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TRANSLATIONAL STUDIES ON MITOCHONDRIAL FUNCTION AND HYPOXIA IN COMPLICATIONS OF DIABETES MELLITUS

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Translational Studies on Mitochondrial Function and Hypoxia in Complications of Diabetes Mellitus

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my parents, and Simone.

Diabetes /ˌdaɪ.əˈbiːtɪs/ *noun*

From Greek, *διαβαίνειν (diabainein)*, “to pass through”, referring to an excessive amount of urination.

Mellitus /ˈmɛlɪtəs/ *adjective*

Classical Latin, *Mellite* (“honey sweet”), prefix *mel-*, “pertaining to honey”, referring to a sweet taste of urine in untreated diabetes, due to glycosuria.

“It is not within the power of the properly constructed human mind to be satisfied. Progress would cease if this were the case.”

Frederick Banting

ENGLISH

Popular Science Summary

DIABETES AND HOW OUR CELLS BREATHE

Diabetes affects almost one out of every ten humans on earth. Yet, it is an often-overlooked disease. Diabetes is a slow killer. As the world gets more sedentary and overweight, diabetes is an ever-growing challenge, causing massive suffering and costs for individuals and society. Diabetes features high blood sugar. Sugar is processed into energy by mitochondria, little powerhouses inside the cells that make up our bodies. Mitochondria need oxygen to process sugar into energy. This process involves the production of reactive oxygen species (ROS), both as a by-product and as important messenger molecules with which different parts of the cells communicate with each other. Too much ROS, however, damages DNA, cells and leads to sickness. It is well known that high blood sugar, such as in diabetes, leads to increased ROS. This is the main cause of the various complications of diabetes that can occur over time if the diabetes is not treated well enough. These complications include heart attacks, strokes, blindness, kidney failure and wounds that risk becoming permanent and lead to limb amputations.

The body contains many mechanisms to reduce damage caused by ROS. Since diabetes leads to increased ROS, the antioxidant defense of the body is insufficient to prevent ROS-inflicted damage. That is why we also investigated Coenzyme Q, an antioxidant that is also a part of the mitochondria powerhouse. We looked at how Coenzyme Q levels in different diabetic patients could explain the difference in complication rates and disease outcome. We also did experiments to see if increasing production of Coenzyme Q could improve diabetic wound healing in diabetic mice.

When oxygen levels are lower in cells, for example when we consume more oxygen while exercising, the result is so called hypoxia. The cell adapts to hypoxia by adjusting many different cellular systems. An important part of these systems is hypoxia-inducible factor (HIF). Since oxygen is so important but at the same time potentially harmful in the form of ROS, HIF is a fine-tuned machinery designed to meet the challenge of oxygen levels going up and down in a cell. Many of these adjustments involve mitochondria, since mitochondria are the main consumer of oxygen in our cells. However, in diabetes this machinery is faulty because of HIF being negatively affected by high sugar levels. Temporary hypoxia happens all the time in our bodies. Then, if HIF is damaged, ROS will cause damage which builds up over time and lead to complications.

We therefore set out to investigate how dysfunctional HIF can be restored to working order and so lessen the harmful effects of diabetes. Our translational studies involved subjects with diabetes, laboratory mice and cell cultures. We investigated how mitochondrial energy production, HIF and ROS interconnect in diabetes, and what can be done to correct the mechanisms which cause damage. Our findings resulted in better understanding of the nature of ROS and energy metabolism in diabetes. This newfound knowledge will contribute to future research efforts in the clinical treatment of severe complications such as diabetic foot ulcers and diabetic kidney disease.

SVENSKA

Populärvetenskaplig Sammanfattning

DIABETES OCH VÅRA CELLERS ANDNING

Nästan en av tio människor i världen lider av diabetes. Trots detta är diabetes en ofta bortglömd sjukdom, då den dödar långsamt. I takt med att samhällen blir mer stillasittande och överviktiga har diabetes blivit ett globalt hot mot människors hälsa med enormt lidande och sjukvårdskostnader som resultat. Diabetes kännetecknas av högt blodsocker. Blodsocker används av mitokondrier, våra cellers kraftverk, till att producera energi. Denna process genererar även reaktiva syrespecies (ROS) som en biprodukt. ROS är både viktiga signalmolekyler men har också hög potential att orsaka skador på vårt DNA, celler och vår hälsa. Man känner till sedan tidigare att högt blodsocker vid diabetes leder till ökad produktion av ROS. Detta är en av huvudorsakerna bakom de diabeteskomplikationer som uppträder över tid om sjukdomen ej behandlas tillräckligt bra. Dessa komplikationer inkluderar hjärt-kärlsjukdom, stroke, blindhet, njursvikt och svårläkta sår som riskerar att leda till amputationer.

Vår kropp har många mekanismer för att minska risken för ROS-orsakad skada. Då diabetes leder till ökad ROS är kroppens inbyggda antioxidantförsvar otillräckligt för att förhindra skador. Därför studerade vi Coenzym Q, en kroppsegen antioxidant som dessutom utgör en viktig beståndsdel I mitokondriers energialstrande apparat. Vi undersökte vilket samband olika nivåer av Coenzym Q hos diabetiker har för risken att utveckla olika komplikationer. Vi utförde även experiment för att se om en metod för att öka kroppens tillverkning av Coenzym Q kunde förbättra sårläkning hos diabetiska möss.

Mängden syre I våra celler varierar, till exempel när vi tränar. När syrenivån är lägre inträffar ett tillstånd som heter hypoxi. Våra celler anpassar sig till hypoxi genom att justera en mängd olika funktioner. En viktig del av kroppens styrsystem vid hypoxi är hypoxia-inducible factor (HIF). Syre är livsviktigt men potentiellt farligt i form av ROS. HIF är därför ett mycket exakt maskineri som är byggt för att möta olika syrenivåer med precisa justeringar i cellens energialstrande mekanismer. Då mitokondrier star för lejonparten av vår syreförbrukning involverar HIFs reglermekanismer många av mitokondriens funktioner. I diabetes är HIF-maskineriet felaktigt kalibrerat då HIF påverkas negativt av höga sockernivåer. Övergående hypoxi är en vanlig och normal händelse I våra kroppar. Ett felaktigt HIF-system leder till ROS-orsakade skador och därmed diabeteskomplikationer.

Vi ville därför studera hur och om skadat HIF kan förbättras eller återställas för att minska de skadliga effekterna av diabetes. Våra olika studier hade ett translationellt fokus som involverade patienter med diabetes, diabetiska möss och cellodlingar. Vi undersökte hur mitokondriell energiproduktion, HIF och ROS relaterar till varandra i ett diabetiskt sammanhang, och vad som kan göras för att rätta till de skadliga mekanismerna. Våra fynd förbättrar den rådande kunskapen om ROS och energiomsättning i diabetes. Denna kunskap kommer att bidra till framtida forskning om och behandling av diabetiska senkomplikationer såsom diabetesfotsår och diabetisk njursjukdom.

中文

科普摘要

糖尿病以及细胞如何呼吸

全球平均每 10 人中就有 1 人患有糖尿病。然而，糖尿病已成为一种经常被忽视的疾病因为它是个慢性杀手。随着人们变得越来越久坐不动和超重，糖尿病已成为一个全球性日益增长的威胁，给人类健康和社会经济都造成巨大的伤害和负担。糖尿病以高血糖为主要特征，众所周知，如果糖尿病病人没有得到及时和充分的治疗，随着时间的流逝形成各种并发症的危险会增加。这些并发症包括心血管疾病, 例如心肌梗塞，脑中风, 眼底血管病失明和肾功能衰竭, 还有糖尿病末梢血管神经病变引起的糖尿病足, 难以愈合的糖尿病伤口会有长期反复感染并可导致肢体截肢。我们的机体是通过细胞的线粒体, 细胞内部的小能源工厂来将糖转化为能量。糖在线粒体内转化时需要氧气并且在转化过程中有活性氧（ROS）产生，ROS 既是衍生物又是细胞内各个部分相互通信的重要信使分子。但过多的 ROS 会损坏 DNA 和细胞功能并导致疾病。所以糖尿病高血糖导致的 ROS 升高是糖尿病各种并发症形成的主要原因。

我们的机体有许多机制来防止 ROS 对细胞造成伤害。但机体内置的抗氧化防御功能不足以阻止糖尿病导致 ROS 升高造成的损害。这就是为什么我们研究辅酶 Q（机体内一种特异性的抗氧化物质，并且是线粒体能量转换结构中的重要组成部分）的原因。我们研究了糖尿病患者中不同的辅酶 Q 水平与发生不同并发症的风险之间的关系。 我们还进行了小鼠实验，以查看一种增加机体辅酶 Q 产生的方法是否可以改善糖尿病小鼠的伤口愈合。

当细胞中的氧气含量较低时，例如当我们在运动时消耗了更多的氧气就会导致所谓的细胞缺氧状况，我们的细胞会通过调节多种功能来适应缺氧。在缺氧时控制系统中的一个重要组成部分是缺氧诱导因子（HIF）。氧气至关重要，但以 ROS 的形式存在时有潜在的危险，HIF 就象是一个组建好的非常精密的仪器，可以通过精细调节细胞能量产生的机制来应对细胞中氧气水平的变化。由于线粒体是细胞中氧气的主要消耗者。因此 HIF 调节机制涉及许多线粒体功能，糖尿病高糖水平对 HIF 产生负面影响，致使 HIF 的校准不正确。 短暂性缺氧是我们体内常见的正常事件，但错误的 HIF 系统无法阻止 ROS 引起的伤害，从而导致糖尿病并发症。

因此我们着手研究如何将受损的 HIF 恢复正常从而减轻 ROS 的有害影响。我们的转化研究涉及糖尿病患者，实验室小鼠和细胞培养。我们研究了糖尿病体内线粒体能量产生，HIF 和 ROS 如何相互联系，以及如何纠正导致损伤的机制。我们的发现使人们对 ROS 的本质和糖尿病患者的能量代谢有了更多的了解。这些新知识将有助于将来对糖尿病严重并发症, 如糖尿病足和糖尿病肾病的临床治疗的研究。

DEUTSCH

Populärwissenschaftliche Zusammenfassung

DIABETES UND WIE UNSERE ZELLEN ATMEN

Beinahe einer in zehn Menschen leidet weltweit an Diabetes. Trotzdem wird die Krankheit oft übersehen, da es sich um eine schleichende Entwicklung handelt. Da die Welt sesshafter und übergewichtiger wird, ist Diabetes eine ständig wachsende Herausforderung. Diabetes zeichnet sich durch hohen Blutzucker aus. Zucker wird von Mitochondrien, kleinen Kraftwerken in den Zellen, aus denen unser Körper besteht, zu Energie umgewandelt. Mitochondrien brauchen Sauerstoff, um Zucker in Energie umzuwandeln. In diesem Prozess wird reaktiver Sauerstoff (ROS) als Nebenprodukt hergestellt. ROS dient zum Teil als wichtiges Botenstoffmolekül, zwischen verschiedenen Zellen. Zu viel ROS schädigt jedoch unsere DNA und führt zu Krankheiten. Es ist bekannt, dass ein hoher Blutzucker, wie bei Diabetes zu einem erhöhten ROS führt. Dies ist die Hauptursache für verschiedene Komplikationen von Diabetes. Zu diesen Komplikationen gehören Herzinfarkte, Schlaganfälle, Blindheit, Nierenversagen und Wunden, die chronisch werden und zu Amputationen der Gliedmassen führen können.

Der Körper hat viele Mechanismen, um durch ROS verursachte Schäden zu reduzieren. Da Diabetes zu einem erhöhten ROS führt, reicht die antioxidative Abwehr des Körpers nicht aus, um die durch ROS verursachten Schäden zu verhindern. Aus diesem Grund haben wir Coenzym Q untersucht, ein Antioxidans, das ebenfalls Teil des Mitochondrien-Kraftwerks ist. Wir haben untersucht, wie die Coenzym-Q-Spiegel bei verschiedenen Diabetikern den Unterschied in der Komplikationsrate erklären kann. Wir haben auch Experimente durchgeführt, um zu sehen, ob eine Erhöhung der Produktion von Coenzym Q die Wundheilung bei diabetischen Mäusen verbessern kann.

Wenn der Sauerstoffgehalt in Zellen niedriger ist, beispielsweise wenn wir während des Trainings mehr Sauerstoff verbrauchen, ist das Ergebnis eine sogenannte Hypoxie. Die Zelle passt sich der Hypoxie an, indem sie viele verschiedene Zellsysteme anpasst. Ein wichtiger Teil dieser Systeme ist der Hypoxia-inducible factor (HIF). Da Sauerstoff wichtig ist, aber gleichzeitig potenziell schädlich in Form von ROS, ist HIF eine fein abgestimmte Maschinerie, die entwickelt wurde, um die Herausforderung zu bewältigen, dass der Sauerstoffgehalt in einer Zelle steigt und fällt. Viele dieser Anpassungen betreffen Mitochondrien, da Mitochondrien der Hauptverbraucher von Sauerstoff in unseren Zellen sind. Bei Diabetes ist diese Maschinerie jedoch fehlerhaft, da HIF durch hohe Zuckerwerte negativ beeinflusst wird. Wenn dann HIF beschädigt ist, verursacht ROS Schäden, die sich im Laufe der Zeit aufbauen und zu Komplikationen führen. Wir wollten daher untersuchen, wie beschädigter HIF wieder funktionsfähig werden kann und so die schädlichen Auswirkungen von Diabetes verringert werden können. Unsere Translationsstudien umfassten Diabetiker, Labormäuse und Zellkulturen. Wir untersuchten, wie mitochondriale Energieerzeugung, HIF und ROS bei Diabetes miteinander in Verbindung stehen und was getan werden kann, um die Mechanismen zu korrigieren, die Schäden verursachen. Unsere Ergebnisse führten zu einem besseren Verständnis von ROS und des Energiestoffwechsels bei Diabetes. Dieses neu gewonnene Wissen, wird zur zukünftigen Forschung und klinischen Behandlung schwerer Komplikationen, wie diabetischen Fussgeschwüren und diabetischen Nierenerkrankungen, beitragen.

ABSTRACT

Diabetes Mellitus (DM) is a major concern for societies and healthcare systems globally. DM-associated morbidity and mortality is mediated by diabetic complications. Hypoxia and oxidative stress have emerged as key players in the pathogenesis of various macro- and microvascular complications of DM. This work aimed to investigate different aspects of the relationship between hypoxia, regulation of hypoxia inducible factor-1 (HIF-1), mitochondrial function and the development of common DM complications from a translational perspective.

Paper I investigates the endogenous antioxidant Coenzyme Q10 in a Swedish cohort of DM patients. We explored the association between markers of oxidative stress and the prevalence of vascular complications of DM. Our results showed hyperlipidemia, hyperglycemia, and inflammation to be associated with markers of oxidative stress, which in turn was correlated to the prevalence of diabetes complications such as peripheral neuropathy.

In **Paper II**, we investigated a novel epoxidated Tocotrienol derivative exhibiting antioxidative properties affecting mitochondrial function. Mono-epoxy-tocotrienol-alpha was seen to stimulate pro-wound healing processes such as fibroblast migration rates and endothelial tube formation *in vitro*. The compound was also shown to increase wound closure rates in diabetic mice. This paper demonstrated experimentally that modulating mitochondrial function can improve factors underlying deficient wound healing in DM.

The association between hypoxia regulation, wound healing and cellular bioenergetics in DM was studied in **Paper III**. HypoxamiR-210 (miR-210) is induced by HIF-1 in response to hypoxia. We found that hyperglycaemia reduced hypoxia-dependent miR-210 induction. miR-210 increased the rate of wound healing in diabetic mice. The same treatment reduced oxygen consumption rate and ROS production in wound tissue. We thus showed that the hypoxia associated dysregulation in wound healing can be reversed through miR-210-mediated improvement of cellular metabolism.

Finally, **Paper IV** explored the relationship between HIF-1 repression by hyperglycemia and overproduction of ROS in DM. Using a translational approach employing cell cultures, mice and human subjects exposed to hypoxic conditions, we showed that HIF-1-deficient induction of ROS production is specific feature in DM. Reversing this process was shown to be a protective factor against diabetic nephropathy. Hence, reversing impaired HIF-1 function is a potential therapeutic target in the treatment of DM complications.

LIST OF SCIENTIFIC PAPERS

I. Elisabete Forsberg*, **Cheng Xu***, Jacob Grünler, Johan Frostegård, Michael Tekle, Kerstin Brismar, Lars Kärvestedt. **Coenzyme Q10 and oxidative stress, the association with peripheral sensory neuropathy and cardiovascular disease in type 2 diabetes mellitus.**
J Diabetes Compliations. Nov-Dec 2015;29(8):1152-8.
doi: 10.1016/j.jdiacomp.2015.08.006.

II. **Cheng Xu**, Magnus Bentinger, Octavian Savu, Ali Mosfegh, Vivekananda Sunkari, Gustav Dallner, Ewa Swiezewska, Sergiu-Bogdan Catrina, Kerstin Brismar, Michael Tekle. **Mono-epoxy-tocotrienol-alpha enhances wound healing in diabetic mice and stimulates in vitro angiogenesis and cell migration.**
J Diabetes Complications. 2017 Jan;31(1):4-12.
doi: 10.1016/j.jdiacomp.2016.10.010.

III. Sampath Narayanan, Sofie Eliasson, **Cheng Xu**, Jacob Grünler, Allan Zhao, Wan Zhu, Ning Xu Landén, Mona Ståhle, Jingping Zhang, Mircea Ivan, Raluca Georgiana Maltesen, Ileana Ruxandra Botusan, Neda Rajamand Ekberg, Xiaowei Zheng, Sergiu-Bogdan Catrina. **HypoxamiR-210 accelerates wound healing in diabetes by improving cellular metabolism.**
Comms Bio. Accepted Manuscript

IV. Xiaowei Zheng*, Sampath Narayanan*, **Cheng Xu***, Sofie Eliasson, Jacob Grünler, Allan Zhao, Alessandro di Torro, Luciano Bernardi, Massimiliano Massone, Peter Carmeliet, Marianna del Sole, Giancarlo Solaini, Kerstin Brismar, Tomas A. Schiffer, Ileana Ruxandra Botusan, Neda Rajamand Ekberg, Fredrik Palm, Sergiu-Bogdan Catrina. **Repression of Hypoxia inducible Factor 1 (HIF-1) in diabetes contributes to the increase of mitochondrial radical oxygen species (ROS) production**
Manuscript

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OTHER PUBLICATIONS

- Robin Fröbom, Felix Sellberg, **Cheng Xu**, Allan Zhao, Catharina Larsson, Wenn-Onn Lui, Inga-Lena Nilsson, Erik Berglund, Robert Bränström. **Biochemical inhibition of DOG1/TMEM16A achieves antitumoral effects in human gastrointestinal stromal tumor cells in vitro.**
  Anticancer Research 2019;39(7):3433-3442.
  doi: 10.21873/anticanres.13489

- Xiaowei Zheng, Sampath Narayanan, Vivekananda Gupta Sunkari, Sofie Eliasson, Ileana Ruxandra Botusan, Jacob Grünler, Anca Irinel Catrina, Freddy Radtke, **Cheng Xu**, Allan Zhao, Neda Rajamand Ekberg, Urban Lendahl, Sergiu-Bogdan Catrina. **Triggering of a Dll4-Notch1 loop impairs wound healing in diabetes.**
  Proc Natl Acad Sci U S A 2019 Apr 2;116(14):6985-6994.
  doi: 10.1073/pnas.1900351116.

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LIST OF ABBREVIATIONS

2DG	2-Deoxyglucose
2OG	α-Ketoglutarate / 2-Oxoglutarate
4HNE	4-Hydroxynonenal
AGE	Advanced Glycated End-Product
AGO	Argonaute RNAse III
ATP	Adenosine Triphosphate
BAT	Brown Adipose Tissue
BMI	Body Mass Index
BNIP3	BCL2/Adenovirus E1B 19 kDa Protein-Interacting Protein 3
BSA	Bovine Serum Albumin
Ca	Calcium
cDNA	Complementary DNA
CMH	1-Hydroxy-3-Methoxycarbonyl-Tetramethylpyrrolidine
cRNA	Complementary RNA
CoQ	Coenzyme Q10
COX4	Cytochrome C Oxidase Subunit 4
COX10	Cytochrome C Oxidase Assembly Protein
CP	3-Carboxy-Proxyl (Oxygen Radical)
CPH	1-Hydroxy-3-Carboxy- 2,2,5,5-Tetramethylpyrrolidine
Crea	Creatinine
CVD	Cardiovascular Disease
CVL	Cerebrovascular Lesion
CysC	Cystatin C
DAPI	4,6-Diamidine-2-Phenylindole Dihydrochloride
db/db	C57BL/KsJm/Leptdb transgenic mouse
DETC	Diethyldithiocarbamate
DFX	Deferoxamine
DFU	Diabetic Foot Ulcer
DGCR8	DiGeorge Syndrome Critical Region 8
DIG	Digoxigenin

DM	Diabetes Mellitus
DMEM	Dulbecco's Modified Eagle's Medium
DMOG	Dimethyloxallyl Glycine
ECAR	Extracellular Acidification Rate
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
eNOS	Endothelial Nitric Oxide Synthase
EPO	Erythropoietin
EPR	Electron Paramagnetic Resonance Spectroscopy
ER	Endoplasmic Reticulum
ETC	Electron Transport Chain
FBS	Fetal Bovine Serum
FFPE	Formalin-fixed, Paraffin-embedded Tissue
FGF	Fibroblast Growth Factor
FORT	Free Oxygen Radicals Test
fpG	Fasting Plasma Glucose
G6PD	Glucose-6-Phosphate Dehydrogenase
GCSF	Granulocyte Colony-stimulating Factor
GLUT	Glucose Transporter
GRX	Glutaredoxin
GSH	Glutathione
GSHPx	Glutathione Peroxidase
GSSG	Oxidized Glutathione
GWAS	Genome-wide Association Studies
H_2O_2	Hydrogen Peroxide
HbA1c	Glycated Hemoglobin
HBO	Hyperbaric Oxygen
HDF	Human Dermal Fibroblast
HDL / HDLc	High-Density Lipoprotein / HDL-Cholesterol
HDMVEC	Human Dermal Microvascular Endothelial Cells
HIF	Hypoxia-inducible factors
HIF-1 / HIF-1α	Hypoxia-inducible factor 1 / Hypoxia-inducible factor 1α
HPLC	High Performance Liquid Chromatography

HRE	Hypoxia Response Element
hsCRP	High-sensitive C-reactive Protein
HT	Hypertension
IMCD-3	Mouse Inner Medulla Collecting Tubular Cells
KGF	Keratinocyte Growth Factor
KHB	Krebs-Henseleit Buffer
KIM-1	Kidney Injury Marker 1
IGF-I	Insulin-like Growth Factor I
ISCU	Iron-Sulphur Cluster Scaffold Protein
LADA	Latent Autoimmune Diabetes in Adults
LDHA	Lactate Dehydrogenase A
LDL / LDLc	Low-density Lipoprotein / LDL-Cholesterol
LNA	Locked Nucleic Acid
MCT4	Monocarboxylate Transporter 4
MET3α	Mono-Epoxy-Tocotrienol-α
miR-210	HypoxamiR-210 / MicroRNA 210
miRISC	MicroRNA-induced Silencing Complex
miRNA	MicroRNA
MMP	Matrix Metalloproteinase
MODY	Maturity Onset Diabetes of the Young
mtROS	Mitochondrial Reactive Oxygen Species
NADPH	Nicotinamide Adenine Dinucleotide Phosphate, Reduced
NFκB	Nuclear Factor κB
NGF	Nerve Growth Factor
NPWT	Negative-pressure Wound Therapy
O_2•−	Superoxide
O_2	Oxygen, O_2
OCR	Oxygen Consumption Rate
OGDC	Oxoglutarate Dehydrogenase Complex
ORF	Open Reading Frame
oxLDL	Oxidized LDL Cholesterol
OXPHOS	Oxidative Phosphorylation

PARP	Poly (ADP-ribose) Polymerase
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate-buffered Saline
PDGF	Platelet-derived Growth Factor
PDH	Pyruvate Dehydrogenase
PDK1	Pyruvate Dehydrogenase Kinase 1
PHBA	4-Hydroxybenzoate / P-Hydroxybenzoic Acid
PHD	Prolyl Hydroxylase
PKC	Protein Kinase C
PMN	Polymorphonuclear Neutrophilic Granulocytes
PRP	Platelet-rich Plasma
PSN	Peripheral Sensory Neuropathy
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RT	Room Temperature
SDHD	Succinate Dehydrogenase Subunit D
SEM	Standard Error of Mean
SOD	Superoxide Dismutase
SSC	Saline-Sodium Citrate Buffer
STZ	Streptozotocin
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TCA	Tricarboxylic Acid (Cycle)
TGF-β	Transforming Growth Factor β
TUNEL	Terminal Transferase (TdT) dUTP Nick End Labeling
UAlb	Urinary Albumin
UCP1	Uncoupling Protein-1
VEGF	Vascular Endothelial Growth Factor
VHL	Von Hippel-Lindau Protein
VPT	Vibration Perception Threshold
VSMC	Vascular Smooth Muscle Cells
wt / WT	Wild-type

1 BACKGROUND

1.1 DIABETES AND DIABETIC COMPLICATIONS

1.1.1 Epidemiology and etiology

Diabetes Mellitus (DM) is a major and ongoing health challenge with a global reach. For both Type 1 Diabetes Mellitus (T1DM) and Type 2 Diabetes Mellitus (T2DM) sufferers, the advent of insulin as a treatment option was an important and life-saving breakthrough. Yet, DM has emerged as a relentlessly expanding health problem, with T2DM accounting for roughly 85% of the disease burden (1). A significant increase in DM-associated morbidity and mortality is predicted to occur in the coming decades, with heavy implications for a wide array of issues facing affected societies (2, 3). Due to the heterogenous nature of DM, relatively few symptoms and impairments in early stages of the disease, and inadequate knowledge about its nature, an estimated 50% of all afflicted individuals remain undiagnosed. Better living conditions and growing prosperity are regrettable culprits in DM. These improvements in life quality, associated with longer lifespans, increasingly sedentary lifestyles and higher obesity rates, are recognized as the main drivers for accelerating DM incidence, especially in low- and middle-income countries. The total prevalence of DM is expected to increase significantly in the near future, reaching 700 million worldwide by 2045 (4, 5).

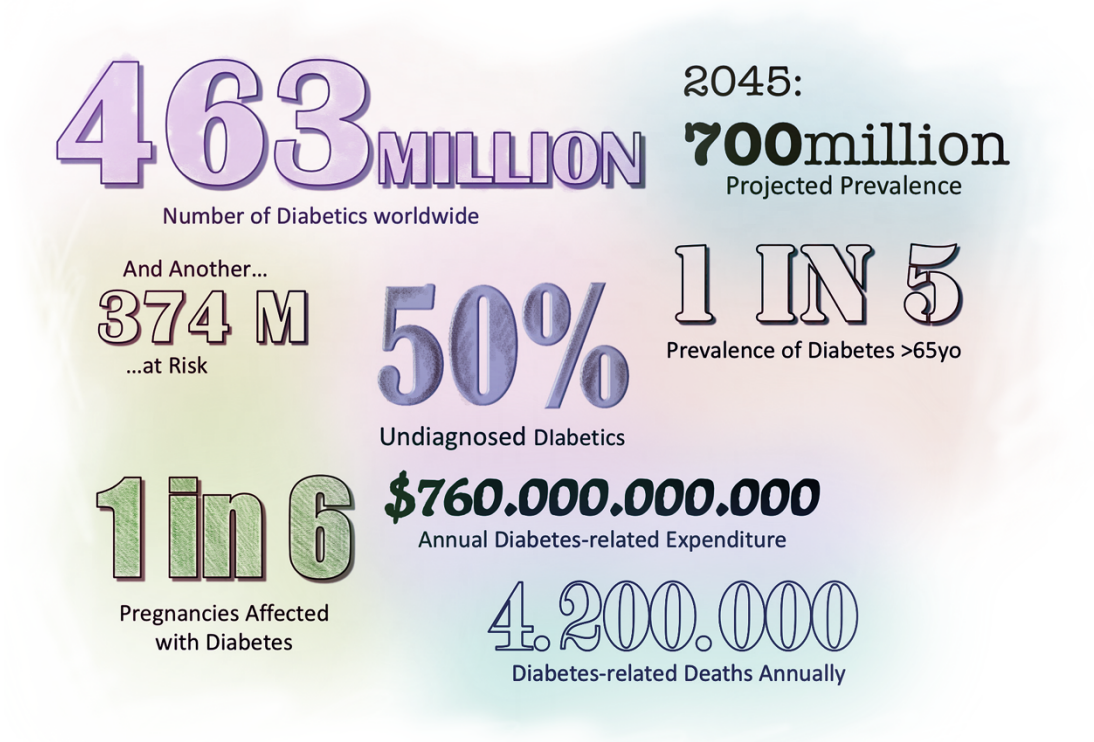


Fig. 1. Diabetes Epidemiology. Source: International Diabetes Federation Diabetes Atlas 9th ed. (6)

1.1.1.1 Type 1 Diabetes Mellitus

Epidemiological data for T1DM is generally considered to be accurate due to the rapid presentation of the disease resulting in early presentation to healthcare facilities and early detection. The incidence of T1DM ranges 0.1 per 100,000 individuals per year in parts of Asia, to 30 per 100,000/year or higher in Scandinavia and Sardinia (7, 8). T1DM incidence peaks during childhood and teen years. It arises due to a combination of genetic and environmental factors (9, 10). There has been a steady increase of incidence of T1DM in children and adolescents during the last decades, a trend which is expected to continue (11).

1.1.1.2 Type 2 Diabetes Mellitus

Due to a long time lag between the onset of hyperglycaemia and time of diagnosis, T2DM epidemiological data is patchy compared to that of T1DM. Prevalence of T2DM is unevenly spread, with a correlation between the adoption of "western" lifestyles and T2DM prevalence (4). The main etiological risk factors of T2DM are advanced age, obesity, family history of DM and a sedentary lifestyle. T2DM has a strong heritable component, though it is spread over a large number of identified genetic risk markers which additionally have variable penetrance depending on environmental and lifestyle factors (5, 7, 12). Dietary pattern, the consumption of red meat, sugared drinks as well as low consumption of fruits and vegetables have been shown to correlate with increased risk of T2DM (13-15).

1.1.1.3 Other Types of Diabetes

Various other modalities of DM exist. Gestational diabetes is considered the third main form of DM. Late-onset autoimmune diabetes in adults (LADA), hereditary maturity onset diabetes of the young (MODY), and brittle diabetes due to pancreatic surgery are some examples of other DM subtypes (16). These are outside the scope of the current thesis.

Table 1. Type 1 and 2 Diabetes Characteristics.

	Type 1 Diabetes (T1DM)	Type 2 Diabetes (T2DM)
Age of onset	Any age, more common in youth	More common later in life
Genetic component	Weaker, often sporadic	Strong polygenic heredity
Beta cell antibodies	Present	Absent
C-peptide	Low or undetectable	Normal or high
Insulin production	Absent	Normal or low
Obesity	Less common	Very common
Diabetic ketoacidosis	High risk	Usually low risk
First line treatment	Insulin	Oral antidiabetic medication

1.1.2 Pathophysiology

Incomplete understanding of the underlying disease mechanisms in DM frustrates attempts to apply research outcomes on a wide therapeutic scale (5, 17). Reduced to its essence, DM is a pathological lack or insufficiency of insulin. The main differences between T1DM and T2DM concern the nature of how insulin fails to exert its regulatory functions on a cellular and sub-cellular level (5, 18). The cardinal symptom of DM is hyperglycaemia resulting from a lack of said insulin action. When cells are exposed to hyperglycaemia for an extended period of time, increased glucose uptake leads to a cascade of events through four major pathological pathways: increased flux through the polyol and hexosamine pathways, increased production of advanced glycated end-products (AGE), and increased activation of protein kinase C (PKC) (19). These four pathways effect a large host of downstream consequences which ultimately increase the production of reactive oxygen species (ROS). In addition, hyperglycaemia induces glycosylation in endothelial nitric oxide synthase (eNOS) and downregulates eNOS activity, thus promoting the production of reactive nitrogen species (RNS). ROS and RNS, as reactive molecules which in sufficient quantities are capable of causing DNA damage and long-term detrimental effects in cells and organs. The resulting damage underpins the development of numerous and serious complications of DM. (17, 20, 21)

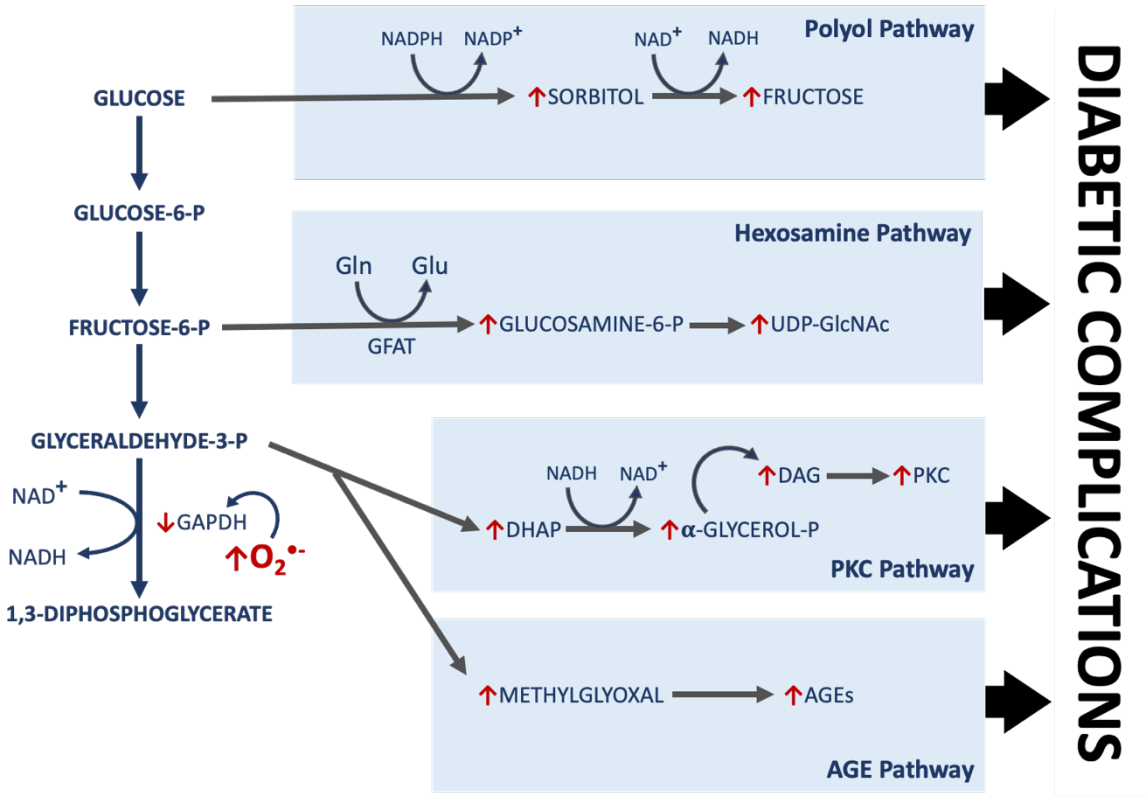


Fig. 2. The Four Pathways of Diabetic Complications. Increased glycolytic substrate availability due to hyperglycemia increases flux through the Polyol, Hexosamine, PKC and AGE pathways, in turn leading to cellular damage and subsequent development of diabetic complications (17, 19)

1.1.3 Macro- and microvascular complications

The spectrum of chronic complications in DM can be divided into microvascular and macrovascular etiologies. Macrovascular complications of DM include cardiovascular disease (CVD), cerebrovascular disease, and peripheral arterial disease, whereas microvascular complications include diabetic retinopathy and diabetic kidney disease (22). In addition, diabetic neuropathy is both a category in itself and also related to microvascular causes. Certain diabetic complications, such as diabetic foot ulcers (DFU), represent a mixed etiology wherein both micro- and macrovascular factors as well as neuropathy and concurrent hyperglycaemia contribute to their development (23). A compromised or dysregulated immune response in DM is also an important factor influencing disease progression (24). DM in itself and its treatment also pose additional perils in the form of acute complications such as Diabetic Ketoacidosis (DKA), acute hypoglycaemia, lactic acidosis and Hyperosmolar Hyperglycaemic State (25).

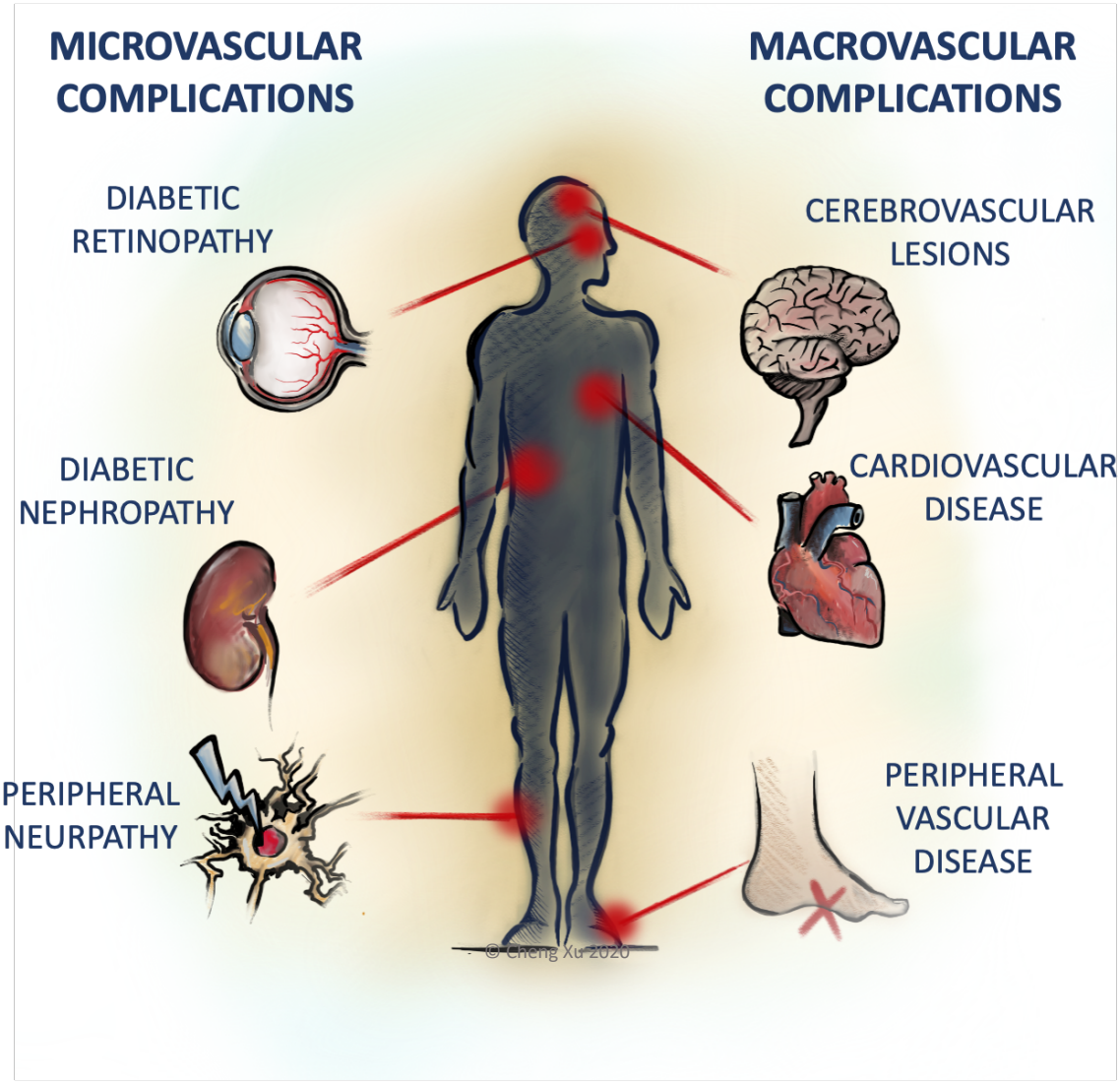


Fig. 3. Diabetic Complications. Complications of DM are divided into macrovascular and microvascular etiologies, although some, such as neuropathy and diabetic wounds, represent a mixed etiology influenced by both large and small vessel disease (26)

1.2 NORMAL AND DIABETIC WOUND HEALING

1.2.1 The wound healing process

Wound healing is a multifactorial and complex process involving a cascade of cellular responses to a disruption in the epidermal and dermal layers of the skin (27-29). Wound healing can be classified in terms of four distinct but timewise overlapping phases: hemostasis, inflammation, proliferation and remodeling. Wound healing is a fragile process, and all components must work in a proper and coordinated manner in order to ensure a successful recovery of the damaged tissue (29-31).

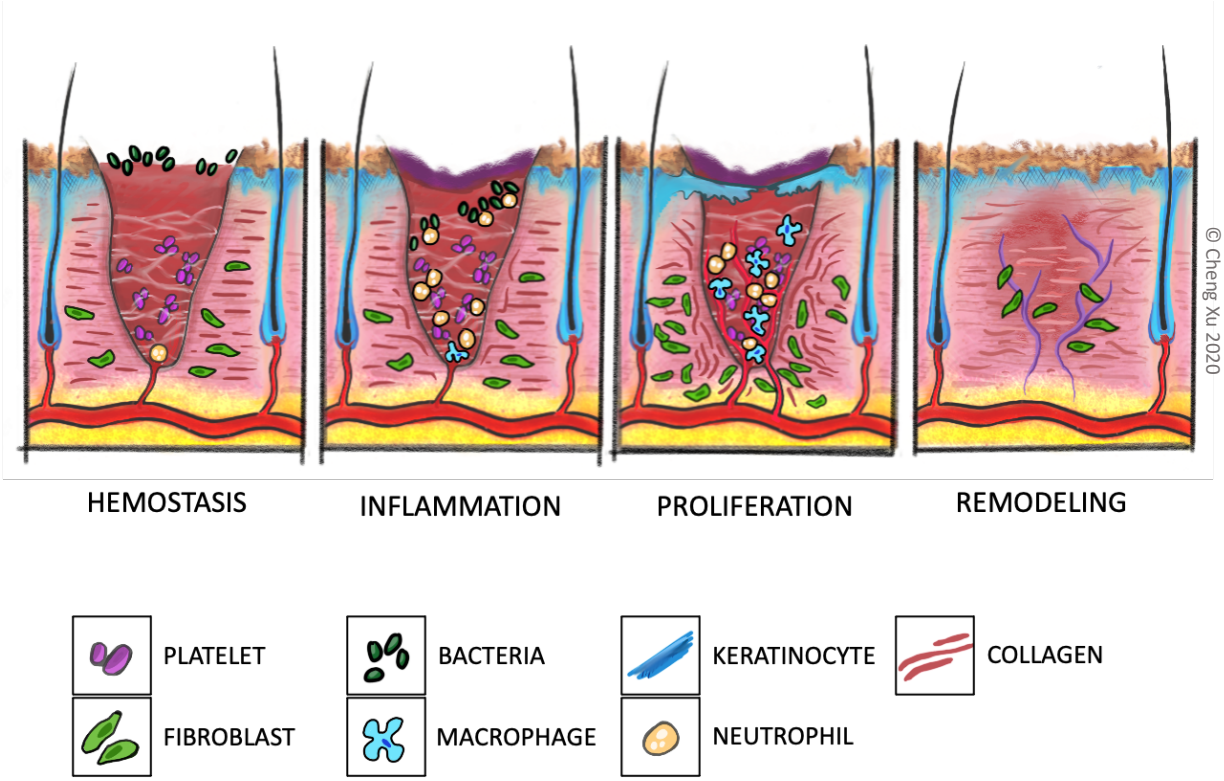


Fig. 4. The Four Phases of Wound Healing. The four phases of physiological wound healing have distinct characteristics yet overlap in time, involving many signaling pathways and cell types (31).

1.2.1.1 Hemostasis and Coagulation

Following a breach of the outer epidermal and dermal layers of the skin barrier, vascular smooth muscle cells (VSMC) constrict the disrupted blood vessels in order to prevent further loss of blood. This is followed by the clotting cascade whereas a fibrin plug is formed. Platelets, rapidly aggregating at the wound site, release clotting factors and, together with the fibrin plug, form a scaffold onto which other cells involved in downstream processes can migrate (32). Activation of platelets results in the production of various growth factors including transforming growth factor-β (TGF-β), epidermal growth factor (EGF) and platelet-derived growth factors (PDGF). These and other signals provide cues for neutrophils, macrophages, fibroblasts and endothelial precursor cells to migrate towards the wound area, setting the stage for the inflammatory phase of wound healing (27, 33).

1.2.1.2 Inflammatory Phase

Polymorphonuclear neutrophilic granulocytes (PMN) are the predominating cell type in the early inflammatory phase of wound healing. PMN are attracted by cytokines and other signaling factors secreted in the wound site during the hemostasis and coagulation phase. PMNs debride damaged tissue through secretion of proteases and phagocytosis, thereby killing pathogens and preparing the wound site for the recruitment of monocytes. Monocytes recruited peripherally can then mature into macrophages, which constitute the predominating inflammatory cell type present during the latter part of the inflammatory phase (29, 30). The inflammatory phase last for as long as debris and pathogens remain in the wound site. An inadequate or misdirected immune response may thus prolong the inflammatory phase, retarding progression into the proliferative phase and result in the emergence of a chronic wound (28, 34-36).

1.2.1.3 Proliferative Phase

The proliferative phase of wound healing starts with dermal fibroblasts migrating towards the wound site as soon as 48-72 hours after the time of wounding. This happens in response to cytokines released from platelets and inflammatory cells, and fibroblast migration thus overlaps with previous wound healing phases. The proliferative phase is characterized by the formation of granulation tissue. Fibroblasts secrete collagen, proteoglycans and fibronectin, providing for a newly formed extracellular matrix (ECM), enabling re-epithelialization by keratinocytes migrating into the ECM. In response to TGF-β stimulation, fibroblasts differentiate into myofibroblasts which contract the wound using smooth muscle-type actin-myosin complexes. (37, 38). Local hypoxia in the wound tissue, caused by the disruption of blood supply during the wounding and subsequent healing process, stimulates the release of vascular endothelial growth factor (VEGF) from macrophages and other cells. VEGF stimulation promotes endothelial cell migration, proliferation and capillary tube formation. As this vascularization and epithelialization process subsides, the resulting granulation tissue gradually matures, dries and forms a scar. Wound healing then progresses into the remodelling phase (31, 39).

1.2.1.4 Remodeling Phase

The remodeling phase is usually defined as starting when collagen secretion and degradation during wound healing reach an equilibrium. During the remodeling phase, Collagen III, the predominating collagen subtype during the proliferative phase, is replaced by Collagen I in a process which also rearranges cross-linked and chaotic collagen matrices into ordered structures aligned with the skin's tension lines (29, 40). Further remodeling and maturation proceeds for an extended period of time, with maximum post-wounding tensile strength reaching 80% of pre-wound levels around 3 months into the healing process. Reduced wound healing activity and thus reduced oxygen requirements locally will eventually lead resorption of superfluous blood vessels through apoptosis. This process reduces the erythematous appearance of scars. The remodeling phase, depending on conditions, can continue for as long as a year or even longer (31, 37, 40).

1.2.2 Wound healing in diabetes and the diabetic foot ulcer

1.2.2.1 Diabetic Foot Ulcers

Normal wound healing functions in a highly concerted and predictable way, according to the distinct yet overlapping phases described above. If this progression of wound healing phases fails, the result may be a non-healing, chronic wound, or that of pathological scarring processes such as in keloid formation. (41, 42). In DM, a host of factors conspire and interact, resulting in impaired wound healing progression and the emergence of a diabetic foot ulcer (DFU). These factors include intrinsic ones such as neuropathy, macro- and microangiopathy, hyperglycemia and hypoxia. Additionally, extrinsic factor such as external pressure on the skin, various infections and poor personal care due to old age or visual impairment may act as culprits in contributing to causing and sustaining DFUs. (28, 43).

1.2.2.2 A Sequence of Unfortunate Events

Sensory neuron loss in diabetic neuropathy causes sensory deprivation. Motor neuron loss leads to anatomical changes, suboptimal ergonomics and clumsy movement. Autonomic neuropathy leads to skin dryness. All these factors in DM increase the risk of wounds (44). Diabetic conditions impair healing through slower cell migration, prolonged inflammation and concurrent infections in wound sites (45). Increased expression of pro-inflammatory cytokines, increased matrix metalloprotease activity, and lower levels of pro-angiogenetic growth factors such as PDGF and VEGF in the wound site. The remodelling phase of wound healing is delayed in DM. This ultimately leads to a slower overall rate of complete wound closure and a significantly raised risk of re-ulceration in locations such as the foot, where there is constant shear stress exerted on the tissue (46, 47).

1.2.2.3 Diabetic Wounds and Disturbed Signaling Pathways

On a molecular level, wound healing dysfunction in DM can in part be attributed to inadequate cytokine signaling (45). One such example of a failed cascade involves reduced fibroblast migration causing a deficient provisional scaffold. This reduces immune responses by macrophages and neutrophils, leading to diminished VEGF signaling, which in turn diminishes the recruitment of endothelial precursor cells (EPC) from the bone marrow. Deficient EPC homing hampers endothelial tube formation, revascularization and proper progression from the inflammatory to the proliferation and remodeling phases of wound healing (30, 36, 46, 48). Other pleiotropic growth factors and cytokines, including insulin-like growth factor 1 (IGF-I), TGF-β, PDGF, nerve growth factor (NGF) and keratinocyte growth factor (KGF), have previously been shown to be reduced in diabetic wounds (49-54). The action of matrix metalloproteinases (MMP), essential for facilitating cytokine signaling and ECM resorption, are also known to be unbalanced in DM (55, 56). Of special consideration in the current body of work is the disturbed signaling pathways that characterize hypoxia regulation in DM, especially in regard to HIF-1 and micro-RNA 210 (miR-210). These are explored further in dedicated chapters below.

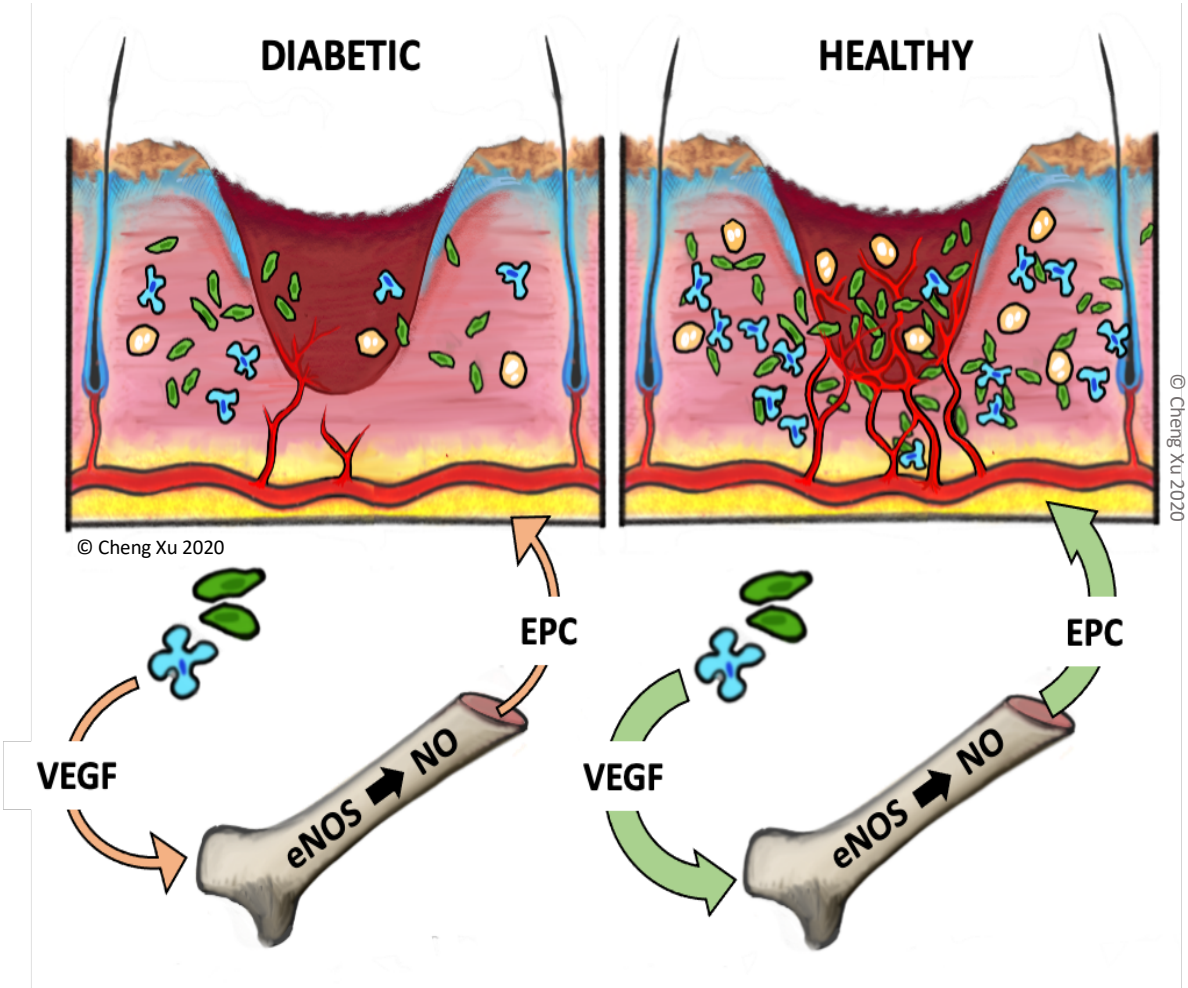


Fig. 5. Diabetic and Normal Wound Healing. Signaling pathways in diabetic wound healing are disturbed, leading to reduced infiltration of cells needed to generate and sustain the protein scaffolding, cytokine signaling and neovascularization characterizing normal wound healing (28). A pictogram legend indicating different cell types can be found in Fig.4.

1.2.3 Therapeutic considerations

DFU represent a major confluence of risk factors and adverse outcome predictors in DM. A manifest DFU often suggests the presence of advanced neuropathy, macro- and microvasculopathy, deranged immunological wound infection responses and ongoing poor glycemic control. Thus, DFUs are also associated with a pessimistic prognosis regarding future morbidity and mortality, and have emerged as a major cause of disability worldwide (57-59). Current therapies focus on mechanical debridement, off-loading, and wound dressing. More advanced current treatment options include negative pressure wound therapy (NPWT), bioengineered skin substitutes and hyperbaric oxygen (HBO). Other approaches, such as PDGF- β substitution, ECM protein supplementation, and MMP modulators point to a large potential for future developments. Other therapies targeting DM-related deranging of signaling pathways in wound healing include local treatment using platelet-rich plasma (PRP), granulocyte colony-stimulating factor (GCSF), EGF and fibroblast growth factor (FGF) (60, 61). The results of these efforts vary and are sometimes conflicting, thus highlighting the need for a better understanding of the molecular aspects underlying impaired wound healing in DM.

1.3 HYPOXIA AND ITS REGULATION IN DIABETES

1.3.1 Hypoxia regulation and dysregulation

1.3.1.1 Hypoxia, Overview

Hypoxia is the physiological state of an absolute or relative lack of oxygen in relation to the physiological requirements of a particular organism, organ or tissue. The ability to maintain oxygen homeostasis is essential for all aerobic organisms, given the status of oxygen (O_2) as a critical substrate in cellular metabolism and signalling (62). Oxygen homeostasis works in a framework wherein different parts of the body, depending on metabolic role, oxygen turnover and distance from the alveolar gas exchange in the lungs, seek to maintain different oxygen concentrations, ranging from somewhat close to the atmospheric oxygen levels of around 20%, down to levels of between 1 and 10% in most peripheral tissues (63, 64). Table 2 summarizes approximate oxygen pressures in different tissues and organs. However, it must be noted that oxygenation is highly heterogenous in many organs, e.g. in kidneys (65). Hypoxia is implicated in several of the most common causes of disability and death in humans, including cardiovascular disease, ischemic stroke, tumorigenesis and, as will be explored further below, in DM (46, 66, 67). These pathological conditions all represent situations where physiological systems evolved for oxygen sensing and adaptation to hypoxia prove inadequate (68).

Table 2. Partial O_2 pressures. Given values are averages as measured in previously published research. Actual oxygen tension in tissues and organs can vary considerably depending on physiological factors.

	pO_2 mmHg	pO_2 %	Reference
Air, ambient	160	21.1	(64)
Air, trachea	150	19.7	(64)
Air, alveolar	110	14.5	(64)
Blood arterial	100	13.2	(69)
Blood, venous	40	5.3	(69)
Lung	42.8	5.6	(70)
Liver	40.6	5.4	(71)
Intestine	61.0	8.0	(72)
Kidney	72.0	9.5	(73)
Brain	35.0	4.6	(74)
Bone Marrow	54.9	7.1	(75)
Skin, epidermis	8.0	1.1	(76)
Skin, papillae	24.0	3.2	(76)
Skin, plexus	35.2	4.6	(76)

1.3.1.2 Hypoxia-inducible Factors

Hypoxia inducible factors (HIFs) are evolutionarily conserved proteins. HIF was first discovered as a nuclear factor binding to a promoter of the erythropoietin (EPO) gene (77). HIFs play a vital role in the regulation of oxygen sensing and adaptation to lower oxygen pressures, and thus represent a very basal survival function in all metazoans (78). HIF consists of two α and β subunits. Three isoforms of the α subunit are known. HIF-1α is ubiquitously expressed in most cells, whereas HIF-2α and HIF-3α are more tissue specific in comparison (68). When combined into a heterodimer with HIF-1β, the resulting complex is termed HIF-1, HIF-2 or HIF-3 depending on which α-subunit comprises the other half of the complex. While HIF-1 and HIF-2 have partly overlapping functions, HIF-1 is thought to be a forceful responder to acute hypoxia, with peak expression reached within 24 hours of the hypoxic insult. In contrast, HIF-2 seems to have a more chronic pattern of activation in response to prolonged hypoxia (79). HIF-3α was initially thought to be a regulatory component wherein the HIF-3 complexes it formed seemed to downregulate HIF transcriptional activities, although other HIF-3-regulated pathways have since been identified (80, 81). Different HIF isoforms have been shown to regulate different processes, e.g. depending on tissue localization (82, 83).

1.3.1.3 HIF Regulation

HIF-1α is regulated through post-translational hydroxylation of specific proline residues by Proline Hydroxylases (PHDs). Pro402 and Pro564 are hydroxylated by PHD proteins in HIF-1α (65). Out of the three known PHDs (PHD1-3), PHD2 is considered to be the main HIF-regulating PHD (84). PHD activity is regulated by O_2 availability, and have been proposed as the *de facto* oxygen sensors linking O_2 concentrations to downstream effects of HIF, the "master regulator" of hypoxia (85). In normoxic conditions, HIF α-subunits are continuously hydroxylated by PHDs due to an abundance of O_2. When in a hydroxylated state, HIF-1α binds to the Von Hippel-Lindau (VHL) protein, a ubiquitin ligase, with a >1000-fold increase in affinity compared to HIF in its non-hydroxylated form. The HIF-VHL complex is then ubiquitylated and thus flagged for rapid proteasomal degradation, thus keeping HIF downstream activity to an appropriate minimum in normoxia (86). In hypoxic conditions, PHD activity is supressed, resulting in an increase in stabilized HIF-1α which then dimerizes with HIF-1β, forming an active HIF complex. This is accomplished by shifting relative substrate availability, such as that of α-ketoglutarate or 2-oxoglutarate (2OG) (87). The HIF heterodimer then binds to hypoxia response elements (HRE) on target DNA sequences. Another separate oxygen-dependent regulatory mechanism in the form of a HIF hydroxylase known as factor inhibiting HIF (FIH) has also been described (88). HIF expression and activity is also affected by transcriptional and translational regulators, post-translational modifications and ligand binding mechanisms (65). Genes affected by HIF will increase transcriptional activities, which promotes functions such as erythropoiesis, cell proliferation, angiogenesis, glucose homeostasis, and other adaptive responses (89). Genome-wide association studies (GWAS) suggest a gene regulatory role of HIF affecting more than 1000 genes (78). In addition to the classical HIF pathway, HIF also regulates several non-coding RNAs (90). Figure 6 on the following page shows a graphic overview of HIF regulation.

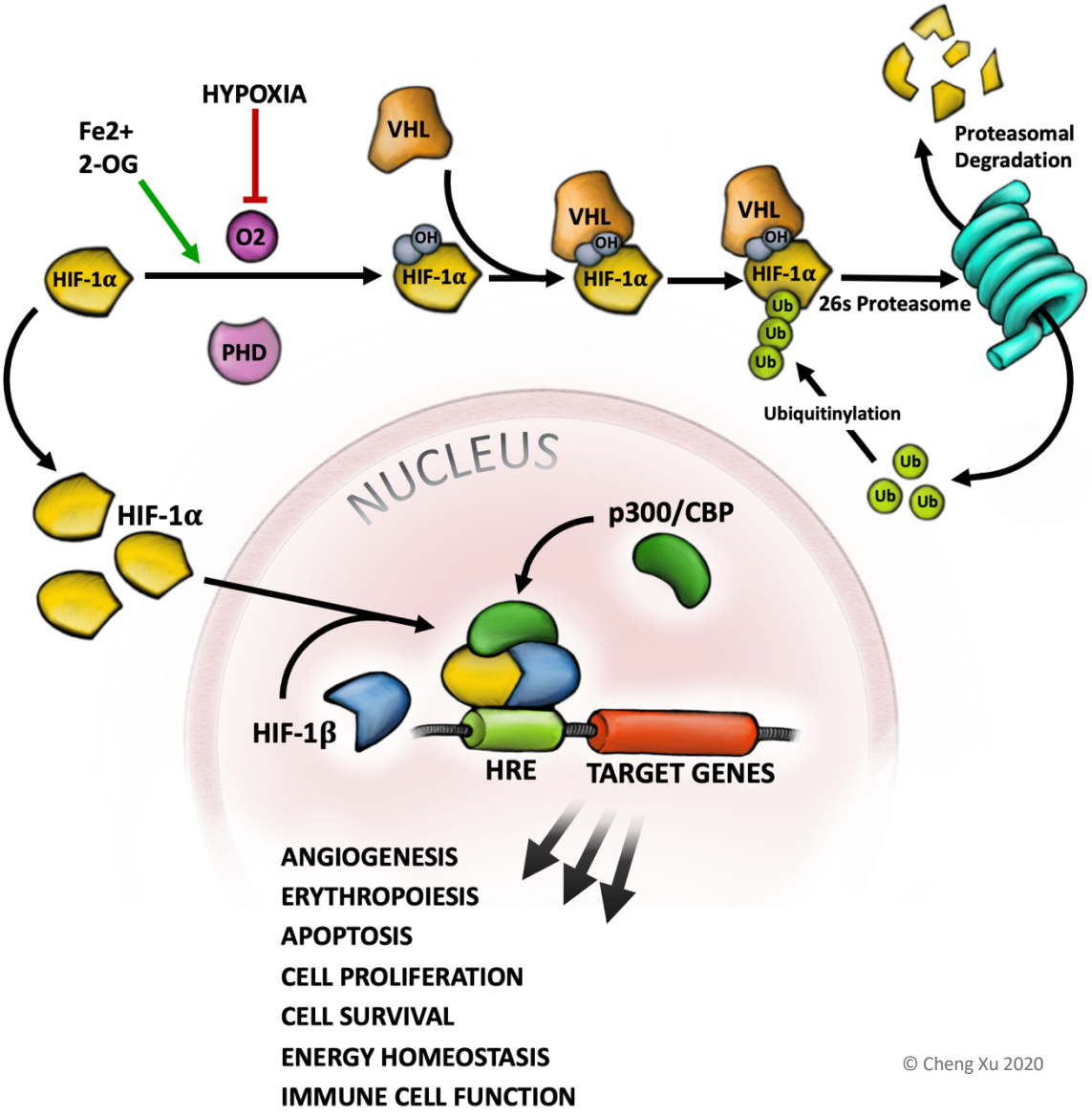


Fig. 6. Regulation of HIF-1 activity. HIF-1α activity is regulated by the oxygen sensing properties of PHDs. In the absence of hypoxia, PHDs will hydroxylate HIF-1α which induces binding by VHL, and subsequent ubiquitylation and proteasomal degradation. In hypoxic conditions, PHDs are inhibited and HIF-1α is instead able to combine into a heterodimer with HIF-1β, forming the HIF-1 complex together with the p300/CBP coactivator, which then binds to HREs of target genes. This in turn regulates a plethora of downstream effects intended to adapt cell response to acute and chronic hypoxia (78).

1.3.1.4 HIF-1 and Diabetic Wound Healing

Hypoxia features in all wounds due to disrupted blood flow and infiltration of inflammatory cells which are highly aerobic and cause inflammatory edema (91-93). Diabetes has long been known to be associated with absolute and relative tissue hypoxia, owing to a combination of substrate consumption imbalances and vascular damage (94). Whereas hyperglycaemia remains the main driver of diabetes complications, hypoxia has emerged as an important factor in DM with implications for wounds, kidney and retina (46). Impaired HIF-1-mediated responses to hypoxia partly explains the pathogenesis of impaired wound healing in DM (95).

Hyperglycaemia destabilizes HIF-1α through increased VHL-dependent degradation. While the exact mechanism is not fully understood, inhibition of PHDs using deferoxamine (DFX), an iron chelator, has been shown to reduce VHL-dependent degradation of HIF in hyperglycaemic environments. This process does not explain the entirety of HIF dysfunction in hyperglycaemia, as the effect seen on HIF stabilization when inhibiting PHDs and VHL only partially restores HIF functionality (95). Accumulation of methylglyoxal (MGO) has been proposed as an alternative mechanism by which HIF is degraded independently of PHD and VHL (96). In addition to reducing HIF expression, the activity of HIF is also affected by hyperglycaemia. This effect has been suggested to occur through interference with HIF-1 dimerization and recruitment of co-activators, as well as indirectly through hypoxia-associated ROS excess (46). Reduced HIF expression and availability result in impaired wound healing in DFU (97). Stabilization of HIF in DM may thus be a therapeutic target for preventing and treating diabetic complications including diabetic wounds (98, 99).

1.3.2 MicroRNAs and hypoxia

1.3.2.1 MicroRNAs

MicroRNAs (miRNA) are a group of small, non-coding RNA oligonucleotide molecules averaging 22 nucleotides (100). More than 2500 human miRNAs have been described, in most cases exerting their effect through binding to the 3′untranslated region (UTR) of target mRNAs, causing translational repression of gene expression (101). Other effector mechanisms are known, in these cases often resulting in a potentiating effect on gene expression (100). The mRNA representing a majority of the human transcriptome are thought to be miRNA targets (102). Aside from intracellular signalling and regulatory functions, miRNAs have also been seen to be ejected into ECM and the bloodstream where a number of miRNAs have been investigated as possible biomarkers for disease (103, 104). miRNAs have been identified as involved in a large number of conditions, including wound healing regulation in DM (105).

1.3.2.2 MicroRNA Biogenesis

MiRNA synthesis is a multi-step process. In the canonical pathway of miRNA biogenesis, miRNA genes are transcribed by RNA Polymerase II into pri-miRNA, a long primary transcript with a hairpin structure containing the miRNA sequence. A microprocessor complex, consisting of the ribonuclease Drosha and the co-factor DiGeorge Syndrome Critical Region 8 (DGCR8), cleaves pri-miRNA into pre-miRNA. Pre-miRNA is then exported into the cytoplasm trough an Exportin 5/Ran-GTP-dependent process where further modification takes place. In the cytoplasm RNase enzymes DICER and Argonaute (AGO) is responsible for cleaving pre-miRNA into mature miRNA (106, 107). The miRNA eventually forms part of a miRNA-induced silencing complex (miRISC), which binds and inhibits target mRNA (108). Several additional, non-canonical miRNA biogenesis pathways bypassing one or more of the mechanisms mentioned above have also been described (109).

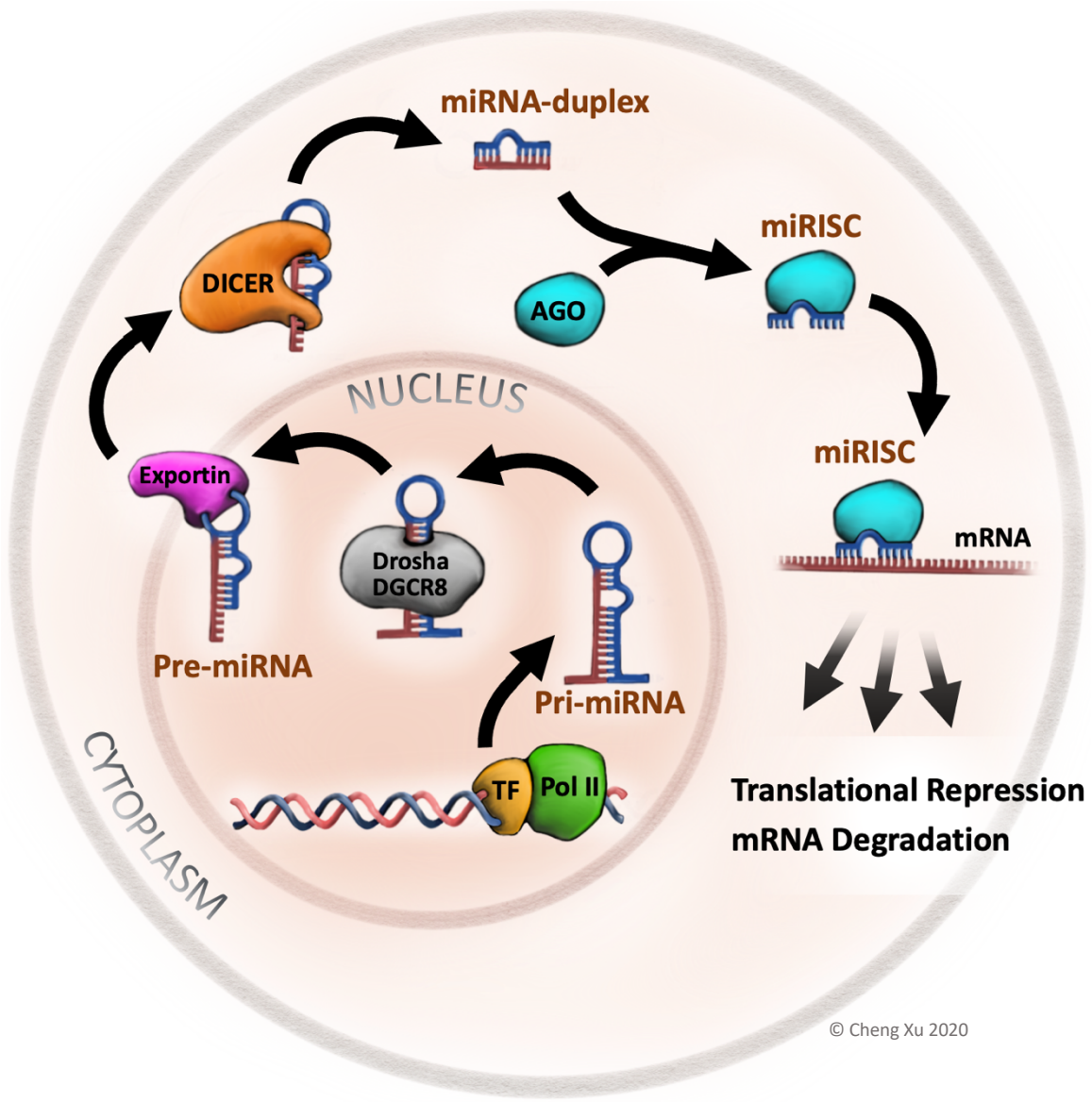


Fig. 7. Standard microRNA Biogenesis. miRNA genes are transcribed by RNA Polymerase II (Pol II), together with general or specific transcription factors (TF). The resulting Pri-miRNA is cleaved into pre-miRNA b the Drosha/DGCR8 complex and transported into the cytoplasm by Exportin. DICER and Argonaute (AGO) then produce the mature miRNA which then binds to target gene mRNA, thus either directly inhibiting its effect and/or flagging it for degradation.

1.3.2.3 MicroRNAs and Diabetes

The ubiquitous nature of miRNAs is reflected in DM and DM complications. Several miRNAs exert effects on insulin resistance, insulin secretion, retinopathy, peripheral neuropathy, diabetic kidney disease, cardiovascular disease and wound healing (110-112). Various miRNAs have been proposed as possible therapeutic targets as well as disease biomarkers in DM (113). These include miR-126 for predicting future DM risk, miR-1249, miR-320b, and miR-572 for early diagnosis, miR-21, miR-29a/b/c, and miR-192 for progression of diabetic nephropathy, as well as miR-132 for wound healing (114-118). Other studies have shown miRNA involvement in DM-associated cardiovascular pathogenesis (119, 120). However, the promiscuous miRNA targeting of mRNA, their mode of expression and frequent tissue-specific expression, leave large unknowns in this area (118, 119, 121-126).

1.3.2.4 MicroRNAs, Wound Healing and HypoxamiRs

Several miRNAs have been identified as important components of the three latter phases of wound healing (127). Of special interest to this thesis are the hypoxamiRs, a label used for miRNAs which are upregulated in hypoxia. HypoxamiRs can be grouped according to how they relate to HIF. HIF-dependent miRNAs include miR-210 and miR-373. miR-20b, miR-199a and miR-424 instead respond to hypoxia independently but affect HIF expression. A third category are not hypoxamiRs in the strict sense since they affect HIF expression independent of hypoxia, such as p53-induced miR-107 inhibiting HIF-β (128, 129).

1.3.3 MicroRNA-210

1.3.3.1 The "Master HypoxamiR"

miR-210 stands out among hypoxamiRs in several ways. miR-210 is induced in hypoxic conditions in many cell types and, notably, in solid tumours (130). miR-210 possesses an HRE structure in its promoter region, thus enabling direct interaction with HIF. Its gene has binding sites for transcription factors such as nuclear factor κB (NFκB) and PPARγ, suggesting a regulatory role in cellular metabolism, apoptosis and cell differentiation (131).

1.3.3.2 miR-210 Functions

One of the most well-studied functions of HIF-1 is its repression of mitochondrial respiration. A lack of oxygen is met by adaptive responses seeking to reduce O_2-intense mitochondrial respiration, instead shifting adenosine tri-phosphate (ATP)-generation towards anaerobic glycolysis (78, 132). Apart from direct HIF-1 actions to achieve this, such as repression of complex I activity through upregulating NDUFA4L2, an indirect effect is exerted through HIF-1 upregulation of miR-210 (133). miR-210 downregulates iron-sulphur cluster scaffold proteins 1 and 2 (ISCU1/2), which are essential components in the tricarboxylic acid cycle (TCA cycle) and the mitochondrial electron transport chain (ETC) (134). MiR-210 also targets the cytochrome c oxidase assembly protein (COX10) and succinate dehydrogenase subunit D (SDHD) (135, 136). Inhibition of COX10 leads to repression of complexes I and IV, whereas inhibition of SDHD inhibits complex II. The net effect of miR-210 on cellular metabolism is a comprehensive downregulation of mitochondrial respiration, thus acting as a complement, potentiator and non-redundant fine tuning mechanism alongside HIF-1 (130).

1.3.3.3 miR-210 and Angiogenesis, Cell Cycle and DNA Repair

In addition to its role in regulating cell bioenergetics, miR-210 augments endothelial cell responses to hypoxia, thus upregulating angiogenesis. This points to miR-210 as a potentially important factor in diabetic wound healing, CVD and cancer tumorigenesis (137, 138). miR-210 also stimulates osteoblast and adipocyte differentiation (139, 140). miR-210 is known to attenuate keratinocyte cell proliferation, as well as a capability to arrest DNA repair processes (141, 142). Elevated expression of miR-210 have previously been identified in several cancers as well as in acute kidney injury. There are ongoing efforts to investigate miR-210 as a potential blood biomarker for diagnosis and prognostic monitoring (130, 143, 144)

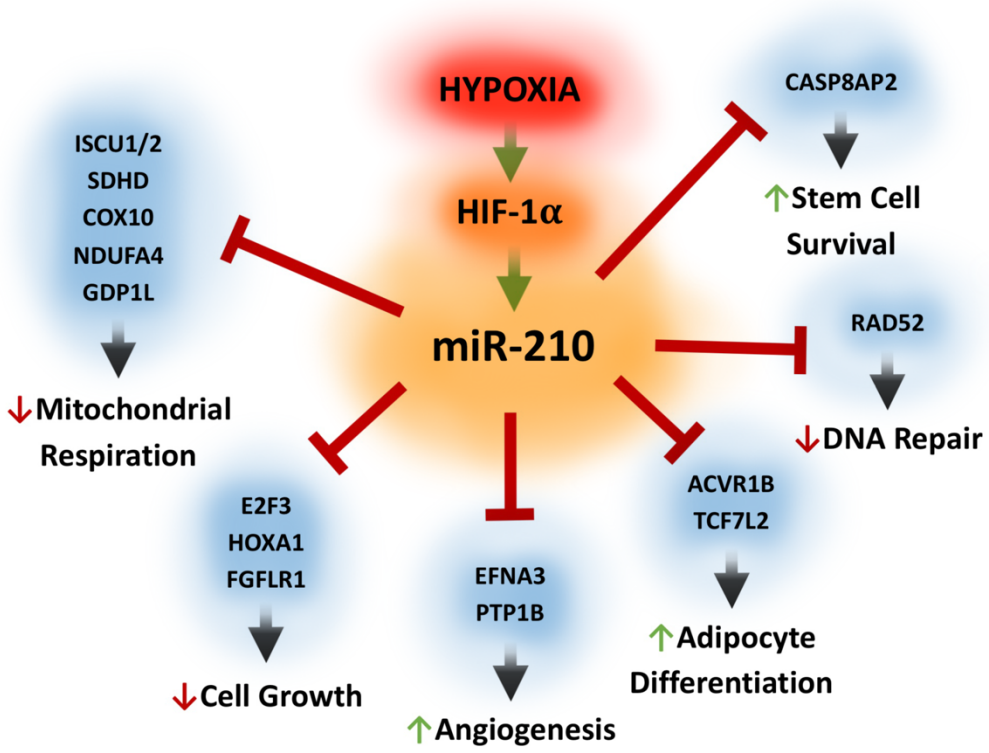


Fig. 8. miR-210 Targets and Effects. miRNA-210 is directly regulated by HIF-1, through an HRE region on the miR-210 gene. miR-210 in turn regulates a number of different processes through targeting mRNA of above listed genes in the manner as described in section 1.3.2. miR-210 function complements, potentiates and fine-tunes HIF-1 regulation of hypoxia responses.

1.3.3.4 miR-210 and Diabetic Wound Healing

Inhibition of miR-210 and PHD2 using target-specific silencing oligonucleotides can improve diabetic wound healing (145). This is in line with reports of elevated miR-210 levels in ischemic wounds (141) Previous work in our lab has shown the importance of HIF-1 derangement as a pathological driver of impaired wound healing in DM (95, 97). Given the HIF-dependent regulation of miR-210 expression, it is unclear whether miR-210 exerts desirable or undesirable effects in diabetic wound healing. This raises the question of whether reports of elevated levels of miR-210 in diabetic and ischemic wounds reflect underlying hypoxia or a manifest imbalance and overexpression of miR-210 with detrimental effects for wound healing in DM. Further investigations on the role of miR-210, both as a complement to and downstream effector of HIF-1 at the intersection between hypoxia and cellular energy turnover in DM, are explored in Paper III.

1.4 CELLULAR BIOENERGETICS IN DIABETES AND HYPOXIA

1.4.1 Mitochondria in brief

1.4.1.1 Powerhouse of the Cell

Mitochondria are complex intracellular organelles of purported endosymbiotic bacterial origin, which facilitate aerobic respiration in all eukaryotic cells. Mitochondria are unique among sub-cellular structures in possessing a double membrane and its own coding DNA. The structural and functional components in mitochondria are not completely coded by mitochondrial DNA, and mitochondrial assembly is impossible without ample input from many nuclear genes (146). Immortalized in popular culture, the phrase "powerhouse of the cell" is a powerful reminder of the basic prerequisite for multicellular life that mitochondria represent (147). Mitochondria produce ATP as a means of energy storage derived from high-energy bonds in macronutrients and oxygen. O_2-dependent aerobic respiration is an order of magnitude more efficient than anaerobic, O_2 and mitochondria-independent fermentation of glucose (148).

1.4.1.2 Beyond the Powerhouse

Mitochondria perform vital functions in many cellular functions in addition to generating ATP. Uncoupling protein 1 (UCP1) regulates ETC uncoupling and heat generation instead of ATP production. UCP1 is especially abundant in brown adipose tissue (BAT), where it regulates non-shivering thermogenesis (149). Mitochondria store intracellular calcium (Ca), interacting with the endoplasmic reticulum (ER) to maintain calcium homeostasis (150). Mitochondria have also been identified as key players in the regulation of cell cycle progression and apoptosis, various cell signaling pathways, as well as in steroid hormone synthesis and signaling (151-156). Additionally, mitochondria can have organ specific functions. One example is mitochondrial detoxification of ammonia in hepatocytes (157).

1.4.2 Mitochondria, ROS and hypoxia

1.4.2.1 Mitochondria and Hypoxia

Due to their natural position, mitochondria are affected by hypoxia more than most other cellular components. Mitochondria react to hypoxia by altering mitochondrial fusion and fission, reduce mitochondrial mass by increased mitophagy as well as by modulating oxidative phosphorylation (OXPHOS) through reducing TCA cycle and electron transfer chain (ETC) activity (158, 159). Several mitochondrial adaptations in hypoxia have been identified as being regulated by HIF-1. These include BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3)-associated mitophagy and mitochondrial morphology (160, 161). Most notably, a hypoxic environment has been shown to affect the tightly regulated production of ROS through reductive carboxylation. This increases ROS production and exposes the cell to potential damage. Transient hypoxia also contributes to increased ROS during ischemia reperfusion (I/R) and reoxygenation (162-164). The discovery of a HIF-regulated "fire hose", NDUFA4L2, to reduce OXPHOS and minimize ROS production in hypoxia, led to efforts to better understand the crosstalk between mitochondria and HIF-1 (133).

1.4.2.2 Mitochondrial ROS and Hypoxia regulation

HIF-1 downregulates production of mitochondrial ROS (mtROS) in hypoxia by reducing mitochondrial respiration. Absence of HIF-1 leads to cell death due to mtROS-induced oxidative stress and improper ROS signaling (165). HIF-1 reduces mtROS production by shunting pyruvate away from the ETC. In addition to Complex I inhibition through upregulating NDUFA4L2 and through regulating miR-210, HIF-1 also reduces mtROS by modulating cytochrome c oxidase 4 (COX4) isoforms (166). HIF-1 activation in hypoxia seeks to equilibrate O_2 homeostasis (78). This is mediated by three mechanisms. Firstly, HIF-1 promotes anaerobic glycolysis by upregulating glucose transporter (GLUT) proteins and glycolytic enzymes (167). HIF-1 also directly upregulates expressions of lactate dehydrogenase A (LDHA) and monocarboxylate transporter 4 (MCT4), which convert pyruvate to lactate, and shuttle lactate out of the cell, respectively (168). Second, HIF-1 increases the expression of pyruvate dehydrogenase kinase 1 (PDK1). PDK1 inhibits pyruvate dehydrogenase (PDH). Decreased PDH activity inhibits pyruvate conversion to acetyl-CoA thus reducing TCA cycle inputs (165). An additional level of potentiation sees 2OG availability increased through HIF-mediated proteasomal degradation of the oxoglutarate dehydrogenase complex (OGDC). This reduces TCA cycle metabolites and upregulates HIF-1 through 2OG-induced PHD inhibition (168, 169). Dimethyloxallyl glycine (DMOG), a 2OG analogue and competitive inhibitor, is used in research to downregulate PHD activity (170).

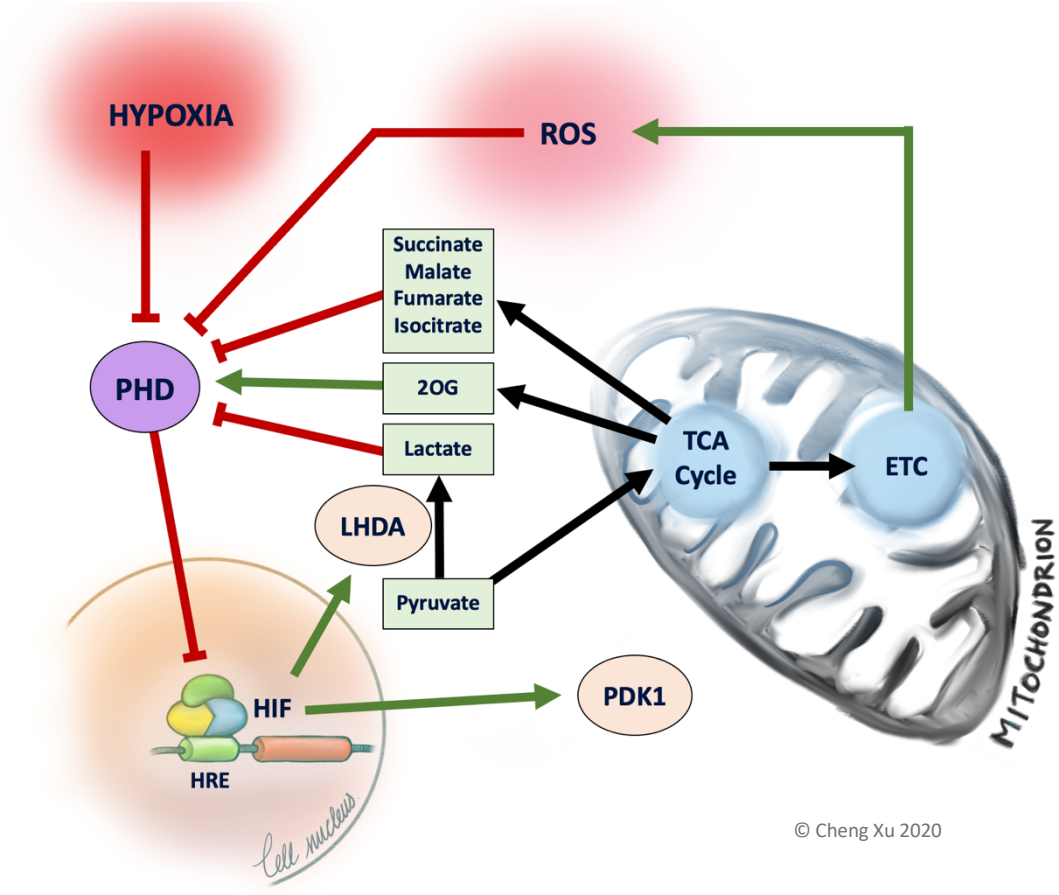


Fig. 9. Mitochondria and HIF-1 crosstalk. HIF-1 exerts direct regulatory functions on mitochondrial respiration. Different intermediate substrates of respiration, and mtROS, have feedback effects on HIF-1 activity through activation or inhibition of PHDs (167).

1.4.2.3 HIF and the Warburg Effect

Hypoxia is an inherent and almost universal characteristic of solid tumors, due to factors such as rapid, disorderly growth with insufficient vascularization leading to tumor hypoxia (171). The tendency of cancer cells to rely on glycolysis rather than aerobic respiration even under normoxic conditions is known as the Warburg effect (172). This effect is often mediated by aberrant HIF signaling, such as when the cancer exhibits loss of VHL, or due to cellular pseudohypoxia (173). Efforts to elucidate the precise role of HIF in cancer tumorigenesis and its potential as a therapeutic target are ongoing (174).

1.4.2.4 ROS Signaling

Perceptions of ROS as a harmful and undesirable side product of OXPHOS have changed in recent years (175). mtROS can feedback-regulate HIF-1, increasing HIF-1 by inhibiting PHDs in acute hypoxia, inducing adaptations in anticipation of chronic hypoxia (176). The details of mtROS signaling are complex with remaining gaps in current knowledge. It is clear, however, that the downstream effects of mtROS signaling depends on mtROS concentration, which mitochondrial complex the mtROS is generated from and which subcellular space is affected (177-179). The targets of mtROS signaling processes from mitochondrial morphology to whole organism survival and longevity (180, 181). A consequentially large array of processes are thus affected by mtROS signaling (182-185).

1.4.3 Diabetes and mitochondrial dysfunction

Deficiencies in mitochondrial function have been linked to many pathological conditions (186, 187). Excessive ROS production in DM has been suggested as a trigger of all major DM complications through the AGE, PKC, hexosamine and polyol flux pathways (19). Additional pathways downstream have also been investigated. These include AGE-induced ROS production from reduced nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase in endothelial cells, and the production of RNS due to hyperglycaemia-dependent glycosylation of endothelial nitric oxide synthase (eNOS) (20, 188). Mitochondrial dysfunction may also manifest in other ways in DM, exacerbating processes such as neuropathy and cell regeneration (189, 190). Skeletal muscle in T2DM contains fewer and smaller mitochondria (191). Additionally, mitochondrial dysfunction in immune cells also play a role in the emergence of DM complications (192). Mitochondrial dysfunction has also been linked to T2DM-associated defects in lipid metabolism (193). There also exists a separate mitochondrial diabetes subtype arising due to a known mutation in mitochondrial DNA (194). Even though outside the scope of this thesis, mitochondrial function in β-cell function, insulin homeostasis and lipolysis in DM is an important field of research in and of itself (195). The role of ROS as both a driver of DM complications and an important component in cellular signalling is an intriguing issue in the study of mitochondrial aspects in DM (146, 196, 197). Between the areas of ROS signalling, HIF regulation of mtROS in hypoxia, and HIF destabilization in DM due to hyperglycaemia, many gaps in the current body of knowledge remain. In Paper IV, the relationship between mitochondrial ROS and impaired HIF activity is further explored experimentally.

1.5 OXIDANTS, ANTIOXIDANTS AND DIABETES

1.5.1 Oxidative stress

The reactivity of oxygen allows it to act as a component in high-energy electron transfers, thereby supporting the generation of ATP through OXPHOS. This enables a mighty leverage of energy underpinning the evolution of all multi-cellular life forms. However, the same reactivity also makes proteins, DNA and other organic molecules highly susceptible to oxidative attack. In order to counteract this, sophisticated antioxidant defenses are vital in keeping oxidative stress in check (78, 196, 198). The previous section established the importance of ROS as signaling molecules. Yet at the same time, they are highly reactive and can cause oxidative damage to DNA, thus leading to cellular damage (180, 199). Oxidative stress refers to an imbalance between the amount of ROS present and the ability of endogenous and exogenous systems' ability to eliminate, neutralize and ameliorate the detrimental effects of ROS, reflecting an insult to redox homeostasis. Oxidative stress and cellular respiration can be measured by various direct and indirect methods including cell respirometry, quantification of oxidized cell components, and measuring real-time ROS production (200, 201).

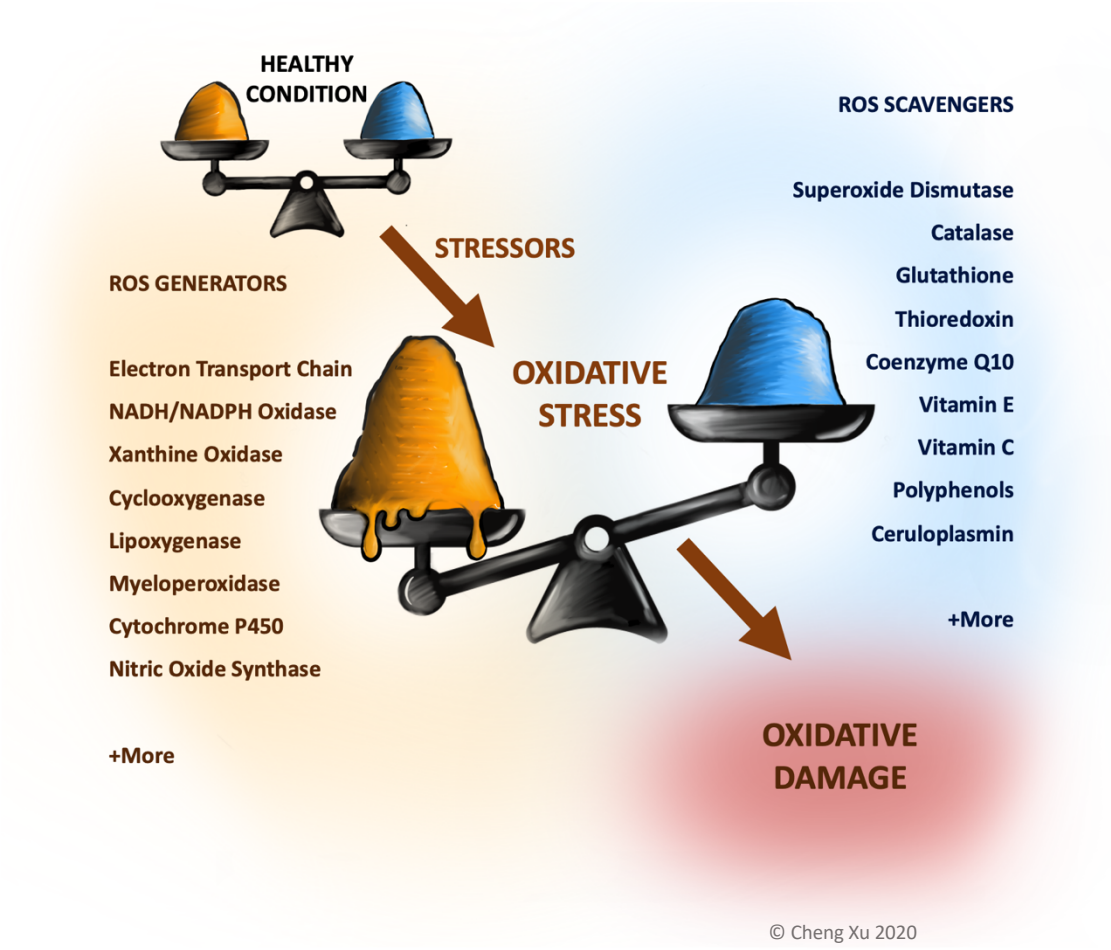


Fig. 10. Redox Homeostasis. Cells and organisms are exposed to a continuous production of ROS from physiological sources. The role of antioxidative systems is to maintain oxidative homeostasis and react to acute insults to the same. Increased oxidative pressure that is not compensated over time will lead to oxidative damage of cellular components (196).

1.5.2 Oxidants and antioxidants

1.5.2.1 Redox Homeostasis

The delineation between endogenous and exogenous sources of oxidants places normal cellular ROS- and RNS-producing processes as endogenous, whereas exogenous factors with oxidizing potential and those which stimulate endogenous ROS production are called external sources (202). Known exogenous sources of oxidants include tobacco smoke, ozone exposure, ionizing radiation, heavy metal ions and arsenic poisoning (203-206). ROS is eliminated by cells using enzymatic and nonenzymatic processes. The large group of heterogenous antioxidants active in humans include glutathione (GSH), superoxide dismutase (SOD), thioredoxins, peroxiredoxins, catalase, vitamins A, B1, B2, B6, B12, C, E, folic acid, and coenzyme Q (CoQ) (207). The canonical main pathway for the body to clear away mtROS in the form of superoxide (O_2•−) is catalysed by SOD. This process results in hydrogen peroxide (H_2O_2), itself an important signalling molecule and potent oxidant, which is then reduced to water (H_2O) by catalase and glutathione peroxidases (GSHPx) using GSH as a substrate. The conversion of oxidized glutathione (GSSG) to GSH by GSH reductase requires NAPDH. This makes glucose-6-phosphate dehydrogenase (G6PD) essential for maintaining the GSSG/GSH ratio. G6PD is the first rate-limiting enzyme in the pentose phosphate pathway which maintains the NADP+/NADPH balance. This ratio must be continuously defended in order to enable scavenging of both physiological and pathological levels of H_2O_2 and O_2•−. (208, 209).

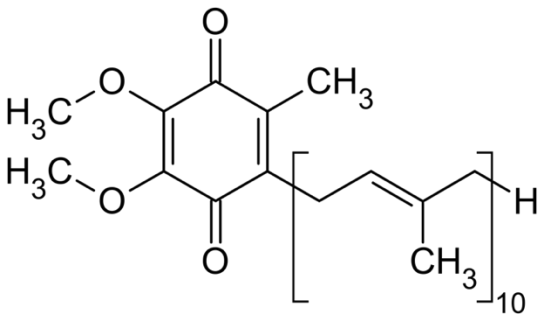
1.5.2.2 Oxidative Damage

Cells respond to oxidative stress by altering the expression of redox enzymes, structural proteins and signalling molecules (202, 210). If cellular adaptations to increased oxidative stress fail, the resulting excess of ROS and other oxidants will cause structural damage to cellular components. ROS can cause damage to DNA through direct modification of nucleotide bases, single- and double-stranded DNA breaks, mutations and protein cross-links. These processes have implications for carcinogenesis, neurodegeneration, metabolic issues and longevity (211). Lipid peroxidation due to oxidative stress may result in disruption of lipid bilayers, affecting membrane integrity and membrane-bound proteins (212). Products of lipid peroxidation, such as 4-hydroxynonenal (4HNE), can act as inducers of further ROS production (213). Proteins are susceptible to oxidative damage as well, and oxidative damage can also cause carbonyl groups to form on amino acid residue side chains (214). Oxdative stress is also known to affect various signalling pathways such as that of NFκB. The results may be intended physiological adaptations to oxidative stress, but could also lead to detrimental effects such as chronic inflammation (215).

1.5.3 Coenzyme Q10

1.5.3.1 Basics and Structure

Coenzyme Q10 is an endogenously synthesized quinone with a long isoprenyl side chain. Its ubiquity in all vertebrates and most other eukaryotes is evident in the alternative name ubiquinone/ubiquinol, referring CoQ in its oxidized and reduced states, respectively. CoQ is constitutively expressed in all human cells and serves as an integral part of oxidative phosphorylation in the mitochondrial respiratory chain (216, 217). CoQ10 is a quinone with 10 isoprenyl subunits in its side chain and the most common CoQ found in humans. Other CoQ frequently encountered in nature and relevant for research include CoQ6 in yeast, and CoQ9 in mice (218). CoQ is an integral part of the ETC and one of its main roles is to act as an electron carrier from complexes I and II, to complex III (219). Consequently, the highest concentration of CoQ are found in tissues where energy requirements are high, such as in cardiac muscle, kidney, liver, and skeletal muscle (217). CoQ uptake by cells is limited, which necessitates biosynthesis of CoQ in nearly all cell types, predominantly on the inner mitochondrial membrane, although parts of the assembly is also thought to occur in the cytoplasm and ER (220).



CoQ10 molecular structure

1.5.3.2 CoQ Biosynthesis

CoQ biosynthesis in eukaryotes begins with the mevalonate pathway, which is shared with the synthesis pathways of dolichol, heme A, cholesterol, cholesterol-derived steroid hormones and proteins with isoprenylate modifications (221). The basic building block of the isoprenoid side chain is farnesyl pyrophosphate (FPP), which is the end product of acetyl-CoA going into the mevalonate pathway (222). Meanwhile, the benzoquinone ring structure deriving from 4-hydroxybenzoate or P-hydroxybenzoic acid (PHBA) originally derives from the amino acid tyrosine. The remaining steps of *de novo* synthesis of CoQ is a not yet fully elucidated multi-step process involving a complex of assembly proteins including PDSS1, PDSS2, and COQ3 through COQ10 commonly called the CoQ synthome or "Complex Q" (223). Most knowledge of CoQ biosynthesis stems from research on brewer's yeast, *Saccharomyces cerevisiae*, with human homologues identified later but with as of yet incomplete attribution of functions and genes. (224). The mevalonate pathway is famously regulated and rate-limited by HMG CoA-reductase, the target for statin class drugs aiming to reduce the influx of cholesterol synthesis precursors. The other synthesis pathways into which the mevalonate pathway feeds its products are likely saturated despite statin-effected inhibition of HMG CoA-reductase, although downstream effects on these pathways have been suggested to be involved in known side effects of statin treatment (225, 226) Apart from insights gained from studies on yeast models, regulatory pathways for CoQ synthome proteins are not well understood as of now (227).

1.5.3.3 CoQ in the Electron Transfer Chain and as an Antioxidant

ETC complexes are arranged in supercomplexes in order to facilitate efficient electron transfer and minimize mtROS production (228). CoQ is a fully integrated part of these supercomplexes, with separate, dedicated CoQ pools for NADH-mediated and FADH2-mediated reactions (229). Indeed, the ratio of reduced versus oxidized CoQ has been suggested as a proxy for ETC fitness (163). CoQ is also involved in the regulation of UCP activity, and the protection of mitochondrial membrane integrity (230, 231). Being lipid-soluble and located close to the source of mtROS, CoQ in its reduced ubiquinol form is very effective at preventing ROS-mediated oxidation of DNA, lipids and proteins (219, 232). CoQ can also restore "spent" vitamin E radicals into their reduced, antioxidant form (233). Separately from its antioxidant properties, CoQ also has a direct effect on ROS signalling, previously found to promote longevity in fruit flies (234). CoQ is a powerful intracellular antioxidant.

1.5.3.4 Tocotrienols and Stimulation of CoQ Biosynthesis

The evidence for oral CoQ raising the amount of bioavailable CoQ is not altogether convincing (222, 235). Certain epoxidated polyisoprenoids are known to significantly increase CoQ biosynthesis (216). Low CoQ levels correlate with peripheral neuropathy in type 2 diabetics (236). Tocotrienols are members of the vitamin E group. Out of the eight vitamin E variants including α-, β-, γ- and δ-tocopherols and -tocotrienols, only α-tocopherol has specific transport proteins, enabling a bodily reserve whereas other variants are rapidly excreted (237). Tocotrienols are less abundant than tocopherols in naturally occurring foods, and have been investigated with regards to their effects on diabetes, obesity and other diseases (238-240). Tocotrienols were found to be suitable for epoxidation modifications, creating epoxidated polyisoprenoids that stimulate CoQ biosynthesis (216, 241). This is achieved by inhibiting oxidosqualene synthase and upregulating of COQ1 (complex of PDSS1 and PDSS2) as well as COQ2 (216, 222). The effect of one of these compounds was investigated in Paper II (201).

1.5.4 CoQ, antioxidants and diabetes

The precise role of antioxidant activity in diabetes and its therapeutic potential is not fully understood (242). The reliance on the pentose phosphate pathway to maintain GSSG/GSH ratios also implicates dysfunction of the same pathway in DM, one of the four pathways to the development of DM complications (19, 243). Evidence suggests that DM may be associated with lower total antioxidant capacity compared to healthy controls (244-247). DM is associated with dysfunctional regulation of SOD, glutathione and ascorbate (248-250). Studies have shown CoQ as being of potential therapeutic use in DM (251). Due to the issue of continuous overproduction of ROS in diabetes, treatment using exogenous antioxidants (250, 252-255). Alternative approaches seek to augment catalytic antioxidant processes such as increasing SOD activity, or by targeting Poly (ADP-ribose) Polymerase (PARP) with PARP inhibitors (256-258). On the whole, the field of antioxidants in DM is still rife with uncertainties. In Paper I CoQ as a biomarker for oxidative stress is investigated further.

2 AIMS

The main objective of my thesis work was to better understand the connection between hypoxia, mitochondrial dysfunction and complications of DM. The thesis work was intended to display translational aspects in regard to diabetic complications, with a special focus on diabetic wound healing. To this end, the following specific aims were pursued:

1. To investigate the epidemiological association between CoQ, oxidative stress and metabolic control in complications of DM **(Paper I)**.

2. To investigate the association between stimulation of CoQ biosynthesis and availability, and mitochondrial function in aspects relating to diabetic wound healing **(Paper II)**.

3. To investigate the effect of miR-210 regulation in hypoxia in the context of diabetic wound healing, its underlying mechanisms and possible therapeutic aspects **(Paper III)**.

4. To investigate the relationship between impaired HIF-1 activity in DM, mitochondrial ROS production and diabetic nephropathy **(Paper IV)**.

3 MATERIALS AND METHODS

3.1 HUMAN STUDIES

3.1.1 Paper I – Study layout

3.1.1.1 Study Recruitment and Data Collection

Subjects were recruited from the municipality of Sundbyberg, Sweden. We included men and women aged 40-70, with T2DM diagnosis at age <35yo. The study population was 61% male and 39% female, 95% Caucasian, aged 62 ± 7 years. Elapsed time since T2DM diagnosis was 7 ± 5.7 years. BMI was 29.2 ± 4.8 and HbA1c 6.4 ± 1.3% (46 ± 14.2 mmol/mol IFCC). The prevalence of complications in the study cohort was previously published (259-261).

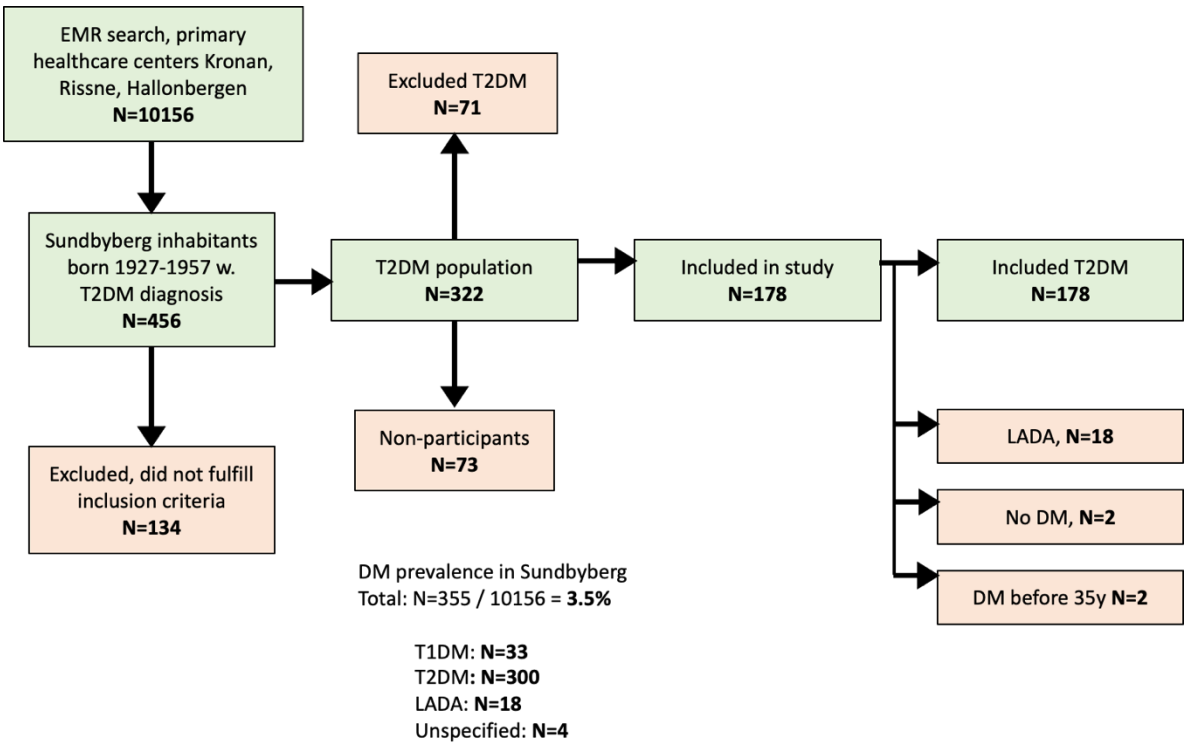


Fig 11. (Paper I – Fig.1). Subject inclusion flowchart.

3.1.1.2 Clinical examination and scoring

Clinical examination of peripheral sensory neuropathy (PSN) was performed using a size 5.07 monofilament tool (10 grams). Vibration perception thresholds (VPT) were assessed with a neurothesiometer. Values were adjusted for gender, age, and body height, and expressed as Z-score (259). PSN was defined as loss of touch discrimination and/or a Z-score of >2 SD compared to age-adjusted normal values (261). CVD was defined as a history of myocardial infarction and/or pathological ECG. Cerebrovascular lesions (CVL) were defined according to diagnosis codes in medical records or demonstrated through cerebral CT scans. Waist index was defined as waist circumference divided by 94 cm in men and by 80 cm in women.

3.1.2 Paper III – Human wound biopsies

8 volunteer subjects with DFU (age: 69.1 ± 9.9 years old; HbA1c: 59.71 ± 11.52 mmol / mol) and 7 volunteer subjects with non-diabetic venous ulcers (age: 78.4 ± 6.9 years old) were recruited from among the patient population at the diabetology and dermatology departments, respectively, at the Karolinska University Hospital. Epidermal and dermal wound biopsies were collected from the marginal zone of the wound using a 4 mm circular biopsy punch with local anesthesia. Collected biopsies were preserved in liquid nitrogen and/or processed prior to preservation or experiments as required by protocols.

3.1.3 Paper IV – Hypoxia in human subjects

Study subjects with T1DM aged 20-50 years with a disease duration of 10-20 years with insufficient glycemic control (defined as HbA1c of >60mmol/L) were recruited from the endocrinology department at the Karolinska University Hospital. Healthy controls were recruited through physical announcements and social media. Exclusion criteria included all major late-stage DM complications, excessively high HbA1c, CVD and malignancy. A full list of exclusion criteria have previously been published (262). Subjects and healthy controls were exposed to systemic hypoxia by inhaling an air mixture containing 13% O_2 intermittently according to a 6-minute normoxic, 6-minute hypoxic cycle for one hour, after which blood samples were collected for analysis of ROS production in peripheral blood.

3.2 ANIMAL STUDIES

3.2.1 Paper II, III and IV – Animal models for diabetic wounds

3.2.1.1 A Diabetic Mouse Model

The db/db mouse, most commonly using the C57BLKS/J strain, is currently the most used mouse strain in the study of DM pathogenesis, obesity and DM complications. Due to a mutation leading to the loss of Leptin receptors, db/db mice develop a T2DM-like phenotype that progresses with age. Obesity, insulin resistance, hyperglycemia, hyperlipidemia and β-cell dysfunction are features seen in this strain (263)

3.2.1.2 Animals and Treatment - Paper II

C57BL/KsJm/Leptdb (db/db) mice and their normoglycemic heterozygous wild-type (wt) littermates (Charles River Laboratories, Brussels, Belgium) were housed five animals per cage in a 12 h light/12 h dark cycle at 22 °C and provided *ad libitum* with standard laboratory chow diet and water supply. The animals were held individually for one week and handled daily. Wounds were made as outlined below (95, 97). A transparent wound dressing was applied to cover the wounds after topical application of either 1 μmol of mono-epoxy-tocotrienol-α (MET3α) for the db/db treatment group and vehicle only for wt and db/db control groups. Wound photographs were taken at the day of surgery and every 48h for 21 days with renewed wound dressing and MeT3α or vehicle treatment at every time point. A circular reference was used to correct for the distance between the wound and the camera. The wound area size was expressed as percentage of the original wound area using and calculated using Image J v1.47 (NIH; Bethesda, MD, USA).

3.2.1.3 Animals and Treatment - Paper III

BKS(D)-Leprdb/JOrIRj (db/db) mice and their corresponding normoglycemic wild-type (wt) control mice (Janvier Labs, Saint-Berthevin, France) were housed up to five per cage in a 12-h light/dark cycle at 22°C and were provided with standard laboratory chow diet and water *ad libitum*. The animals were caged individually and handled daily for one week before wounding as outlined below and previously described (95, 97). Mice were randomized to control microRNA mimic or miR-210 mimic groups according to matching levels of HbA1c, body weight and blood glucose. 0.125mmol per wound of miR-210 mimic or control and their respective controls was injected intradermally around the wound edges on the day of wounding and again 6 days after wounding. In experiments using DMOG, 100μL DMOG (2mM) or vehicle were applied locally every other day. The mice were housed individually and received intraperitoneal buprenorphine (0.03 mg/kg) twice daily for 2 days post-wounding in order to reduce physical distress from the procedure. Wounds were photographed every 48h until 95% wound closure was observed. A circular reference was used to correct for the distance between the wound and the camera. The wound area size was expressed as percentage of the original wound area using and calculated using Image J v1.47 (NIH). Wounds were harvested at sacrifice 8 days post-wounding for further analysis.

3.2.1.4 Wounding Procedure

After measuring blood glucose and HbA1c, mice were anesthetized using 3% isoflurane. Fur on the dorsal surface was removed using an electric shaver and depilatory cream. After disinfection with 70% ethanol, two full-thickness wounds were made extending through the panniculus carnosus on either side of the dorsal midline, using a 6mm circular biopsy punch.

3.2.2 Paper IV – Animal model for diabetic nephropathy

Diabetic male BKS-Leprdb/db/JOrlRj db/db mice (16-20 weeks old) and their healthy controls were from Janvier Labs. Db/db mice with HbA1c >55 mmol/mol, or blood glucose >15mM when HbA1c levels were between 45-55 mmol/mol, were included. The mice were randomized into groups according to their HbA1c levels. The mice were injected intraperitoneally with 320 mg/Kg body weight DMOG 5 and 1 days before sacrifice for the analysis of mitochondrial function. For other analyses, db/db mice were injected with 50 mg / Kg body weight DMOG every second day for one month before sacrifice. PHD2-haploinsufficient (PHD2+/-) mice and their wild-type (WT) littermates were generated as previously described (264). DM was induced in male PHD2+/- and WT mice (12-16 weeks old) with streptozotocin (STZ) i.p. injection at 50 mg/kg body weight daily for five consecutive days and the mice were considered diabetic when their blood glucose levels were above 15 mM. The PHD2+/- and WT mice had been diabetic for 6 weeks before sacrifice for analysis.

3.3 CELL CULTURES AND IN VITRO STUDIES

3.3.1 Cell lines and cell cultures

3.3.1.1 Paper II – Cell Cultures – HDF, HepG2, HDMVEC

HepG2 and human dermal fibroblast (HDF) cells (ATCC, Manassas VA, USA) were grown, unless otherwise stated, in Dulbecco's modified Eagle's medium (DMEM) (ThermoFisher Scientific, Waltham MA, USA) containing 5 mM glucose. HepG2 cells were cultured in RPMI medium containing 11 mM glucose. Media for both cell types were supplemented with 10% fetal bovine serum (FBS), penicillin (100IU/mL), and streptomycin (100mg/mL). Human dermal microvascular endothelial cells (HDMVEC) were cultured in endothelial growth media-2 microvascular (EGM-2MV) (both Lonza, Walkersville MD, USA).

3.3.1.2 Paper III – Cell Cultures – HDF and miR-210 mimic transfection

Primary HDF cells (ATCC) were cultured in DMEM with 5.5mM glucose supplemented with penicillin, streptomycin, and 10% FBS as per section 3.3.1.1. Cells were maintained in a humidified atmosphere with 5% CO_2 at 37 °C. Passages 4 to 9 were used for experiments. Cells were cultured under normoxic (21% O_2) or hypoxic (1% O_2) conditions in a hypoxia workstation (INVIVO2, Baker, Sanford ME, USA). HDFs were transfected with miR-210 mimic or negative control mimic using Lipofectamine RNAiMAX (ThermoFisher Scientific). Cells were harvested 24-48 h after transfection.

3.3.1.3 Paper IV – Cell Cultures – IMCD-3

Mouse Inner Medullary Collecting Duct-3 (IMCD3) cells (ATCC® CRL2123™, ATCC, USA) were cultured in DMEM with 5.5 mM glucose supplemented with penicillin, streptomycin, and 10% FBS as per section 3.3.1.1. The cells were maintained in a humidified atmosphere with 5% CO_2 at 37 °C. Cells were cultured under normoxia and hypoxia as per section 3.3.1.2.

3.3.2 *In vitro* wound healing experiments

3.3.2.1 Paper II – HDMVEC Endothelial Tube Formation Assay

HDMVEC were plated on ECMatrixTM ECM625 (MilliporeSigma, St Louis MO, USA) according to published protocols (265). The cells were grown in endothelial cell growth media, 150µL/well in 96-well microplates. 10,000 cells/well mixed with media containing 10 nmol MeT3α dissolved in ethanol or ethanol alone were seeded onto the surface of polymerized ECMatrixTM. After 6h incubation at 37 °C, tube formation was observed by inverted light microscopy at 40x–200x. For semi-quantitative analysis, a numerical value which is associated with degree of angiogenesis progression was assigned to each pattern. 3 randomly selected viewing-fields per well were examined and the values averaged.

3.3.2.2 Paper II – HDF In vitro Wound Healing Assay

HDF migration was studied using a standard "scratch" assay with minor modifications (266). Plates were precoated with 50 µg/mL collagen I, washed twice with PBS and blocked by 3% bovine serum albumin (BSA) for 1h. Wells were washed with PBS x2 and dried. Cells were then seeded and left to reach confluence. Prior to assay, cells were serum starved overnight. Scratches were made the following day using micropipette tips. Floating cells and debris were removed by PBS washing. Cells were incubated in media containing 0.2% BSA with 20 nmol MeT3α or ethanol. Mitomycin C 10 µg/mL was added to the media to inhibit cell proliferation in selected conditions. Scratches were photographed with an inverted phase microscope before and after 6 and 36 h treatment and cell migration was quantified with ImageJ v1.47 (NIH).

3.3.2.3 Paper III – HDF In vitro Wound Healing Assay

HDF migration was studied using the basic setup of a scratch assay as laid out above. Cells were transfected with miR-210 mimic or negative control mimic as described in section 3.3.1.2. Scratches were made 24h after transfection. The cells were rinsed and treated with normal (5.5 mM) or high glucose (30 mM) medium supplemented with 0.2% FBS and Mitomycin C 10 µg/mL. Plates were placed in normoxia or hypoxia. Digital pictures were obtained immediately after scratching and after 16 h using EVOS XL Core Cell Imaging System (ThermoFisher Scientific). Images were analyzed using ImageJ v1.47 software (NIH). The experiment was done in triplicates and 3 images from each replicate were used for analysis.

3.4 LABORATORY METHODS

3.4.1 Histology and fluorescent immunohistochemistry

3.4.1.1 Histology

Histology experiments were performed on formalin-fixed, paraffin-embedded 5 μm thick sections. Prepared slides were stained with Hematoxylin and Eosin per standard protocols after deparaffinization and rehydration. The granulation area of the wound was then quantified. Image analysis and quantification were performed using smart segmentation features on Image Pro-Premier v9.2 (Media Cybernetics, Rockville, MD, USA). A minimum of two images from each slide were manually analyzed, with 4-6 slides prepared for each condition. Granulation of the wound area was measured as the ratio of the number of cells to the total area of the granulation layer in a given image.

3.4.1.2 Fluorescent Immunohistochemistry

Formalin-fixed, paraffin-embedded tissues (FFPE) were deparaffinized and rehydrated. Antigen retrieval was performed by microwave at 800W for 10min using a standard citrate buffer. Slides were subsequently washed with Phosphate-buffered Saline (PBS)-T containing 0.1% tween 3x3 min. Sections were blocked with goat serum or 5% BSA in PBS for 30 minutes, room temperature (RT), and incubated with primary antibodies (overnight at 4℃). Sections were incubated with fluorochrome-conjugated secondary antibodies for 1 hour at RT in darkness. After another PBS-T 3x3min washing procedure slides were treated for 10 minutes with 0.1% Sudan Black-B solution (MilliporeSigma) to quench autofluorescence. the sections were incubated with 1:5000 diluted 4,6-Diamidine-2-phenylindole dihydrochloride (DAPI) (ThermoFisher Scientific) in PBS followed by washing in PBS-T. Mounted sections were stored at 4℃. Fluorescent images were acquired using a Leica TCS SP5 and SP8 model confocal microscopes (Leica Microsystems, Wetzlar, Germany). Image analysis was performed using Image-Pro Premier v9.2.

3.4.1.3 HIF Detection

To detect HIF-1α, Tyramide Superboost kit (Thermofisher Scientific) was used to enhance fluorescent signals. Following antigen retrieval, the sections were briefly blocked with 3% H_2O_2 before blocking with goat serum. Sections were incubated with primary antibodies and washed with PBS-T 3x3 min followed by incubation with an HRP-conjugated rabbit antibody for 1 hour at RT. The sections were then washed and incubated with tyramide reagent for 10 minutes at RT in dark. The reaction was stopped through incubation in stop solution for 5 minu followed by PBS-T washing 3x3 min. The sections were then treated with 0.1% Sudan Black-B solution, followed by counterstaining with DAPI (1:5000) for 5 min. Finally, the sections were washed with PBS-T and mounted.

3.4.1.4 TUNEL Staining

Apoptosis in kidneys were detected using In Situ Cell Death Detection Kit (Roche Diagnostics, Basel, Switzerland) Briefly, FFPE sections were deparaffinized and rehydrated and blocked with 3% H_2O_2 to quench endogenous peroxidase activity. The sections were permeabilized with 0.1% Triton X-100, 0.1% sodium citrate solution and blocked with 3% BSA in PBS. The sections were then incubated with the TUNEL mixture for 1 hour at 37°C. The sectioned were rinsed in PBS, treated with 0.1% Sudan Black-B solution to quench autofluorescence and counterstained with DAPI. The sections were mounted and stored in 4°C. Image acquisition and analysis were performed as in section 3.3.3.2.

3.4.2 *In situ* hybridization

For the detection of miR-210 in FFPE sections, Exiqon miRCURY locked nucleic acid (LNA)-digoxigenin (DIG) labelled probe was used as previously described with minor modifications (126). Briefly, FFPE slides 5µm thick were deparaffinized and RNAs were demasked using 15 µg/mL proteinase K treatment for 10 min at 37°C. Probes were hybridized with Mmu-miR-210-3p specific LNA-probes (Exiqon, Vedbaek, Denmark) at a concentration of 250nM for 2h at 53°C. A sequence of washes were carried out at 53°C with decreasing concentrations of Saline-Sodium Citrate buffer (SSC); once in 5X SSC, twice in 1X SSC and twice in 0.2X SSC for 5 min each followed by a final wash with 0.2X SSC at RT. The sections were then blocked and incubated with an alkaline phosphatase-conjugated antibody specific to DIG at 1:1000 (Roche) for 1h at RT. miR-210 signals were visualized by addition of an AP substrate, NBT/BCIP (ThermoFisher Scientific) and the slides were counterstained with nuclear fast red stain (Vectorlabs, Burlingame, CA, USA).

3.4.3 Masson-Goldner trichrome staining

FFPE sections of harvested wound samples were deparaffinized with 2 passes in Xylene for 3 min each and rehydrated in sequential passes of 100% and 95% Ethanol for 3 min x2 times each. Slides were treated according to manufacturer's instructions to obtain Masson-Goldner Trichrome staining (MilliporeSigma). Images were obtained using aLeica DM3000 LED fluorescence microscope (Leica Microsystems) using the transmitted light. Collagen stained areas in the wounds were analyzed and quantified using the Smart Segmentation feature on Image Pro-Premier software v9.2 (Media Cybernetics). A minimum of 2-4 images from 3-4 tissues from each condition were analyzed. Collagen staining was expressed as the percentage of area stained by collagen (green).

3.4.4 Protein expression assays

3.4.4.1 Paper II – Western Blotting of LSS, PDGFB and KIF26A

HepG2 and HDF cells were incubated with MeT3α or vehicle for 3h, washed twice with PBS and harvested in pellet form. Cells were lysed in 100µL of RIPA buffer followed by centrifugation at 20,000 rpm for 20min at 4 °C. Protein concentration was determined by Bradford assay using BSA as a standard. 50µg of protein was loaded on each well and separated

on 12% SDS-PAGE gel electrophoresis and transferred to a nitrocellulose membrane (Hybond-C, Amersham Biosciences, Uppsala, Sweden) and blocked overnight with 5% non-fat milk in 0.1% Tween PBS at 4 °C. Primary antibodies used were mouse ant-KIF26A, rabbit anti-LSS (1:500, MilliporeSigma), rabbit anti-PDGFB (1:1000, Abcam) and rabbit anti-β-actin (1:3000, Neomarkers, Fremont CA, USA). The membranes were incubated with horseradish peroxidase conjugated anti-rabbit IgG secondary antibody and detected using enhanced chemiluminescence (GE Healthcare, Uppsala, Sweden).

3.4.4.2 Paper IV – Protein Extraction and Western Blotting

Cell pellets and kidney biopsies were homogenized and centrifuged. Protein concentrations were determined using Bradford Protein Assay (BioRad, Hercules CA, USA). The extracts were separated by SDS-PAGE and blotted. After blocking, blots were incubated with anti-KIM-1 (Novus Biologicals, Centennial CO, USA) and anti-α-tubulin antibodies (Abnova, Walnut CA, USA). After several washes, the membranes were incubated with IRDye 800 goat anti-rabbit or IRDye 680 goat anti-mouse secondary antibodies and scanned using an Odyssey Clx Imaging System (LI-COR, Lincoln NE, USA).

3.4.5 Gene expression and miRNA assays

3.4.5.1 Paper II – Microarray and qPCR

Affymetrix U133 plus 2.0 GeneChips Human array (ThermoFisher Scientific) was used for gene expression analysis. Samples were analyzed according to manufacturer's instructions. RNA was isolated from HepG2 cells treated with 10nmol MeT3α for 2h and controls using a QIAGEN Mini RNeasy plus minikit (Qiagen, Hilden, Germany). RNA integrity was controlled with Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara CA, USA). Complementary DNA (cDNA) was used for isolation of biotin-labeled complementary RNA (cRNA), which was then fragmented to a mean length of ~50–100 nucleotides. Triplicate samples were used for each experimental condition and the raw data files were analyzed by GeneSpring v7.2 (Agilent Technologies). Functional pathway analysis was per- formed using ingenuity pathway analysis (NIH). The LSS gene was used as a reference. For qPCR, 0.1–0.5μg of total RNA was reverse-transcribed using random hexamer oligos (Applied Biosystems, Foster City CA, USA). cDNA was mixed with SYBR Green specific primers. PCR analysis was performed with ABI Prism 7300 sequence detector (Applied Biosystems).

3.4.5.2 Paper III – RNA Purification and qRT-PCR

Total RNA including microRNAs were extracted from cells and tissues using a miRNeasy RNA extraction kit (Qiagen). To detect mRNA expression, High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific) was used. q RT-PCR was performed on a 7300 or QuantStudio 6 and 7 (Applied Biosystems) using SYBR Green Master Mix or Taqman Gene Expression Assays (ThermoFisher Scientific). The internal controls for mRNA expression were PBGD and Actin.

3.4.5.3 miRNA Detection

TaqMan microRNA Reverse Transcription kit and TaqMan miRNA assays (ThermoFisher Scientific) were used for the analysis of miR-210, U6 snRNA and snoRNA55 expression. U6 was used as internal control for HDF and keratinocytes, and snoRNA55 was used as control for mouse tissues. To detect miR-210 expression in HDMEC and human wounds, cDNA was produced using TaqMan Advanced miRNA cDNA Synthesis Kit and microRNA expression was detected using TaqMan Advanced miRNA assays (both ThermoFisher Scientific), where miR-103a or the average of miR-16, miR-23a, and miR-24 were used as internal controls.

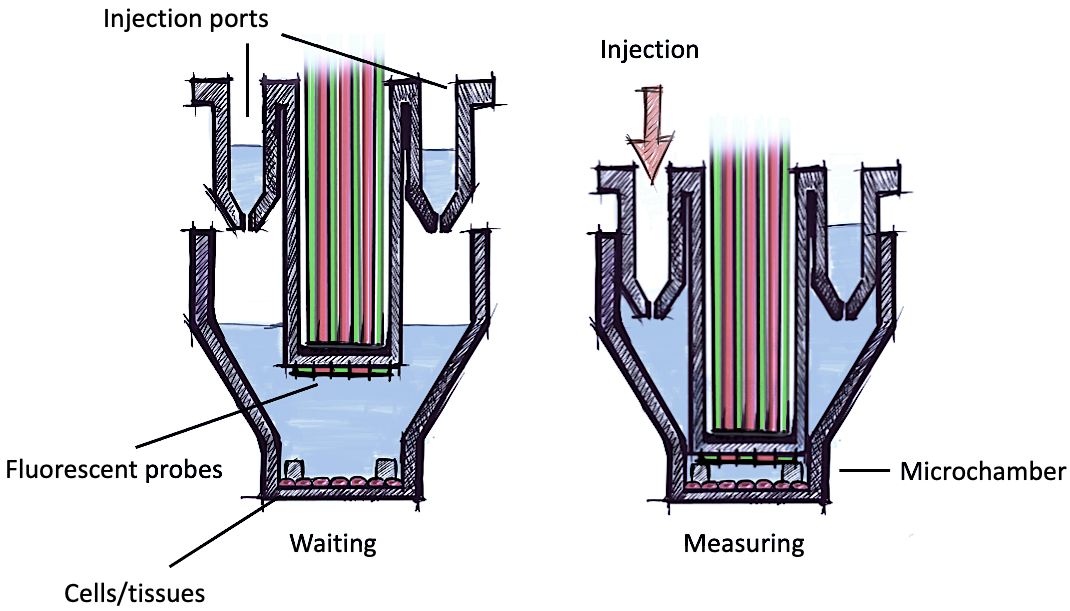
3.4.6 Cellular respirometry

3.4.6.1 Seahorse Respirometry - General Principles

Seahorse cell analysis is a high-throughput method for measuring cellular bioenergetics in intact cells, tissue samples and isolated mitochondria (267). All machines and reagents used for Seahorse cell respirometry experiments were obtained from Agilent Technologies unless otherwise stated. In our experiments the XF24-3 and XF96e machine models were used. Cell and tissue plates used in our experiments include XF24 PS-7, XF24 Islet Capture and XF96e microplates. Plate/cartridge combinations enable cycles of measurement wherein a small (3-7μL) transient microchamber is formed. Fluorescent probes on the cartridge will record oxygen and proton gradients, enabling measurement of oxygen consumption rate (OR) and extracellular acidification rate (ECAR), a proxy for glycolysis rate. Fig.12 shows the basic layout of plate wells in ambient and measurement modes, respectively. Other inferred measurements achieved by measuring OCR and ECAR before and after injection of different mitochondrial inhibitors, substrates and treatments yields values for ATP production, proton leak, mitochondrial reserve capacity and non-mitochondrial respiration. Standard protocols frequently involve injectables such as oligomycin, an inhibitor of ATP synthase, Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), a mitochondrial uncoupler, Rotenone, an inhibitor of complex I, antimycin, an inhibitor of complex III and 2-deoxyglucose, (2DG) an inhibitor of glycolysis. Fig.12 shows standard OCR and ECAR readouts from a Seahorse respirometric experiment.

3.4.6.2 Paper II – Seahorse Respirometry in HepG2 and HDF cells

Basal OCR, ECAR, and ATP production rate were measured using XF Cell Mito Stress Test kit. HepG2 and HDF cells treated with either vehicle,10μM CoQ, or 10μM MeT3α once every 48h for 14 days prior to analysis. Cells were seeded in 24-well Seahorse XF24 plates at a density of 30,000 (HepG2) or 40,000 (HDF) cells per well in standard growth medium and incubated at 37 °C overnight before running the assay according to optimized protocols.



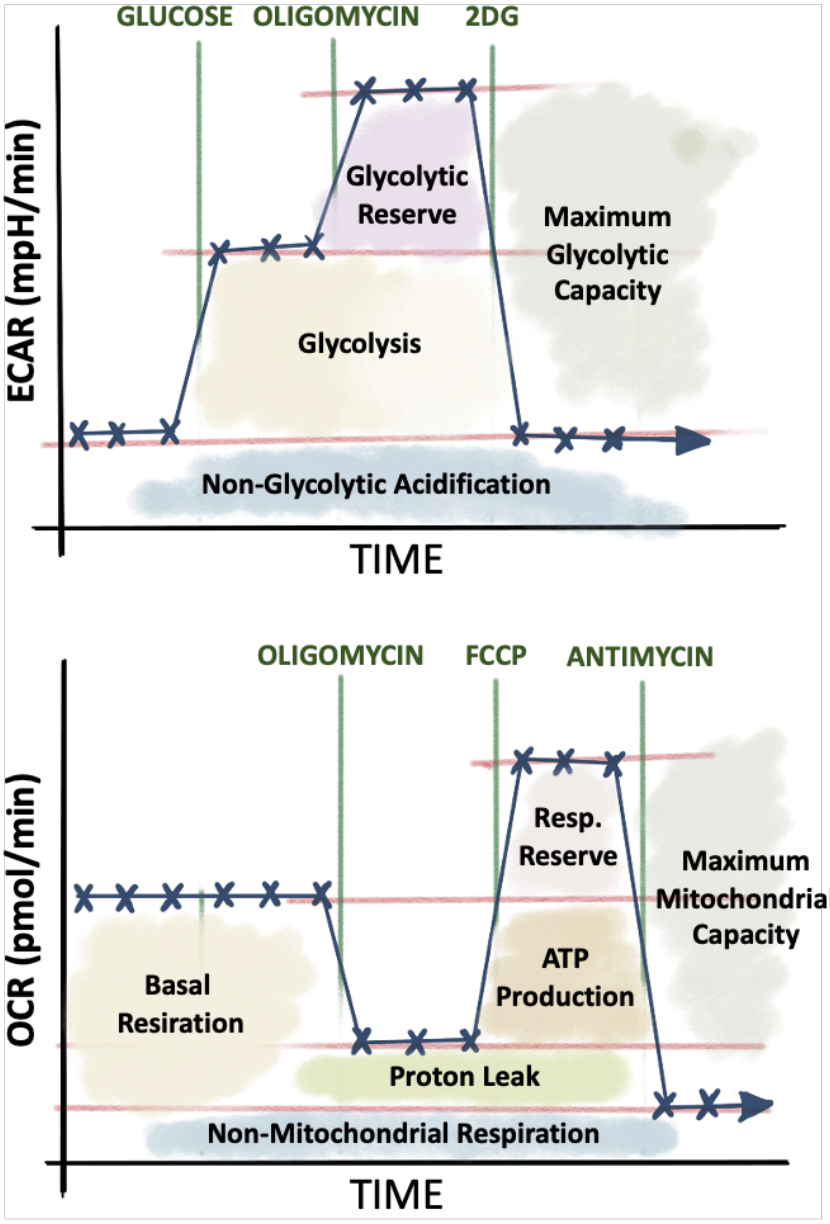


Fig 12. Seahorse cell/tissue plate structure and assay readout.

3.4.6.3 Paper III – Seahorse Respirometry in Tissue and Cells

Basal oxygen consumption rate (OCR), extracellular acidification rate (ECAR), and ATP production rate were measured using XF Cell Mito Stress Test kit, XF Glycolytic Rate Assay kit, and XF Real-Time ATP Rate Assay kit on Seahorse XF Analyzer. The sensor cartridges used for measuring the oxygen flux was equilibrated in an XF Calibrant for 16-24h before the experiment in a 0%-CO_2 37°C incubator. The granulation tissue from the wounds taken from mice after 8 days of wounding was carefully dissected and rinsed with unbuffered Krebs-Henseleit buffer (KHB) media. The tissue was placed at the bottom of the XF24 Islet Capture Microplate and covered with a mesh. 450µL KHB medium was added to each well containing the tissue and equilibrated in a 0%-CO_2 incubator for 30 min prior to running the assay according to optimized protocols. For analysis in cells, HDFs were transfected with negative control mimic or miR-210 mimic and treated with normal (5.5mM) or high (30mM) glucose levels in normoxia or hypoxia. The cells from each condition were then seeded onto an XF24 or XFe96 Cell Culture Microplate. Results were normalized to protein concentration or cell number as indicated.

3.4.6.4 Paper IV – Kidney Mitochondrial Function High Resolution Respirometry

Mitochondria were isolated from mouse kidneys and mitochondrial function was determined using high resolution respirometry (Oxygraph 2k, Oroboros) as described previously (268).

3.4.7 EPR spectrometry

3.4.7.1 General Principles

Direct detection of ROS is very difficult at RT or physiological conditions because of very short half-lives of most common ROS (O_2•- and H_2O_2). Electron spin or electron paramagnetic resonance spectrometry (EPR) is a powerful method for detecting molecules with unpaired electrons. By using a designed spin probe, such as the 1-hydroxy-3-methoxycarbonyl-tetramethylpyrrolidine (CMH) and 1-Hydroxy-3-carboxy- 2,2,5,5-tetramethylpyrrolidine (CPH), with a very high affinity for binding O_2•- and other mtROS, it is possible to obtain stable spin probe radicals which have longer half-lives and whose concentrations are proportional to those of O_2•-. Living samples from cells, tissues, supernatant or other sources, when controlled for sample mass, temperature, shear stress, surface area and incubation time, can yield accurate data on ROS production in real time (200). Calibration curves and positive controls can be made using known quantities of 3-carboxy-proxyl (CP) radical. Obtained samples can be analyzed directly or preserved in liquid nitrogen for later analysis. EPR-grade Krebs HEPES buffer supplemented with iron chelator Deferoxamine (DFX) and copper chelator diethyldithiocarbamate (DETC) were used in order to avoid radical formation by metal ions. Unless otherwise stated, all reagents were obtained the same supplier (Noxygen, Elzach, Germany). The EPR machine used for experiments listed below was a Bruker E-Scan M system (Bruker, Billerica MA, USA). EPR spectrum readouts yield an amplitude which are then correlated to a CP radical calibration curve.

3.4.7.2 EPR Spectrometry in Cultured Cells

ROS levels in HDF cells were measured using a CMH spin probe (200 mM) and a CP radical standard curve, using EPR spectrometer (Noxygen). HDF cells were transfected with miR-210 mimic or control mimic and treated with normal or high glucose and hypoxia or normoxia 16h after transfection. Following 24h of treatment, media was removed, and the cells were washed twice with PBS. 700µL CMH buffer (200 mM) was added to the cells and were incubated for 30 min. Cells were collected in CMH buffer and frozen in liquid nitrogen prior to measurement.

3.4.7.3 EPR Spectrometry in Blood

Peripheral blood samples were mixed with CPH spin probe (200 mM) and incubated at 37°C for 30 min before being frozen in liquid nitrogen prior to measurement.

3.4.8 Other laboratory methods and chemicals

3.4.8.1 Paper I

CoQ and vitamin E were measured in human serum samples using high-performance liquid chromatography (HPLC) according to previously published protocols (219). Levels of high-sensitive C-reactive protein (hsCRP) was measured using an immunonephelometric kit (Siemens Dade Behring, Marburg, Germany). Glycated hemoglobin (HbA1c) was measured using the Unimate system (Roche Diagnostics). Oxidized low-density lipoprotein (LDL) cholesterol (oxLDL) was measured using a commercial ELISA kit (Mercodia, Uppsala, Sweden). Oxidative stress markers in blood was measured using a colorimetric free oxygen radicals test (FORT) assay (269). Urinary albumin (UAlb), s-Creatinine (Crea), s-Cystatin C (CysC) and plasma lipid profiles including total cholesterol, LDL and high-density lipoprotein cholesterol (HDL) were assayed using standard hospital laboratory procedures. Glutaredoxin (GRX) activity in serum was determined using a fluorescent grx1 assay (IMCO Corporation, Stockholm, Sweden) as previously described (208).

3.4.8.2 Paper III - Chemicals

Custom stabilized miRIDIAN mmu-microRNA-210-3p mimic (C-310570-5) and custom stabilized miRIDIAN negative control mimic #1 (CN-001000-01) (Dharmacon Lafayette CO, USA) were used for miR-210 mimic experiments. MaxSuppressorTM In Vivo RNA-LANCEr II (BIOO Scientific, Austin TX, USA) and RNAlaterTM Stabilization (ThermoFisher Scientific) 10% neutral buffered Formalin solution (MilliporeSigma), Mayers Hemotoxylin and 0.2% Eosin from (Histolab, Stockholm Sweden), Mmu-miR-210-3p specific LNA-probes (Exiqon/Qiagen) 2-deoxy-D- Glucose, Syrosingopine and sodium oxamate (MilliporeSigma) were used as required by experiments performed for Paper III.

3.4.8.3 Paper III and IV – 4HNE and Lactate Assays

4HNE was measured in frozen wound lysates using commercially available kits OxiSelect STA-838 and STA-310 (Cell Biolabs, San Diego CA, USA) according to the manufacturer's instructions. Lactate production was measured using the Lactate Colorimetric Assay kit

(Biovision, Milpitas CA, USA) according to the manufacturer's instructions. Wound tissues were homogenized in lactate assay buffer provided in the kit and lactate levels were measured and normalized to the protein levels.

3.4.8.4 Paper IV – ROS Detection with Flow Cytometry

mIMCD3 cells, after exposure to experimental conditions, were stained with a MitoSOX™ Red Mitochondrial Superoxide Indicator (ThermoFisher Scientific) at a 5μM working concentration at 37 °C for 10 min. After washing. cells were suspended in KHB and analyzed using flow cytometry on a CyanTM ADP analyzer (Beckman Coulter, Brea CA, USA). Analysis was performed using FlowJo software (FlowJo, Ashland OR, USA). ROS levels were expressed as percentage of MitoSOX Red fluorescence intensity.

3.4.8.5 Paper IV – Plasmid Transfection

Plasmids encoding HRE-driven luciferase reporter, Renilla luciferase, GFP, and GFP-HIF-1α were described previously (270). Plasmid transfections were performed using Lipofectamine reagent (ThermoFisher Scientific) according to the manufacturer's protocol.

3.4.8.6 Paper IV – HRE-driven Luciferase Reporter Assay

mIMCD3 cells were transfected with plasmids encoding HRE-driven firefly luciferase and Renilla luciferase using Lipofectamine reagent (ThermoFisher Scientific). The cells were then cultured in normal (5.5 mM) or high (30 mM) glucose and exposed to normoxia or hypoxia for 40 hours. The cells were harvested and luciferase activities were measured using Dual luciferase assay system in a GloMax luminometer (Both Promega Biotech, Madison WI, USA) per manufacturer's instructions. The HRE-driven firefly luciferase activity was normalized to Renilla luciferase activity and expressed as relative luciferase activity.

3.4.8.7 Paper IV – RNA Interference

siRNA for mouse VHL (Flexitube Gene Solution GS22346, Qiagen) and AllStars negative control siRNA (Ambion, Austin TX; USA) were transfected using Lipofectamine RNAiMAX Transfection Reagent (ThermoFisher Scientific) according to the manufacturer's protocol. 24 hours post-transfection, the cells were exposed to 5.5 mM or 30 mM glucose in normoxia or hypoxia for 24 h before harvest.

3.5 STATISTICAL ANALYSIS

3.5.1.1 Paper I

Data were analyzed using Statistica (Dell, Round Rock, TX, USA). Parameters are expressed as mean±SD unless otherwise stated. Parameters with non-normal distributions were transformed, and log-normalized values were used for significance testing. Mann– Whitney U testing was used when log-normalization was not possible. Levels of significance were tested with Fisher's exact two-tailed test for simple frequency and Pearson chi-squared test for multiple frequencies. One-way ANOVA was used for parametric variables in two groups or more. A p-value <0.05 was considered significant.

3.5.1.2 Paper II

Data from animal and cell experiments were analyzed in GraphPad Prism v6 (GraphPad Software, San Diego CA, USA). Analyses used included one-way ANOVA followed by Tukey's multiple comparison test. A P-value of $p<0.05$ was considered significant.

3.5.1.3 Paper III

Statistical analysis and graphing were performed using GraphPad Prism v6 (GraphPad Software). Outliers were identified using Grubbs' test. Normality of distribution was analyzed using Kolmogorov-Smirnov test. Differences between two groups were analyzed using two-sided Student's t-test for data with normal distribution, and nonparametric test was used for data that was not normally distributed. Multiple comparisons of three or more groups were performed using one-way ANOVA or two-way ANOVA followed by Bonferroni's post hoc test. A p-value of $p<0.05$ was considered statistically significant. All the in vitro experiments were performed at least 3 times independently. Data is presented as mean ± standard error of the mean (SEM).

3.5.1.4 Paper IV

Statistical analysis were performed using GraphPad Prism v6 (GraphPad). Outliers were identified using Grubbs' test. Differences between two groups were analyzed using two-sided Student's t-test. Multiple comparisons of three or more groups were performed using one-way ANOVA followed by Bonferroni's post hoc test or Holm Sidak's test. $P<0.05$ was considered statistically significant. All the in vitro experiments were performed at least 3 times independently. Data is presented as mean ± SEM.

3.6 ETHICAL CONSIDERATIONS

All human and animal studies included in the present body of work was approved after ethical review by requisite and relevant ethical review boards and government agencies. All participants in human studies provided written consent after receiving relevant information in print regarding the studies in which they participated. All human studies were conducted according to rules, regulations and guidelines as mandated by Karolinska Institutet, grant organizations, relevant government agencies and the World Medical Association declaration of Helsinki (271). Human samples were handled according to local and national biobank regulations. All animal experiments were conducted in line with existing rules, regulations and ethical guidelines as mandated by Karolinska Institutet, the Swedish 3R-council, and the Swedish Board of Agriculture (272).

4 RESULTS AND DISCUSSION

4.1 PAPER I

4.1.1 Clinical characteristics

4.1.1.1 Clinical Characteristics, Study Population

There were no sex differences regarding age, disease duration, fasting plasma glucose (fpG), glycated hemoglobin (HbA1c) or body mass index (BMI). Women had a higher waist index and hsCRP, total, LDL and HDL cholesterol, and FORT levels. Men had higher UAlb, S-Crea and CoQ10/chol ratio. Fisher's exact test revealed no gender difference in major complications.

Table 3. (Paper I – Table 1). Clinical Characteristics of Study Participants with T2DM.

		Men n=95		Women n=61		
		Mean	SD	Mean	SD	p
Age	year	61.6	7.3	61.9	7.1	NS
Diabetes duration	year	6.4	5.3	8.0	6.2	0.1
fP-glucose	mmol/L	8.8	3.1	9.0	2.7	NS
HbA1c	%	6.3	1.3	6.6	1.3	0.1
HbA1c	mmol/mol	45	14.2	49	14.2	0.1
BMI	kg/m2	29.0	4.9	29.6	4.7	NS
Waist index		1.10	0.13	1.20	0.14	< 0.001
BP systolic	mmHg	147	19	150	20	NS
BP diastolic	mmHg	84	10	80	9	0.04
Pulse pressure	mmHg	63	15	69	20	0.05
VPT	V	23.0	10.1	19.0	8.0	0.01
Z score VPT	SD	1.32	1.18	1.24	0.18	NS
hsCRP	mg/L	2.37	3.20	3.58	3.48	< 0.01
Cholesterol	mmol/L	4.75	1.10	5.29	0.97	< 0.01
LDLc	mmol/L	2.91	0.88	3.31	0.86	< 0.01
HDLc	mmol/L	1.15	0.35	1.32	0.39	< 0.01
TG	mmol/L	1.77	1.39	1.75	0.98	NS
Ualb	mg/L	82	246	69	354	< 0.01
Creatinine	µmol/L	85	23	67	12	< 0.001
Cystatin C	mg/L	0.79	0.36	0.74	0.34	NS
FORT	U/L	267	57	326	70	< 0.001
CoQ10	nmol/mL	1.88	0.86	1.73	0.49	NS
CoQ10/cholesterol		0.46	0.13	0.39	0.10	0.01
Vitamin E	µg/mL	12.78	4.47	13.54	4.08	NS
oxLDLc	U/L	57.51	27.07	67.80	37.16	0.1

p *refers to ANOVA one-way and Mann–Whitney U-test 2*1 sided.*

4.1.1.2 Clinical Characteristics, CVD vs. no CVD

The prevalence of CVD in our study population was 59% and 66% for men and women, respectively (p = 0.5). Comparisons of characteristics for subjects with (n = 96) and without (n = 60) CVD are shown in Table 4 below. Those with CVD were older, had longer disease durations, higher Ualb, and higher VPT. Women with CVD had higher oxLDLc compared to those without CVD. In the group with CVD, the women had a higher waist index (p = 0.003), higher values hsCRP (p = 0.03), total cholesterol (p < 0.01) LDLc (p < 0.01) and (p < 0.01), FORT (p < 0.001) and oxLDL (p = 0.01).

Table 4. (Paper I – Table 2). Clinical Characteristics, T2DM patients with CVD vs. no CVD.

		No CVD n=60		CVD n=96			Men[a]	Women[a]
		Mean	SD	Mean	SD	p	p	p
Age	year	60.5	8.5	62.5	6.2	0.1	NS	0.002
Diabetes duration	year	5.6	4.5	8.0	6.2	0.01	0.09	0.09
fP-glucose	mmol/L	8.6	2.8	9.0	3.0	NS	NS	NS
HbA1c	%	6.3	1.4	6.5	1.2	NS	NS	NS
HbA1c	mmol/mol	45	15.3	48	13.1	NS	NS	NS
BMI	kg/m2	28.7	5.0	29.6	4.7	NS	0.1	NS
Waist index		1.11	0.15	1.15	0.14	0.1	0.08	NS
BP systolic	mmHg	145	20	150	18	0.09	0.05	NS
BP diastolic	mmHg	81	9	83	10	NS	0.09	NS
VPT	V	19.5	8.1	22.6	10.1	0.08	NS	0.01
Z score VPT	SD	1.51	0.81	1.72	1.04	NS	NS	NS
hsCRP	mg/L	2.50	2.48	3.05	3.79	NS	NS	NS
Cholesterol	mmol/L	5.06	0.98	4.89	1.14	NS	NS	NS
LDLc	mmol/L	3.17	0.77	2.99	0.96	NS	0.09	NS
HDLc	mmol/L	1.23	0.37	1.21	0.38	NS	NS	NS
TG	mmol/L	1.68	1.02	1.81	1.36	NS	NS	NS
Ualb	mg/L	34	107	103	359	0.03	0.1	0.04
Creatinine	µmol/L	77	17	79	24	NS	NS	0.01
Cystatin C	mg/L	0.733	0.262	0.791	0.397	NS	NS	NS
FORT	U/L	284	62	293	72	NS	NS	NS
CoQ10	nmol/L	1.89	0.77	1.78	0.76	NS	NS	NS
CoQ10/cholesterol		0.44	0.13	0.43	0.12	NS	NS	NS
Vitamin E	µg/ml	13.70	3.83	12.56	4.65	0.07	0.03	NS
oxLDLc	U/L	57.46	30.57	63.97	32.20	NS	NS	0.02
Age	year	60.5	8.5	62.5	6.2	0.1	NS	0.002

p *refers to ANOVA one-way and Mann–Whitney U-test 2*1 sided.*

a *Refers to with vs. without CVD in men and women respectively.*

4.1.1.3 Clinical Characteristics, PSN vs. no PSN

PSN prevalence was 38% in men and 25% in women (p = 0.1). Comparisons of characteristics for subjects with (n = 48) and without (n = 96) PSN are shown in Table 5. Subjects with PSN had longer disease duration and had higher levels of hsCRP, UAlb and CysC. Women with PSN had higher waist indices (p = 0.005), FORT levels (p = 0.02) and oxLDLc (p = 0.04) compared to men with PSN.

Table 5. (Paper I – Tale 3). Clinical Characteristics, T2DM patients with PSN vs no PSN.

		No PSN n=96		PSN n=48			Men[a]	Women[a]
		Mean	SD	Mean	SD	p	p	p
Age	year	61.0	7.4	62.4	6.8	NS	NS	NS
Diabetes duration	year	5.9	4.8	9.0	6.4	0.002	0.002	NS
fP-glucose	mmol/L	8.4	2.7	9.5	3.3	0.08	0.1	NS
HbA1c	%	6.2	1.2	6.6	1.4	0.07	NS	0.09
HbA1c	mmol/mol	44	13.1	49	15.3	0.07	NS	0.09
BMI	kg/m^2	29.4	4.9	29.2	4.7	NS	NS	NS
Waist index		1.13	0.15	1.15	0.14	NS	NS	NS
BP systolic	mmHg	148	20	149	18	NS	NS	NS
BP diastolic	mmHg	83	8	83	11	NS	NS	NS
VPT	V	16.9	5.2	30.7	9.6	< 0.001	< 0.001	< 0.001
Z score VPT	SD	1.18	0.66	2.59	0.77	< 0.001	< 0.001	< 0.001
hsCRP	mg/L	2.46	2.38	3.71	4.84	0.02	0.006	NS
Cholesterol	mmol/L	4.97	1.06	4.98	1.15	NS	NS	NS
LDLc	mmol/L	3.05	0.91	3.08	0.83	NS	NS	NS
HDLc	mmol/L	1.27	0.39	1.13	0.33	0.05	NS	0.02
TG	mmol/L	1.73	1.11	1.92	1.58	NS	NS	NS
Ualb	mg/L	45	181	150	459	0.003	NS	0.003
Creatinine	µmol/L	76	16	82	30	NS	NS	NS
Cystatin C	mg/L	0.695	0.274	0.909	0.432	0.002	0.03	0.03
FORT	U/L	289	69	296	70	NS	NS	NS
CoQ10	nmol/mL	1.82	0.70	1.91	0.93	NS	NS	NS
CoQ10/cholesterol		0.44	0.12	0.44	0.14	NS	NS	NS
Vitamin E	µg/ml	12.90	4.11	13.38	5.21	NS	NS	NS
oxLDLc	U/L	59.80	28.21	71.15	37.35	0.1	NS	< 0.05
Age	year	61.0	7.4	62.4	6.8	NS	NS	NS

***p** refers to ANOVA one-way and Mann–Whitney U-test 2*1 sided.*

PSN defined as Z score > 2 SD and/or pathological filament.

***a** Refers to with vs. without CVD in men and women respectively.*

4.1.2 Markers of oxidative stress

Our overall study population had satisfactory glycemic control, and no correlation was seen between glycemic control and complication rates in our study population. There was however a correlation between HbA1c, fpGlu, hsCRP and BMI, and and signs of oxidative stress as measured by FORT and oxLDL. We found that FORT levels were correlated with hsCRP ($p = 0.0002$) and CVL ($p = 0.02$) and PSN as previously published (261). A positive correlation between FORT and LDLc was found in men ($p = 0.04$) but not in women. There was a strong overall correlation between cholesterol and oxLDL ($p < 0.001$). Increased oxLDL was independently associated with CVD ($p = 0.02$). Women with CVD ($p = 0.02$) and PSN ($p = 0.04$) had higher levels of oxLDL compared to those without CVD and PSN, respectively. The corresponding differences were not seen in men. The higher levels of hsCRP, lipid risk factors and FORT, and sex differences in antioxidant responses, may explain the observed discrepancy between men and women in our material (273, 274).

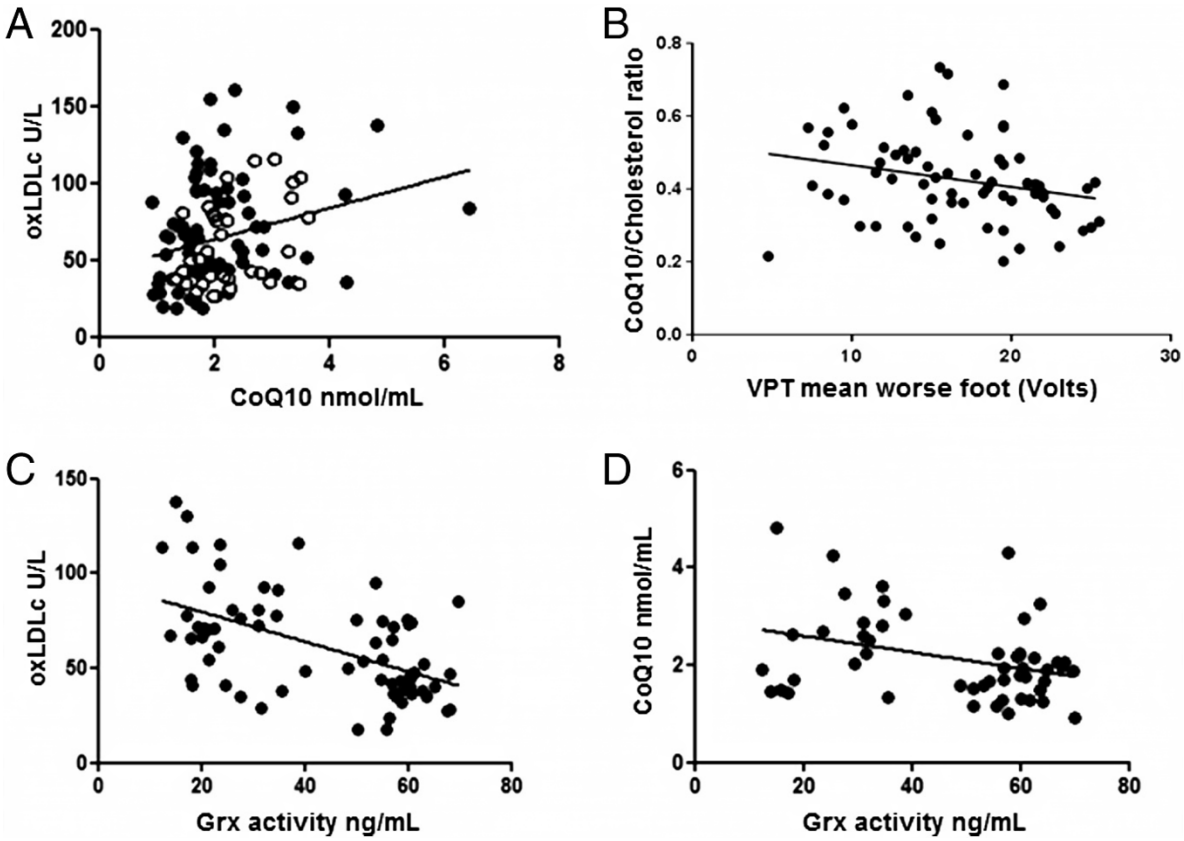


Fig. 13. (Paper I – Fig.2) Oxidative Stress and CoQ correlations. A: Correlation oxLDLc vs. CoQ10 (nmol/ml) in all subjects ($r = 0.34$, $p = 0.0005$), ●= subjects with statin treatment ($r = 0.383$, $p = 0.031$); ○= no statin treatment ($r = 0.253$, $p = 0.018$). B: The relationship between CoQ10/chol and PSN in women with T2DM ($r = -0.174$, $p = 0.046$). C: Correlation between Grx activity and oxLDLc ($r = -0.28$, $p < 0.001$). D: Correlation between Grx activity and CoQ10 levels ($r = -0.23$, $p < 0.01$).

4.1.3 Coenzyme Q10 is increased in response to oxidative stress

We found a significant correlation between CoQ and oxLDLc in the total population (Paper I – Fig.2A), as well as between as well as between cholesterol and CoQ10, between cholesterol and vitamin E, and between vitamin E and the ratio CoQ10/cholesterol. There was also correlation between BMI and the ratio CoQ10/chol ($r = 0.23$, $p = 0.02$) in the whole study population. In women there was a significant inverse correlation between VPT and CoQ10/chol ratio ($r = -0.174$, $p = 0.046$) (2B). Additionally, we found inverse correlations between oxLDL, CoQ and Grx activity (Fig 2C and 2D, respectively). Low Grx activity was found to be negatively correlated to higher oxLDLc, implying Grx activity being associated with an oxidative environment that prevails in the plasma in diabetics. There was a correlation between CoQ10 and Grx activity, where high CoQ is matched by lower Grx activity. The plasma levels of CoQ were significantly higher in our type 2 diabetes patients as compared to healthy controls. CoQ levels in DM have been seen to both be elevated and reduced, and represent a sum of both oxidized and reduced forms (251, 273). Higher levels of CoQ in blood are thought to represent a higher level of continuous oxidative stress, even though studies have shown benefits with CoQ supplementation(251, 275, 276). In men, we found increased levels of the ratio CoQ10/chol as a response to increased ROS production. In previous work, our lab showed that CoQ supplementation led to reduced Grx activity and total antioxidant capacity peripherally but increased intracellular Grx activity (208).

4.1.4 Discussion

The results of Paper I suggest that increased oxidative stress in T2DM as determined by FORT, CoQ10, and oxLDLc is correlated with the presumed causative factors hyperlipidemia, hyperglycemia and inflammation (236). This increased oxidative stress was in turn seen to correlate with PSN and CVD, having implications for both micro- and macrovascular complications of DM. CoQ was seen to be increased in T2DM. This could be due to both an artifact reflecting a shift in ubiquinone/ubiquinol ratio, or an indication that the endogenous increase of circulating CoQ in response to DM-associated oxidative stress is not enough to reduce harmful effects. This led to the need for further studies on ways to increase the amount of bioavailable and actively antioxidative CoQ, and how this affects disease progression in DM. This was the focus in the studies underpinning Paper II (201).

4.2 PAPER II

4.2.1 Mono-epoxy-tocotrienol-α enhances wound healing

4.2.1.1 MeT3α Promotes Fibroblast Cell Migration and Endothelial tube formation

MeT3α treated HDF cells show increased migration rates in starved HDF cells exposed to hyperglycemic conditions *in vitro* (Paper II – Fig.2). This effect was more pronounced when Mitomycin C was used to inhibit cell proliferation, demonstrating that MeT3α primarily exerts its effect in this scratch assay by stimulating cell migration rather than proliferation. MeT3α was also shown to increase the rate of endothelial tube formation in HMVEC (Paper II Fig.3). This suggests that MeT3α plays a role in reversing impaired fibroblast migration and revascularization in hyperglycemic conditions (201). It also implies a possible CoQ role in this process due to the known CoQ-stimulatory effect of MeT3α (216).

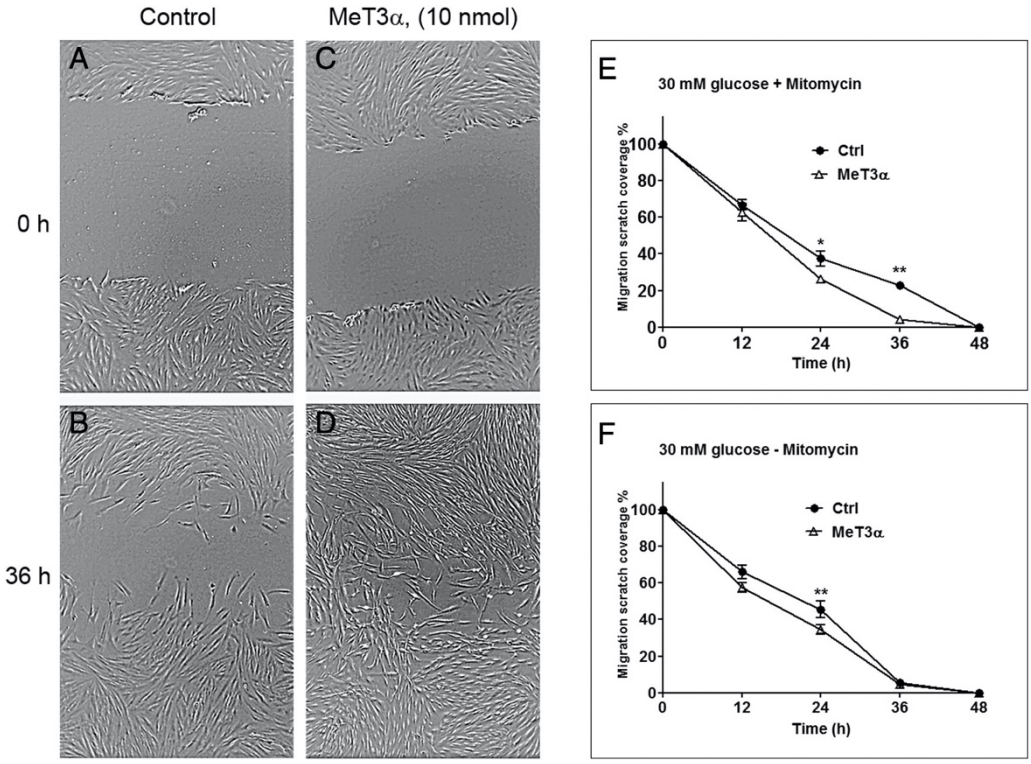


Fig. 14. (Paper II – Fig.2). MeT3α treatment effect on HDF migration. In vitro wound healing "scratch assay". Wound/scratch at the start 0h are seen for untreated (A) and MeT3α treated (C) conditions. Migration after 36h is depicted in (B) and (D), respectively. (E) compiled results of multiple experiments (n=5). (F) Compiled results of multiple experiments without Mitomycin C.

4.2.1.2 MeT3α modulates expressions of genes involved in wound healing

Microarray analysis of MeT3α in HeG2 cells showed an 11-fold decrease of LSS expression and a 2.5-fold decrease of akt1 and akt2 expression. LSS encodes oxidosqualene synthase, which regulates cholesterol biosynthesis (216). Akt1 and Akt2 have important functions in cell migration, proliferation, differentiation and cell cycle regulation (277). Other changes seen included IF26A with a 20-fold increase, 2-fold increases in TRIM54, TOR2A and COX7A, while that of MYH14 showed a 250-fold increase. KIF26A, MYH14 and TRIM54 are involved

in cell growth and motility. Ingenuity Pathway Analysis provided a graphical network representation of genes affected by MeT3α treatment (Paper II Fig. 4). Microarray data was validated by qPCR showing similar patterns, wherein LSS, AKT1, AKT2 and AGT were downregulated, while KIF26A, TRIM54, TOR2A, COX7A, SREBP1, PPARγ, and LPT were upregulated. This indicates an effect of MeT3α on carbohydrate and lipid metabolism. Further analyses also showed an increase in PDGFB and VEGFA gene (Paper II, Fig. 5) and protein (Paper II, Fig. 6) expression, thus showing an effect of MeT3α on angiogenesis.

4.2.1.3 MeT3α enhances in vivo wound healing

Topical MeT3α of wounds on db/db mice improved wound closure rate compared to untreated controls ($p<0.05$). The observed effect was not fully restorative compared to healthy mice, likely owing to the multifactorial nature of diabetic wounds.

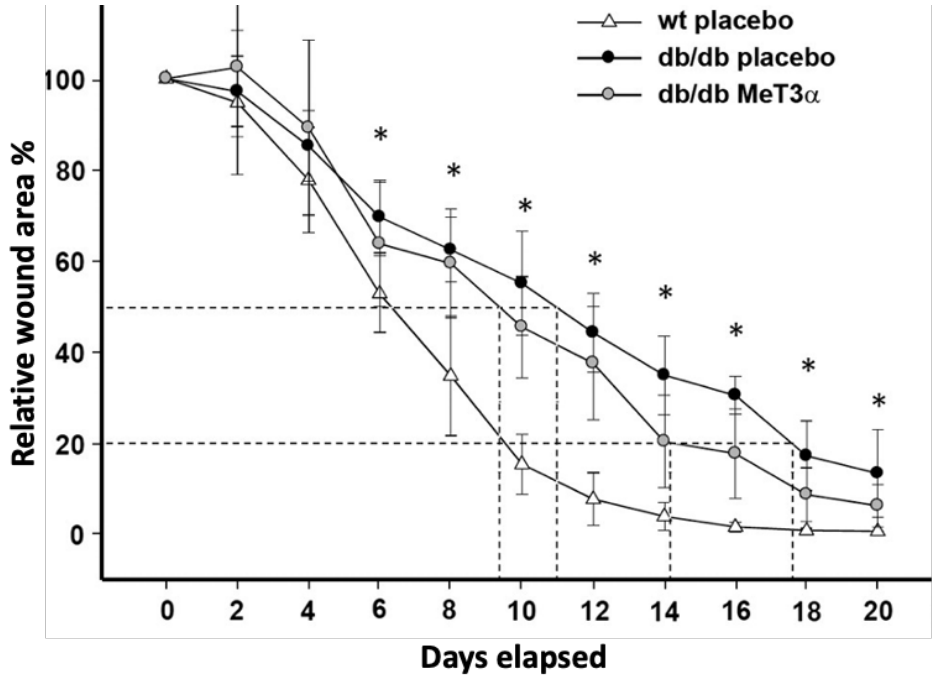


Fig. 15. (Paper II – Fig.8). MeT3α treatment on cutaneous wounds in vivo. Effect of local MeT3α treatment on wound healing in db/db mice. values are means ± SEM. The hatched lines denote the time to elapsed at 50% and 80% wound closure, respectively.

4.2.2 MeT3α and mitochondrial function

MeT3α treatment in HDF and HepG2 cell cultures resulted in increased basal OCR and reserve mitochondrial capacity (Paper II, Fig. 7). The was not significantly different from that of direct CoQ supplementation. This upregulation of OCR is opposite of what normal HIF regulation of hypoxia is usually directed towards, perhaps a possible explanation in part for the incomplete reversal of wound healing as a result of MeT3α treatment. Since MeT3α stimulates CoQ and CoQ having a dual effect as a part of the ETC and as an antioxidant, we next investigated mitochondrial function and diabetic wound healing in relation to hyperglycemia-hypoxia interactions previously established (95).

4.3 PAPER III

4.3.1 Inhibition and reconstitution of miR-210 in diabetic wounds

HDF, HDMVEC and Keratinocytes exposed to hyperglycemia had reduced expression miR-210 (Paper III – Fig.1). miR-210 expression was increased in wounds of WT, normoglycemic mice compared to WT skin. Wounds from db/db mice were deficient in miR-210 (Paper III – Fig.2A-B). We also found miR-210 levels to be lower in human DFU compared to venous ulcers (Paper III – Fig.2C-D). This effect was mediated by impaired HIF-1 signaling as shown by the restoration of miR-210 levels when HIF-1 was stabilized using DMOG (Paper III – Fig.2E-F). This finding aligns with previous work on HIF-1 stabilization in diabetic wounds (95). Intradermal injection of stabilized miR-210 mimic improved wound healing in db/db wounds (Paper III – Fig.3A-C). Treatment had no effect on WT wounds (D-E). miR-210 mimic injection increased granulation tissue deposition in db/db (F-G). Treatment also improved markers for angiogenesis, cell proliferation and inflammation (H-J).

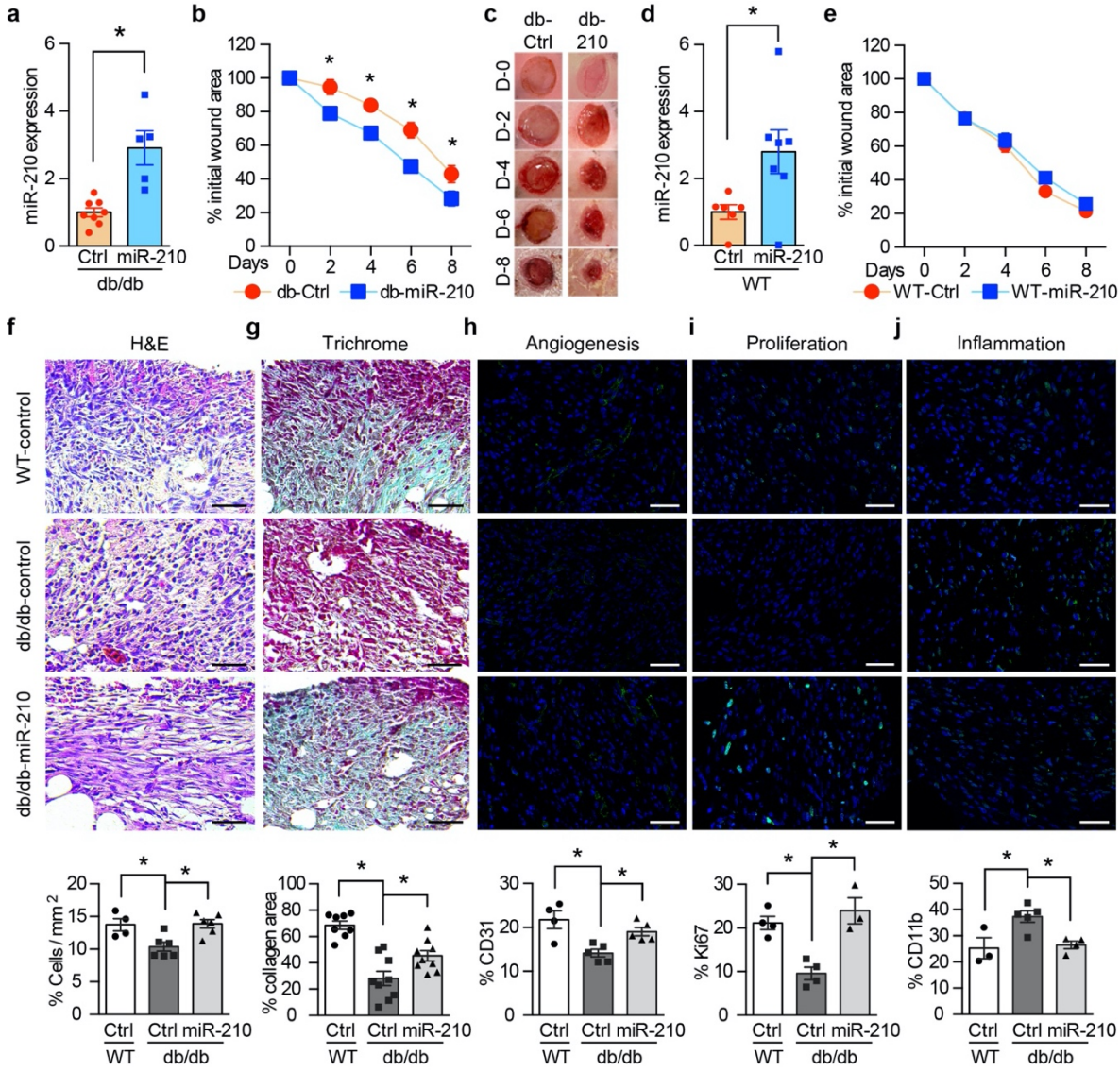


Fig. 16: (Paper III Fig.3). miR-210 reconstitution and diabetic wound healing.
For full figure legends please refer to the original paper.

4.3.2 miR-210, wound healing and metabolic reprogramming

In five validated potential mitochondrial target genes of miR-210 (105), local administration of miR-210 repressed the expression of ISCU and SDHD in db/db wounds (Paper III – Fig. 4A) and HDF cells (Paper III – Fig. 5A) In line with this finding, we also found that administration of miR-210 reduced db/db wound tissue OCR to normal levels found in WT mice (4B), while increasing ECAR, indicating an increase in glycolysis (4C). This was accompanied by a reduction in ROS production and oxidative stress as measured by 4HNE (4D). Corresponding changes were seen in HDF cells, with miR-210 effecting a reduction in OCR (5B), increase in ECAR (5C) and reduction of ROS production in the form of O_2•− (5D). A shift in metabolic phenotype was seen wherein the proportion of ATP produced through glycolysis was increased in HDF cells after miR-210 administration (5E). Adding glycolysis inhibitors abrogated the increase in miR-210-induced HDF migration rate (5F).

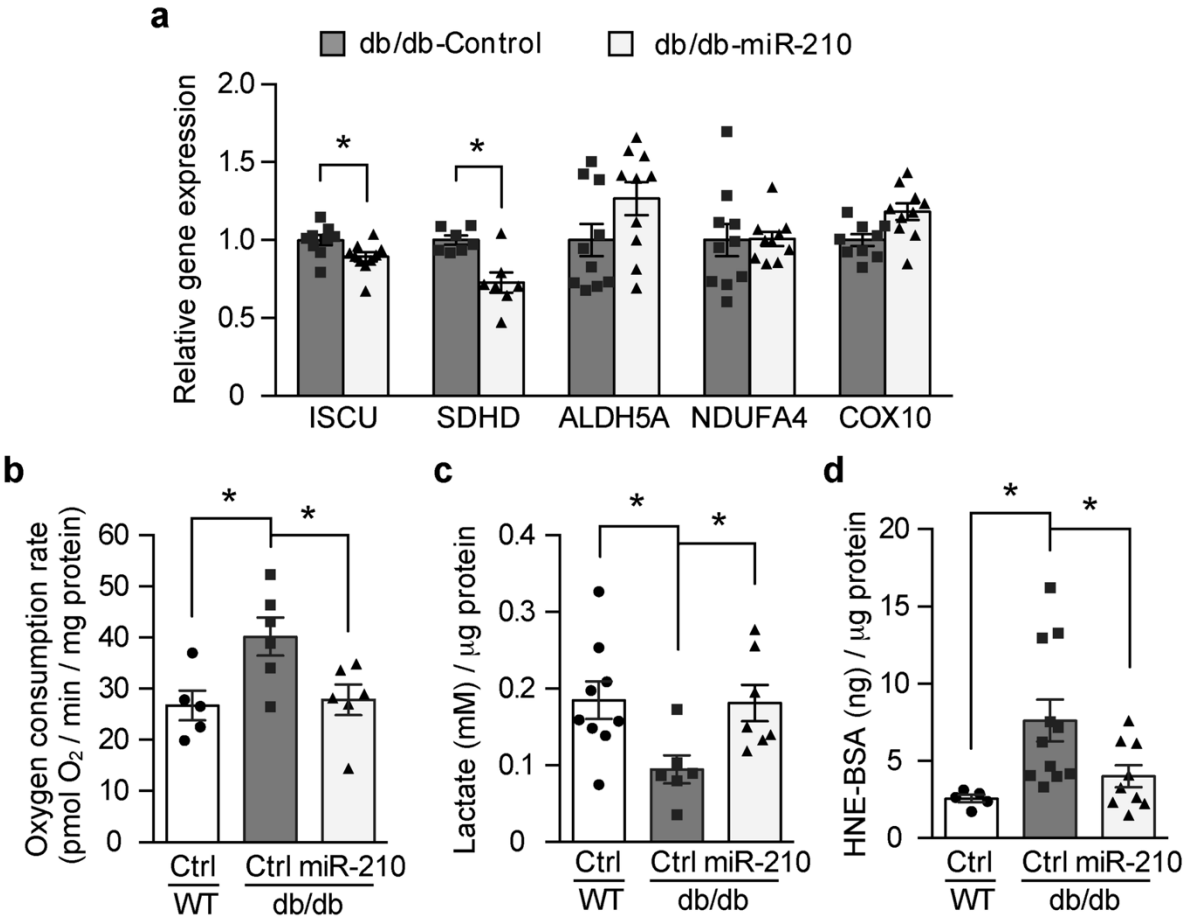


Fig. 17: (Paper III Fig.4). miR-210 and metabolic reprogramming in db/db mice.
For full figure legends please refer to the original paper.

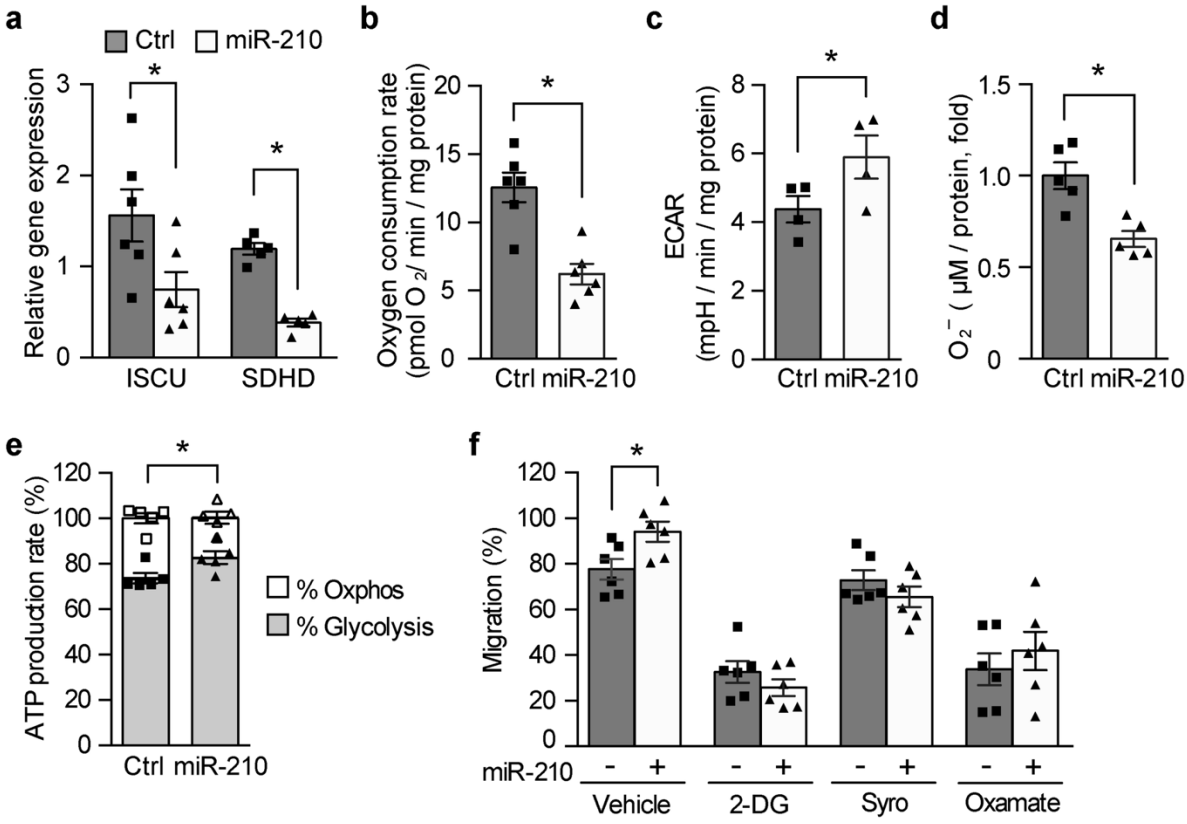


Fig. 18. (Paper III Fig.5). miR-210 and metabolic programming in HDF cells.
For full figure legends please refer to the original paper

4.3.3 Discussion

HIF-1 targets a broad array of genes and modulate pathways other than those which are relevant for wound healing. We sought to investigate a narrower hypoxic signature more specific for hypoxic wounds. In Paper III, we show that miR-210, which directly regulates approximately 30 genes, improves impaired wound healing in DM (130). The regulatory role of HIF-1 in miR-210 expression puts our findings in perspective with the knowledge that HIF signaling is repressed by hyperglycemia in DM (46, 95). Previous research has showed, however, that DM is associated with increased miR-210 levels (278-280), and that keratinocyte proliferation is repressed by miR-210 (141). We theorize that these apparent contradictions are due to hypoxia in DM affected tissues leading to elevated miR-210 levels, not reflecting a continued relative lack of hypoxic signaling. The same is true for the work for Biswas et. al, wherein extremely high miR-210 levels due to ischemia would likely lead to different net effects than the context of DM wound healing which we set out to investigate. Paper III also shows that a miR-210-induced glycolytic shift and reduction of mtROS production is at least partly responsible for the improved wound healing process in diabetic mice. Having established a clear connection between mitochondrial function in hypoxia and diabetic wound healing, we then set out to investigate the implications of this finding in a wider context, touching upon systemic hypoxia in diabetics and diabetic nephropathy, another complication deeply affected by tissue hypoxia in DM. The findings of these efforts are presented in Paper IV.

4.4 PAPER IV

4.4.1 HIF-1α function mediates ROS dysregulation in diabetes

The effect of transient systemic hypoxia in T1DM and healthy controls was investigated according to a protocol (Paper IV – Fig. 1A) modified from previous studies (281, 282). After hypoxic exposure, ROS in peripheral blood were increased in T1DM but not in normoglycemic controls (1B). Oxygen tension is known to be affected in diabetic kidney disease (283). The effect of increased ROS due to hypoxia in DM was evaluated in IMCD-3 cells, where hypoxia was seen to increase HIF-1α expression, which was subsequently reduced by concurrent hyperglycemia (Paper IV- Fig. 2A). This effect was mediated by a reduction in HIF transcription activity as measured by an HRE-driven luciferase assay (2B). PHD inhibition by DMOG treatment restored HIF-1 expression (2A) and activity (2B). mtROS production followed a corresponding pattern wherein combined hyperglycemia and hypoxia resulted in increased mtROS, and DMOG treatment reversed the same (Paper IV – Fig. 3A). Silencing of VHL (3B) and HIF-1α overexpression through transfection (3C) showed the same effect.

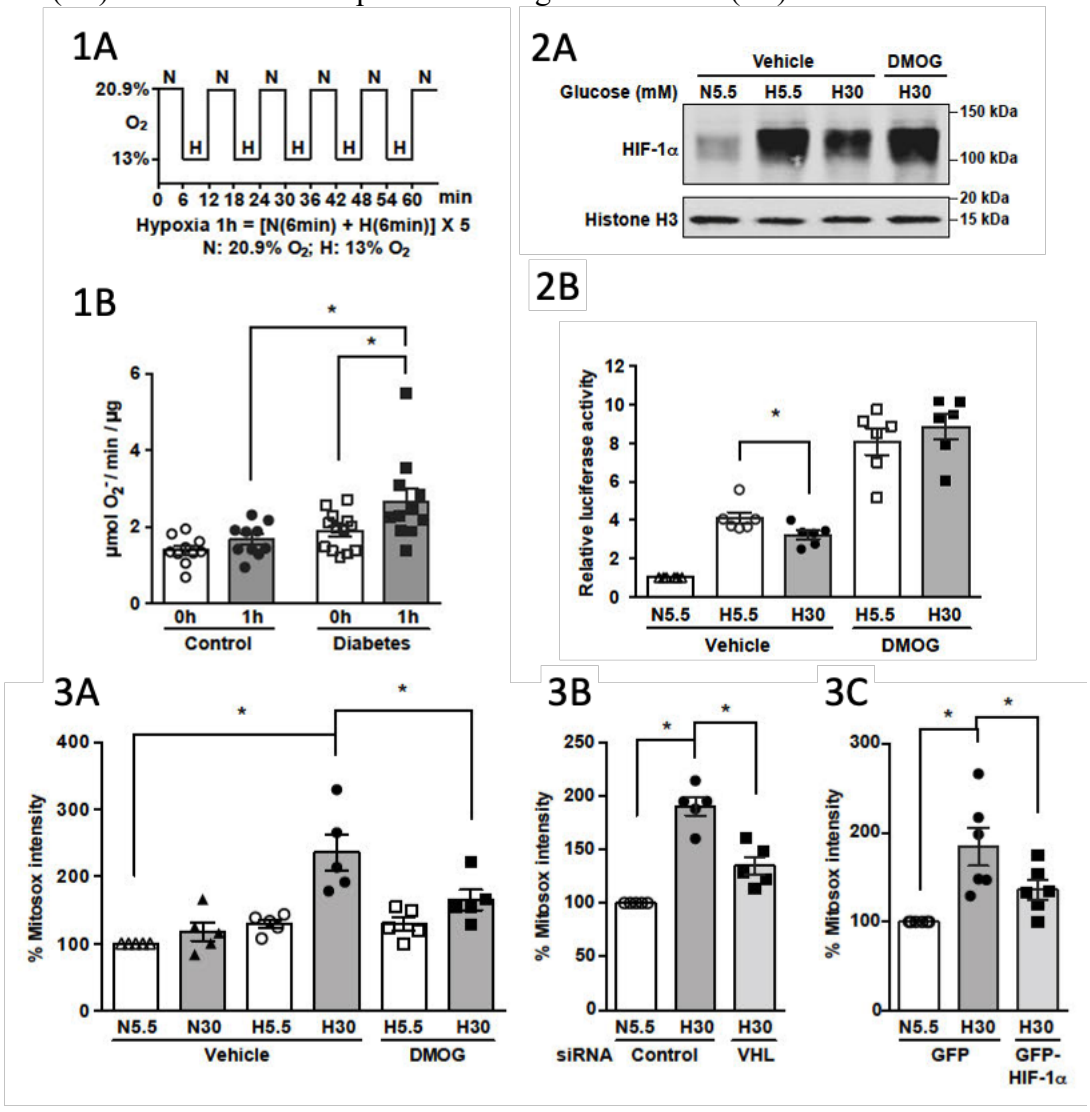


Fig. 19. (Paper IV – Fig.1-3). HIF-1α and ROS production in DM
For full figure legends please refer to the original paper.

Having established a clear relationship between hypoxia, HIF-1 destabilization and ROS production in a diabetic context, we continued by investigating it in an experimental model of mouse diabetic nephropathy. In accordance with our findings *in vitro*, HIF-1 signaling was repressed in kidney tissue from db/db and STZ-induced models of T2DM and T1DM, respectively (Paper IV- Fig. 4A and 4C). Higher 4HNE levels were seen in both diabetic models compared to WT mice (4B and 4D). Importantly, restoration of HIF-1 activity through DMOG in db/db (4B) and through genetic PHD2+/- STZ-induced mice (4D) led to lower ROS levels. An increase in complex-specific respiration and proton leak was observed in both animal models Activation of HIF-1 in the same tissues normalized mitochondrial respiration (4E and 4F).

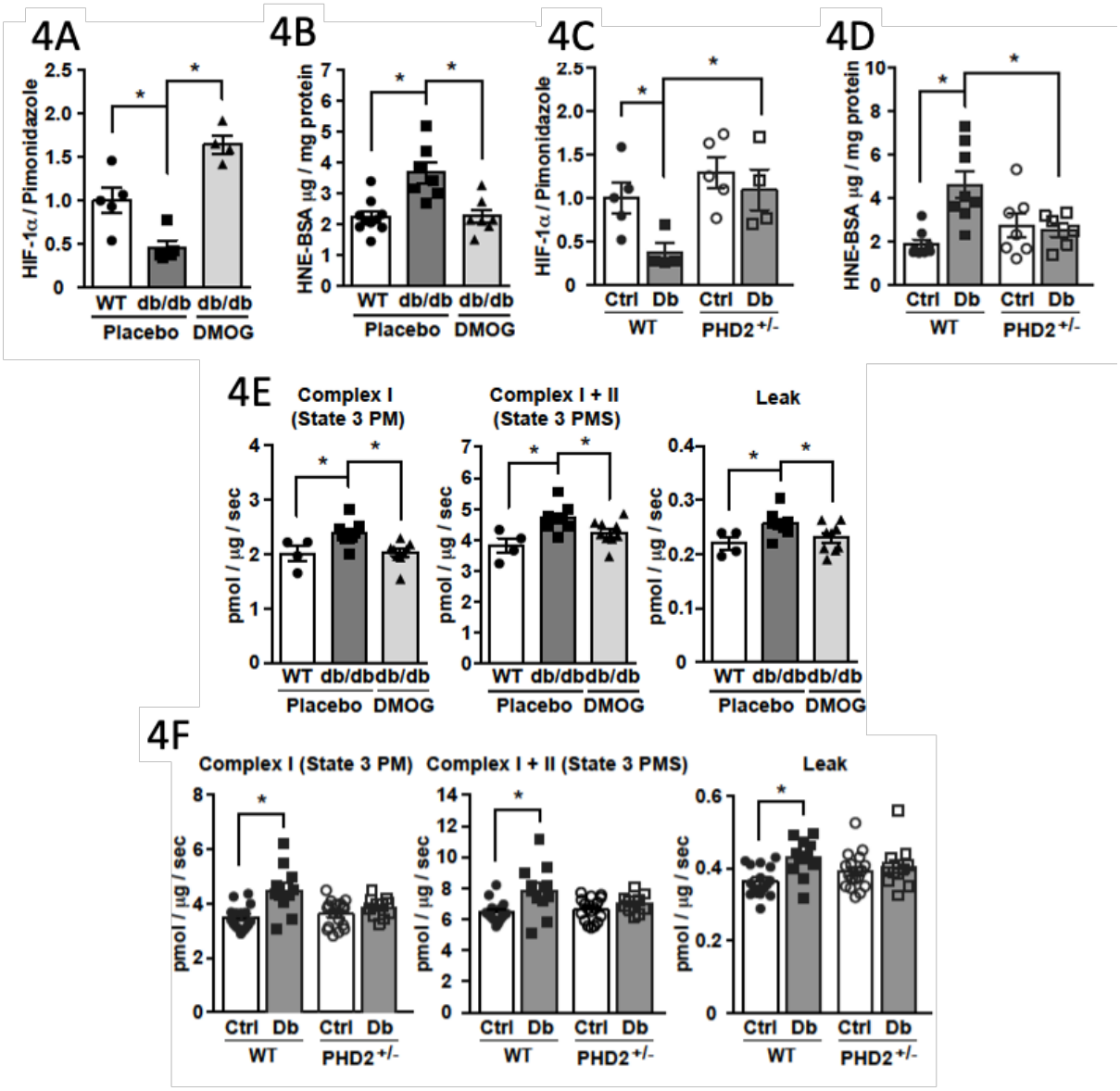


Fig. 20. (Paper IV – Fig.4). HIF-1α and ROS in mouse models of diabetic nephropathy.
Note: Histological stainings in 4A and 4C omitted. See Paper IV for full figure.
For full figure legends please refer to the original paper.

4.4.2 HIF-1α activation is a protective factor in diabetic nephropathy

We next investigated if suppression of the ROS accumulation through HIF-1 activation could have kidney-protective effects. In both db/db mice and in STZ-induced diabetes mice, reversal of the HIF-1 function was followed by prevention of typical diabetic kidney lesions as assessed through Kidney Injury Marker-1 (KIM-1) in db/db (Paper IV – Fig. 5A and 5B) and PHD2$^{+/-}$ (5D and 5E), and apoptosis as measured through TUNEL staining in db/db (5C). These findings were further corroborated by a decrease in UAlb/Crea ratios (5F and 5G).

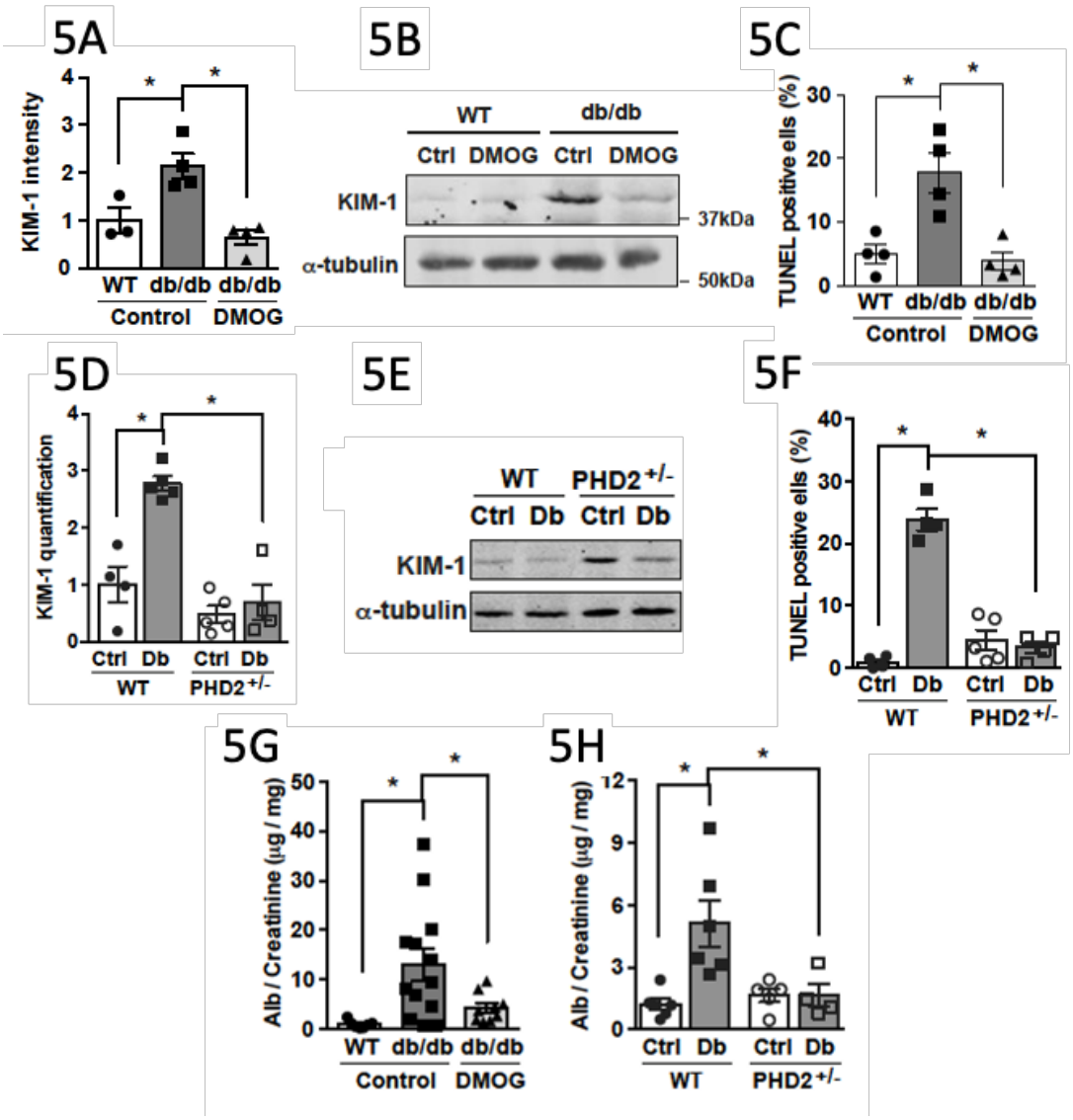


Fig. 21. (Paper IV – Fig.5). HIF-1 activation and markers of diabetic nephropathy.
Note: Histological stainings in 5A, 5C, 5D and 5F omitted. See Paper IV for full figure.
For full figure legends please refer to the original paper.

4.4.3 Discussion

In Paper IV, we demonstrated that HIF-1 repression induced by high glucose is an important mechanism for the excessive production of mitochondrial ROS in diabetes. We show that the observed increase in ROS is not solely due to increased ETC substrate availability. Hypoxia regulation is a continuous process that is always activated to some extent, due to tissue oxygenation levels typically being low compared to atmospheric O_2 pressure even in normal, normoxic conditions (64). Small perturbances in tissue oxygenation could thus contribute to ROS production and complications in DM (284). Increased substrate availability in hyperglycemia and deficient HIF signaling leads to higher mtROS production (89, 133). Increased mitochondrial proton leak as seen in DM is a rescue mechanism intended to reduce ETC throughput thus reducing mtROS production (285). In our experiments, ROS production could be reversed by HIF-1 activation despite persisting hyperglycemia both *in vitro* and *in vivo*. Restoring HIF-1 function, as shown in mouse models of both T1DM and T2DM, is thus sufficient to normalize ROS levels and to protect against diabetes nephropathy. This has potential implications for DM management, as we show the importance of hypoxic regulation in DM not just topically as in Paper III. Oral PHD inhibition has recently received regulatory approval for the treatment of anemia in chronic kidney disease (286, 287). Our findings in Paper IV pave the way for further research into the possibility of modulating pathogenetic mechanisms in complications of DM.

5 CONCLUSIONS

We set out to study the complex interplay between DM, hypoxia and mitochondrial function in the context of DM complications. Our findings are presented in Paper I-IV. Based on these findings, and in accordance other research conducted in our lab and in association with various collaborators (47, 98, 126, 251, 288-290), we draw the following conclusions:

- Hyperlipidemia, hyperglycemia and inflammation are associated with markers of oxidative stress such oxLDL and FORT in T2DM patients. Markers of oxidative stress are correlated to the prevalence of DM complications including CVD and PSN. CoQ was positively correlated with oxLDL, reflecting increased oxidative stress in DM.

- Increasing endogenous CoQ through treatment with MeT3α results in an upregulation of fibroblast migration and angiogenesis, thus promoting wound healing in DM. CoQ was at the same time seen to increase cellular respiration.

- miR-210 expression is reduced by hyperglycemia in DM. Treatment with reconstituted miR-210 improves wound healing and reduces ROS production and oxidative stress in diabetic wounds. This treatment effect is due to modulation of cellular respiration by miR-210.

- Inadequate HIF-1 acitivty due to HIF-1 destabilization by hyperglycemia is responsible for an increased rate of ROS production in DM. Reversing this process reduces ROS, and is protective against diabetic nephropathy.

6 FUTURE PERSPECTIVES

Research efforts in diabetes and diabetes complications are progressing rapidly. Several new discoveries have been brought to market and the multifactorial nature of T2DM in particular enables many angles of attack (291-293).

Due to its ubiquitous nature yet difficulty to take up when supplemented orally, Current knowledge on CoQ supplementation is inconclusive (294, 295). Alternative mechanisms for increasing CoQ availability are currently being investigated and could potentially lead to new treatment options in DM, in particular metabolic control in T2DM.

Ongoing efforts are investigating the feasibility of applying the knowledge gained from our results in clinical practice. These include the possibility of increasing local HIF expression and/or stability as a treatment option for DM complications (46). A clinical study is underway to investigate the systemic effect of HIF stabilization using DFX upon hypoxia responses in T1DM patients (296). Another study has been initiated to investigate the effects of topical DFX in the treatment of DFU (297). Since DFX is a well proven and safe treatment for other indications, positive outcomes could lead to these treatment strategies being adopted in clinical practice with relative ease.

With HIF being a critical component in regulating cell bioenergetics and DM featuring both hyperglycemia and hypoxia, it is unsurprising that other aspects of DM treatment also touch upon HIF regulation. This includes one of the great successes in novel diabetes treatments in recent years, the SGLT2-inhibitors, whose scope of use has been widened due to well documented effects on reducing CVD risk, although conflicting results have also been reported (291, 298). With the nascent approval of PHD inhibitors, further research directions will have a wider array of experimental treatments to investigate (287).

In a larger perspective, cellular metabolism and ROS have increasingly been implicated and shown to play a central role in almost all aspects of DM including areas of research such as beta cell dysfunction, inflammation regulation, adipocyte cell signaling and obesity (299-302). Further research directions to investigate the precise role of hypoxia regulation and antioxidant systems in these areas will hopefully pave the way for improved understanding of diabetes and novel options for preventing, assessing and treating diabetes and its complications.

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To my wife **Simone**, who supported me through this entire journey by way of example and by being the most loving and considerate partner imaginable. This book will proudly sit next to yours, reminding us of how we met, the good times we had, challenges we've faced together, and experiences gained. I will always cherish this special time in our lives as we look towards the future and many wonderful endeavors ahead.

8 REFERENCES

1. Quianzon CC, Cheikh I. History of insulin. J Community Hosp Intern Med Perspect. 2012;2(2).

2. Bommer C, Sagalova V, Heesemann E, Manne-Goehler J, Atun R, Bärnighausen T, et al. Global Economic Burden of Diabetes in Adults: Projections From 2015 to 2030. Diabetes Care. 2018;41(5):963-70.

3. Collaborators GDaH. Global, regional, and national disability-adjusted life-years (DALYs) for 359 diseases and injuries and healthy life expectancy (HALE) for 195 countries and territories, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. Lancet. 2018;392(10159):1859-922.

4. Zheng Y, Ley SH, Hu FB. Global aetiology and epidemiology of type 2 diabetes mellitus and its complications. Nat Rev Endocrinol. 2018;14(2):88-98.

5. Tuomi T, Santoro N, Caprio S, Cai M, Weng J, Groop L. The many faces of diabetes: a disease with increasing heterogeneity. Lancet. 2014;383(9922):1084-94.

6. IDF Diabetes Atlas. 9th edition ed. Brussels, Belgium.: International Diabetes Federation; 2019.

7. Forouhi NG, Wareham NJ. Epidemiology of diabetes. Medicine (Abingdon). 2014;42(12):698-702.

8. Group DP. Incidence and trends of childhood Type 1 diabetes worldwide 1990-1999. Diabet Med. 2006;23(8):857-66.

9. Todd JA. Etiology of type 1 diabetes. Immunity. 2010;32(4):457-67.

10. Hyppönen E, Läärä E, Reunanen A, Järvelin MR, Virtanen SM. Intake of vitamin D and risk of type 1 diabetes: a birth-cohort study. Lancet. 2001;358(9292):1500-3.

11. Patterson CC, Karuranga S, Salpea P, Saeedi P, Dahlquist G, Soltesz G, et al. Worldwide estimates of incidence, prevalence and mortality of type 1 diabetes in children and adolescents: Results from the International Diabetes Federation Diabetes Atlas, 9th edition. Diabetes Res Clin Pract. 2019;157:107842.

12. Morris AP, Voight BF, Teslovich TM, Ferreira T, Segrè AV, Steinthorsdottir V, et al. Large-scale association analysis provides insights into the genetic architecture and pathophysiology of type 2 diabetes. Nat Genet. 2012;44(9):981-90.

13. Consortium I. Adherence to predefined dietary patterns and incident type 2 diabetes in European populations: EPIC-InterAct Study. Diabetologia. 2014;57(2):321-33.

14. O'Connor L, Imamura F, Lentjes MA, Khaw KT, Wareham NJ, Forouhi NG. Prospective associations and population impact of sweet beverage intake and type 2 diabetes, and effects of substitutions with alternative beverages. Diabetologia. 2015;58(7):1474-83.

15. Bendinelli B, Palli D, Masala G, Sharp SJ, Schulze MB, Guevara M, et al. Association between dietary meat consumption and incident type 2 diabetes: the EPIC-InterAct study. Diabetologia. 2013;56(1):47-59.

16. Maraschin JdF. Classification of Diabetes. In: Ahmad SI, editor. Diabetes: An Old Disease, a New Insight. New York, NY: Springer New York; 2013. p. 12-9.

17. Brownlee M. Biochemistry and molecular cell biology of diabetic complications. Nature. 2001;414(6865):813-20.

18. Skyler JS, Bakris GL, Bonifacio E, Darsow T, Eckel RH, Groop L, et al. Differentiation of Diabetes by Pathophysiology, Natural History, and Prognosis. Diabetes. 2017;66(2):241-55.

19. Brownlee M. The pathobiology of diabetic complications: a unifying mechanism. Diabetes. 2005;54(6):1615-25.

20. Vanhoutte PM, Zhao Y, Xu A, Leung SW. Thirty Years of Saying NO: Sources, Fate, Actions, and Misfortunes of the Endothelium-Derived Vasodilator Mediator. Circ Res. 2016;119(2):375-96.

21. Nishikawa T, Brownlee M, Araki E. Mitochondrial reactive oxygen species in the pathogenesis of early diabetic nephropathy. J Diabetes Investig. 2015;6(2):137-9.

22. Forbes JM, Cooper ME. Mechanisms of diabetic complications. Physiol Rev. 2013;93(1):137-88.

23. Ansurudeen I, Sunkari VG, Grunler J, Peters V, Schmitt CP, Catrina SB, et al. Carnosine enhances diabetic wound healing in the db/db mouse model of type 2 diabetes. Amino Acids. 2012;43(1):127-34.

24. Lavery LA, Armstrong DG, Wunderlich RP, Mohler MJ, Wendel CS, Lipsky BA. Risk factors for foot infections in individuals with diabetes. Diabetes Care. 2006;29(6):1288-93.

25. Rewers A. Acute metabolic complications in diabetes. Diabetes in America, 3rd edn National Institutes of Health, NIH Pub. 2017(17-1468):17-1.

26. Forbes JM, Cooper ME. Mechanisms of diabetic complications. Physiol Rev. 2013;93(1):137-88.

27. Singer AJ, Clark RA. Cutaneous wound healing. N Engl J Med. 1999;341(10):738-46.

28. Falanga V. Wound healing and its impairment in the diabetic foot. Lancet. 2005;366(9498):1736-43.

29. Guo S, Dipietro LA. Factors affecting wound healing. J Dent Res. 2010;89(3):219-29.

30. Werner S, Grose R. Regulation of wound healing by growth factors and cytokines. Physiol Rev. 2003;83(3):835-70.

31. Lindley LE, Stojadinovic O, Pastar I, Tomic-Canic M. Biology and Biomarkers for Wound Healing. Plast Reconstr Surg. 2016;138(3 Suppl):18S-28S.

32. Velnar T, Bailey T, Smrkolj V. The wound healing process: an overview of the cellular and molecular mechanisms. J Int Med Res. 2009;37(5):1528-42.

33. Martin P, Nunan R. Cellular and molecular mechanisms of repair in acute and chronic wound healing. Br J Dermatol. 2015;173(2):370-8.

34. Stadelmann WK, Digenis AG, Tobin GR. Physiology and healing dynamics of chronic cutaneous wounds. Am J Surg. 1998;176(2A Suppl):26S-38S.

35. Nunan R, Harding KG, Martin P. Clinical challenges of chronic wounds: searching for an optimal animal model to recapitulate their complexity. Dis Model Mech. 2014;7(11):1205-13.

36. Landén NX, Li D, Ståhle M. Transition from inflammation to proliferation: a critical step during wound healing. Cell Mol Life Sci. 2016;73(20):3861-85.

37. Darby IA, Hewitson TD. Fibroblast differentiation in wound healing and fibrosis. Int Rev Cytol. 2007;257:143-79.

38. Nolte SV, Xu W, Rennekampff HO, Rodemann HP. Diversity of fibroblasts--a review on implications for skin tissue engineering. Cells Tissues Organs. 2008;187(3):165-76.

39. Pastar I, Stojadinovic O, Yin NC, Ramirez H, Nusbaum AG, Sawaya A, et al. Epithelialization in Wound Healing: A Comprehensive Review. Adv Wound Care (New Rochelle). 2014;3(7):445-64.

40. Greenhalgh DG. The role of apoptosis in wound healing. Int J Biochem Cell Biol. 1998;30(9):1019-30.

41. Vinaik R, Barayan D, Auger C, Abdullahi A, Jeschke MG. Regulation of glycolysis and the Warburg effect in wound healing. JCI Insight. 2020;5(17).

42. Xu X, Gu S, Huang X, Ren J, Gu Y, Wei C, et al. The role of macrophages in the formation of hypertrophic scars and keloids. Burns Trauma. 2020;8:tkaa006.

43. Frykberg RG. Diabetic foot ulcers: pathogenesis and management. Am Fam Physician. 2002;66(9):1655-62.

44. Boulton AJ. The diabetic foot: grand overview, epidemiology and pathogenesis. Diabetes Metab Res Rev. 2008;24 Suppl 1:S3-6.

45. Brem H, Tomic-Canic M. Cellular and molecular basis of wound healing in diabetes. J Clin Invest. 2007;117(5):1219-22.

46. Catrina SB, Zheng X. Disturbed hypoxic responses as a pathogenic mechanism of diabetic foot ulcers. Diabetes Metab Res Rev. 2016;32 Suppl 1:179-85.

47. Zheng X, Narayanan S, Sunkari VG, Eliasson S, Botusan IR, Grünler J, et al. Triggering of a Dll4-Notch1 loop impairs wound healing in diabetes. Proc Natl Acad Sci U S A. 2019;116(14):6985-94.

48. Thangarajah H, Yao D, Chang EI, Shi Y, Jazayeri L, Vial IN, et al. The molecular basis for impaired hypoxia-induced VEGF expression in diabetic tissues. Proc Natl Acad Sci U S A. 2009;106(32):13505-10.

49. Werner S, Breeden M, Hübner G, Greenhalgh DG, Longaker MT. Induction of keratinocyte growth factor expression is reduced and delayed during wound healing in the genetically diabetic mouse. J Invest Dermatol. 1994;103(4):469-73.

50. Anand P, Terenghi G, Warner G, Kopelman P, Williams-Chestnut RE, Sinicropi DV. The role of endogenous nerve growth factor in human diabetic neuropathy. Nat Med. 1996;2(6):703-7.

51. Pierce GF, Mustoe TA, Lingelbach J, Masakowski VR, Griffin GL, Senior RM, et al. Platelet-derived growth factor and transforming growth factor-beta enhance tissue repair activities by unique mechanisms. J Cell Biol. 1989;109(1):429-40.

52. Blakytny R, Jude EB, Martin Gibson J, Boulton AJ, Ferguson MW. Lack of insulin-like growth factor 1 (IGF1) in the basal keratinocyte layer of diabetic skin and diabetic foot ulcers. J Pathol. 2000;190(5):589-94.

53. Jude EB, Blakytny R, Bulmer J, Boulton AJ, Ferguson MW. Transforming growth factor-beta 1, 2, 3 and receptor type I and II in diabetic foot ulcers. Diabet Med. 2002;19(6):440-7.

54. Blakytny R, Jude E. The molecular biology of chronic wounds and delayed healing in diabetes. Diabet Med. 2006;23(6):594-608.

55. Muller M, Trocme C, Lardy B, Morel F, Halimi S, Benhamou PY. Matrix metalloproteinases and diabetic foot ulcers: the ratio of MMP-1 to TIMP-1 is a predictor of wound healing. Diabet Med. 2008;25(4):419-26.

56. Lobmann R, Schultz G, Lehnert H. Proteases and the diabetic foot syndrome: mechanisms and therapeutic implications. Diabetes Care. 2005;28(2):461-71.

57. Apelqvist J, Larsson J, Agardh CD. Long-term prognosis for diabetic patients with foot ulcers. J Intern Med. 1993;233(6):485-91.

58. Boulton AJ, Vileikyte L, Ragnarson-Tennvall G, Apelqvist J. The global burden of diabetic foot disease. Lancet. 2005;366(9498):1719-24.

59. Lazzarini PA, Pacella RE, Armstrong DG, van Netten JJ. Diabetes-related lower-extremity complications are a leading cause of the global burden of disability. Diabet Med. 2018.

60. Alexiadou K, Doupis J. Management of diabetic foot ulcers. Diabetes Ther. 2012;3(1):4.

61. Aldana PC, Khachemoune A. Diabetic Foot Ulcers: Appraising Standard of Care and Reviewing New Trends in Management. Am J Clin Dermatol. 2020;21(2):255-64.

62. Michiels C. Physiological and pathological responses to hypoxia. Am J Pathol. 2004;164(6):1875-82.

63. McKeown SR. Defining normoxia, physoxia and hypoxia in tumours-implications for treatment response. Br J Radiol. 2014;87(1035):20130676.

64. Carreau A, El Hafny-Rahbi B, Matejuk A, Grillon C, Kieda C. Why is the partial oxygen pressure of human tissues a crucial parameter? Small molecules and hypoxia. J Cell Mol Med. 2011;15(6):1239-53.

65. Schodel J, Ratcliffe PJ. Mechanisms of hypoxia signalling: new implications for nephrology. Nat Rev Nephrol. 2019;15(10):641-59.

66. Giaccia AJ, Simon MC, Johnson R. The biology of hypoxia: the role of oxygen sensing in development, normal function, and disease. Genes Dev. 2004;18(18):2183-94.

67. Catrina SB. Impaired hypoxia-inducible factor (HIF) regulation by hyperglycemia. J Mol Med (Berl). 2014;92(10):1025-34.

68. Semenza GL. Oxygen sensing, homeostasis, and disease. N Engl J Med. 2011;365(6):537-47.

69. Ortiz-Prado E, Dunn JF, Vasconez J, Castillo D, Viscor G. Partial pressure of oxygen in the human body: a general review. Am J Blood Res. 2019;9(1):1-14.

70. Le QT, Chen E, Salim A, Cao H, Kong CS, Whyte R, et al. An evaluation of tumor oxygenation and gene expression in patients with early stage non-small cell lung cancers. Clin Cancer Res. 2006;12(5):1507-14.

71. Leary TS, Klinck JR, Hayman G, Friend P, Jamieson NV, Gupta AK. Measurement of liver tissue oxygenation after orthotopic liver transplantation using a multiparameter sensor. A pilot study. Anaesthesia. 2002;57(11):1128-33.

72. Thermann M, Jostarndt L, Eberhard F, Richter H, Sass W. [Oxygen supply of the human small intestine in mechanical ileus]. Langenbecks Arch Chir. 1985;363(3):179-84. Die Sauerstoffversorgung des menschlichen Dunndarmes im mechanischen Ileus.

73. Muller M, Padberg W, Schindler E, Sticher J, Osmer C, Friemann S, et al. Renocortical tissue oxygen pressure measurements in patients undergoing living donor kidney transplantation. Anesth Analg. 1998;87(2):474-6.

74. Dings J, Meixensberger J, Jager A, Roosen K. Clinical experience with 118 brain tissue oxygen partial pressure catheter probes. Neurosurgery. 1998;43(5):1082-95.

75. Harrison JS, Rameshwar P, Chang V, Bandari P. Oxygen saturation in the bone marrow of healthy volunteers. Blood. 2002;99(1):394.

76. Wang W, Winlove CP, Michel CC. Oxygen partial pressure in outer layers of skin of human finger nail folds. J Physiol. 2003;549(Pt 3):855-63.

77. Semenza GL, Wang GL. A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol Cell Biol. 1992;12(12):5447-54.

78. Semenza GL. Oxygen sensing, hypoxia-inducible factors, and disease pathophysiology. Annu Rev Pathol. 2014;9:47-71.

79. Holmquist-Mengelbier L, Fredlund E, Lofstedt T, Noguera R, Navarro S, Nilsson H, et al. Recruitment of HIF-1alpha and HIF-2alpha to common target genes is differentially regulated in neuroblastoma: HIF-2alpha promotes an aggressive phenotype. Cancer Cell. 2006;10(5):413-23.

80. Makino Y, Cao R, Svensson K, Bertilsson G, Asman M, Tanaka H, et al. Inhibitory PAS domain protein is a negative regulator of hypoxia-inducible gene expression. Nature. 2001;414(6863):550-4.

81. Zhang P, Yao Q, Lu L, Li Y, Chen PJ, Duan C. Hypoxia-inducible factor 3 is an oxygen-dependent transcription activator and regulates a distinct transcriptional response to hypoxia. Cell Rep. 2014;6(6):1110-21.

82. Samanta D, Semenza GL. Maintenance of redox homeostasis by hypoxia-inducible factors. Redox Biol. 2017;13:331-5.

83. Hubbi ME, Semenza GL. Regulation of cell proliferation by hypoxia-inducible factors. Am J Physiol Cell Physiol. 2015;309(12):C775-82.

84. Berra E, Benizri E, Ginouves A, Volmat V, Roux D, Pouyssegur J. HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1alpha in normoxia. EMBO J. 2003;22(16):4082-90.

85. Kaelin WG, Jr., Ratcliffe PJ. Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. Mol Cell. 2008;30(4):393-402.

86. Semenza GL. Hypoxia-inducible factor 1 and cardiovascular disease. Annu Rev Physiol. 2014;76:39-56.

87. Burr SP, Costa ASH, Grice GL, Timms RT, Lobb IT, Freisinger P, et al. Mitochondrial Protein Lipoylation and the 2-Oxoglutarate Dehydrogenase Complex Controls HIF1 alpha Stability in Aerobic Conditions. Cell Metabolism. 2016;24(5):740-52.

88. Mahon PC, Hirota K, Semenza GL. FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. Genes Dev. 2001;15(20):2675-86.

89. Yang C, Jiang L, Zhang H, Shimoda LA, DeBerardinis RJ, Semenza GL. Analysis of hypoxia-induced metabolic reprogramming. Methods Enzymol. 2014;542:425-55.

90. Choudhry H, Harris AL. Advances in Hypoxia-Inducible Factor Biology. Cell Metab. 2018;27(2):281-98.

91. Peyssonnaux C, Boutin AT, Zinkernagel AS, Datta V, Nizet V, Johnson RS. Critical role of HIF-1 alpha in keratinocyte defense against bacterial infection. Journal of Investigative Dermatology. 2008;128(8):1964-8.

92. Zhang XJ, Liu LX, Wei XF, Tan YS, Tong LN, Chang R, et al. Impaired angiogenesis and mobilization of circulating angiogenic cells in HIF-1 alpha heterozygous-null mice after burn wounding. Wound Repair and Regeneration. 2010;18(2):193-201.

93. Semenza GL. Targeting hypoxia-inducible factor 1 to stimulate tissue vascularization. J Investig Med. 2016;64(2):361-3.

94. Williamson JR, Chang K, Frangos M, Hasan KS, Ido Y, Kawamura T, et al. Hyperglycemic pseudohypoxia and diabetic complications. Diabetes. 1993;42(6):801-13.

95. Botusan IR, Sunkari VG, Savu O, Catrina AI, Grünler J, Lindberg S, et al. Stabilization of HIF-1alpha is critical to improve wound healing in diabetic mice. Proc Natl Acad Sci U S A. 2008;105(49):19426-31.

96. Bento CF, Fernandes R, Ramalho J, Marques C, Shang F, Taylor A, et al. The Chaperone-Dependent Ubiquitin Ligase CHIP Targets HIF-1 alpha for Degradation in the Presence of Methylglyoxal. PLoS One. 2010;5(11).

97. Catrina SB, Okamoto K, Pereira T, Brismar K, Poellinger L. Hyperglycemia regulates hypoxia-inducible factor-1alpha protein stability and function. Diabetes. 2004;53(12):3226-32.

98. Sunkari VG, Lind F, Botusan IR, Kashif A, Liu ZJ, Ylä-Herttuala S, et al. Hyperbaric oxygen therapy activates hypoxia-inducible factor 1 (HIF-1), which contributes to improved wound healing in diabetic mice. Wound Repair Regen. 2015;23(1):98-103.

99. Semenza GL. Pharmacologic Targeting of Hypoxia-Inducible Factors. Annu Rev Pharmacol Toxicol. 2019;59:379-403.

100. O'Brien J, Hayder H, Zayed Y, Peng C. Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation. Front Endocrinol. 2018;9.

101. Cai Y, Yu X, Hu S, Yu J. A brief review on the mechanisms of miRNA regulation. Genomics Proteomics Bioinformatics. 2009;7(4):147-54.

102. Friedman RC, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. Genome Res. 2009;19(1):92-105.

103. Pereira-da-Silva T, Coutinho Cruz M, Carrusca C, Cruz Ferreira R, Napoleao P, Mota Carmo M. Circulating microRNA profiles in different arterial territories of stable atherosclerotic disease: a systematic review. Am J Cardiovasc Dis. 2018;8(1):1-13.

104. Roderburg C, Luedde T. Circulating microRNAs as markers of liver inflammation, fibrosis and cancer. J Hepatol. 2014;61(6):1434-7.

105. Bertero T, Rezzonico R, Pottier N, Mari B. Impact of MicroRNAs in the Cellular Response to Hypoxia. Int Rev Cell Mol Biol. 2017;333:91-158.

106. Gebert LFR, MacRae IJ. Regulation of microRNA function in animals. Nat Rev Mol Cell Biol. 2019;20(1):21-37.

107. Hutvagner G, McLachlan J, Pasquinelli AE, Balint E, Tuschl T, Zamore PD. A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. Science. 2001;293(5531):834-8.

108. Ha M, Kim VN. Regulation of microRNA biogenesis. Nat Rev Mol Cell Biol. 2014;15(8):509-24.

109. Yang JS, Lai EC. Alternative miRNA biogenesis pathways and the interpretation of core miRNA pathway mutants. Mol Cell. 2011;43(6):892-903.

110. Guay C, Roggli E, Nesca V, Jacovetti C, Regazzi R. Diabetes mellitus, a microRNA-related disease? Transl Res. 2011;157(4):253-64.

111. McClelland AD, Kantharidis P. microRNA in the development of diabetic complications. Clin Sci (Lond). 2014;126(2):95-110.

112. Dey N, Das F, Mariappan MM, Mandal CC, Ghosh-Choudhury N, Kasinath BS, et al. MicroRNA-21 orchestrates high glucose-induced signals to TOR complex 1, resulting in renal cell pathology in diabetes. J Biol Chem. 2011;286(29):25586-603.

113. Feng J, Xing W, Xie L. Regulatory Roles of MicroRNAs in Diabetes. Int J Mol Sci. 2016;17(10).

114. Zhang T, Lv C, Li L, Chen S, Liu S, Wang C, et al. Plasma miR-126 is a potential biomarker for early prediction of type 2 diabetes mellitus in susceptible individuals. Biomed Res Int. 2013;2013:761617.

115. Li D, Wang A, Liu X, Meisgen F, Grünler J, Botusan IR, et al. MicroRNA-132 enhances transition from inflammation to proliferation during wound healing. J Clin Invest. 2015;125(8):3008-26.

116. Yan SL, Wu ST, Yin MC, Chen HT, Chen HC. Protective effects from carnosine and histidine on acetaminophen-induced liver injury. J Food Sci. 2009;74(8):H259-65.

117. Chien HY, Chen CY, Chiu YH, Lin YC, Li WC. Differential microRNA Profiles Predict Diabetic Nephropathy Progression in Taiwan. Int J Med Sci. 2016;13(6):457-65.

118. Wu J, Li X, Li D, Ren X, Li Y, Herter EK, et al. MicroRNA-34 Family Enhances Wound Inflammation by Targeting LGR4. J Invest Dermatol. 2020;140(2):465-76 e11.

119. Raitoharju E, Lyytikainen LP, Levula M, Oksala N, Mennander A, Tarkka M, et al. miR-21, miR-210, miR-34a, and miR-146a/b are up-regulated in human atherosclerotic plaques in the Tampere Vascular Study. Atherosclerosis. 2011;219(1):211-7.

120. Al-Kafaji G, Al-Mahroos G, Abdulla Al-Muhtaresh H, Sabry MA, Abdul Razzak R, Salem AH. Circulating endothelium-enriched microRNA-126 as a potential biomarker for coronary artery disease in type 2 diabetes mellitus patients. Biomarkers. 2017;22(3-4):268-78.

121. Zhou B, Li C, Qi W, Zhang Y, Zhang F, Wu JX, et al. Downregulation of miR-181a upregulates sirtuin-1 (SIRT1) and improves hepatic insulin sensitivity. Diabetologia. 2012;55(7):2032-43.

122. Karolina DS, Armugam A, Sepramaniam S, Jeyaseelan K. miRNAs and diabetes mellitus. Expert Rev Endocrinol Metab. 2012;7(3):281-300.

123. Kovacs B, Lumayag S, Cowan C, Xu S. MicroRNAs in early diabetic retinopathy in streptozotocin-induced diabetic rats. Invest Ophthalmol Vis Sci. 2011;52(7):4402-9.

124. Denby L, Ramdas V, McBride MW, Wang J, Robinson H, McClure J, et al. miR-21 and miR-214 are consistently modulated during renal injury in rodent models. Am J Pathol. 2011;179(2):661-72.

125. Baker MA, Davis SJ, Liu P, Pan X, Williams AM, Iczkowski KA, et al. Tissue-Specific MicroRNA Expression Patterns in Four Types of Kidney Disease. J Am Soc Nephrol. 2017;28(10):2985-92.

126. Li X, Li D, Wang A, Chu T, Lohcharoenkal W, Zheng X, et al. MicroRNA-132 with Therapeutic Potential in Chronic Wounds. J Invest Dermatol. 2017;137(12):2630-8.

127. Barutta F, Bellini S, Mastrocola R, Bruno G, Gruden G. MicroRNA and Microvascular Complications of Diabetes. Int J Endocrinol. 2018;2018:6890501.

128. Loscalzo J. The cellular response to hypoxia: tuning the system with microRNAs. J Clin Invest. 2010;120(11):3815-7.

129. Mulholland EJ, Dunne N, McCarthy HO. MicroRNA as Therapeutic Targets for Chronic Wound Healing. Mol Ther Nucleic Acids. 2017;8:46-55.

130. Ivan M, Huang X. miR-210: fine-tuning the hypoxic response. Adv Exp Med Biol. 2014;772:205-27.

131. Huang X, Le QT, Giaccia AJ. MiR-210--micromanager of the hypoxia pathway. Trends Mol Med. 2010;16(5):230-7.

132. Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proc Natl Acad Sci U S A. 1995;92(12):5510-4.

133. Tello D, Balsa E, Acosta-Iborra B, Fuertes-Yebra E, Elorza A, Ordonez A, et al. Induction of the mitochondrial NDUFA4L2 protein by HIF-1alpha decreases oxygen consumption by inhibiting Complex I activity. Cell Metab. 2011;14(6):768-79.

134. Chan SY, Zhang YY, Hemann C, Mahoney CE, Zweier JL, Loscalzo J. MicroRNA-210 controls mitochondrial metabolism during hypoxia by repressing the iron-sulfur cluster assembly proteins ISCU1/2. Cell Metab. 2009;10(4):273-84.

135. Chen Z, Li Y, Zhang H, Huang P, Luthra R. Hypoxia-regulated microRNA-210 modulates mitochondrial function and decreases ISCU and COX10 expression. Oncogene. 2010;29(30):4362-8.

136. Puissegur MP, Mazure NM, Bertero T, Pradelli L, Grosso S, Robbe-Sermesant K, et al. miR-210 is overexpressed in late stages of lung cancer and mediates mitochondrial alterations associated with modulation of HIF-1 activity. Cell Death Differ. 2011;18(3):465-78.

137. Fasanaro P, D'Alessandra Y, Di Stefano V, Melchionna R, Romani S, Pompilio G, et al. MicroRNA-210 modulates endothelial cell response to hypoxia and inhibits the receptor tyrosine kinase ligand Ephrin-A3. J Biol Chem. 2008;283(23):15878-83.

138. Hu S, Huang M, Li Z, Jia F, Ghosh Z, Lijkwan MA, et al. MicroRNA-210 as a novel therapy for treatment of ischemic heart disease. Circulation. 2010;122(11 Suppl):S124-31.

139. Qin L, Chen Y, Niu Y, Chen W, Wang Q, Xiao S, et al. A deep investigation into the adipogenesis mechanism: profile of microRNAs regulating adipogenesis by modulating the canonical Wnt/beta-catenin signaling pathway. BMC Genomics. 2010;11:320.

140. Mizuno Y, Tokuzawa Y, Ninomiya Y, Yagi K, Yatsuka-Kanesaki Y, Suda T, et al. miR-210 promotes osteoblastic differentiation through inhibition of AcvR1b. FEBS Lett. 2009;583(13):2263-8.

141. Biswas S, Roy S, Banerjee J, Hussain SR, Khanna S, Meenakshisundaram G, et al. Hypoxia inducible microRNA 210 attenuates keratinocyte proliferation and impairs closure in a murine model of ischemic wounds. Proc Natl Acad Sci U S A. 2010;107(15):6976-81.

142. Crosby ME, Kulshreshtha R, Ivan M, Glazer PM. MicroRNA regulation of DNA repair gene expression in hypoxic stress. Cancer Res. 2009;69(3):1221-9.

143. Greither T, Wurl P, Grochola L, Bond G, Bache M, Kappler M, et al. Expression of microRNA 210 associates with poor survival and age of tumor onset of soft-tissue sarcoma patients. Int J Cancer. 2012;130(5):1230-5.

144. Lorenzen JM, Kielstein JT, Hafer C, Gupta SK, Kumpers P, Faulhaber-Walter R, et al. Circulating miR-210 predicts survival in critically ill patients with acute kidney injury. Clin J Am Soc Nephrol. 2011;6(7):1540-6.

145. Dallas A, Trotsyuk A, Ilves H, Bonham CA, Rodrigues M, Engel K, et al. Acceleration of Diabetic Wound Healing with PHD2- and miR-210-Targeting Oligonucleotides. Tissue Eng Part A. 2019;25(1-2):44-54.

146. Brand MD. The role of mitochondria in longevity and healthspan. Longev Healthspan. 2014;3:7.

147. Siekevitz P. Powerhouse of the Cell. Scientific American. 1957;197(1):131-44.

148. Voet D VJ, Pratt CW Fundamentals of Biochemistry John Wiley and Sons, Inc. ; 2006.

149. Mozo J, Emre Y, Bouillaud F, Ricquier D, Criscuolo F. Thermoregulation: what role for UCPs in mammals and birds? Biosci Rep. 2005;25(3-4):227-49.

150. Pizzo P, Pozzan T. Mitochondria-endoplasmic reticulum choreography: structure and signaling dynamics. Trends Cell Biol. 2007;17(10):511-7.

151. Wang C, Youle RJ. The role of mitochondria in apoptosis*. Annu Rev Genet. 2009;43:95-118.

152. Li X, Fang P, Mai J, Choi ET, Wang H, Yang XF. Targeting mitochondrial reactive oxygen species as novel therapy for inflammatory diseases and cancers. J Hematol Oncol. 2013;6:19.

153. Hajnoczky G, Csordas G, Das S, Garcia-Perez C, Saotome M, Sinha Roy S, et al. Mitochondrial calcium signalling and cell death: approaches for assessing the role of mitochondrial Ca2+ uptake in apoptosis. Cell Calcium. 2006;40(5-6):553-60.

154. Rossier MF. T channels and steroid biosynthesis: in search of a link with mitochondria. Cell Calcium. 2006;40(2):155-64.

155. Klinge CM. Estrogenic control of mitochondrial function and biogenesis. J Cell Biochem. 2008;105(6):1342-51.

156. McBride HM, Neuspiel M, Wasiak S. Mitochondria: more than just a powerhouse. Curr Biol. 2006;16(14):R551-60.

157. Degli Esposti D, Hamelin J, Bosselut N, Saffroy R, Sebagh M, Pommier A, et al. Mitochondrial roles and cytoprotection in chronic liver injury. Biochem Res Int. 2012;2012:387626.

158. Fuhrmann DC, Brune B. Mitochondrial composition and function under the control of hypoxia. Redox Biol. 2017;12:208-15.

159. Kubli DA, Gustafsson AB. Mitochondria and mitophagy: the yin and yang of cell death control. Circ Res. 2012;111(9):1208-21.

160. Maiuri MC, Zalckvar E, Kimchi A, Kroemer G. Self-eating and self-killing: crosstalk between autophagy and apoptosis. Nat Rev Mol Cell Biol. 2007;8(9):741-52.

161. Plecita-Hlavata L, Jezek P. Integration of superoxide formation and cristae morphology for mitochondrial redox signaling. Int J Biochem Cell Biol. 2016;80:31-50.

162. Wise DR, Ward PS, Shay JE, Cross JR, Gruber JJ, Sachdeva UM, et al. Hypoxia promotes isocitrate dehydrogenase-dependent carboxylation of alpha-ketoglutarate to citrate to support cell growth and viability. Proc Natl Acad Sci U S A. 2011;108(49):19611-6.

163. Guaras A, Perales-Clemente E, Calvo E, Acin-Perez R, Loureiro-Lopez M, Pujol C, et al. The CoQH2/CoQ Ratio Serves as a Sensor of Respiratory Chain Efficiency. Cell Rep. 2016;15(1):197-209.

164. Chouchani ET, Pell VR, Gaude E, Aksentijevic D, Sundier SY, Robb EL, et al. Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS. Nature. 2014;515(7527):431-5.

165. Kim JW, Tchernyshyov I, Semenza GL, Dang CV. HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. Cell Metab. 2006;3(3):177-85.

166. Fukuda R, Zhang H, Kim JW, Shimoda L, Dang CV, Semenza GL. HIF-1 regulates cytochrome oxidase subunits to optimize efficiency of respiration in hypoxic cells. Cell. 2007;129(1):111-22.

167. Schonenberger MJ, Kovacs WJ. Hypoxia signaling pathways: modulators of oxygen-related organelles. Front Cell Dev Biol. 2015;3:42.

168. Papandreou I, Cairns RA, Fontana L, Lim AL, Denko NC. HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. Cell Metab. 2006;3(3):187-97.

169. Sun RC, Denko NC. Hypoxic regulation of glutamine metabolism through HIF1 and SIAH2 supports lipid synthesis that is necessary for tumor growth. Cell Metab. 2014;19(2):285-92.

170. Selvaraju V, Parinandi NL, Adluri RS, Goldman JW, Hussain N, Sanchez JA, et al. Molecular mechanisms of action and therapeutic uses of pharmacological inhibitors of HIF-prolyl 4-hydroxylases for treatment of ischemic diseases. Antioxid Redox Signal. 2014;20(16):2631-65.

171. Muz B, de la Puente P, Azab F, Azab AK. The role of hypoxia in cancer progression, angiogenesis, metastasis, and resistance to therapy. Hypoxia (Auckl). 2015;3:83-92.

172. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science. 2009;324(5930):1029-33.

173. Hayashi Y, Yokota A, Harada H, Huang G. Hypoxia/pseudohypoxia-mediated activation of hypoxia-inducible factor-1alpha in cancer. Cancer Sci. 2019;110(5):1510-7.

174. Courtnay R, Ngo DC, Malik N, Ververis K, Tortorella SM, Karagiannis TC. Cancer metabolism and the Warburg effect: the role of HIF-1 and PI3K. Mol Biol Rep. 2015;42(4):841-51.

175. Jones DP, Sies H. The Redox Code. Antioxid Redox Signal. 2015;23(9):734-46.

176. Sena LA, Chandel NS. Physiological roles of mitochondrial reactive oxygen species. Mol Cell. 2012;48(2):158-67.

177. Bleier L, Wittig I, Heide H, Steger M, Brandt U, Drose S. Generator-specific targets of mitochondrial reactive oxygen species. Free Radic Biol Med. 2015;78:1-10.

178. Brand MD. Mitochondrial generation of superoxide and hydrogen peroxide as the source of mitochondrial redox signaling. Free Radic Biol Med. 2016;100:14-31.

179. Quinlan CL, Perevoshchikova IV, Hey-Mogensen M, Orr AL, Brand MD. Sites of reactive oxygen species generation by mitochondria oxidizing different substrates. Redox Biol. 2013;1:304-12.

180. Hamanaka RB, Chandel NS. Mitochondrial reactive oxygen species regulate cellular signaling and dictate biological outcomes. Trends Biochem Sci. 2010;35(9):505-13.

181. Ristow M, Zarse K. How increased oxidative stress promotes longevity and metabolic health: The concept of mitochondrial hormesis (mitohormesis). Exp Gerontol. 2010;45(6):410-8.

182. Redza-Dutordoir M, Averill-Bates DA. Activation of apoptosis signalling pathways by reactive oxygen species. Biochim Biophys Acta. 2016;1863(12):2977-92.

183. Li Z, Xu X, Leng X, He M, Wang J, Cheng S, et al. Roles of reactive oxygen species in cell signaling pathways and immune responses to viral infections. Arch Virol. 2017;162(3):603-10.

184. Hanson ES, Leibold EA. Regulation of the iron regulatory proteins by reactive nitrogen and oxygen species. Gene Expr. 1999;7(4-6):367-76.

185. Finkel T. Signal transduction by reactive oxygen species. J Cell Biol. 2011;194(1):7-15.

186. Montgomery MK, Turner N. Mitochondrial dysfunction and insulin resistance: an update. Endocr Connect. 2015;4(1):R1-R15.

187. Pinti MV, Fink GK, Hathaway QA, Durr AJ, Kunovac A, Hollander JM. Mitochondrial dysfunction in type 2 diabetes mellitus: an organ-based analysis. Am J Physiol Endocrinol Metab. 2019;316(2):E268-E85.

188. Kakehi T, Yabe-Nishimura C. NOX enzymes and diabetic complications. Semin Immunopathol. 2008;30(3):301-14.

189. Xie L, Zhu X, Hu Y, Li T, Gao Y, Shi Y, et al. Mitochondrial DNA oxidative damage triggering mitochondrial dysfunction and apoptosis in high glucose-induced HRECs. Invest Ophthalmol Vis Sci. 2008;49(9):4203-9.

190. Schrauwen P, Hesselink MK. Oxidative capacity, lipotoxicity, and mitochondrial damage in type 2 diabetes. Diabetes. 2004;53(6):1412-7.

191. Kelley DE, He J, Menshikova EV, Ritov VB. Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. Diabetes. 2002;51(10):2944-50.

192. Hartman ML, Shirihai OS, Holbrook M, Xu G, Kocherla M, Shah A, et al. Relation of mitochondrial oxygen consumption in peripheral blood mononuclear cells to vascular function in type 2 diabetes mellitus. Vasc Med. 2014;19(1):67-74.

193. Kulkarni SS, Salehzadeh F, Fritz T, Zierath JR, Krook A, Osler ME. Mitochondrial regulators of fatty acid metabolism reflect metabolic dysfunction in type 2 diabetes mellitus. Metabolism. 2012;61(2):175-85.

194. Karaa A, Goldstein A. The spectrum of clinical presentation, diagnosis, and management of mitochondrial forms of diabetes. Pediatr Diabetes. 2015;16(1):1-9.

195. Fex M, Nicholas LM, Vishnu N, Medina A, Sharoyko VV, Nicholls DG, et al. The pathogenetic role of beta-cell mitochondria in type 2 diabetes. J Endocrinol. 2018;236(3):R145-R59.

196. Pizzino G, Irrera N, Cucinotta M, Pallio G, Mannino F, Arcoraci V, et al. Oxidative Stress: Harms and Benefits for Human Health. Oxid Med Cell Longev. 2017;2017:8416763.

197. Shadel GS, Horvath TL. Mitochondrial ROS signaling in organismal homeostasis. Cell. 2015;163(3):560-9.

198. Betteridge DJ. What is oxidative stress? Metabolism. 2000;49(2 Suppl 1):3-8.

199. Johansen JS, Harris AK, Rychly DJ, Ergul A. Oxidative stress and the use of antioxidants in diabetes: linking basic science to clinical practice. Cardiovasc Diabetol. 2005;4:5.

200. Dikalov SI, Kirilyuk IA, Voinov M, Grigor'ev IA. EPR detection of cellular and mitochondrial superoxide using cyclic hydroxylamines. Free Radic Res. 2011;45(4):417-30.

201. Xu C, Bentinger M, Savu O, Moshfegh A, Sunkari V, Dallner G, et al. Mono-epoxy-tocotrienol-alpha enhances wound healing in diabetic mice and stimulates in vitro angiogenesis and cell migration. J Diabetes Complications. 2017;31(1):4-12.

202. Birben E, Sahiner UM, Sackesen C, Erzurum S, Kalayci O. Oxidative stress and antioxidant defense. World Allergy Organ J. 2012;5(1):9-19.

203. Church DF, Pryor WA. Free-radical chemistry of cigarette smoke and its toxicological implications. Environ Health Perspect. 1985;64:111-26.

204. Narayanan PK, Goodwin EH, Lehnert BE. Alpha particles initiate biological production of superoxide anions and hydrogen peroxide in human cells. Cancer Res. 1997;57(18):3963-71.

205. Stohs SJ, Bagchi D. Oxidative mechanisms in the toxicity of metal ions. Free Radic Biol Med. 1995;18(2):321-36.

206. Hiltermann JT, Lapperre TS, van Bree L, Steerenberg PA, Brahim JJ, Sont JK, et al. Ozone-induced inflammation assessed in sputum and bronchial lavage fluid from asthmatics: a new noninvasive tool in epidemiologic studies on air pollution and asthma. Free Radic Biol Med. 1999;27(11-12):1448-54.

207. Maritim AC, Sanders RA, Watkins JB, 3rd. Diabetes, oxidative stress, and antioxidants: a review. J Biochem Mol Toxicol. 2003;17(1):24-38.

208. Montano SJ, Grunler J, Nair D, Tekle M, Fernandes AP, Hua X, et al. Glutaredoxin mediated redox effects of coenzyme Q10 treatment in type 1 and type 2 diabetes patients. BBA Clin. 2015;4:14-20.

209. Holmgren A. Antioxidant function of thioredoxin and glutaredoxin systems. Antioxid Redox Signal. 2000;2(4):811-20.

210. Dalton TP, Shertzer HG, Puga A. Regulation of gene expression by reactive oxygen. Annu Rev Pharmacol Toxicol. 1999;39:67-101.

211. Cooke MS, Evans MD, Dizdaroglu M, Lunec J. Oxidative DNA damage: mechanisms, mutation, and disease. Faseb J. 2003;17(10):1195-214.

212. Ayala A, Munoz MF, Arguelles S. Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. Oxid Med Cell Longev. 2014;2014:360438.

213. Uchida K, Shiraishi M, Naito Y, Torii Y, Nakamura Y, Osawa T. Activation of stress signaling pathways by the end product of lipid peroxidation. 4-hydroxy-2-nonenal is a potential inducer of intracellular peroxide production. J Biol Chem. 1999;274(4):2234-42.

214. Stadtman ER, Moskovitz J, Levine RL. Oxidation of methionine residues of proteins: biological consequences. Antioxid Redox Signal. 2003;5(5):577-82.

215. Gilmore TD. Introduction to NF-kappaB: players, pathways, perspectives. Oncogene. 2006;25(51):6680-4.

216. Bentinger M, Tekle M, Brismar K, Chojnacki T, Swiezewska E, Dallner G. Polyisoprenoid epoxides stimulate the biosynthesis of coenzyme Q and inhibit cholesterol synthesis. J Biol Chem. 2008;283(21):14645-53.

217. Bentinger M, Brismar K, Dallner G. The antioxidant role of coenzyme Q. Mitochondrion. 2007;7 Suppl:S41-50.

218. Acosta MJ, Vazquez Fonseca L, Desbats MA, Cerqua C, Zordan R, Trevisson E, et al. Coenzyme Q biosynthesis in health and disease. Biochim Biophys Acta. 2016;1857(8):1079-85.

219. Turunen M, Olsson J, Dallner G. Metabolism and function of coenzyme Q. Biochim Biophys Acta. 2004;1660(1-2):171-99.

220. Miles MV. The uptake and distribution of coenzyme Q10. Mitochondrion. 2007;7 Suppl:S72-7.

221. Buhaescu I, Izzedine H. Mevalonate pathway: a review of clinical and therapeutical implications. Clin Biochem. 2007;40(9-10):575-84.

222. Bentinger M, Tekle M, Brismar K, Chojnacki T, Swiezewska E, Dallner G. Stimulation of coenzyme Q synthesis. Biofactors. 2008;32(1-4):99-111.

223. Stefely JA, Pagliarini DJ. Biochemistry of Mitochondrial Coenzyme Q Biosynthesis. Trends Biochem Sci. 2017;42(10):824-43.

224. Tran UC, Clarke CF. Endogenous synthesis of coenzyme Q in eukaryotes. Mitochondrion. 2007;7 Suppl:S62-71.

225. Low P, Andersson M, Edlund C, Dallner G. Effects of mevinolin treatment on tissue dolichol and ubiquinone levels in the rat. Biochim Biophys Acta. 1992;1165(1):102-9.

226. Bentinger M, Tekle M, Dallner G. Coenzyme Q--biosynthesis and functions. Biochem Biophys Res Commun. 2010;396(1):74-9.

227. Kawamukai M. Biosynthesis of coenzyme Q in eukaryotes. Biosci Biotechnol Biochem. 2016;80(1):23-33.

228. Genova ML, Lenaz G. Functional role of mitochondrial respiratory supercomplexes. Biochim Biophys Acta. 2014;1837(4):427-43.

229. Lapuente-Brun E, Moreno-Loshuertos R, Acin-Perez R, Latorre-Pellicer A, Colas C, Balsa E, et al. Supercomplex assembly determines electron flux in the mitochondrial electron transport chain. Science. 2013;340(6140):1567-70.

230. Echtay KS, Winkler E, Frischmuth K, Klingenberg M. Uncoupling proteins 2 and 3 are highly active H(+) transporters and highly nucleotide sensitive when activated by coenzyme Q (ubiquinone). Proc Natl Acad Sci U S A. 2001;98(4):1416-21.

231. Papucci L, Schiavone N, Witort E, Donnini M, Lapucci A, Tempestini A, et al. Coenzyme q10 prevents apoptosis by inhibiting mitochondrial depolarization independently of its free radical scavenging property. J Biol Chem. 2003;278(30):28220-8.

232. Stocker R, Bowry VW, Frei B. Ubiquinol-10 protects human low density lipoprotein more efficiently against lipid peroxidation than does alpha-tocopherol. Proc Natl Acad Sci U S A. 1991;88(5):1646-50.

233. Mukai K, Kikuchi S, Urano S. Stopped-flow kinetic study of the regeneration reaction of tocopheroxyl radical by reduced ubiquinone-10 in solution. Biochim Biophys Acta. 1990;1035(1):77-82.

234. Scialo F, Sriram A, Fernandez-Ayala D, Gubina N, Lohmus M, Nelson G, et al. Mitochondrial ROS Produced via Reverse Electron Transport Extend Animal Lifespan. Cell Metab. 2016;23(4):725-34.

235. Sen CK, Khanna S, Roy S. Tocotrienols in health and disease: the other half of the natural vitamin E family. Mol Aspects Med. 2007;28(5-6):692-728.

236. Forsberg E, Xu C, Grunler J, Frostegard J, Tekle M, Brismar K, et al. Coenzyme Q10 and oxidative stress, the association with peripheral sensory neuropathy and cardiovascular disease in type 2 diabetes mellitus. J Diabetes Complications. 2015;29(8):1152-8.

237. Sato Y, Hagiwara K, Arai H, Inoue K. Purification and characterization of the alpha-tocopherol transfer protein from rat liver. FEBS Lett. 1991;288(1-2):41-5.

238. Mishima K, Tanaka T, Pu F, Egashira N, Iwasaki K, Hidaka R, et al. Vitamin E isoforms alpha-tocotrienol and gamma-tocopherol prevent cerebral infarction in mice. Neurosci Lett. 2003;337(1):56-60.

239. Budin SB, Othman F, Louis SR, Bakar MA, Das S, Mohamed J. The effects of palm oil tocotrienol-rich fraction supplementation on biochemical parameters, oxidative stress and the vascular wall of streptozotocin-induced diabetic rats. Clinics (Sao Paulo). 2009;64(3):235-44.

240. Nakagawa K, Shibata A, Yamashita S, Tsuzuki T, Kariya J, Oikawa S, et al. In vivo angiogenesis is suppressed by unsaturated vitamin E, tocotrienol. J Nutr. 2007;137(8):1938-43.

241. Colombo ML. An update on vitamin E, tocopherol and tocotrienol-perspectives. Molecules. 2010;15(4):2103-13.

242. Montonen J, Knekt P, Jarvinen R, Reunanen A. Dietary antioxidant intake and risk of type 2 diabetes. Diabetes Care. 2004;27(2):362-6.

243. Ge T, Yang J, Zhou S, Wang Y, Li Y, Tong X. The Role of the Pentose Phosphate Pathway in Diabetes and Cancer. Front Endocrinol (Lausanne). 2020;11:365.

244. Vessby J, Basu S, Mohsen R, Berne C, Vessby B. Oxidative stress and antioxidant status in type 1 diabetes mellitus. J Intern Med. 2002;251(1):69-76.

245. Feillet-Coudray C, Rock E, Coudray C, Grzelkowska K, Azais-Braesco V, Dardevet D, et al. Lipid peroxidation and antioxidant status in experimental diabetes. Clin Chim Acta. 1999;284(1):31-43.

246. van der Schaft N, Schoufour JD, Nano J, Kiefte-de Jong JC, Muka T, Sijbrands EJG, et al. Dietary antioxidant capacity and risk of type 2 diabetes mellitus, prediabetes and insulin resistance: the Rotterdam Study. Eur J Epidemiol. 2019;34(9):853-61.

247. Mancini FR, Affret A, Dow C, Balkau B, Bonnet F, Boutron-Ruault MC, et al. Dietary antioxidant capacity and risk of type 2 diabetes in the large prospective E3N-EPIC cohort. Diabetologia. 2018;61(2):308-16.

248. Sindhu RK, Koo JR, Roberts CK, Vaziri ND. Dysregulation of hepatic superoxide dismutase, catalase and glutathione peroxidase in diabetes: response to insulin and antioxidant therapies. Clin Exp Hypertens. 2004;26(1):43-53.

249. Sekhar RV, McKay SV, Patel SG, Guthikonda AP, Reddy VT, Balasubramanyam A, et al. Glutathione synthesis is diminished in patients with uncontrolled diabetes and restored by dietary supplementation with cysteine and glycine. Diabetes Care. 2011;34(1):162-7.

250. Davison GW, Ashton T, George L, Young IS, McEneny J, Davies B, et al. Molecular detection of exercise-induced free radicals following ascorbate prophylaxis in type 1 diabetes mellitus: a randomised controlled trial. Diabetologia. 2008;51(11):2049-59.

251. Shi TJ, Zhang MD, Zeberg H, Nilsson J, Grunler J, Liu SX, et al. Coenzyme Q10 prevents peripheral neuropathy and attenuates neuron loss in the db-/db- mouse, a type 2 diabetes model. Proc Natl Acad Sci U S A. 2013;110(2):690-5.

252. Giacco F, Brownlee M. Oxidative stress and diabetic complications. Circ Res. 2010;107(9):1058-70.

253. Mortensen SA, Rosenfeldt F, Kumar A, Dolliner P, Filipiak KJ, Pella D, et al. The effect of coenzyme Q10 on morbidity and mortality in chronic heart failure: results from Q-SYMBIO: a randomized double-blind trial. JACC Heart Fail. 2014;2(6):641-9.

254. Balbi ME, Tonin FS, Mendes AM, Borba HH, Wiens A, Fernandez-Llimos F, et al. Antioxidant effects of vitamins in type 2 diabetes: a meta-analysis of randomized controlled trials. Diabetol Metab Syndr. 2018;10:18.

255. Rabbani N, Alam SS, Riaz S, Larkin JR, Akhtar MW, Shafi T, et al. High-dose thiamine therapy for patients with type 2 diabetes and microalbuminuria: a randomised, double-blind placebo-controlled pilot study. Diabetologia. 2009;52(2):208-12.

256. Szabo C, Biser A, Benko R, Bottinger E, Susztak K. Poly(ADP-ribose) polymerase inhibitors ameliorate nephropathy of type 2 diabetic Leprdb/db mice. Diabetes. 2006;55(11):3004-12.

257. Salvemini D, Wang ZQ, Zweier JL, Samouilov A, Macarthur H, Misko TP, et al. A nonpeptidyl mimic of superoxide dismutase with therapeutic activity in rats. Science. 1999;286(5438):304-6.

258. Vincent AM, Russell JW, Sullivan KA, Backus C, Hayes JM, McLean LL, et al. SOD2 protects neurons from injury in cell culture and animal models of diabetic neuropathy. Exp Neurol. 2007;208(2):216-27.

259. Karvestedt L, Martensson E, Grill V, Elofsson S, von Wendt G, Hamsten A, et al. Peripheral sensory neuropathy associates with micro- or macroangiopathy: results from a population-based study of type 2 diabetic patients in Sweden. Diabetes Care. 2009;32(2):317-22.

260. Gu HF, Gu T, Hilding A, Zhu Y, Karvestedt L, Ostenson CG, et al. Evaluation of IGFBP-7 DNA methylation changes and serum protein variation in Swedish subjects with and without type 2 diabetes. Clin Epigenetics. 2013;5(1):20.

261. Karvestedt L, Martensson E, Grill V, Elofsson S, von Wendt G, Hamsten A, et al. The prevalence of peripheral neuropathy in a population-based study of patients with type 2 diabetes in Sweden. J Diabetes Complications. 2011;25(2):97-106.

262. Rajamand Ekberg N. Studies on the Adaptive Responses to Hypoxia (HYKRAND), ClinicalTrials.gov Identifier: NCT02629406. 2015 [cited 2020 20 Feb].

263. Kobayashi K, Forte TM, Taniguchi S, Ishida BY, Oka K, Chan L. The db/db mouse, a model for diabetic dyslipidemia: molecular characterization and effects of Western diet feeding. Metabolism. 2000;49(1):22-31.

264. Mazzone M, Dettori D, de Oliveira RL, Loges S, Schmidt T, Jonckx B, et al. Heterozygous deficiency of PHD2 restores tumor oxygenation and inhibits metastasis via endothelial normalization. Cell. 2009;136(5):839-51.

265. Salani D, Taraboletti G, Rosano L, Di Castro V, Borsotti P, Giavazzi R, et al. Endothelin-1 induces an angiogenic phenotype in cultured endothelial cells and stimulates neovascularization in vivo. Am J Pathol. 2000;157(5):1703-11.

266. Liang CC, Park AY, Guan JL. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. Nat Protoc. 2007;2(2):329-33.

267. Rogers GW, Brand MD, Petrosyan S, Ashok D, Elorza AA, Ferrick DA, et al. High throughput microplate respiratory measurements using minimal quantities of isolated mitochondria. PLoS One. 2011;6(7):e21746.

268. Schiffer TA, Gustafsson H, Palm F. Kidney outer medulla mitochondria are more efficient compared with cortex mitochondria as a strategy to sustain ATP production in a suboptimal environment. Am J Physiol Renal Physiol. 2018;315(3):F677-F81.

269. Lorgis L, Zeller M, Dentan G, Sicard P, Richard C, Buffet P, et al. The free oxygen radicals test (FORT) to assess circulating oxidative stress in patients with acute myocardial infarction. Atherosclerosis. 2010;213(2):616-21.

270. Zheng X, Ruas JL, Cao R, Salomons FA, Cao Y, Poellinger L, et al. Cell-type-specific regulation of degradation of hypoxia-inducible factor 1 alpha: role of subcellular compartmentalization. Mol Cell Biol. 2006;26(12):4628-41.

271. World Medical A. World Medical Association Declaration of Helsinki. Ethical principles for medical research involving human subjects. Bull World Health Organ. 2001;79(4):373-4.

272. Statens jordbruksverks föreskrifter och allmänna råd om försöksdjur, Stat. SJVFS 2019:9 Saknr L50 (2019-03-29, 2019).

273. Qiao Q, Jousilahti P, Eriksson J, Tuomilehto J. Predictive properties of impaired glucose tolerance for cardiovascular risk are not explained by the development of overt diabetes during follow-up. Diabetes Care. 2003;26(10):2910-4.

274. Vincent AM, Callaghan BC, Smith AL, Feldman EL. Diabetic neuropathy: cellular mechanisms as therapeutic targets. Nat Rev Neurol. 2011;7(10):573-83.

275. Giannubilo SR, Tiano L, Cecchi S, Principi F, Tranquilli AL, Littarru GP. Plasma coenzyme Q10 is increased during gestational diabetes. Diabetes Res Clin Pract. 2011;94(2):230-5.

276. Lim SC, Lekshminarayanan R, Goh SK, Ong YY, Subramaniam T, Sum CF, et al. The effect of coenzyme Q10 on microcirculatory endothelial function of subjects with type 2 diabetes mellitus. Atherosclerosis. 2008;196(2):966-9.

277. Irie HY, Pearline RV, Grueneberg D, Hsia M, Ravichandran P, Kothari N, et al. Distinct roles of Akt1 and Akt2 in regulating cell migration and epithelial-mesenchymal transition. J Cell Biol. 2005;171(6):1023-34.

278. Osipova J, Fischer DC, Dangwal S, Volkmann I, Widera C, Schwarz K, et al. Diabetes-associated microRNAs in pediatric patients with type 1 diabetes mellitus: a cross-sectional cohort study. J Clin Endocrinol Metab. 2014;99(9):E1661-5.

279. Assmann TS, Recamonde-Mendoza M, Punales M, Tschiedel B, Canani LH, Crispim D. MicroRNA expression profile in plasma from type 1 diabetic patients: Case-control study and bioinformatic analysis. Diabetes Res Clin Pract. 2018;141:35-46.

280. Nielsen LB, Wang C, Sorensen K, Bang-Berthelsen CH, Hansen L, Andersen ML, et al. Circulating levels of microRNA from children with newly diagnosed type 1 diabetes and healthy controls: evidence that miR-25 associates to residual beta-cell function and glycaemic control during disease progression. Exp Diabetes Res. 2012;2012:896362.

281. Duennwald T, Bernardi L, Gordin D, Sandelin A, Syreeni A, Fogarty C, et al. Effects of a single bout of interval hypoxia on cardiorespiratory control in patients with type 1 diabetes. Diabetes. 2013;62(12):4220-7.

282. Duennwald T, Gatterer H, Groop PH, Burtscher M, Bernardi L. Effects of a single bout of interval hypoxia on cardiorespiratory control and blood glucose in patients with type 2 diabetes. Diabetes Care. 2013;36(8):2183-9.

283. Palm F. Intrarenal oxygen in diabetes and a possible link to diabetic nephropathy. Clin Exp Pharmacol Physiol. 2006;33(10):997-1001.

284. Bernardi L, Gordin D, Bordino M, Rosengard-Barlund M, Sandelin A, Forsblom C, et al. Oxygen-induced impairment in arterial function is corrected by slow breathing in patients with type 1 diabetes. Sci Rep. 2017;7(1):6001.

285. Miwa S, Brand MD. Mitochondrial matrix reactive oxygen species production is very sensitive to mild uncoupling. Biochem Soc Trans. 2003;31(Pt 6):1300-1.

286. Chen N, Hao C, Peng X, Lin H, Yin A, Hao L, et al. Roxadustat for Anemia in Patients with Kidney Disease Not Receiving Dialysis. N Engl J Med. 2019;381(11):1001-10.

287. Dhillon S. Roxadustat: First Global Approval. Drugs. 2019;79(5):563-72.

288. Narayanan SK. A multifactorial approach to targeting signalling pathways in diabetic foot ulcers. 48 sidor p.

289. Savu O, Sunkari VG, Botusan IR, Grunler J, Nikoshkov A, Catrina SB. Stability of mitochondrial DNA against reactive oxygen species (ROS) generated in diabetes. Diabetes Metab Res Rev. 2011;27(5):470-9.

290. Botusan IR, Zheng X, Narayanan S, Grünler J, Sunkari VG, Calissendorff FS, et al. Deficiency of liver-derived insulin-like growth factor-I (IGF-I) does not interfere with the skin wound healing rate. PLoS One. 2018;13(3):e0193084.

291. Verma S, McMurray JJV. SGLT2 inhibitors and mechanisms of cardiovascular benefit: a state-of-the-art review. Diabetologia. 2018;61(10):2108-17.

292. American Diabetes A. 11. Microvascular Complications and Foot Care: Standards of Medical Care in Diabetes-2020. Diabetes Care. 2020;43(Suppl 1):S135-S51.

293. Perez-Favila A, Martinez-Fierro ML, Rodriguez-Lazalde JG, Cid-Baez MA, Zamudio-Osuna MJ, Martinez-Blanco MDR, et al. Current Therapeutic Strategies in Diabetic Foot Ulcers. Medicina (Kaunas). 2019;55(11).

294. Jafari M, Mousavi SM, Asgharzadeh A, Yazdani N. Coenzyme Q10 in the treatment of heart failure: A systematic review of systematic reviews. Indian Heart J. 2018;70 Suppl 1:S111-S7.

295. Zhang SY, Yang KL, Zeng LT, Wu XH, Huang HY. Effectiveness of Coenzyme Q10 Supplementation for Type 2 Diabetes Mellitus: A Systematic Review and Meta-Analysis. Int J Endocrinol. 2018;2018:6484839.

296. Catrina S-B. Desferal Administration to Improve the Impaired Reaction to Hypoxia in Diabetes (DESIRED), ClinicalTrials.gov Identifier: NCT03085771. 2018.

297. Effect of Deferoxamine on Wound Healing Rate in Patients With Diabetes Foot Ulcers (DEFEHU), ClinicalTrials.gov Identifier: NCT03137966 [database on the Internet]. 2017.

298. Bessho R, Takiyama Y, Takiyama T, Kitsunai H, Takeda Y, Sakagami H, et al. Hypoxia-inducible factor-1alpha is the therapeutic target of the SGLT2 inhibitor for diabetic nephropathy. Sci Rep. 2019;9(1):14754.

299. Haythorne E, Rohm M, van de Bunt M, Brereton MF, Tarasov AI, Blacker TS, et al. Diabetes causes marked inhibition of mitochondrial metabolism in pancreatic beta-cells. Nat Commun. 2019;10(1):2474.

300. Kim J. Regulation of Immune Cell Functions by Metabolic Reprogramming. J Immunol Res. 2018;2018:8605471.

301. Keane KN, Calton EK, Carlessi R, Hart PH, Newsholme P. The bioenergetics of inflammation: insights into obesity and type 2 diabetes. Eur J Clin Nutr. 2017;71(7):904-12.

302. Chen J, Stimpson SE, Fernandez-Bueno GA, Mathews CE. Mitochondrial Reactive Oxygen Species and Type 1 Diabetes. Antioxid Redox Signal. 2018;29(14):1361-72.