1458-1486.

Brahimaj, A., S. Ligthart, M. A. Ikram, A. Hofman, O. H. Franco, E. J. Sijbrands, M. Kavousi and A. Dehghan (2017). "Serum Levels of Apolipoproteins and Incident Type 2 Diabetes: A Prospective Cohort Study." Diabetes Care **40**(3): 346-351.

Brahm, A. J. and R. A. Hegele (2015). "Chylomicronaemia--current diagnosis and future therapies." Nat Rev Endocrinol **11**(6): 352-362.

Brem, H., A. Kodra, M. S. Golinko, H. Entero, O. Stojadinovic, V. M. Wang, C. M. Sheahan, A. D. Weinberg, S. L. Woo, H. P. Ehrlich and M. Tomic-Canic (2009). "Mechanism of sustained release of vascular endothelial growth factor in accelerating experimental diabetic healing." J Invest Dermatol **129**(9): 2275-2287.

Brem, H. and M. Tomic-Canic (2007). "Cellular and molecular basis of wound healing in diabetes." J Clin Invest **117**(5): 1219-1222.

Broughton, G., 2nd, J. E. Janis and C. E. Attinger (2006). "The basic science of wound healing." Plast Reconstr Surg **117**(7 Suppl): 12s-34s.

Brown, W. V., R. I. Levy and D. S. Fredrickson (1969). "Studies of the proteins in human plasma very low density lipoproteins." J Biol Chem **244**(20): 5687-5694.

Brunet-Maheu, J. M., J. C. Fernandes, C. A. de Lacerda, Q. Shi, M. Benderdour and P. Lavigne (2009). "Pluronic F-127 as a cell carrier for bone tissue engineering." J Biomater Appl **24**(3): 275-287.

Burdett, H. (2016). "Antisense inhibition of apolipoprotein C-III in patients with hypertriglyceridemia." Ann Clin Biochem **53**(3): 415.

Camont, L., M. J. Chapman and A. Kontush (2011). "Biological activities of HDL subpopulations and their relevance to cardiovascular disease." Trends Mol Med **17**(10): 594-603.

Carnevale Schianca, G. P., R. Pedrazzoli, S. Onolfo, E. Colli, E. Cornetti, L. Bergamasco, G. P. Fra and E. Bartoli (2011). "ApoB/apoA-I ratio is better than LDL-C in detecting cardiovascular risk." Nutr Metab Cardiovasc Dis **21**(6): 406-411.

Caron, S., A. Verrijken, I. Mertens, C. H. Samanez, G. Mautino, J. T. Haas, D. Duran-Sandoval, J. Prawitt, S. Francque, E. Vallez, A. Muhr-Tailleux, I. Berard, F. Kuipers, J. A. Kuivenhoven, S. B. Biddinger, M. R. Taskinen, L. Van Gaal and B. Staels (2011). "Transcriptional activation of apolipoprotein CIII expression by glucose may contribute to diabetic dyslipidemia." Arterioscler Thromb Vasc Biol **31**(3): 513-519.

Cavanagh, P. R. and S. A. Bus (2010). "Off-loading the diabetic foot for ulcer prevention and healing." J Vasc Surg **52**(3 Suppl): 37s-43s.

Chang, P.-Y., C.-M. Lee, H.-C. Hsu, H.-J. Lin, K.-L. Chien, M.-F. Chen, C.-H. Chen, Y.-T. Lee and C.-Y. Yang (2012). "Identification of the HDL-ApoCIII to VLDL-ApoCIII ratio as a predictor of coronary artery disease in the general population: The Chin-Shan Community Cardiovascular Cohort (CCCC) study in Taiwan." Lipids in Health and Disease **11**: 162-162.

Chatterjee, S., K. Khunti and M. J. Davies (2017). "Type 2 diabetes." The Lancet **389**(10085): 2239-2251.

Chatterjee, S. S. (2012). "Venous ulcers of the lower limb: Where do we stand?" Indian J Plast Surg **45**(2): 266-274.

Chen, L. and L. A. DiPietro (2017). "Toll-Like Receptor Function in Acute Wounds." Advances in Wound Care **6**(10): 344-355.

Chen, L., S. Guo, M. J. Ranzer and L. A. DiPietro (2013). "Toll-like receptor 4 has an essential role in early skin wound healing." J Invest Dermatol **133**(1): 258-267.

Chen, M., J. L. Breslow, W. Li and T. Leff (1994). "Transcriptional regulation of the apoC-III gene by insulin in diabetic mice: correlation with changes in plasma triglyceride levels." J Lipid Res **35**(11): 1918-1924.

Chen, Y.-J., C.-C. Chen, T.-K. Li, P.-H. Wang, L.-R. Liu, F.-Y. Chang, Y.-C. Wang, Y.-H. Yu, S.-P. Lin, H. J. Mersmann and S.-T. Ding (2012). "Docosahexaenoic acid suppresses the expression of FoxO and its target genes." The Journal of Nutritional Biochemistry **23**(12): 1609-1616.

Claudel, T., Y. Inoue, O. Barbier, D. Duran-Sandoval, V. Kosykh, J. Fruchart, J. C. Fruchart, F. J. Gonzalez and B. Staels (2003). "Farnesoid X receptor agonists suppress hepatic apolipoprotein CIII expression." Gastroenterology **125**(2): 544-555.

Crawford, D. C., L. Dumitrescu, R. Goodloe, K. Brown-Gentry, J. Boston, B. McClellan, Jr., C. Sutcliffe, R. Wiseman, P. Baker, M. A. Pericak-Vance, W. K. Scott, M. Allen, P. Mayo, N. Schnetz-Boutaud, H. H. Dilks, J. L. Haines and T. I. Pollin (2014). "Rare variant APOC3 R19X is associated with cardio-protective profiles in a diverse population-based survey as part of the Epidemiologic Architecture for Genes Linked to Environment Study." Circ Cardiovasc Genet **7**(6): 848-853.

Crosby, J., G. M. Peloso, P. L. Auer, D. R. Crosslin, N. O. Stitzel, L. A. Lange, Y. Lu, Z. Z. Tang, H. Zhang, G. Hindy, N. Masca, K. Stirrups, S. Kanoni, R. Do, G. Jun, Y. Hu, H. M. Kang, C. Xue, A. Goel, M. Farrall, S. Duga, P. A. Merlini, R. Asselta, D. Girelli, O. Olivieri, N. Martinelli, W. Yin, D. Reilly, E. Speliotes, C. S. Fox, K. Hveem, O. L. Holmen, M. Nikpay, D. N. Farlow, T. L. Assimes, N. Franceschini, J. Robinson, K. E. North, L. W. Martin, M. DePristo, N. Gupta, S. A. Escher, J. H. Jansson, N. Van Zuydam, C. N. Palmer, N. Wareham, W. Koch, T. Meitinger, A. Peters, W. Lieb, R. Erbel, I. R. Konig, J. Kruppa, F. Degenhardt, O. Gottesman, E. P. Bottinger, C. J. O'Donnell, B. M. Psaty, C. M. Ballantyne, G. Abecasis, J. M. Ordovas, O. Melander, H. Watkins, M. Orho-Melander, D. Ardisino, R. J. Loos, R. McPherson, C. J. Willer, J. Erdmann, A. S. Hall, N. J. Samani, P. Deloukas, H. Schunkert, J. G. Wilson, C. Kooperberg, S. S. Rich, R. P. Tracy, D. Y. Lin, D. Altshuler, S. Gabriel, D. A. Nickerson, G. P. Jarvik, L. A. Cupples, A. P. Reiner, E. Boerwinkle and S. Kathiresan (2014). "Loss-of-function mutations in APOC3, triglycerides, and coronary disease." N Engl J Med **371**(1): 22-31.

Curry, J. L., J. Z. Qin, B. Bonish, R. Carrick, P. Bacon, J. Panella, J. Robinson and B. J. Nickoloff (2003). "Innate immune-related receptors in normal and psoriatic skin." Arch Pathol Lab Med **127**(2): 178-186.

Dallinga-Thie, G. M., I. I. L. Berk-Planken, A. H. Bootsma and H. Jansen (2004). "Atorvastatin Decreases Apolipoprotein C-III in Apolipoprotein B-Containing Lipoprotein and HDL in Type 2 Diabetes." A potential mechanism to lower plasma triglycerides **27**(6): 1358-1364.

Dasu, M. R., S. Devaraj, S. Park and I. Jialal (2010). "Increased Toll-Like Receptor (TLR) Activation and TLR Ligands in Recently Diagnosed Type 2 Diabetic Subjects." Diabetes Care **33**(4): 861-868.

Dasu, M. R. and I. Jialal (2013). "Amelioration in wound healing in diabetic toll-like receptor-4 knockout mice." J Diabetes Complications **27**(5): 417-421.

Dasu, M. R. and S. J. Martin (2014). "Toll-like receptor expression and signaling in human diabetic wounds." World Journal of Diabetes **5**(2): 219-223.

Dasu, M. R., R. K. Thangappan, A. Bourgette, L. A. DiPietro, R. Isseroff and I. Jialal (2010). "TLR2 expression and signaling-dependent inflammation impair wound healing in diabetic mice." Lab Invest **90**(11): 1628-1636.

de Seny, D., G. Cobraiville, E. Charlier, S. Neuville, L. Lutteri, C. Le Goff, D. Malaise, O. Malaise, J.-P. Chapelle, B. Relic and M. G. Malaise (2015). "Apolipoprotein-A1 as a Damage-Associated Molecular Patterns Protein in Osteoarthritis: Ex Vivo and In Vitro Pro-Inflammatory Properties." PLOS ONE **10**(4): e0122904.

Digenio, A., R. L. Dunbar, V. J. Alexander, M. Hompesch, L. Morrow, R. G. Lee, M. J. Graham, S. G. Hughes, R. Yu, W. Singleton, B. F. Baker, S. Bhanot and R. M. Crooke (2016). "Antisense-Mediated Lowering of Plasma Apolipoprotein C-III by Volanesorsen Improves Dyslipidemia and Insulin Sensitivity in Type 2 Diabetes." Diabetes Care **39**(8): 1408-1415.

Dinh, T. L. and A. Veves (2006). "The efficacy of Apligraf in the treatment of diabetic foot ulcers." Plast Reconstr Surg **117**(7 Suppl): 152S-157S; discussion 158S-159S.

Driver, V. R., L. A. Lavery, A. M. Reyzelman, T. G. Dutra, C. R. Dove, S. V. Kotsis, H. M. Kim and K. C. Chung (2015). "A clinical trial of Integra Template for diabetic foot ulcer treatment." Wound Repair Regen **23**(6): 891-900.

Dubsky, M., A. Jirkovska, R. Bem, V. Fejfarova, L. Pagacova, B. Sixta, M. Varga, S. Langkramer, E. Sykova and E. B. Jude (2013). "Both autologous bone marrow mononuclear cell and peripheral blood progenitor cell therapies similarly improve ischaemia in patients with diabetic foot in comparison with control treatment." Diabetes Metab Res Rev **29**(5): 369-376.

Dunn, L., H. C. G. Prosser, J. T. M. Tan, L. Z. Vanags, M. K. C. Ng and C. A. Bursill (2013). "Murine Model of Wound Healing." Journal of Visualized Experiments : JoVE(75): 50265.

Eguchi, K., I. Manabe, Y. Oishi-Tanaka, M. Ohsugi, N. Kono, F. Ogata, N. Yagi, U. Ohto, M. Kimoto, K. Miyake, K. Tobe, H. Arai, T. Kadowaki and R. Nagai (2012). "Saturated fatty acid and TLR signaling link beta cell dysfunction and islet inflammation." Cell Metab **15**(4): 518-533.

Elias, P. M. and J. S. Wakefield (2011). Skin Barrier Function. Nutrition for Healthy Skin: Strategies for Clinical and Cosmetic Practice. J. Krutmann and P. Humbert. Berlin, Heidelberg, Springer Berlin Heidelberg: 35-48.

Enoch, S. and D. J. Leaper (2008). "Basic science of wound healing." Surgery - Oxford International Edition **26**(2): 31-37.

Erridge, C., A. Burdess, A. J. Jackson, C. Murray, M. Riggio, D. Lappin, S. Milligan, C. M. Spickett and D. J. Webb (2008). "Vascular cell responsiveness to Toll-like receptor ligands in carotid atheroma." European Journal of Clinical Investigation **38**(10): 713-720.

Fagerberg, B., S. Edwards, T. Halmos, J. Lopatynski, H. Schuster, S. Stender, G. Stoa-Birketvedt, S. Tonstad, S. Halldorsdottir and I. Gause-Nilsson (2005). "Tesaglitazar, a novel dual peroxisome proliferator-activated receptor alpha/gamma agonist, dose-dependently improves the metabolic abnormalities associated with insulin resistance in a non-diabetic population." Diabetologia **48**(9): 1716-1725.

Falanga, V. (2005). "Wound healing and its impairment in the diabetic foot." Lancet **366**(9498): 1736-1743.

Feingold KR, G. C. (2000). "Introduction to Lipids and Lipoproteins. ." Endotext [Internet].

Florvall, G., S. Basu and A. Larsson (2006). "Apolipoprotein A1 Is a Stronger Prognostic Marker Than Are HDL and LDL Cholesterol for Cardiovascular Disease and Mortality in Elderly Men." The Journals of Gerontology: Series A **61**(12): 1262-1266.

Fonder, M. A., G. S. Lazarus, D. A. Cowan, B. Aronson-Cook, A. R. Kohli and A. J. Mamelak (2008). "Treating the chronic wound: A practical approach to the care of nonhealing wounds and wound care dressings." J Am Acad Dermatol **58**(2): 185-206.

Galiano, R. D., O. M. Tepper, C. R. Pelo, K. A. Bhatt, M. Callaghan, N. Bastidas, S. Bunting, H. G. Steinmetz and G. C. Gurtner (2004). "Topical vascular endothelial growth factor accelerates diabetic wound healing through increased angiogenesis and by mobilizing and recruiting bone marrow-derived cells." Am J Pathol **164**(6): 1935-1947.

Galkowska, H., U. Wojewodzka and W. L. Olszewski (2006). "Chemokines, cytokines, and growth factors in keratinocytes and dermal endothelial cells in the margin of chronic diabetic foot ulcers." Wound Repair Regen **14**(5): 558-565.

Game, F. L., R. J. Hinchliffe, J. Apelqvist, D. G. Armstrong, K. Bakker, A. Hartemann, M. Londahl, P. E. Price and W. J. Jeffcoate (2012). "A systematic review of interventions to enhance the healing of chronic ulcers of the foot in diabetes." Diabetes Metab Res Rev **28 Suppl 1**: 119-141.

Gangabadage, C. S., J. Zdunek, M. Tessari, S. Nilsson, G. Olivecrona and S. S. Wijmenga (2008). "Structure and dynamics of human apolipoprotein CIII." J Biol Chem **283**(25): 17416-17427.

Gaudet, D., V. J. Alexander, B. F. Baker, D. Brisson, K. Tremblay, W. Singleton, R. S. Geary, S. G. Hughes, N. J. Viney, M. J. Graham, R. M. Crooke, J. L. Witztum, J. D. Brunzell and J. J. P. Kastelein (2015). "Antisense Inhibition of Apolipoprotein C-III in Patients with Hypertriglyceridemia." New England Journal of Medicine **373**(5): 438-447.

Gaudet, D. and D. Brisson (2015). "Gene-based therapies in lipidology: current status and future challenges." Current Opinion in Lipidology **26**(6): 553-565.

Gaudet, D., D. Brisson, K. Tremblay, V. J. Alexander, W. Singleton, S. G. Hughes, R. S. Geary, B. F. Baker, M. J. Graham, R. M. Crooke and J. L. Witztum (2014). "Targeting APOC3 in the Familial Chylomicronemia Syndrome." New England Journal of Medicine **371**(23): 2200-2206.

Gaudet, D., J. P. Drouin-Chartier and P. Couture (2017). "Lipid Metabolism and Emerging Targets for Lipid-Lowering Therapy." Can J Cardiol **33**(7): 872-882.

Gaudet, D., J. Méthot, S. Déry, D. Brisson, C. Essiembre, G. Tremblay, K. Tremblay, J. de Wal, J. Twisk, N. van den Bulk, V. Sier-Ferreira and S. van Deventer (2013). "Efficacy and long term safety of alipogene tiparvovec (AAV1-LPL(S447X)) gene therapy for lipoprotein lipase deficiency: an open label trial." Gene therapy **20**(4): 361-369.

Giarratana, N., G. Penna, S. Amuchastegui, R. Mariani, K. C. Daniel and L. Adorini (2004). "A Vitamin D Analog Down-Regulates Proinflammatory Chemokine Production by Pancreatic Islets Inhibiting T Cell Recruitment and Type 1 Diabetes Development." The Journal of Immunology **173**(4): 2280-2287.

Giau, V. V., E. Bagyinszky, S. S. A. An and S. Y. Kim (2015). "Role of apolipoprotein E in neurodegenerative diseases." Neuropsychiatric Disease and Treatment **11**: 1723-1737.

Gibson, D. J., L. Pi, S. Sriram, C. Mao, B. E. Petersen, E. W. Scott, A. Leask and G. S. Schultz (2014). "Conditional knockout of CTGF affects corneal wound healing." Invest Ophthalmol Vis Sci **55**(4): 2062-2070.

Gioffredi, E., M. Boffito, S. Calzone, S. M. Giannitelli, A. Rainer, M. Trombetta, P. Mozetic and V. Chiono (2016). "Pluronic F127 Hydrogel Characterization and Biofabrication in Cellularized Constructs for Tissue Engineering Applications." Procedia CIRP **49**: 125-132.

Gordon, K. A., E. A. Lebrun, M. Tomic-Canic and R. S. Kirsner (2012). "The role of surgical debridement in healing of diabetic foot ulcers." Skinmed **10**(1): 24-26.

Gordts, S. C., I. Muthuramu, R. Amin, F. Jacobs and B. De Geest (2014). "The Impact of Lipoproteins on Wound Healing: Topical HDL Therapy Corrects Delayed Wound Healing in Apolipoprotein E Deficient Mice." Pharmaceuticals (Basel, Switzerland) **7**(4): 419-432.

Gouni-Berthold, I. (2017). "The role of antisense oligonucleotide therapy against apolipoprotein-CIII in hypertriglyceridemia." Atherosclerosis Supplements **30**: 19-27.

Gouni-Berthold, I. and H. K. Berthold (2011). "Antisense oligonucleotides for the treatment of dyslipidemia." Curr Pharm Des **17**(9): 950-960.

Graham, M. J., R. G. Lee, T. A. Bell, 3rd, W. Fu, A. E. Mullick, V. J. Alexander, W. Singleton, N. Viney, R. Geary, J. Su, B. F. Baker, J. Burkey, S. T. Crooke and R. M. Crooke (2013). "Antisense oligonucleotide inhibition of apolipoprotein C-III reduces plasma triglycerides in rodents, nonhuman primates, and humans." Circ Res **112**(11): 1479-1490.

Greenhalgh, D. G. (1998). "The role of apoptosis in wound healing." The International Journal of Biochemistry & Cell Biology **30**(9): 1019-1030.

Gruber, P. J., A. Torres-Rosado, M. L. Wolak and T. Leff (1994). "Apo CIII gene transcription is regulated by a cytokine inducible NF-kappa B element." Nucleic Acids Res **22**(12): 2417-2422.

Guariguata, L., D. R. Whiting, I. Hambleton, J. Beagley, U. Linnenkamp and J. E. Shaw (2014). "Global estimates of diabetes prevalence for 2013 and projections for 2035." Diabetes Res Clin Pract **103**(2): 137-149.

Guo, S. and L. A. DiPietro (2010). "Factors Affecting Wound Healing." Journal of Dental Research **89**(3): 219-229.

Gurtner, G. C., S. Werner, Y. Barrandon and M. T. Longaker (2008). "Wound repair and regeneration." Nature **453**: 314.

Han, G. and R. Ceilley (2017). "Chronic Wound Healing: A Review of Current Management and Treatments." Adv Ther **34**(3): 599-610.

Helminen, O., H. Huhta, J. H. Kauppila, P. P. Lehenkari, J. Saarnio and T. J. Karttunen (2017). "Localization of nucleic acid-sensing toll-like receptors in human and mouse pancreas." Apmis **125**(2): 85-92.

Hernandez, C., M. Molusky, Y. Li, S. Li and J. D. Lin (2010). "Regulation of hepatic ApoC3 expression by PGC-1beta mediates hypolipidemic effect of nicotinic acid." Cell Metab **12**(4): 411-419.

Hertz, R., J. Bishara-Shieban and J. Bar-Tana (1995). "Mode of action of peroxisome proliferators as hypolipidemic drugs. Suppression of apolipoprotein C-III." J Biol Chem **270**(22): 13470-13475.

Hiebert, P. R., D. Wu and D. J. Granville (2013). "Granzyme B degrades extracellular matrix and contributes to delayed wound closure in apolipoprotein E knockout mice." Cell Death And Differentiation **20**: 1404.

Hilton, J. R., D. T. Williams, B. Beuker, D. R. Miller and K. G. Harding (2004). "Wound dressings in diabetic foot disease." Clin Infect Dis **39 Suppl 2**: S100-103.

Hiukka, A., M. Stahlman, C. Pettersson, M. Levin, M. Adiels, S. Teneberg, E. S. Leinonen, L. M. Hulten, O. Wiklund, M. Oresic, S. O. Olofsson, M. R. Taskinen, K. Ekroos and J. Boren (2009). "ApoCIII-enriched LDL in type 2 diabetes displays altered lipid composition, increased susceptibility for sphingomyelinase, and increased binding to biglycan." Diabetes **58**(9): 2018-2026.

Hofker, M. H. (2010). "APOC3 deficiency: from mice to man." European Journal of Human Genetics **18**(1): 1-2.

Holdsworth, G., J. Stocks, P. Dodson and D. J. Galton (1982). "An abnormal triglyceride-rich lipoprotein containing excess sialylated apolipoprotein C-III." J Clin Invest **69**(4): 932-939.

Holmberg, R., E. Refai, A. Höög, R. M. Crooke, M. Graham, G. Olivecrona, P.-O. Berggren and L. Juntti-Berggren (2011). "Lowering apolipoprotein CIII delays onset of type 1 diabetes." Proceedings of the National Academy of Sciences **108**(26): 10685-10689.

Holmberg, R., E. Refai, A. Hoog, R. M. Crooke, M. Graham, G. Olivecrona, P. O. Berggren and L. Juntti-Berggren (2011). "Lowering apolipoprotein CIII delays onset of type 1 diabetes." Proc Natl Acad Sci U S A **108**(26): 10685-10689.

Huby, T., C. Doucet, C. Dacet, B. Ouzilleau, Y. Ueda, V. Afzal, E. Rubin, M. J. Chapman and P. Lesnik (2006). "Knockdown expression and hepatic deficiency reveal an atheroprotective role for SR-BI in liver and peripheral tissues." Journal of Clinical Investigation **116**(10): 2767-2776.

Hulkower, K. I. and R. L. Herber (2011). "Cell Migration and Invasion Assays as Tools for Drug Discovery." Pharmaceutics **3**(1): 107.

Humpert, P. M., U. Bartsch, I. Konrade, H. P. Hammes, M. Morcos, M. Kasper, A. Bierhaus and P. P. Nawroth (2005). "Locally applied mononuclear bone marrow cells restore angiogenesis and promote wound healing in a type 2 diabetic patient." Exp Clin Endocrinol Diabetes **113**(9): 538-540.

Ilegems, E., A. Dicker, S. Speier, A. Sharma, A. Bahow, P. K. Edlund, I. B. Leibiger and P. O. Berggren (2013). "Reporter islets in the eye reveal the plasticity of the endocrine pancreas." Proc Natl Acad Sci U S A **110**(51): 20581-20586.

Isaac, A. L. and D. G. Armstrong (2013). "Negative pressure wound therapy and other new therapies for diabetic foot ulceration: the current state of play." Med Clin North Am **97**(5): 899-909.

Jacinto, A., A. Martinez-Arias and P. Martin (2001). "Mechanisms of epithelial fusion and repair." Nat Cell Biol **3**(5): E117-123.

Jacinto, A., A. Martinez-Arias and P. Martin (2001). "Mechanisms of epithelial fusion and repair." Nature Cell Biology **3**: E117.

Jeffcoate, W. J., P. Price and K. G. Harding (2004). "Wound healing and treatments for people with diabetic foot ulcers." Diabetes Metab Res Rev **20 Suppl 1**: S78-89.

Jensen, M. K., E. B. Rimm, J. D. Furtado and F. M. Sacks (2012). "Apolipoprotein C-III as a Potential Modulator of the Association Between HDL-Cholesterol and Incident Coronary Heart Disease." Journal of the American Heart Association: Cardiovascular and Cerebrovascular Disease **1**(2): jah3-e000232.

Jong, M. C., M. H. Hofker and L. M. Havekes (1999). "Role of ApoCs in lipoprotein metabolism: functional differences between ApoC1, ApoC2, and ApoC3." Arterioscler Thromb Vasc Biol **19**(3): 472-484.

Jong, M. C., P. C. Rensen, V. E. Dahlmans, H. van der Boom, T. J. van Berkel and L. M. Havekes (2001). "Apolipoprotein C-III deficiency accelerates triglyceride hydrolysis by lipoprotein lipase in wild-type and apoE knockout mice." J Lipid Res **42**(10): 1578-1585.

Jørgensen, A. B., R. Frikke-Schmidt and B. G. Nordestgaard (2014). "Loss-of-Function Mutations in APOC3 and Risk of Ischemic Vascular Disease." Journal of Vascular Surgery **60**(4): 1096.

Joven, J., E. Vilella, B. Costa, P. R. Turner, C. Richart and L. Masana (1989). "Concentrations of lipids and apolipoproteins in patients with clinically well-controlled insulin-dependent and non-insulin-dependent diabetes." Clin Chem **35**(5): 813-816.

Juntti-Berggren, L., O. Larsson, P. Rorsman, C. Ammala, K. Bokvist, K. Wahlander, P. Nicotera, J. Dypbukt, S. Orrenius, A. Hallberg and et al. (1993). "Increased activity of L-type Ca²⁺ channels exposed to serum from patients with type I diabetes." Science **261**(5117): 86-90.

Juntti-Berggren, L., E. Refai, I. Appelskog, M. Andersson, G. Imreh, N. Dekki, S. Uhles, L. Yu, W. J. Griffiths, S. Zaitsev, I. Leibiger, S. N. Yang, G. Olivecrona, H. Jornvall and P. O. Berggren (2004). "Apolipoprotein CIII promotes Ca²⁺-dependent beta cell death in type 1 diabetes." Proc Natl Acad Sci U S A **101**(27): 10090-10094.

Kant, V., A. Gopal, D. Kumar, S. Bag, N. P. Kurade, A. Kumar, S. K. Tandan and D. Kumar (2013). "Topically applied substance P enhanced healing of open excision wound in rats." Eur J Pharmacol **715**(1-3): 345-353.

Kashyap, M. L., L. S. Srivastava, B. A. Hynd, P. S. Gartside and G. Perisutti (1981). "Quantitation of human apolipoprotein C-III and its subspecies by radioimmunoassay and analytical isoelectric focusing: abnormal plasma triglyceride-rich lipoprotein apolipoprotein C-III subspecies concentrations in hypertriglyceridemia." J Lipid Res **22**(5): 800-810.

Kastelein, J. J., K. C. Maki, A. Susekov, M. Ezhov, B. G. Nordestgaard, B. N. Machielse, D. Kling and M. H. Davidson (2014). "Omega-3 free fatty acids for the treatment of severe hypertriglyceridemia: the EpanoVa fOr Lowering Very high triglyceridEs (EVOLVE) trial." J Clin Lipidol **8**(1): 94-106.

Katagiri, S., K. Park, Y. Maeda, T. N. Rao, M. Khamaisi, Q. Li, H. Yokomizo, A. Mima, L. Lancerotto, A. Wagers, D. P. Orgill and G. L. King (2016). "Overexpressing IRS1 in Endothelial Cells Enhances Angioblast Differentiation and Wound Healing in Diabetes and Insulin Resistance." Diabetes **65**(9): 2760-2771.

Kaupila, J. H., A. E. Mattila, T. J. Karttunen and T. Salo (2013). "Toll-like receptor 5 (TLR5) expression is a novel predictive marker for recurrence and survival in squamous cell carcinoma of the tongue." Br J Cancer **108**(3): 638-643.

Kawakami, A., M. Aikawa, P. Alcaide, F. W. Lusinskas, P. Libby and F. M. Sacks (2006). "Apolipoprotein CIII induces expression of vascular cell adhesion molecule-1 in vascular endothelial cells and increases adhesion of monocytic cells." *Circulation* **114**(7): 681-687.

Kawakami, A., M. Aikawa, P. Libby, P. Alcaide, F. W. Lusinskas and F. M. Sacks (2006). "Apolipoprotein CIII in apolipoprotein B lipoproteins enhances the adhesion of human monocytic cells to endothelial cells." *Circulation* **113**(5): 691-700.

Kawakami, A., M. Aikawa, N. Nitta, M. Yoshida, P. Libby and F. M. Sacks (2007). "Apolipoprotein CIII-induced THP-1 cell adhesion to endothelial cells involves pertussis toxin-sensitive G protein- and protein kinase C alpha-mediated nuclear factor-kappaB activation." *Arterioscler Thromb Vasc Biol* **27**(1): 219-225.

Khattak, S. F., S. R. Bhatia and S. C. Roberts (2005). "Pluronic F127 as a cell encapsulation material: utilization of membrane-stabilizing agents." *Tissue Eng* **11**(5-6): 974-983.

Kim, H. S., M. S. Han, K. W. Chung, S. Kim, E. Kim, M. J. Kim, E. Jang, H. A. Lee, J. Youn, S. Akira and M. S. Lee (2007). "Toll-like receptor 2 senses beta-cell death and contributes to the initiation of autoimmune diabetes." *Immunity* **27**(2): 321-333.

Kim, J., J. M. Basak and D. M. Holtzman (2009). "The Role of Apolipoprotein E in Alzheimer's Disease." *Neuron* **63**(3): 287-303.

Koh, T. J. and L. A. DiPietro (2011). "Inflammation and wound healing: The role of the macrophage." *Expert reviews in molecular medicine* **13**: e23-e23.

Köllisch, G., B. N. Kalali, V. Voelcker, R. Wallich, H. Behrendt, J. Ring, S. Bauer, T. Jakob, M. Mempel and M. Ollert (2005). "Various members of the Toll-like receptor family contribute to the innate immune response of human epidermal keratinocytes." *Immunology* **114**(4): 531-541.

Koopal, C., A. D. Marais and F. L. Visseren (2017). "Familial dysbetalipoproteinemia: an underdiagnosed lipid disorder." *Curr Opin Endocrinol Diabetes Obes* **24**(2): 133-139.

Kosoglou, T., P. Statkevich, J. C. Fruchart, L. J. Pember, L. Reyderman, D. L. Cutler, M. Guillaume, S. E. Maxwell and E. P. Veltri (2004). "Pharmacodynamic and pharmacokinetic interaction between fenofibrate and ezetimibe." *Curr Med Res Opin* **20**(8): 1197-1207.

Laiva, A. L., F. J. O'Brien and M. B. Keogh (2018). "Innovations in gene and growth factor delivery systems for diabetic wound healing." *J Tissue Eng Regen Med* **12**(1): e296-e312.

Lamers, M. L., M. E. S. Almeida, M. Vicente-Manzanares, A. F. Horwitz and M. F. Santos (2011). "High Glucose-Mediated Oxidative Stress Impairs Cell Migration." *PLOS ONE* **6**(8): e22865.

Lan, C. C., I. H. Liu, A. H. Fang, C. H. Wen and C. S. Wu (2008). "Hyperglycaemic conditions decrease cultured keratinocyte mobility: implications for impaired wound healing in patients with diabetes." *Br J Dermatol* **159**(5): 1103-1115.

Larsson, M., E. Vorrsjö, P. Talmud, A. Lookene and G. Olivecrona (2013). "Apolipoproteins C-I and C-III Inhibit Lipoprotein Lipase Activity by Displacement of the Enzyme from Lipid Droplets." *The Journal of Biological Chemistry* **288**(47): 33997-34008.

Lee, R. G., J. Crosby, B. F. Baker, M. J. Graham and R. M. Crooke (2013). "Antisense technology: an emerging platform for cardiovascular disease therapeutics." *J Cardiovasc Transl Res* **6**(6): 969-980.

Lewis, J. and A. Lipp (2013). "Pressure-relieving interventions for treating diabetic foot ulcers." *Cochrane Database Syst Rev*(1): Cd002302.

Li, H., M. Yu, M. Wang, R. Qi, W. Huang, Y. Tang, Y. Han, Y. Wang, G. Liu, X. Zhang, M. Han, Y.-N. Shu, X. Liu and B. Rodrigues (2015). "Aggravated restenosis and atherogenesis in ApoCIII transgenic mice but lack of protection in ApoCIII knockouts: the effect of authentic triglyceride-rich lipoproteins with and without ApoCIII." *Cardiovascular Research* **107**(4): 579-589.

Li, J., L. Chen, Y. Zhang, W. J. Zhang, W. Xu, Y. Qin, J. Xu and D. Zou (2013). "TLR4 is required for the obesity-induced pancreatic beta cell dysfunction." *Acta Biochimica et Biophysica Sinica* **45**(12): 1030-1038.

Li, M., L. Song, X. Gao, W. Chang and X. Qin (2012). "Toll-like receptor 4 on islet β cells senses expression changes in high-mobility group box 1 and contributes to the initiation of type 1 diabetes." Experimental & Molecular Medicine **44**(4): 260-267.

Liang, C.-C., A. Y. Park and J.-L. Guan (2007). "In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro." Nature Protocols **2**: 329.

Lin, Q., D. Fang, J. Fang, X. Ren, X. Yang, F. Wen and S. B. Su (2011). "Impaired wound healing with defective expression of chemokines and recruitment of myeloid cells in TLR3-deficient mice." J Immunol **186**(6): 3710-3717.

Lin, Q., L. Wang, Y. Lin, X. Liu, X. Ren, S. Wen, X. Du, T. Lu, S. Y. Su, X. Yang, W. Huang, S. Zhou, F. Wen and S. B. Su (2012). "Toll-like receptor 3 ligand polyinosinic:polycytidylic acid promotes wound healing in human and murine skin." J Invest Dermatol **132**(8): 2085-2092.

Liu, R., L. Li, M. Yang, G. Boden and G. Yang (2013). "Systematic review of the effectiveness of hyperbaric oxygenation therapy in the management of chronic diabetic foot ulcers." Mayo Clin Proc **88**(2): 166-175.

Liu, Y., D. Min, T. Bolton, V. Nube, S. M. Twigg, D. K. Yue and S. V. McLennan (2009). "Increased matrix metalloproteinase-9 predicts poor wound healing in diabetic foot ulcers." Diabetes Care **32**(1): 117-119.

Lu, M., Q. Lu, Y. Zhang and G. Tian (2011). "ApoB/apoA1 is an effective predictor of coronary heart disease risk in overweight and obesity()." Journal of Biomedical Research **25**(4): 266-273.

Luc, G., C. Fievet, D. Arveiler, A. E. Evans, J. M. Bard, F. Cambien, J. C. Fruchart and P. Ducimetiere (1996). "Apolipoproteins C-III and E in apoB- and non-apoB-containing lipoproteins in two populations at contrasting risk for myocardial infarction: the ECTIM study. Etude Cas Temoins sur 'Infarctus du Myocarde.'" J Lipid Res **37**(3): 508-517.

Luo, M., A. Liu, S. Wang, T. Wang, D. Hu, S. Wu and D. Peng (2017). "ApoCIII enrichment in HDL impairs HDL-mediated cholesterol efflux capacity." Scientific reports **7**(1): 2312-2312.

M., R. H. and B. A. J. M. (2005). "Recent advances in the diagnosis and management of diabetic neuropathy." The Journal of Bone and Joint Surgery. British volume **87-B**(12): 1605-1610.

Madeo, M., C. R. Martin, C. Turner, V. Kirkby and D. R. Thompson (1998). "A randomized trial comparing Arglaes (a transparent dressing containing silver ions) to Tegaderm (a transparent polyurethane dressing) for dressing peripheral arterial catheters and central vascular catheters." Intensive and Critical Care Nursing **14**(4): 187-191.

Mahley, R. W. (2016). "Apolipoprotein E: from cardiovascular disease to neurodegenerative disorders." Journal of molecular medicine (Berlin, Germany) **94**(7): 739-746.

Mahley, R. W., T. L. Innerarity, S. C. Rall, Jr. and K. H. Weisgraber (1984). "Plasma lipoproteins: apolipoprotein structure and function." J Lipid Res **25**(12): 1277-1294.

Marston, W. A. (2004). "Dermagraft, a bioengineered human dermal equivalent for the treatment of chronic nonhealing diabetic foot ulcer." Expert Rev Med Devices **1**(1): 21-31.

Mauger, J. F., P. Couture, N. Bergeron and B. Lamarche (2006). "Apolipoprotein C-III isoforms: kinetics and relative implication in lipid metabolism." J Lipid Res **47**(6): 1212-1218.

Mirza, R. E., M. M. Fang, W. J. Ennis and T. J. Koh (2013). "Blocking interleukin-1 β induces a healing-associated wound macrophage phenotype and improves healing in type 2 diabetes." Diabetes **62**(7): 2579-2587.

Moretti, B., A. Notarnicola, G. Maggio, L. Moretti, M. Pascone, S. Tafuri and V. Patella (2009). "The management of neuropathic ulcers of the foot in diabetes by shock wave therapy." BMC Musculoskeletal Disord **10**: 54.

Morita, K., Y. Miyachi and M. Furuse (2011). "Tight junctions in epidermis: from barrier to keratinization." Eur J Dermatol **21**(1): 12-17.

Moura, L. I., A. M. Dias, E. Carvalho and H. C. de Sousa (2013). "Recent advances on the development of wound dressings for diabetic foot ulcer treatment--a review." Acta Biomater **9**(7): 7093-7114.

Murphy, A. J. (2013). "High Density Lipoprotein: Assembly, Structure, Cargo, and Functions." ISRN Physiology **2013**: 20.

Nackiewicz, D., M. Dan, W. He, R. Kim, A. Salmi, S. Rutti, C. Westwell-Roper, A. Cunningham, M. Speck, C. Schuster-Klein, B. Guardiola, K. Maedler and J. A. Ehses (2014). "TLR2/6 and TLR4-activated macrophages contribute to islet inflammation and impair beta cell insulin gene expression via IL-1 and IL-6." Diabetologia **57**(8): 1645-1654.

Nagai, M. K. and J. M. Embil (2002). "Becaplermin: recombinant platelet derived growth factor, a new treatment for healing diabetic foot ulcers." Expert Opin Biol Ther **2**(2): 211-218.

Nagashima, K., C. Lopez, D. Donovan, C. Ngai, N. Fontanez, A. Bensadoun, J. Fruchart-Najib, S. Holleran, J. S. Cohn, R. Ramakrishnan and H. N. Ginsberg (2005). "Effects of the PPARgamma agonist pioglitazone on lipoprotein metabolism in patients with type 2 diabetes mellitus." J Clin Invest **115**(5): 1323-1332.

Nair, S., R. Guo, R. Nair, C. Zgheib, J. Xu and K. W. Liechty (2017). "Targeting Cathepsin K to Promoted Diabetic Wound Healing." The FASEB Journal **31**(1_supplement): 673.611-673.611.

Newton, D. J., F. Khan, J. J. F. Belch, M. R. Mitchell and G. P. Leese (2002). "Blood flow changes in diabetic foot ulcers treated with dermal replacement therapy." The Journal of Foot and Ankle Surgery **41**(4): 233-237.

Nikolaidou-Neokosmidou, V., V. I. Zannis and D. Kardassis (2006). "Inhibition of hepatocyte nuclear factor 4 transcriptional activity by the nuclear factor kappaB pathway." Biochem J **398**(3): 439-450.

Norata, G. D., S. Tsimikas, A. Pirillo and A. L. Catapano (2015). "Apolipoprotein C-III: From Pathophysiology to Pharmacology." Trends Pharmacol Sci **36**(10): 675-687.

Nunan, R., K. G. Harding and P. Martin (2014). "Clinical challenges of chronic wounds: searching for an optimal animal model to recapitulate their complexity." Dis Model Mech **7**(11): 1205-1213.

O'Reilly, D., A. Pasricha, K. Campbell, N. Burke, N. Assasi, J. M. Bowen, J. E. Tarride and R. Goeree (2013). "Hyperbaric oxygen therapy for diabetic ulcers: systematic review and meta-analysis." Int J Technol Assess Health Care **29**(3): 269-281.

O'Loughlin, A., M. Kulkarni, M. Creane, E. E. Vaughan, E. Mooney, G. Shaw, M. Murphy, P. Dockery, A. Pandit and T. O'Brien (2013). "Topical Administration of Allogeneic Mesenchymal Stromal Cells Seeded in a Collagen Scaffold Augments Wound Healing and Increases Angiogenesis in the Diabetic Rabbit Ulcer." Diabetes **62**(7): 2588-2594.

Ong, Y. H., W. C. A. Koh, M. L. Ng, Z. Y. Tam, S. C. Lim, B. O. Boehm, C. the Adult-Onset Autoimmune Diabetes Mellitus, R. Blagieva, J. Boehm, J. Brückel, S. Claudi-Boehm, T. Haak, M. Haenle, S. Höpfer, G. Jütting, A. Kleger, W. Kratzer, A. Kurkhaus, B. Lippmann-Grob, B. Manfras, S. Merger, S. Rosinger, S. Schilling, T. Seufferlein, R. Sing, G. Trischler, Y. Ali, Y. M. Bee, J. Boehm, D. E. K. Chew, K. S. Chia, R. Dalan, C. M. Khoo, E. S. Lee, M. K. Leow, S. C. Lim, S. P. Ng, X. Sim, D. K. Srinivasan, C. F. Sum, E. S. Tai, Z. Y. Tam, W. E. Tan, S. Tavintharan and M. P. Toh (2017). "Glutamic acid decarboxylase and islet antigen 2 antibody profiles in people with adult-onset diabetes mellitus: a comparison between mixed ethnic populations in Singapore and Germany." Diabetic Medicine **34**(8): 1145-1153.

Ooi, E. M., P. H. Barrett, D. C. Chan and G. F. Watts (2008). "Apolipoprotein C-III: understanding an emerging cardiovascular risk factor." Clin Sci (Lond) **114**(10): 611-624.

Ooi, E. M. M., D. T. Chan, G. F. Watts, D. C. Chan, T. W. K. Ng, G. K. Dogra, A. B. Irish and P. H. R. Barrett (2011). "Plasma apolipoprotein C-III metabolism in patients with chronic kidney disease." Journal of Lipid Research **52**(4): 794-800.

Ooi, E. M. M., G. F. Watts, D. C. Chan, M. M. Chen, P. J. Nestel, D. Sviridov and P. H. R. Barrett (2008). "Dose-Dependent Effect of Rosuvastatin on VLDL-Apolipoprotein C-III Kinetics in the Metabolic Syndrome." Diabetes Care **31**(8): 1656-1661.

Opalenik, S. R. and J. M. Davidson (2005). "Fibroblast differentiation of bone marrow-derived cells during wound repair." Faseb j **19**(11): 1561-1563.

Papanas, N. and E. Maltezos (2007). "Growth factors in the treatment of diabetic foot ulcers: new technologies, any promises?" Int J Low Extrem Wounds **6**(1): 37-53.

Pavlic, M., R. Valero, H. Duez, C. Xiao, L. Szeto, B. W. Patterson and G. F. Lewis (2008). "Triglyceride-rich lipoprotein-associated apolipoprotein C-III production is stimulated by plasma free fatty acids in humans." Arterioscler Thromb Vasc Biol **28**(9): 1660-1665.

Pereira, S. S. and J. I. Alvarez-Leite (2014). "Low-Grade Inflammation, Obesity, and Diabetes." Curr Obes Rep **3**(4): 422-431.

Peters, J. M., N. Hennuyer, B. Staels, J. C. Fruchart, C. Fievet, F. J. Gonzalez and J. Auwerx (1997). "Alterations in lipoprotein metabolism in peroxisome proliferator-activated receptor alpha-deficient mice." J Biol Chem **272**(43): 27307-27312.

Petersen, K. F., S. Dufour, A. Hariri, C. Nelson-Williams, J. N. Foo, X.-M. Zhang, J. Dziura, R. P. Lifton and G. I. Shulman (2010). "Apolipoprotein C3 Gene Variants in Nonalcoholic Fatty Liver Disease." New England Journal of Medicine **362**(12): 1082-1089.

Phan, T. P., L. Alkema, E. S. Tai, K. H. X. Tan, Q. Yang, W.-Y. Lim, Y. Y. Teo, C.-Y. Cheng, X. Wang, T. Y. Wong, K. S. Chia and A. R. Cook (2014). "Forecasting the burden of type 2 diabetes in Singapore using a demographic epidemiological model of Singapore." BMJ Open Diabetes Research & Care **2**(1).

Plikaitis, C. M. and J. A. Molnar (2006). "Subatmospheric pressure wound therapy and the vacuum-assisted closure device: basic science and current clinical successes." Expert Rev Med Devices **3**(2): 175-184.

Png, M. E., J. Yoong, T. P. Phan and H. L. Wee (2016). "Current and future economic burden of diabetes among working-age adults in Asia: conservative estimates for Singapore from 2010-2050." BMC Public Health **16**(1): 153.

Pollin, T. I., C. M. Damcott, H. Shen, S. H. Ott, J. Shelton, R. B. Horenstein, W. Post, J. C. McLenithan, L. F. Bielak, P. A. Peyser, B. D. Mitchell, M. Miller, J. R. O'Connell and A. R. Shuldiner (2008). "A null mutation in human APOC3 confers a favorable plasma lipid profile and apparent cardioprotection." Science **322**(5908): 1702-1705.

Pradhan, L., C. Nabzdyk, N. D. Andersen, F. W. LoGerfo and A. Veves (2009). "Inflammation and Neuropeptides: The Connection in Diabetic Wound Healing." Expert reviews in molecular medicine **11**: e2-e2.

Qamar, A., S. A. Khetarpal, A. V. Khera, A. Qasim, D. J. Rader and M. P. Reilly (2015). "Plasma apolipoprotein C-III levels, triglycerides, and coronary artery calcification in type 2 diabetics." Arteriosclerosis, thrombosis, and vascular biology **35**(8): 1880-1888.

Qu, S., G. Perdomo, D. Su, F. M. D'Souza, N. S. Shachter and H. H. Dong (2007). "Effects of apoA-V on HDL and VLDL metabolism in APOC3 transgenic mice." J Lipid Res **48**(7): 1476-1487.

Reiber, G. E., E. J. Bokyko and D. G. Smith (1995). "Lower extremity foot ulcers and amputations in diabetes. ." In Diabetes in America: 409-428.

Reinke, J. M. and H. Sorg (2012). "Wound repair and regeneration." Eur Surg Res **49**(1): 35-43.

Reiss, M. J., Y. P. Han, E. Garcia, M. Goldberg, Y. K. Hong and W. L. Garner (2010). "Matrix Metalloproteinase-9 Delays Wound Healing in a Murine Wound Model." Surgery **147**(2): 295.

Reverter, J. L., M. Senti, J. Rubiés-Prat, A. Lucas, I. Salinas, E. Pizarro, J. Pedro-Botet and A. Sanmarti (1993). "Lipoprotein composition in the insulin-deficient non-acidotic phase of type I diabetic patients and early evolution after the start of insulin therapy." Clinica Chimica Acta **223**(1): 113-120.

Rice, J. B., U. Desai, A. K. Cummings, H. G. Birnbaum, M. Skornicki and N. B. Parsons (2014). "Burden of diabetic foot ulcers for medicare and private insurers." Diabetes Care **37**(3): 651-658.

Robson, M. C., D. L. Steed and M. G. Franz (2001). "Wound healing: biologic features and approaches to maximize healing trajectories." Curr Probl Surg **38**(2): 72-140.

Rosen, E. D. and B. M. Spiegelman (2006). "Adipocytes as regulators of energy balance and glucose homeostasis." Nature **444**: 847.

Roshan, M. H. K., A. Tambo and N. P. Pace (2016). "The Role of TLR2, TLR4, and TLR9 in the Pathogenesis of Atherosclerosis." International Journal of Inflammation **2016**: 1532832.

S., T. N. and W. Walter (2013). "Studying Wound Repair in the Mouse." Current Protocols in Mouse Biology **3**(3): 171-185.

Sacks, F. M. (2015). "The crucial roles of apolipoproteins E and C-III in apoB lipoprotein metabolism in normolipidemia and hypertriglyceridemia." Curr Opin Lipidol **26**(1): 56-63.

Sacks, F. M., P. Alaupovic, L. A. Moye, T. G. Cole, B. Sussex, M. J. Stampfer, M. A. Pfeffer and E. Braunwald (2000). "VLDL, apolipoproteins B, CIII, and E, and risk of recurrent coronary events in the Cholesterol and Recurrent Events (CARE) trial." Circulation **102**(16): 1886-1892.

Salvador, B., A. Arranz, S. Francisco, L. Córdoba, C. Punzón, M. Á. Llamas and M. Fresno (2016). "Modulation of endothelial function by Toll like receptors." Pharmacological Research **108**: 46-56.

Sarwar, N., J. Danesh, G. Eiriksdottir, G. Sigurdsson, N. Wareham, S. Bingham, S. M. Boekholdt, K. T. Khaw and V. Gudnason (2007). "Triglycerides and the risk of coronary heart disease: 10,158 incident cases among 262,525 participants in 29 Western prospective studies." Circulation **115**(4): 450-458.

Semenkovich, C. F. (2012). 213 - Disorders of Lipid Metabolism. Goldman's Cecil Medicine (Twenty Fourth Edition). L. Goldman and A. I. Schafer. Philadelphia, W.B. Saunders: 1346-1354.

Seo, M.-D., T. J. Kang, C. H. Lee, A.-Y. Lee and M. Noh (2012). "HaCaT Keratinocytes and Primary Epidermal Keratinocytes Have Different Transcriptional Profiles of Cornified Envelope-Associated Genes to T Helper Cell Cytokines." Biomolecules & Therapeutics **20**(2): 171-176.

Shahrara, S., S. R. Pickens, A. Dorfleitner and R. M. Pope (2009). "IL-17 induces monocyte migration in rheumatoid arthritis." J Immunol **182**(6): 3884-3891.

Shen, Y., H. Qin, J. Chen, L. Mou, Y. He, Y. Yan, H. Zhou, Y. Lv, Z. Chen, J. Wang and Y.-D. Zhou (2016). "Postnatal activation of TLR4 in astrocytes promotes excitatory synaptogenesis in hippocampal neurons." The Journal of Cell Biology.

Shi, Y., G. Yang, J. Yu, L. Yu, R. Westenbroek, W. A. Catterall, L. Juntti-Berggren, P. O. Berggren and S. N. Yang (2014). "Apolipoprotein CIII hyperactivates beta cell CaV1 channels through SR-BI/beta1 integrin-dependent coactivation of PKA and Src." Cell Mol Life Sci **71**(7): 1289-1303.

Singer, A. J. and R. A. Clark (1999). "Cutaneous wound healing." N Engl J Med **341**(10): 738-746.

Smiell, J. M., T. J. Wieman, D. L. Steed, B. H. Perry, A. R. Sampson and B. H. Schwab (1999). "Efficacy and safety of becaplermin (recombinant human platelet-derived growth factor-BB) in patients with nonhealing, lower extremity diabetic ulcers: a combined analysis of four randomized studies." Wound Repair Regen **7**(5): 335-346.

Snyder, R. J., R. G. Frykberg, L. C. Rogers, A. J. Applewhite, D. Bell, G. Bohn, C. E. Fife, J. Jensen and J. Wilcox (2014). "The management of diabetic foot ulcers through optimal off-loading: building consensus guidelines and practical recommendations to improve outcomes." J Am Podiatr Med Assoc **104**(6): 555-567.

Song, M. J., K. H. Kim, J. M. Yoon and J. B. Kim (2006). "Activation of Toll-like receptor 4 is associated with insulin resistance in adipocytes." Biochem Biophys Res Commun **346**(3): 739-745.

Song, W.-L. and G. A. FitzGerald (2013). "Niacin, an old drug with a new twist." Journal of Lipid Research **54**(10): 2586-2594.

Staels, B., N. Vu-Dac, V. A. Kosykh, R. Saladin, J. C. Fruchart, J. Dallongeville and J. Auwerx (1995). "Fibrates downregulate apolipoprotein C-III expression independent of induction of peroxisomal acyl coenzyme A oxidase. A potential mechanism for the hypolipidemic action of fibrates." Journal of Clinical Investigation **95**(2): 705-712.

Steckelings, U. M., B. M. Henz, S. Wiehstutz, T. Unger and M. Artuc (2005). "Differential expression of angiotensin receptors in human cutaneous wound healing." Br J Dermatol **153**(5): 887-893.

Steed, D. L., C. Attinger, T. Colaizzi, M. Crossland, M. Franz, L. Harkless, A. Johnson, H. Moosa, M. Robson, T. Serena, P. Sheehan, A. Veves and L. Wiersma-Bryant (2006). "Guidelines for the treatment of diabetic ulcers." Wound Repair Regen **14**(6): 680-692.

Steiner, D. J., A. Kim, K. Miller and M. Hara (2010). "Pancreatic islet plasticity: Interspecies comparison of islet architecture and composition." Islets **2**(3): 135-145.

Storling, J., L. Juntti-Berggren, G. Olivecrona, M. C. Prause, P. O. Berggren and T. Mandrup-Poulsen (2011). "Apolipoprotein CIII reduces proinflammatory cytokine-induced apoptosis in rat pancreatic islets via the Akt prosurvival pathway." Endocrinology **152**(8): 3040-3048.

Streubel, P. N., D. J. Stinner and W. T. Obrebsky (2012). "Use of negative-pressure wound therapy in orthopaedic trauma." J Am Acad Orthop Surg **20**(9): 564-574.

Sun, B. K., Z. Siprashvili and P. A. Khavari (2014). "Advances in skin grafting and treatment of cutaneous wounds." Science **346**(6212): 941-945.

Sundaram, M., S. Zhong, M. B. Khalil, P. H. Links, Y. Zhao, J. Iqbal, M. M. Hussain, R. J. Parks, Y. Wang and Z. Yao (2010). "Expression of apolipoprotein C-III in McA-RH7777 cells enhances VLDL assembly and secretion under lipid-rich conditions." Journal of Lipid Research **51**(1): 150-161.

Sundsten, T., C. G. Ostenson and P. Bergsten (2008). "Serum protein patterns in newly diagnosed type 2 diabetes mellitus--influence of diabetic environment and family history of diabetes." Diabetes Metab Res Rev **24**(2): 148-154.

Takemoto, Y., T.-S. Li, M. Kubo, M. Ohshima, H. Kurazumi, K. Ueda, T. Enoki, T. Murata and K. Hamano (2012). "The Mobilization and Recruitment of C-Kit+ Cells Contribute to Wound Healing after Surgery." PLOS ONE **7**(11): e48052.

Tao, Y., Y. Xiong, H. Wang, S. Chu, R. Zhong, J. Wang, G. Wang, X. Ren and J. Yu (2016). "APOC3 induces endothelial dysfunction through TNF- α and JAM-1." Lipids in Health and Disease **15**(1): 153.

Tecilazich, F., T. L. Dinh and A. Veves (2013). "Emerging drugs for the treatment of diabetic ulcers." Expert Opin Emerg Drugs **18**(2): 207-217.

Thakral, G., J. LaFontaine, B. Najafi, T. K. Talal, P. Kim and L. A. Lavery (2013). "Electrical stimulation to accelerate wound healing." Diabetic Foot & Ankle **4**: 10.3402/dfa.v3404i3400.22081.

Tiaka, E. K., N. Papanas, A. C. Manolakis and E. Maltezos (2011). "The role of nerve growth factor in the prophylaxis and treatment of diabetic foot ulcers." International Journal of Burns and Trauma **1**(1): 68-76.

Tilly, P., C. Sass, M. Vincent-Viry, D. Aguillon, G. Siest and S. Visvikis (2003). "Biological and genetic determinants of serum apoC-III concentration: reference limits from the Stanislas Cohort." Journal of Lipid Research **44**(2): 430-436.

Twisk, J., D. L. Gillian-Daniel, A. Tebon, L. Wang, P. H. R. Barrett and A. D. Attie (2000). "The role of the LDL receptor in apolipoprotein B secretion." Journal of Clinical Investigation **105**(4): 521-532.

Veselits, M., A. Tanaka, S. Lipkowitz, S. O'Neill, R. Sciammas, A. Finnegan, J. Zhang and M. R. Clark (2014). "Recruitment of Cbl-b to B cell antigen receptor couples antigen recognition to Toll-like receptor 9 activation in late endosomes." PLoS One **9**(3): e89792.

Vinik, A., J. Ullal, H. K. Parson and C. M. Casellini (2006). "Diabetic neuropathies: clinical manifestations and current treatment options." Nat Clin Pract Endocrinol Metab **2**(5): 269-281.

Visser, M. E., J. L. Witztum, E. S. G. Stroes and J. J. P. Kastelein (2012). "Antisense oligonucleotides for the treatment of dyslipidaemia." European Heart Journal **33**(12): 1451-1458.

Vives-Pi, M., N. Somoza, J. Fernandez-Alvarez, F. Vargas, P. Caro, A. Alba, R. Gomis, M. O. Labeta and R. Pujol-Borrell (2003). "Evidence of expression of endotoxin receptors CD14, toll-like receptors TLR4 and TLR2 and associated molecule MD-2 and of sensitivity to endotoxin (LPS) in islet beta cells." Clin Exp Immunol **133**(2): 208-218.

Wang, X. (2004). "Lipid signaling." Current Opinion in Plant Biology **7**(3): 329-336.

Wellen, K. E. and G. S. Hotamisligil (2005). "Inflammation, stress, and diabetes." Journal of Clinical Investigation **115**(5): 1111-1119.

Wen, L., J. Peng, Z. Li and F. S. Wong (2004). "The Effect of Innate Immunity on Autoimmune Diabetes and the Expression of Toll-Like Receptors on Pancreatic Islets." The Journal of Immunology **172**(5): 3173-3180.

Werner, S. and R. Grose (2003). "Regulation of wound healing by growth factors and cytokines." Physiol Rev **83**(3): 835-870.

Westwell-Roper, C. Y., J. A. Ehses and C. B. Verchere (2014). "Resident macrophages mediate islet amyloid polypeptide-induced islet IL-1 β production and beta-cell dysfunction." Diabetes **63**(5): 1698-1711.

Wickett, R. R. and M. O. Visscher (2006). "Structure and function of the epidermal barrier." American Journal of Infection Control **34**(10): S98-S110.

Wong, E. and T. Goldberg (2014). "Mipomersen (Kynamro): A Novel Antisense Oligonucleotide Inhibitor for the Management of Homozygous Familial Hypercholesterolemia." Pharmacy and Therapeutics **39**(2): 119-122.

Yao, Z. (2012). "Human apolipoprotein C-III - a new intrahepatic protein factor promoting assembly and secretion of very low density lipoproteins." Cardiovasc Hematol Disord Drug Targets **12**(2): 133-140.

Yazdanpanah, L., M. Nasiri and S. Adarvishi (2015). "Literature review on the management of diabetic foot ulcer." World Journal of Diabetes **6**(1): 37-53.

Zaidi, Z. and S. W. Lanigan (2010). Skin: Structure and Function. Dermatology in Clinical Practice. London, Springer London: 1-15.

Zhang, P., J. Lu, Y. Jing, S. Tang, D. Zhu and Y. Bi (2017). "Global epidemiology of diabetic foot ulceration: a systematic review and meta-analysis (dagger)." Ann Med **49**(2): 106-116.

Zheng, C., V. Azcutia, E. Aikawa, J. L. Figueiredo, K. Croce, H. Sonoki, F. M. Sacks, F. W. Luscinskas and M. Aikawa (2013). "Statins suppress apolipoprotein CIII-induced vascular endothelial cell activation and monocyte adhesion." Eur Heart J **34**(8): 615-624.

Zheng, C., C. Khoo, J. Furtado and F. M. Sacks (2010). "Apolipoprotein C-III and the metabolic basis for hypertriglyceridemia and the dense low-density lipoprotein phenotype." Circulation **121**(15): 1722-1734.

Zhu, Y., A. Kodvawala and D. Y. Hui (2010). "Apolipoprotein E Inhibits Toll-like Receptor (TLR)-3 and TLR-4 Mediated Macrophage Activation through Distinct Mechanisms." The Biochemical journal **428**(1): 47-54.

Zouboulis, C. C. (2009). "The skin as an endocrine organ." Dermato-endocrinology **1**(5): 250-252.