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Studies of Insulin and Cytokine Regulation of Fatty Acid Desaturases, FOXO3A and FOXO3A Target Genes in THP-1 Monocytes

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Let food be thy medicine, thy medicine shall be thy food.

–Hippocrates (460 BC –370 BC)

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Contents

SUMMARY

The increase of obesity that we have experienced during the last decades and its association with insulin resistance, type 2 diabetes and other metabolic diseases has resulted in an enormous interest for understanding the mechanisms underlying these disorders. Tissue inflammation triggered by food with a high glycemic index has been suggested to be an important mediator in the development of insulin resistance. Despite great research efforts lately, more research is needed in order to understand how nutrients interact with the genetic factors that control and triggers the inflammatory responses.

The composition of macronutrients in a diet influences the levels of insulin secretion in the body. Besides controlling the blood glucose concentration, insulin also regulates a range of inflammatory processes. Inflammation is largely dependent on some small cell-signaling molecules called cytokines, as these activate a wide range of inflammatory-related genes.

The objective of this study is to explore the regulatory effects of insulin and cytokines on the transcription of the following selected genes related to inflammation; D5D, D6D, SCD and FOXO3A. In addition, expression of TRAIL, BTG1 and TWIST1 is studied as they all are target genes for FOXO3A, and related to inflammatory processes and/or glucose metabolism. Quantitative-PCR was used to study mRNA expression of relevant genes in THP-1 cells treated with insulin and cytokines.

As the investigation was performed on THP-1 monocytes, it was necessary to optimize the *in vitro* conditions in order to obtain a maximal response from the insulin and cytokine treatments. The concentration of insulin was an important factor in this study, because the regulation of FOXO3A and desaturases (D5D, D6D and SCD) mRNA expression seemed to be dose-dependent. The treatment period was also critical, as a set of time-course experiments revealed that FOXO3A and the desaturases were regulated by insulin and cytokines at different time-points.

In this study, THP-1 cells treated with insulin and/or cytokines revealed significant regulations of the relevant genes. Gene expression of D5D, D6D and SCD was significantly up-regulated in response to insulin. Furthermore, mRNA expression of the transcription factor FOXO3A was significantly down-regulated by insulin, IL-1β and TNF-α. However, neither FOXO3A nor the desaturases were cooperatively regulated by these stimulating factors.

TRAIL, TWIST and BTG1 on the other hand, were significantly up-regulated in a synergistic manner when cells were treated with a combination of insulin, IL-1β and TNF-α.

The observed regulation of gene expressions in THP-1 monocytes treated with insulin and cytokines suggests that insulin may affect the regulation of inflammatory related genes in circulating human monocytes. As insulin is secreted in the bloodstream followed by elevated levels of glucose after a meal, these results may reflect possible diet-induced changes in gene expression.

SAMMENDRAG

Økende tilfeller av fedme og fedme-relaterte lidelser som insulinresistans, type-2 diabetes og andre metabolske sykdommer har ført til en tiltagende interesse for å forstå de molekylærbiologiske mekanismene bak disse tilstandene. Kronisk betennelse trigget av mat med en høy glykemisk indeks antas nå for å være en viktig faktor i utviklingen av insulinresistens. Til tross for en allerede stor forskingsinnsats på dette feltet, kreves det ytterligere forskning for å forstå hvordan de ulike næringsstoffene og sammensetningen av dem påvirker de genetiske faktorene som regulerer inflammatoriske prosesser i kroppen.

Kostholdsammensetningen påvirker insulinkonsentrasjonen i kroppen, ettersom insulin utskilles i blodet som følge av økt glukose nivå. I tillegg til å kontrollere blodsukkernivået i kroppen, er insulin en viktig regulator for en rekke inflammatoriske prosesser. Disse prosessene er i stor grad avhengig av signalmolekyler kalt cytokiner. Cytokiner aktiverer en rekke gen som spiller en viktig rolle i inflammatoriske prosesser.

I dette studiet ble det undersøkt om insulin og cytokiner kunne påvirke transkripsjonen til følgende betennelses-relaterte gen: D5D, D6D, SCD og FOXO3A. I tillegg ble transkripsjon av TRAIL, BTG1 og TWIST1 studert ettersom de alle er målgener for FOXO3A, og i seg selv er relatert til inflammatoriske prosesser og/eller glukosemetabolismen. cDNA fra THP-1 celler stimulert med insulin, IL-1β og TNF-α ble brukt som templat for kvantitativ PCR for å kvantifisere uttrykk av relevante gen i forhold til de ulike stimuleringene.

Ettersom studiet ble utført i THP-1 monocytter, var det nødvendig å optimalisere stimuleringstid og konsentrasjon av stimuleringsfaktorene for å påvise eventuelle reguleringer av transkripsjon som følge av insulin og cytokiner. Insulinkonsentrasjonen viste seg å være en viktig faktor, ettersom regulering av desaturasene (D5D, D6D og SCD) og FOXO3A viste seg å være konsentrasjonsavhengig. Antall timer stimulering var også essensielt, da FOXO3A og desaturasene ble regulert av insulin og cytokiner ved spesifikke tidspunkt.

Genuttrykk av D5D, D6D og SCD viste seg å være betydelig oppregulert i THP-1 celler stimulert med insulin. Transkripsjon av FOXO3A ble derimot betydelig nedregulert som følge av insulin stimulering. Denne nedreguleringen ble også observert i celler stimulert med IL-1β og TNF-α. En synergistisk effekt av insulin og cytokiner ble observert i reguleringen av

TRAIL, TWIST og BTG1 transkripsjon. Det ble i midlertidig ikke observert en kombinert effekt for transkripsjonsregulering av FOXO3A eller desaturasene.

Ettersom genuttrykk ble regulert av insulin og cytokiner i THP-1 celler, er det mulig at disse observasjonene også forekommer i humane monocytter. Ettersom økt glukosekonsentrasjon trigger insulinutskillelse som følge av et måltid, kan disse resultatene reflektere mulige endringer i genuttrykk som følge av kostholdssammensetning.

1. ABBREVATIONS

18s rRNA	18S ribosomal RNA
AA	Arachidonic acid
ALA	α-Linoleic acid
B_2M	Beta-2-microglubin
BLAST	Nucleotide basic local alignment search tool
CCL2	Chemokine (C-C motif) ligand 2
cDNA	Complementary DNA
Cq	Quantitative cycle
D5D	Delta-5-desaturase
D6D	Delta-6-desaturase
DDB1	DNA-damage binding protein 1
DGLA	Dihomo-γ-linoleic acid
DHA	Docosaexaenoic acid
DMSO	Dimethylsulfoxide
FA	Fatty acids
FBS	Fetal bovine serum
FCS	Fetal calf serum
FFA	Free fatty acids
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLA	γ-Linoleic acid
HPRT1	Hypoxanthine phosphoribosyl-transferase 1
IGF-1	Insulin-like growth factor 1
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
IR	Insulin receptor
IRS	Insulin receptor substrate
LA	Linoleic acid
mRNA	Messenger RNA
MUFA	Monounsaturated fatty acid
NF-κB	Nuclear factor κB
OA	Oleic acid
PI3K	Phosphatidylinositol-3 kinase
PIP_2	Phosphatidylinositol-4.5-biphosphate
PIP_3	Phosphatidylinositol-3.4.5-triphosphate

PUFA	Polyunsaturated fatty acid
qPCR	Quantitative real-time polymerase chain reaction
SCD	Stearoyl-CoA desaturase
SRE	Sterol regulatory element
SREBP-1c	Sterol regulatory element binding factor 1c
STA	Stearidonic acid
TG	Triglycerides
T_m	Annealing temperature
TNF-α	Tumor necrosis factor alpha
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
TWIST1	Twist-related protein 1
YBX1	Y box binding protein 1
ω3	Omega 3
ω6	Omega 6

2. INRODUCTION

2.1 Diet related disease risk

Over the last 50 years, the occurrence of human obesity has escalated epidemically worldwide. By 2005, 1.6 billion adults were considered as overweight, and more than 400 million were affected by obesity. As a consequence of this trend, the prevalence of obesity-associated maladies such as type 2 diabetes (T2D), cardiovascular diseases (CVD) and certain forms of cancer have shown a tremendous increase [1].

As an action to the increasing number of obesity cases, the main focus has been to reduce the dietary intake of fat. Both the public and the food industry have followed recommendations given by health organizations worldwide. Although the recommended composition of the three macronutrients in food varies from country to country, the message has been the same; to decrease the dietary intake of fat, while increasing the dietary carbohydrate intake. According to The US department of Health and Human Services and the US Department of Agriculture dietary fat intake decreased from 40 E% to 33 E% from the 1960s to 1995 ,while the carbohydrate intake has increased from 45 E % to 52 E% in the same period. [2, 3]

Even though the American and European population has adapted to the above mentioned recommendations, cardiovascular diseases are still considered as a number one cause of death, and cases of obesity and type 2 diabetes continue to rise. This trend has engaged researchers to examine the effect of the increased proportion of dietary carbohydrates relative to fat and proteins, and its possible harmful effects on human health.

2.2 Function and metabolism of dietary fats

2.2.1 Dietary Fats

There are a variety of types of fat, due to their different chemical structure and physical properties. A major part of dietary fats consists of triglycerides (TG), but fat also include cholesterol, phospholipids and free fatty acids (FFA). TGs are made up by triesters of glycerol and any kind of fatty acids, and constitute the majority of energy stored in the body [4].

Besides being a source of energy, dietary fatty acids have a variety of functions required for normal growth and development. These biocompounds play a major role in a variety of metabolic pathways, and are also incorporated into the lipid bilayer of cell membranes. Fatty acids are divided into two main groups; saturated- and unsaturated acids. Unsaturated acid is further categorized as monounsaturated acids (MUFAs) and polyunsaturated fatty acids (PUFAs). In human beings, not all fatty acids can be synthesized *de novo*, due to lack of certain enzymes. Omega 3 (ω3) and omega 6 (ω6) PUFAs are essential for survival, but require a dietary intake of their precursor; Linoleic Acid (LA) and α-Linoleic Acid (ALA). Thus, these are considered as essential fatty acids [5]

2.2.2 Desaturases in the metabolism of unsaturated fatty acids

The biosynthesis of PUFAs is catalyzed by a set of enzymes known as fatty acid desaturases. There is a variety of desaturases, and they are all specific for the double bonds of the PUFA. Delta-5-desaturase (D5D) and Delta-6-desaturase (D6D) are responsible for the desaturation of LA and ALA in the biosynthesis of various ω6 and ω3 PUFAs [6].

In the first step of the ω6 PUFA pathway, LA is desaturated into γ-Linoleic acid (GLA, 18:3). ALA is desaturated by the same enzyme, but is converted to stearidonic acid (STA, 18:4) [7] (figure 1). The reaction is followed by an elongation step catalyzed by elongase which converts GLA to dihomo-γ-linoleic acid (DGLA, 20:3) and STA to eicosatetraenoic acid (20:4). D5D is responsible for the second desaturation in these two pathways. By introducing a double bond at carbon number 5, DGLA and eicosatetraenoic acid are desaturated to arachidonic acid (AA, 20:4) and eicosapentaenoic acid (EPA, 20:5). As a final step in the ω3-

pathway, docosaexaenoic acid (DHA, 22:6) may be synthesized from EPA through elongation and desaturation [6, 7].

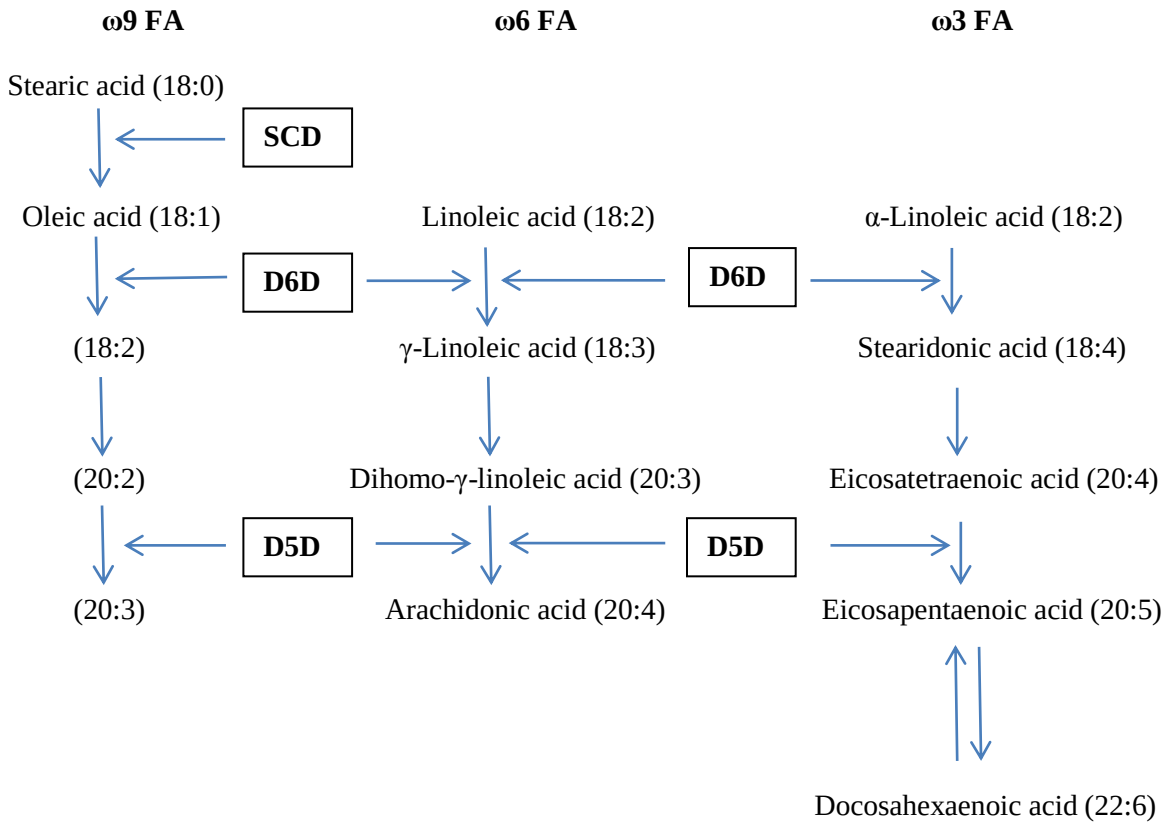

Figure 1: Metabolism of ω9, ω6 and ω3 PUFA in mammals. Modified figure [8].

D5D and D6D do also act in the metabolism of MUFAs. In the biosynthesis of MUFAs, stearic acid (18:0) is desaturated to form oleic acid (OA (18:1) by stearoyl-CoA desaturase (SCD). SCD introduces a double bond at carbon number 9, and is therefore also called delta-9-desaturase. D6D and D5D catalyze the further metabolism of OA [9].

2.2.3 The role of unsaturated fatty acids in inflammation

Inflammation caused by infection, irritation or other injury is a complex response and is characterized by redness, swelling, heat and pain. The classical, acute inflammation is a strictly regulated process, that will continue until the tissue damage is repaired or the pathogen is defeated [10]. PUFAs are important in inflammation as some of theme serve as

precursors for metabolites in the initiation, progression or resolution of the process. Metabolites derived from the ω6 FA AA, such as the 2-series of prostaglandins, thromboxanes and the 4-series of leukotrienes act as pro-inflammatory compounds and activate responses like cytokine production [11]. However, some signal molecules made from AA may also act as key mediators of the resolution of the inflammatory process, like resolvins and lipoxins. This is also true for resolvins and protectins derived from ω3 FAs DGLA and GLA which has a number of anti-inflammatory properties [12].

In modern diet, there are few sources of ω3 FAs, while ω6 FAs can be found in a range of different food items. Thus, most people obtain a high ω6 to ω3 ratio through their meals. As metabolites derived from PUFAs have opposite roles regarding inflammation, there should be a balance between these. As D5D promote the formation of pro-inflammatory compounds as well as anti-inflammatory (see section 2.2.2), it is reasonable to believe that regulation of D5D should have antagonistic properties in the inflammatory process. But due to the low presence of ALA in most people's diet, the anti-inflammatory outcome from D5D activity is almost negligible.

The imbalance of ω6 to ω3 FAs may be part of the explanation of the significant rise of diseases like atherosclerosis, psoriasis, cancer, arthritis and chronic pulmonary diseases which are all associated with a condition called chronic low grade inflammation [13]. In acute inflammation, the inflammatory conditions will ideally terminate when the immune system has responded to and accomplished the damage. Chronic low grade inflammation on the other hand, may continue for year, decades or even a life time [14]. Chronic inflammation will be further discussed in section 2.4.3

2.3 Cellular signaling pathways and gene control – an overview

Cell signaling is a complex system where cells are able to respond to changes in the environment, and facilitate intracellular- and extracellular communication. Basically, signaling involves an interaction between a soluble molecule (ligand) and a cell membrane-bound protein (receptor) (figure 2). There are an enormous number of different signaling pathways, and they typically get initiated when the ligand binds to its receptor inside or outside of the cell. A water soluble signal requires a receptor to transmit the message into the cell, while a membrane soluble ligand may diffuse through the cell membrane. When a ligand

binds to its specific receptor, it activates a series of events inside the cell. Intracellular enzymes like protein kinases and protein phosphatases are commonly found in signal pathways. Kinases and phosphatases activate certain transduction proteins in the signal cascade through phosphorylation and dephosphorylation respectively. Once an enzyme is activated, it may activate up to several downstream target enzymes and thereby amplify the intensity of a signal. The signal cascade will eventually result in protein synthesis, protein secreting, metabolism alterations or differentiation of the cell [15].

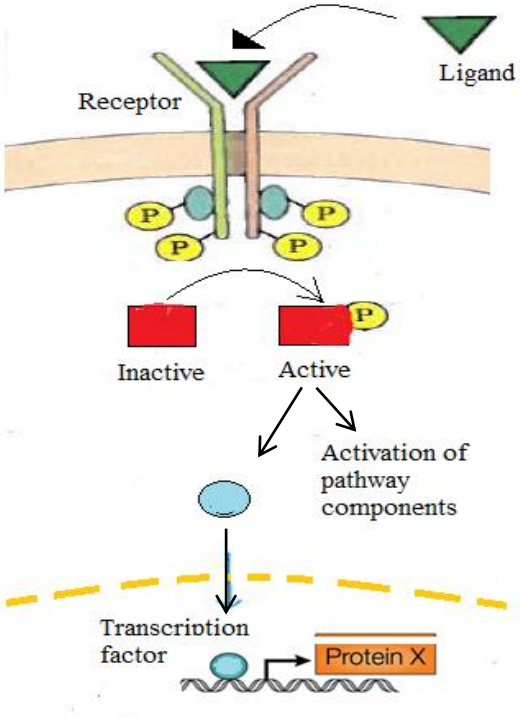

Figure 2: Signal transduction. A ligand binds to its receptor and activates a signal cascade pathway. The signal may eventually activate a transcription factor and the initiation of protein synthesis [15]. Modified figure [15].

The synthesis of a protein is a very complex and strictly regulated process. In brief, protein synthesis is initiated by transcription of nuclear DNA into messenger RNA (mRNA). The mRNA is translated into a polypeptide which ideally forms into a functional protein [15].

The initiation of gene transcription requires proteins called transcription factors. Transcription factors are able to bind to specific DNA sequences (regulatory regions), to other transcription factors or even both. However, they all regulate the transcription of genes into mRNAs by either promoting or inhibiting the transcription process. The activity of a transcription factor is regulated at several levels; like all proteins, transcription factors are transcribed from a

chromosomal gene into an mRNA and translated from an mRNA into a protein. All these steps are strictly regulated, and the transcription factors may even regulate themselves by being their own repressor or activator. The translation of mRNA takes place in the cytoplasm, so the newly synthesized transcription factor needs to be transported into the nucleus where it promotes or inhibits the transcription of other genes. Several enzymes act to facilitate this relocalization through post-translational events such as phosphorylation, acetylation, methylation or ubiquitination. On the other hand, such modifications may also cause transportation out of the nucleus for some transcription factors, and thereby keeping them inactive (see section 2.6). The ability of DNA-binding or interactions with other transcription factors may also be regulated through such intracellular events. This multiple layer of regulation ensures a great specificity in the transcription of genes and production of proteins [16].

2.4 Insulin, a hormone with multiple effects

Insulin is a peptide hormone that regulates energy storage and the metabolism for fat and carbohydrates in the body. Insulin is secreted from the islet of Langerhans in pancreas at low levels on a continuous basis, but will increase in response to high blood glucose [17]. Secreted insulin stimulates the cellular uptake of glucose by facilitating the translocation of the intracellular glucose transporter GLUT4 to the cell surface [18]. Besides being a regulator for energy metabolism, insulin acts in the regulation of gene expressions and cell morphology alterations through several signaling pathways (Figure 3).

2.4.1 Insulin activates the PI3-kinase/Akt pathway

When insulin binds to the insulin receptor (IR) on the cell surface, it triggers the activation of at least nine intracellular substrates with different roles [19, 20]. The IR belongs to the tyrosine kinase receptors that catalyze the phosphorylation of its target proteins. Among these target substrates, four of them belong to the insulin-receptor substrate (IRS) family [19]. The phosphorylation of one of the IRS leads to an activation of phosphatidylinositol-3 kinase (PI3K), which has a major role in the insulin signaling. Activated PI3K will increase levels of phosphatidylinositol-3,4,5-triphosphate (PIP_3), by phosphorylate phosphatidylinositol-4.5-

biphosphate (PIP_2). PIP_3 can activate Akt, an important serine/threonine kinase. Akt plays a key role in the regulation of cellular growth and in the many responses to insulin [16]. Translocation of GLUT4 to the cell surface [21] and inhibition of the biological activity of transcription factor FOXO3A [22] as shown in figure 3, is two of many downstream events of Akt. The latter pathway is highlighted in blue, and will be discussed in section 2.6.

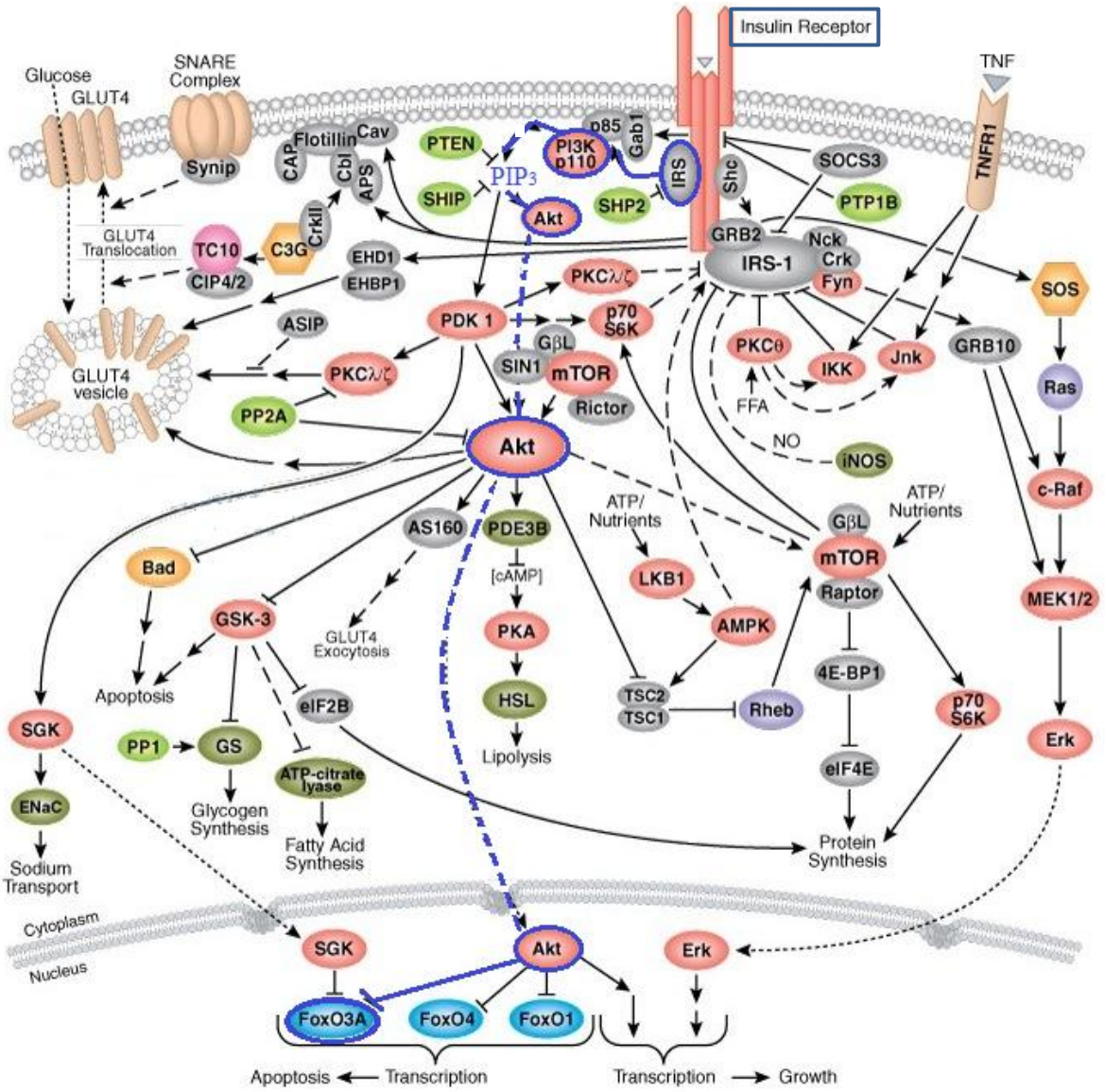

Figure 3: Signaling pathways in response to insulin. The PI3-kinase/Akt pathway is highlighted in blue. Insulin binds to the insulin receptor (IR) which will activate phosphatidylinositol-3 kinase (PI3K) through insulin receptor substrate (IRS). Active PI3K leads to an increase in levels of phosphatidylinositol-3.4.5-triphosphate (PIP_3), which activates Akt. Akt is translocated into the nucleus where it inhibits the activity of several transcription factors [16]. Modified figure [23].

2.4.2 Insulin and regulation of desaturases

An important transcription factor induced by insulin is the sterol regulatory element binding transcription factor 1c (SREBP-1c). The binding site of SREBP-1c, the sterol regulatory element (SRE) has been reported in the promoter regions of D5D, D6D and SCD [24, 25]. Several reports have demonstrated how insulin affects transcription of desaturases; Experiments performed on rats showed an increase in expression of D5D, D6D and SCD as a response to insulin through SREBP-1c [25]. It has been suggested that insulin activates SREBP-1c through the PI3-kinase/Akt pathway [26]. More recently, Arbo *et al* demonstrated an induction in expression of the mentioned desaturases in human monocytes stimulated with insulin [27]. These findings illustrate how insulin might affect the metabolism of unsaturated fatty acids in mammals.

2.4.3 Insulin resistance and chronic low grade inflammation

As mentioned in section 2.2.3, some lifestyle diseases are strongly associated with chronic low-grade inflammation, a condition which may continue for several years [14]. The metabolic state that leads to an increase in expression of markers and mediators of chronic inflammation may also increase insulin resistance [28, 29]. Insulin resistance is a condition where the response to insulin is insufficient, which may lead to development of T2D [30]. How insulin resistance originates is not fully understood, but there is a link between insulin resistance and inflammatory processes. Inflammatory cytokines such as IL-1β and TNF-α may act as a mediator for insulin resistance by impairing the tyrosine kinase activity of IR and IRS, and thereby inhibit insulin signaling responses [31-33]. Chronic inflammation and insulin resistance are common in a numerous diseases like CVD, fatty liver diseases, dyslipidemia, hypertension, asthma and certain forms of cancer [34-36]. Obesity may trigger this type of condition as a response to excess nutrients and energy [37]. It has been estimated that every year, 35 million people will die due to chronic diseases. In fact, chronic inflammatory conditions are responsible for two thirds of global morbidity and about half of global deaths [13].

2.5 Cytokines –inflammatory agents

The immune response is largely dependent on small regulatory proteins called cytokines. These molecules are released from a wide range of cells in order to modulate cellular responses important for inflammation. Cytokines act through receptors on the surface of the target cell, and induce intracellular responses depending on cell type, environment and timing. [38].

The cytokine tumor necrosis factor alpha (TNF-α) is known to be one of the major agents in the inflammation process, and acts directly towards recovery from damage and destruction of tissues [39]. Induction of TNF-α will induce an inflammatory cascade and activate other inflammatory proteins where the injury or infection is located [39]. Although TNF-α has been shown to have an important therapeutic role, this cytokine have paradoxical features in relation to diseases: Due to the action of silent inflammation, TNF-α has been implicated in several diseases including ovarian cancer, neuroblastoma and AIDS [40-42]. TNF-α has been considered as a therapeutic agent in the treatment of cancer due to its ability to induce apoptosis in endothelium cells of tumor blood vessels [43, 44]. Later experiments have also shown some pro-cancer properties of TNF-α. Steps involved in tumorigenesis, including cellular transformation, proliferation and tumor promotion have all been linked to TNF-α [45-47].

Another proinflammatory cytokine, interleukin-1 beta (IL-1β), affects almost every cell type by stabilizing mRNA or by initiating transcription of target genes [48]. IL-1β is mainly produced by macrophages and monocytes, but also by a variety of other cells [49]. Like TNF-α, IL-1β stimulates a wide range of inflammatory and immune responses [50].

TNF-α and IL-1β are both responsible for the activation of the nuclear factor κB (NF-κB) [51]. NF-κB is a major transcription factor that regulates a wide range of genes involved in inflammatory processes. When TNF-α or IL-1β binds to specific receptors on the cell surface, it triggers the phosphorylation of IκB, an inhibitor of NF-κB. Phosphorylated IκB gets degraded through the ubiquitin system, and the free NF-κB is able to promote transcription of its target genes. Thus, incorrect regulation of NF-κB is associated with several inflammatory and autoimmune diseases [15].

2.6 FOXO3A

FOXO transcription factors are members of the Forkhead family of proteins. These transcriptions factors have an important role in a variety of pathological and physiological processes, as they regulate the expression of several genes involved in development, differentiation, metabolism, cell growth and longevity [52-54]. In mammals, four members of the FOXO subgroup have been detected; FOXO1, FOXO2, FOXO3A and FOXO6 [55]. All FOXO proteins contain the characteristic Forkhead domain consisting of a 100-amino-acid, monomeric DNA-binding domain which folds up to a helix-turn-helix motif made up by three α helices and two large loops [56].

FOXO3A (previously known as FKHRL1) has an important role in a variety of signaling pathways involved in apoptosis, cell cycle control, DNA repair, longevity and the immune system. As a transcription factor, FOXO3A acts to promote or inhibit expression of a wide range of genes. For instance, FOXO3A has been reported to induce a delay in the G_2-M phase, and trigger DNA repair pathway through regulation of Gadd45a protein [54]. FOXO3A's ability to control the cell cycle has also been shown in G_1 and M-phase [57, 58].

In mice FOXO3A has been reported to function as a NF-κB antagonist; FOXO3A overexpression was demonstrated to inhibit TNF-induced nuclear translocation of NF-κB, and thereby inhibit T-cell activity [59]. In the absence of FOXO3A, mice developed a spontaneous inflammatory syndrome associated with an increased NF-κB activity [59]. In neutrophilic inflammation however, FOXO3A is required to maintain the proinflammatory environment by suppressing neutrophilic apoptosis through inhibition of FASL [60]. Recently, the transcription factor Twist-related protein 1 (TWIST1) has attracted great attention due to its role in inflammation. TWIST1 activity has been reported to regulate expression and secretion of inflammatory adipokines (cytokines secreted by adipose tissue) in human white adipocytes [61]. Studies have also revealed a correlation between elevated TWIST1 levels and cancer metastasis [62]. Interestingly, FOXO3A has recently been reported to inhibit expression of TWIST1 in human bladder cancer cells, and thereby suppress urothelial cancer invasiveness [63]. Other reported FOXO3A downstream target genes include DNA-damage binding protein 1 (DDB1) [64], B cell translocation gene 1 (BTG1) [65, 66], tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) [67, 68], p130 [69] and Y box binding protein 1 (YBX1) [63].

FOXO proteins are strictly regulated in response to environmental conditions. The stability, subcellular location, gene target specificity of FOXO3A and its DNA binding activity are controlled by post-transcriptional modifications including methylation, phosphorylation, acetylation and ubiquitination [70]. As mentioned in section 2.4.1, activity of FOXO3A is regulated by Akt through the insulin/PI3K/Akt signaling pathway in response to insulin and growth factors (figure 4). FOXO3A is directly phosphorylated at three conserved residues by Akt. Phosphorylated FOXO3A binds to a protein called 14-3-3, which will lead to an export of FOXO3A from the nucleus and out in the cytoplasm [22, 71].

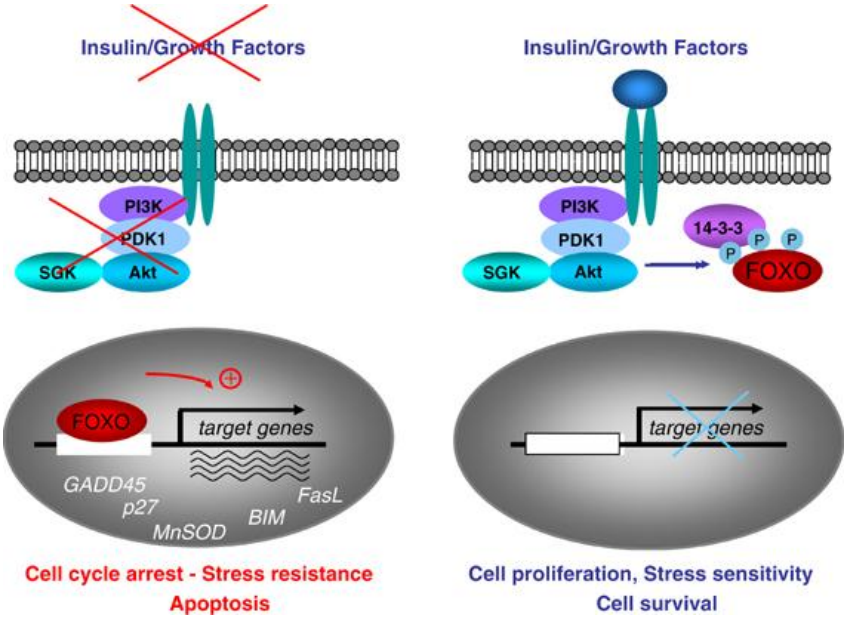

Figure 4: regulation of FOXO transcription factors. Insulin and growth hormones activates the PI3K/Akt signaling pathway. Akt is translocated into the nucleus where it phosphorylates FOXO transcription factors on three conserved residues. Phosphorylated FOXO factors are recognized by 14-3-3 and translocated from nucleus into cytoplasm. In the absence of insulin and growth factor, FOXO transcription factors will continue the regulation of their target genes [71]. Figure [71].

Although the post-transcriptional regulations of FOXO3A activity have been well investigated, the investigation of the regulation of FOXO3A mRNA expression has barely begun. During mRNA expression analysis, one must pay attention to the possibilities of presence pseudogenes. A pseudogene is a noncoding sequence sharing close similarity to a known gene present in the genome of an organism [72]. The first pseudogene was reported during investigation of the genome of *Xenopus laevis* in 1977 [73], and has subsequently been identified in bacteria, plants, insects and other vertebrates [74-76]. The nucleotide sequence

of a pseudogene differs from the coding gene at essential points as a result of gene duplication, either by retrotransposition or duplication of genomic DNA [72]. Initially, pseudogenes were considered to be nonfunctional. However, as some research groups have come across cases where pseudogenes may be involved in regulating transcription of other genes [77, 78], it has been suggested to establish a new term for pseudogenes that are involved in meaningful biological interactions. A pseudogene for FOXO3A has been found in humans located on chromosome 17, and is called FOXO3B [79].

2.7 Monocytes

Monocytes are circulating blood cells with essential roles in the innate immune system of humans. In line with all blood cells, monocytes arise from hematopoetic stem cells located in the bone marrow. Promonocytes, developed from granulocyte-monocyte progenitors in the bone marrow, enter the bloodstream and differentiate into mature monocytes. In response to an inflammatory signal, monocytes are able to move quickly to the site of infection to elicit an immune response. They circulate and enlarge in the bloodstream for about 8 hours, before migrating into infected tissues and differentiate into macrophages [80].

2.7.1 The monocytic cell line THP-1

Investigations of the function and features of human monocytes in the cardiovascular system are often performed in THP-1 monocytes. The monocytic cell line THP-1 is derived from the blood of a human male with acute monocytic leukemia. As these cells are cultured *in vitro* there are certain required condition factors that need to be fulfilled: A temperature at 37 °C and a gas mixture at 5 % CO_2 are good conditions for achieving cell growth [81]. THP-1 cells have an average doubling time at 35 to 50 hours, and should be kept in cultures of minimum $2x10^5$ cells/mL and not more than $1x10^6$. Recommended medium is the RPMI1640 and 10% fetal bovine serum (FBS).

Cells usually grow in a standard pattern of growth consisting of three phases; lag phase, log phase and plateau phase. During the lag phase there is minor growth or no growth at all. This is the first phase after subculture, and the cells need some time to adapt to the medium. The

cells are in its most reproducible condition during the log-phase. Here, the cells are in exponential growth, until they reach the plateau phase where cell growth is more or less equal to cell death. Adhesive monolayer cells will stop dividing when they reach a saturated density, due to the contact with other cells. Suspension cells, such as THP-1 do not necessarily show the same plateau in growth, but will eventually reach this phase due to exhausting of the medium. Prior to experiments, cells should be in the log-phase where the growth fraction is high [82].

During log-phase THP-1 cells are randomly distributed in the cell cycle. Prior to any experiments, the cells should be synchronized [82]. This can be done by restricting the growth and manage the cells "out of cycle", a state called G_0. Previous research showed that THP-1 cells kept in 0.5% Fetal calf serum (FCS) for 16 hours prior to the experiment, revealed a significantly higher expression of desaturases (D5D, D6D and SCD) compared to cells kept in 10% FCS [27].

THP-1 cells are one of the most widely used cell lines to investigate the regulation and function of monocytes and macrophages. Several reposts have demonstrated that this cell line is suitable for mimicking human monocytes in inflammation-and diabetes-related studies [83, 84]. However, it is important to know that THP-1 cells and circulating monocytes do have some significant differences [84]. Careful consideration is therefore needed when results are generalized to monocytes and macrophages.

2.7.2 Passage number

The cell line THP-1 can provide continuous culture, which means that they are able to be propagated in vitro for an indefinite time. However, subculturing may change the properties of a cell line over time. The degree of subculturing is often expressed as "passage number" It has been demonstrated that a high passage number may influence cell morphology, growth rate and gene expression compared to cells with a low passage number [85, 86]. The aging effect is cell type dependent, and has been shown to have a variable impact on different genes [86]. Continuous cell lines are widely used as research tools, and the quality of the cell line is crucial to obtain successful experiments. To ensure reproducible and reliable results, it is important to use cells with a low passage number and pay attention to any alteration over time.

2.8 Quantitative real-time PCR (qPCR)

The regulation of gene expression makes it possible for an organism to adapt to variable conditions and stimuli, such as exposure to hormones or cytokines [87, 88]. These changes in levels of expressed genes may be analyzed by a variant of polymerase chain reaction (PCR) called quantitative real-time polymerase chain reaction (qPCR). qPCR is a sensitive and precise method where complementary DNA (cDNA) synthesized from total RNA, is used as template for the amplification reactions. This method is based on detections of fluorescence signals from a reporter molecule, which will increase during each cycle of cDNA amplification. A quantitative threshold for the DNA-based signal to be detected is set just above the background. The quantitative cycle (Cq) is defined as the number of cycles needed for the fluorescence signal to reach a fixed threshold, and represent the amount of target gene in the sample [89].

As the amount of RNA added to the reverse transcript reaction may vary, the PCR reaction needs to be normalized by a suitable reference gene. Ideally, a reference gene should be unaffected by the experimental procedure [89]. Several housekeeping genes, like GAPH, β-actin, β_2-microglobulin and rRNA have been used for this purpose, but it has been reported that these genes may be influenced by various experimental settings [90]. Therefore, the selection of an appropriate reference gene should be done prior to qPCR analysis.

2.9 Primer design

Prior to any qPCR analyses, it is essential to choose a suitable primer pair to achieve successful amplification of a target gene. When designing primers it is important to check them for specificity to avoid amplification of other genes with similar sequence. NCBI's Primer BLAST (Nucleotide Basic Local Alignment Search Tool) is a useful tool during primer selection.

Primer BLAST uses Primer3 [91] to design PCR primers and then submits them to a BLAST search against the GenBank database to ensure specificity. BLAST also allows the primer pair to be complementary to each side of an intron on the DNA sequence, in order to distinguish between amplification of genomic DNA and cDNA. The primer 3 software makes it possible to select options like product size and annealing temperature (T_m). Such factors are important

to consider as certain guidelines needs to followed when designing primer pairs [92] ; Each primer should consist of 18-25 bases, where 40-60% of these should include the bases cytosine (G) an guanine (C). The primer sequence end should not contain 3 or more Cs or Gs, but should end with a G/C/GC or CG. To avoid the synthesis primer diming, the 3'ends should be uncomplimentary and self-complementary primers must be excluded. Finally, the T_m should be between 55-80 °C [92].

To evaluate the qPCR products given by the primer pairs, analyses of the dissociation curve data can be very useful. Products from the amplification should have the same melting point, which can be seen as a single, sharp peak at the melting temperature of the amplicon. If the dissociation curve reveals a series of different peaks, the discrimination between specific and non-specific products are not sufficient [93]. In addition, qPCR products may be evaluated by gel-electrophoresis. If the qPCR results in one product per primer pair and product size correspond to an expected length, the primer pair is considered as suitable.

2.10 The $2^{-\Delta\Delta Cq}$ Method

Data from quantitative qPCR analyses may be calculated by the $2^{-\Delta\Delta Cq}$ method in order to analyze relative changes in gene expression.

First, the average Cq value is calculated for each target- and reference gene. The average Cq value is further used to find ΔCq, where $\Delta Cq = Cq_{\text{target gene}} - Cq_{\text{reference gene}}$. The ΔΔCq is calculated from $\Delta Cq_{\text{sample x}} - \Delta Cq_{\text{control}}$, which is used to find $2^{-\Delta\Delta Cq}$ [89]

The fold change of expression of the target gene in treated samples relative to the untreated samples (control) is indicated by the evaluation of $2^{-\Delta\Delta Cq}$ [89].

A prerequisite for the $2^{-\Delta\Delta Cq}$ method is that the target gene and the reference gene should have similar amplification efficiency. The efficiency may be evaluated by investigating the ΔCq using various cDNA dilutions. When ΔCq-values are plotted against log cDNA dilutions, the slope of regression line should be close to zero [89].

2.11 Aims of thesis

Recently, a human intervention study was performed in order to investigate changes in gene expression in response to nutrient composition [94]. Microarray analyses revealed diet-specific changes in leukocyte gene expression when slightly overweight individuals went through diets with different carbohydrate fraction relative to fat and protein. A diet with a reduced amount of carbohydrates seemed to reduce processes associated with chronic inflammation compared to a high carbohydrate diet relative to protein and fat. Among several findings, an upregulation of FOXO3A mRNA expression was reported in the diet with a reduced fraction of carbohydrates relative to protein and fat.

At the same time the group demonstrated an upregulation in the fatty acids desaturases D5D, D6D and SCD mRNA expression in THP-1 monocytes treated with insulin [27]. These findings demonstrated that THP-1 monocytes may work well as a model-system for circulating blood cells in the study of nutrients responses *in vitro*.

The aim of this thesis is to investigate the regulation of gene expression related to inflammation in response to insulin and/or cytokines in THP- monocytes. The main focus is to reproduce the regulative insulin-effect on desaturases expression that was recently shown in THP-1 cells by Arbo *et al* [27], and to investigate potential regulating effects of insulin related to FOXO3A mRNA expression. Furthermore, it is of interest to look at mRNA expression levels of FOXO3A and desaturases in response to cytokines, and the effect of insulin in combination with cytokines. Finally, expression levels of FOXO3A target genes are investigated in order to demonstrate potential effects of insulin, cytokines and the combination of insulin and cytokines. In addition, the following secondary objectives were included:

-Optimization of qPCR parameters

-Designing and evaluate relevant primers

-Choosing suitable reference genes

-Determine the cell concentration at which the THP-1 cells should be during the experiments

-Investigation of responsiveness in low-passage and high-passage THP-1 monocytes

3. MATERIAL AND METHODS

3.1 Reagents

Information about essential reagents used in the current study can be found in table 1.

Table 1: Reagents are presented with lot number and provider.

Materials	Provider	Lot number
6x Orange Loading Dye Solution	Fermentas	0802
Agarose	Sigma	059K0033
Deoxyribonucleotide triphosphate (dNTPs)	Sigma Aldrich	011M0489
Distilled Water (dH_2O), DNase/RNase Free	Gibco/Invitrogen	722035
Dithiothreito (DTT)	Invitrogen	1009479
DNA ladder (100 bp)	Promega	24974001
Fetal Bovine Serum	Gibco	41G5893P
Gel red	Biotium	10G0428
Gentamicin	Sigma Aldrich	031M0851
Insulin solution, human recombinant	Sigma Aldrich	011M8410
Interleukin-1 Beta (IL-1β)	Roche	131926000
L-glutamin	Sigma Aldrich	F7524
Moloney Murine Leukemia Virus Reverse Transcription (M-MuLV RT)	Invitrogen	1102594
Random Hexamer Primer	Promega	0000001274
Recombinant RNasin Ribonuclease inhibitor (RNasin)	Promega	29458001
RNA isolation kit; RNeasy Mini Kit	Qiagen	N/A
RPMI 1640-medium	Sigma Aldrich	RNBB6748
SYBR-green Jump Start Taq Redy Mix	Sigma Aldrich	N/A
Tumor necrosis factor alpha (TNF-α)	R&D systems	N/A
β-mercaptoetanol	Sigma Aldrich	N/A

3.2 Cell culture

3.2.1 Cell cultivation

All experiments were performed on human monocytes from the monocytic cell line THP-1 (American Type Culture Collection, Manassas, VA, Catalog No. TIB-202). The concentration of cells was kept under 1 x 10^6 at all-time by subculturing the cells to 2 x 10^5 cells/mL every 3^{rd}-4^{th} day. Cells were grown in suspension of RPMI-1640 medium supplemented with heat-inactivated 10 % fetal bovine serum (FBS), 0.002 % gentamicin, 1 % L-glutamine and 0.05 mM β-mercaptoetanol. To ensure optimal conditions for achieving cell growth, the THP-1 cells were cultured in tissue culture flask at 37˚C in 5 % CO_2.

3.2.2 Freezing and thawing of cells

After a certain number of passages, the shape and properties of THP-1 cells may be altered [82]. In order to keep the passage number to a similar level during the experiments, cells were frozen in suspension with cryoprotectant dimethylsulfoxide (DMSO) at -80°C. DMSO was used to slow down the cooling rate and reduce the formation of ice crystal inside the cell [82].

The frozen cells suspension were thawed on water bath holding 37°C, and supplemented with 10% RPMI medium (10 mL, 37°C). The DMSO-medium was replaced with 10 mL preheated RPMI medium after centrifugation (700 rpm, 5min) of the cells. Cells were placed in a 25 cm^2 tissue flask, and incubated at 37°C in 5% CO_2 for 24 hours. To remove all remains of the DMSO, the cells were centrifuged and resuspended in preheated RPMI medium the next day. After 3 days, the cells were once again resuspended to 2x10^5 cells/mL and cultured as normal.

3.2.3 Stimulation of THP-1 monocytes

Prior to all experiments, cells were subcultured to 3x10^5 cells/mL and grown in 10 % serum. Then, within the next 2-3 days the cells were grown to a concentration of ~6x10^5 cells/mL, centrifuged (700 rpm, 5min) and resuspended in 0.5% FBS for serum starvation. The cells were starved in 0.5% FBS for 16 hours prior to the various treatments.

Cells were stimulated with human recombinant insulin (0.01 µM), human IL-1β (10 ng/mL) and human TNF-α (10 ng/mL) in 0.5% FBS. The concentrations of IL-1β and TNF-α that were chosen were based on previous research [95-97]. Dose dependent experiments were performed in order to find the optimal concentration of insulin. The various insulin concentrations were chosen based on previous research [27]. To optimize the period of treatment, time-course experiments were performed for all the listed stimulation factors and for the different target genes.

3.3 Isolation of total RNA

Qiagen RNeasy Mini kit was used to isolate total RNA from THP-1 monocytes, in accordance with the manufacturer's instructions [98]. Only RNA of high quality (A260/A280>2, A260/A230>1.8) was used for further analysis. The quality and quantity (ng/µL) of RNA were measured by using Nanodrop ND-1000 Spectrophotometer (NanoDrop®). Samples of RNA were always kept on ice during experiments in order to prevent degradation. The RNA samples were stored at -80°C between the analyses.

3.4 cDNA synthesis from total-RNA

1 µg of total RNA was used for each first strand complementary DNA (cDNA) synthesis. Distilled water was added to the RNA to adjust the volume to a total of 9 µL. A mix of reagents containing 5x First strand buffer (4 µL), DTT (10 mM, 2 µL), dNTP (10 mM, 2 µL), Random hexamer primer (100 µg/mL, 1 µL) and M-MuLV-RT (1 µL) was made for each sample of RNA/dH_2O. After adding the mix of reagents, the samples were incubated at 25°C for 10 minutes to start a primer extension, then at 37 °C for 1 hour for first strand synthesis and finally at 95°C for 5 minutes to obtain a reaction termination. The cDNA was diluted 1:6 with ultra-pure water, and stored at -20°C for further analysis.

3.5 Primers for qPCR

Primers for D5D, D6D and SCD were chosen based on previous research [99, 100], while primers for FOXO3A, BTG1, DDB1, TRAIL, P130 and TWIST1, YBX1 were all designed according to guidelines described in section 2.9 with the assistance from Sigma Aldrich. Oligoname and sequences for each primer pairs are listed in table 2. A Primer BLAST search was performed for all primers to confirm gene specificity.

Table 2: Oligoname and sequences for primer pairs. The underlined G in reverse primer for D6D is different from the sequence of Cho et al.[99]. The correct base should be a G as shown here, according to the sequence of accession number AF126799.

Oligoname	Forward primer	Reverse primer
BTG1	CTGGCACAAGATAGAATGGTAA	ACTTGGACTCACAGGCTAT
D5D	GAATAAAGAGCTGACAGATGAG	CCTGAACTGCACTGAGCA
D6D	GGCAAGAACTCAAAGATCAC	GAGAGGTAGCAAGGACAAAG
DDB1	GTCACTCTCAAGGATCTC	AACACAACACCATTATCAAG
FOXO3A	AGGAAGGGGAAGTGGGCAAAGC	TGCTGGTTAGGAAAATGGCGTGG
P130	TTGCTAACAGACTGAAAGA	GCTCAATAACAGATTCTAATACT
SCD	ATCTCTAGCTCCTATACCACC	CCCAAAGCCAGGTGTAGAAC
TRAIL	TCAGGATGATACACTATGAAGATG	GTTGTGGCTGCTCTACTC
TWIST1	ACCATCCTCACACCTCTG	GATTGGCACGACCTCTTG
YBX1	CTTACCATCTCTACCATCAT	AGCACTTTAGGTCTTCAG

3.6 qPCR

Fluorescence-based qPCR analyses were performed for all the genes of interest on a Mx3000P instrument (Stratagene, La Jolla, CA, USA). All the reactions contained 5 µL cDNA diluted 1:6, 0.3 µM of forward and reverse primer and 12.5 µL SYBR®Green JumpStart Taq ReadyMix (0.4 mM of each dNTP, 20 mM Tris-HCl (pH 8.3), 100 nM KCl, 7 mM $MgCl_2$, 0,05 U/µL Taq DNA polymerase, JumpStart antibody, SYBR green 1 dye and stabilizers). Ultra-pure water was added to adjust the volume to a total of 25µL.

The cycling program of Mx3000P started at 95°C for 3 minutes to initiate a denaturation step, followed by 40 cycles of 95°C for 30 seconds, 60°C (65°C for FOXO3A) for 30 seconds and finally 72°C for 30 seconds to perform denaturation, annealing and extension respectively. Mx Pro^{TM} Q-PCR Software (Stratagene, La Jolla, CA, USA) were used to analyze the data.

3.7 Gel-electrophoresis

The products from the qPCR reaction were separated and visualized by gel electrophoresis. The products were run on a 3% agarose gel, made up by agarose diluted to a 3% concentration in 1 x TAE buffer (a mixture of Tris base, acetic acid and EDTA). GelRed^{TM} DNA stain were added to the agarose solution (1:10 000). Bands were visualized by UV light on a Molecular Imager gel Doc XR System (BioRad), and compared to a 100 bp DNA ladder.

3.8 Calculations and Statistical analysis

The $2^{-\Delta\Delta Cq}$ method was used to analyze the gene expression data from the qPCR experiments for all genes.

Paired, two-tailed Student T-test was used to compare the mean of the ΔCq-value for treated cells with the ΔCq-value for untreated cells (control) in relation to the variation in the data.

A difference between groups of $p<0.05$ was considered as significant. When several T-tests are being performed, the probability that the result of a test is random will increase. The Bonferroni correction is a method used to counteract this problem. It simply divides the p-value on the number of independent experiments performed. P-values for the T-tests performed in this study are presented in the Appendix.

Since the T-test requires data to be approximately normally distributed, the $2^{-\Delta\Delta Cq}$ values were log-transformed. This is common a practice in analyses of mRNA gene expression data.

To present the differences in treated- and untreated THP-1 cells as fold change, ΔΔCq was transformed to $2^{-\Delta\Delta Cq}$, where the control is set to the value 1.0.

4 RESULTS

4.1 Optimization of the qPCR parameters

4.1.1 Validation of primers

In this study, quantitative PCR (qPCR) were used to detect the mRNA expression of selected genes. This method requires optimization of certain parameters in order to obtain successful results, including the validation of selected primer pairs. As mentioned in section 2.9, the dissociation curve from qPCR can be useful to ensure that the amplicon of interest is detected.

The dissociation curve was examined for each primer pair after every qPCR to exclude primer-dimer, contaminating DNA and qPCR products from misannealed primers. When primers for FOXO3A were added to 12 separate samples of cDNA, the resulting dissociation curves from qPCR revealed a single peak at the melting temperature for the FOXO3A amplicon (figure 5). This was also observed for all the primers used in this study (not shown).

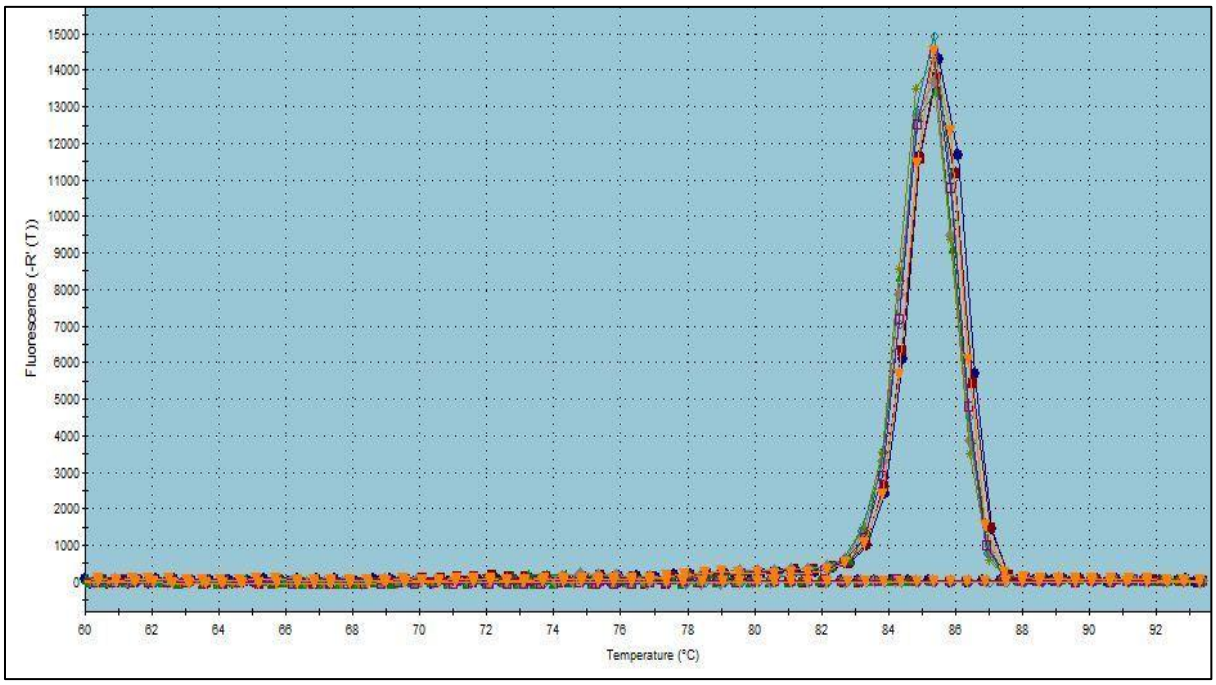

Figure 5: Dissociation curve for qPCR product from 12 samples with primers for FOXO3A. Taken together, all 12 samples results in a single peak at the melting temperature for the amplicon, which reveals a pure and correct amplification.

4.1.2 Finding the optimal primer concentration for qPCR

The optimization of the primers is essential as each set of primers are most efficient under different conditions. Primer concentrations need to be optimized to ensure an accurate and specific qPCR reaction. The primer concentration in the amplification reaction should be somewhere between 0.1 and 0.5 µM [101].

Primer concentrations of 0.2 µM, 0.3 µM and 0.4 µM were validated to find the most efficient concentration for qPCR. cDNA from 1 µg total RNA isolated from non-stimulated THP-1 monocytes was used as template for the reaction. The amplification plots were compared and the FOXO3A primer concentration that gave the lowest Cq-value, 0.3 µM, was chosen for qPCR (figure 6).

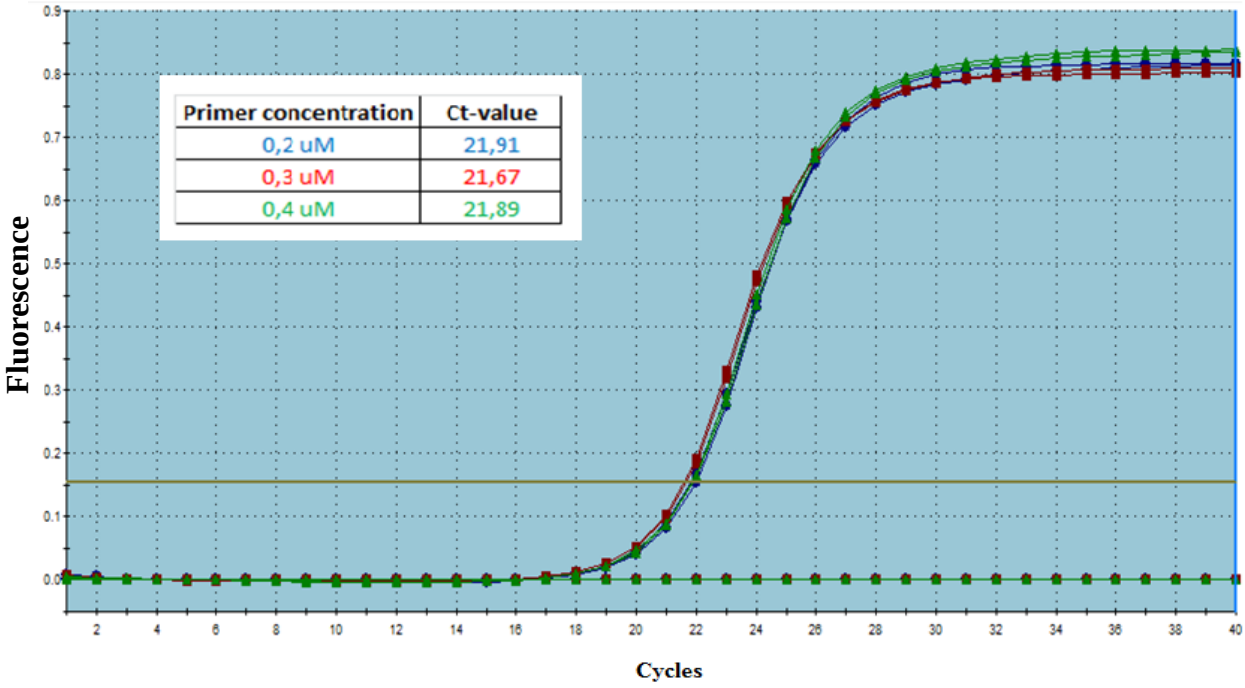

Figure 6: Validation of primer concentration for FOXO3A: Amplification plots and Cq-values from a qPCR reaction with various concentrations of primers for FOXO3A (0.2 µM, 0.3 µM and 0.4 µM).

Even though there was only a small difference in the Cq-value between the three concentrations, 0.3 µM was selected as the primer concentration for further experiments. 0.3 µM was also selected as the proper concentration for the same primer pairs used in previous study of D5D, D6D and SCD mRNA expression [27]. Consequently, this primer concentration was selected for all target genes in current study.

4.1.3 Visualization of qPCR products

As different cells may express different sets of genes and due to the fact that a primer pair may recognize more than one target gene, it was desirable to ensure that the templates used for qPCR were indeed the genes of interests. Products from qPCR were separated on a 1.5% agarose gel and visualized with UV-light after staining with GelRed (figure 7). A 100-bp DNA ladder was used to indicate the sizes of the different qPCR products. Single, clear bands visualized on the three separate gels in figure 7 indicate pure qPCR products. The size of the fragments was verified based on the DNA ladder, and they all revealed bands with expected sizes. FOXO3A, D5D, D6D, SCD, DDB1, TRAIL, BTG1 and TWIST1 were therefore considered as expressed in THP-1 monocytes. Neither P130 nor YBX1 were detected by qPCR, and were therefore excluded in further experiments.

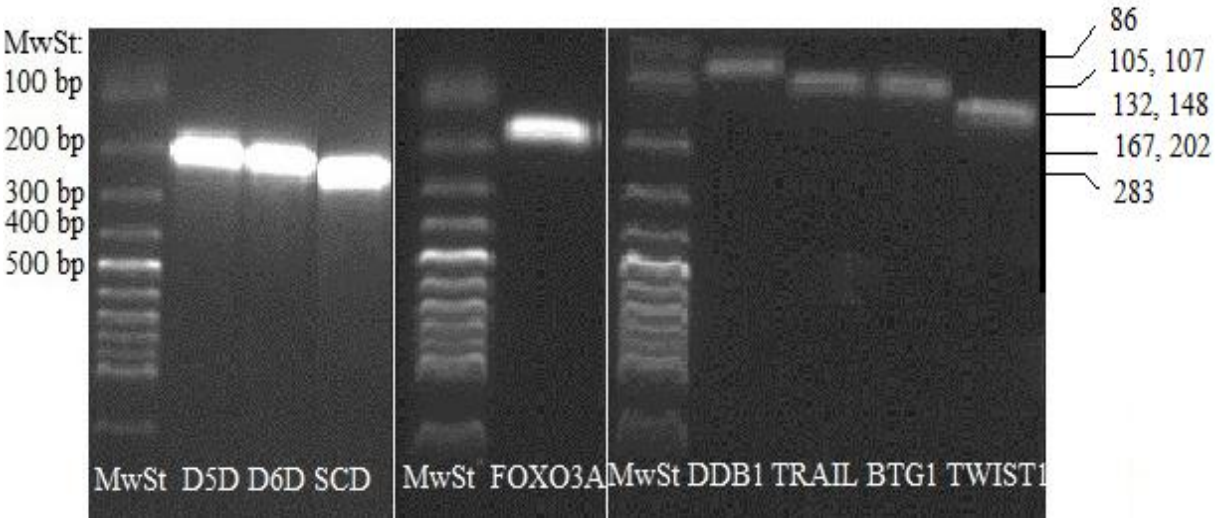

Figure 7: Products form the qPCR with primers for the target genes. Gel electrophoresis (3% agarose gel) was used to separate the products after qPCR amplification, and bands were visualized by UV-light. The molecular weight standard (MwSt) indicates a marker lane to the left where DNA fragments with a known base pair (bp) size are visualized and can be compared with the qPCR products with primers for D5D (202 bp), D6D (167 bp), SCD (283 bp), FOXO3A (148 bp), DDB1 (86 bp), TRAIL (107 bp), BTG1 (105 bp) and TWIST1(132 bp).

4.1.4 Finding a suitable reference gene

There is a challenge associated with qPCR; the uncertainty about the precise amount of amplifiable cDNA present in the reaction. As described in section 2.10, this can be solved by amplifying a second gene used as a reference gene [89]. Four genes were selected as potential reference genes for this study; Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Beta-2-microglobulin (B_2M), Hypoxanthine phosphoribosyl-transferase I (HPRT1) and 18S ribosomal RNA (18s rRNA). When the ΔΔCq method is being used to analyze relative changes in gene expression, the amplification efficiency between the target and the reference gene should be similar (section 2.10) [89]. The amplification efficiency was determined for all target genes.

cDNA from 1 µg of total RNA isolated from untreated THP-1 cells were diluted over a 1000 fold range and used as template for the qPCR. qPCR were performed for each dilution, with primers for the target genes and the reference genes. ΔCq was calculated for each reference gene compared to every target gene, and plotted against log cDNA dilution. The ΔΔCq method can be used to analyze changes in gene expression only if the absolute value of the slope is close to zero.

GAPDH, 18sRNA, B_2M and HRTP were all suitable reference genes for D6D mRNA expression analysis according to the amplification efficiency test (figure 8). Due to biological and technical reasons (discussed in section 5.2), 18s rRNA was chosen as reference gene for further experiments. 18s rRNA was also considered to be a suitable reference gene for D5D, SCD, FOXO3A and TRAIL (not shown).

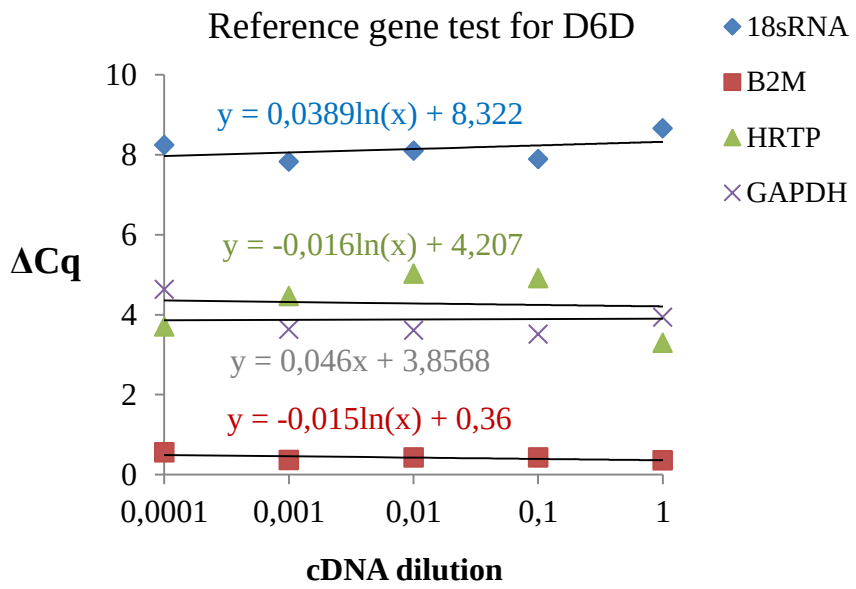

Figure 8: Slop of the regression lines for D6D compared to potential reference genes. The efficiency of amplification of D6D and potential reference genes (18s rRNA, B_2M, HRTP and GAPDH) was evaluated using qPCR. cDNA diluted over a 1000 fold range (where the most concentrated sample was diluted 1:2) from 1 µg RNA was used as template for the qPCR. The resulting ΔCq was calculated for D6D and each reference gene and plotted against cDNA dilution.

4.2 Cell growth of THP-1 monocytes

Prior to the experiments it was of interest to study the growth of the THP-1 monocytes, as the properties of a cell culture vary between lag, log and the plateau phase. Consequently, the relationship between cell state and cell number was investigated. THP-1 cells were subcultured in three different culture tissue flasks with an initial number of $1.5x10^5$, $2.0x10^5$ and $2.5x10^5$ cells/mL. Cell number was counted on a Bürker counting chamber every 24^{th} hours for 6 days without replacing the medium. Cell numbers from the three different culture tissue flasks were plotted against time to illustrate a growth curve (figure 9). THP-1 cells that were subcultured to $2.5x10^5$ cells/mL went immediately to the log-phase, while cells with an initial concentration of $1.5x10^5$ and $2x10^5$ cells/mL were in the lag-phase the first 24 hours. A plateau phase can be observed when the cells reach a concentration of $7\text{-}8x10^5$ cells/mL. Cells for experimental use should be taken in late log phase to ensure that cells are in a proliferative state before serum starvation. As mentioned in section 2.7.1, THP-1 cells should never exceed 1 x 10^6 cells/ml in order to ensure exponential growth [81]. Consequently, cells used

in further experiments were always on a concentration of ~6x10^5 cells/mL during serum starvation, and hence 7-8x10^5 cells/mL during stimulation.

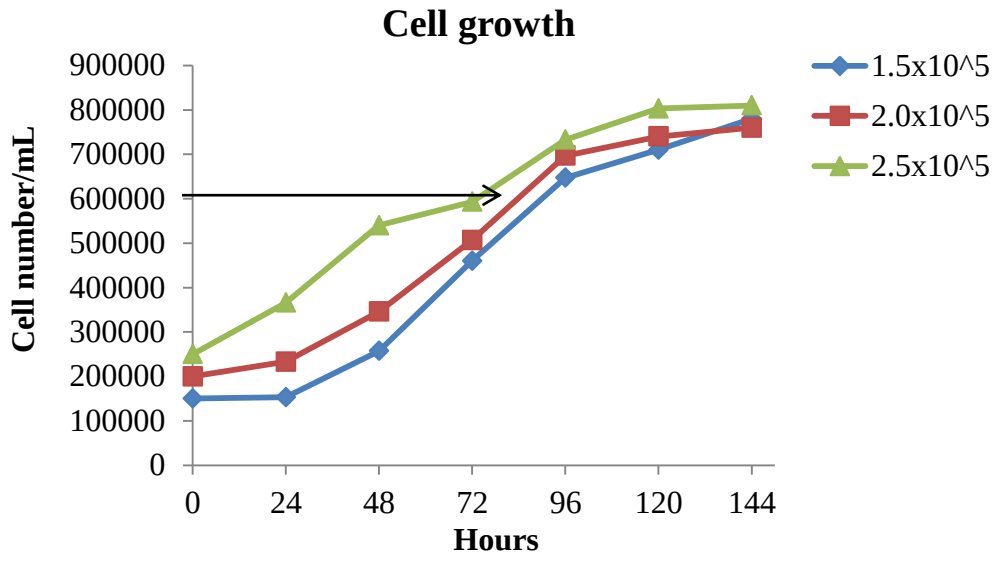

Figure 9: Determination of cell growth and cell state. THP-1 cells were subcultured to a concentration of 1.5x10^5, 2.0x10^5 and 2.5x10^5 per mL in RPMI medium with 10% FBS. The cell number was counted every 24th hour for 6 days and plotted against time. The arrow indicates the cell concentration that was used in further experiments (late log-phase).

4.3 D5D, D6D and SCD gene expression is regulated by insulin

4.3.1 Expression of desaturases is dose-dependently regulated by insulin in THP-1 monocytes.

The enzymes delta-5-desaturase (D5D), Delta-6-desaturase (D6D) and stearoyl-CoA desaturase (SCD) participate in the biosynthesis of PUFAs in humans [6, 9]. Recently, nutrient composition has been suggested to influences the transcription of these desaturases as mRNA expression has been reported to increase in response to insulin [27, 102-104]. In these former experiments, different concentrations of insulin were used to demonstrate this regulative effect.

In order to optimize *in vitro* conditions for studying alterations in desaturase mRNA expression in response to insulin in THP-1 monocytes, a series of dose-response experiments

was performed. THP-1 cells were treated with various concentrations of insulin (0.1 µM, 1 µM, 10 µM, 100 µM and 1000 µM) chosen based on concentrations used in Arbo *et al* [27].

THP-1 cells treated with 0.1 µM insulin seemed to be more responsive compared to cells treated with higher insulin concentrations. Consequently, it was of interest to investigate the regulation of desaturase mRNA expression in response to insulin concentrations beneath 0.1 µM.

Consequently, the next experiment included a minimum concentration of 0.001 µM insulin. Results from these experiments indicated that 0.01, 0.1 and 1 µM insulin were all good inducers for desaturases mRNA expression in THP-1 cells (figure 10). The experiment was repeated and resulted in similar observations (not shown). 0.01 µM insulin was used to investigate changes in expression of D5D, D6D and SCD in further experiments as this concentration also seemed to induce regulation of FOXO3A mRNA expression (see section 4.4.1).

A time-course experiment was performed in order to verify that the 24-hours treatment period chosen based on previous work [27, 102-105] was optimal for inducing desaturase expression. THP-1 cells were treated with insulin for several treatment periods (2, 4, 8, 12, 24, 48, 72 hours). Cells treated with insulin for 24 hours were indeed more responsive than cells treated for a longer or shorter period (not shown). Consequently, THP-1 cells were treated with insulin for 24 hours in further experiments in order to investigate regulation of D5D, D6D and SCD mRNA expressions induced by insulin.

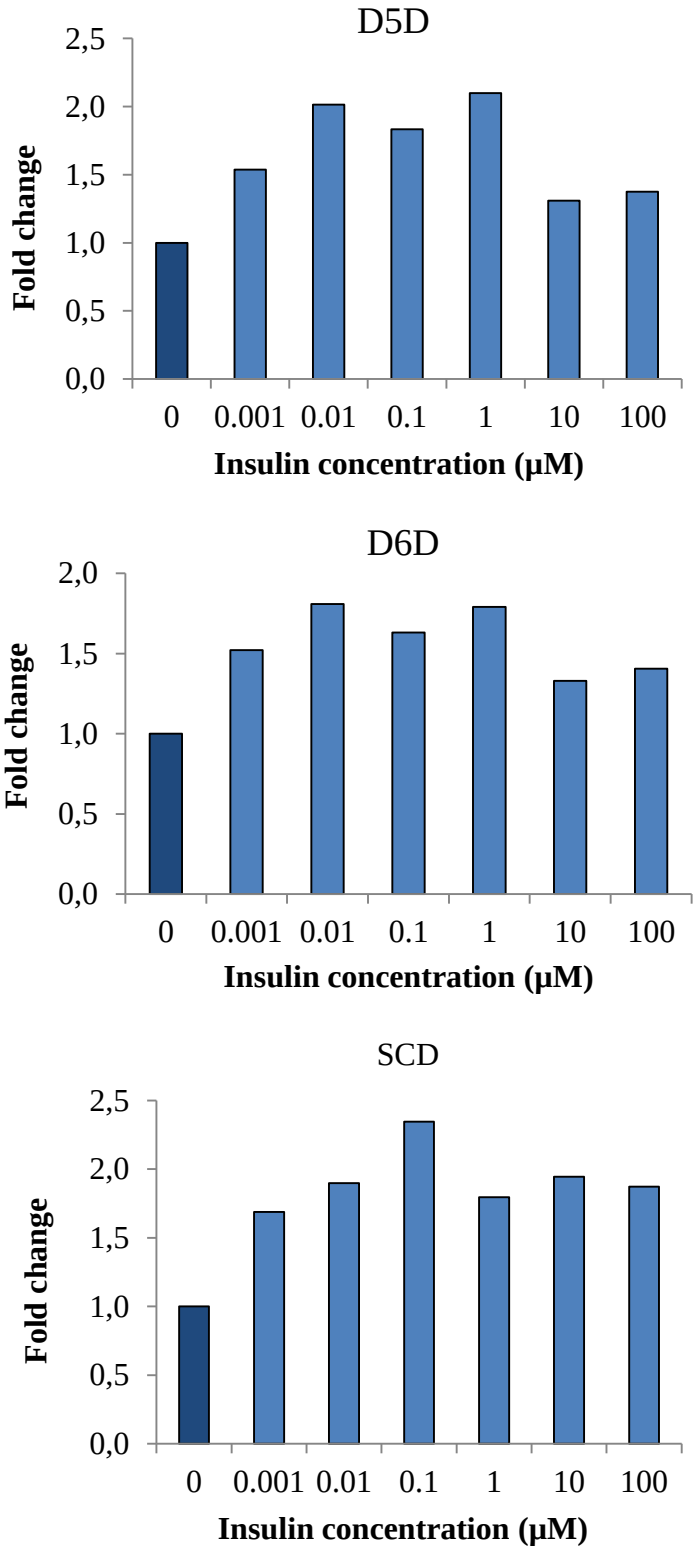

Figure 10: Fold change of D5D, D6D and SCD mRNA expression compared to control (0). THP-1 cells (29 passages) were stimulated with indicated concentrations of insulin in 0.5% FBS for 24 hours after serum starvation (0.5% FBS, 16 hours). Expression of D5D, D6D and SCD is normalized with 18s rRNA, and fold change for each gene is calculated by the $2^{-\Delta\Delta Cq}$ *method. The experiment were repeated and revealed similar results.*

4.3.2 D5D, D6D and SCD gene expression is significantly up-regulated by insulin

THP-1 cells stimulated with insulin for 24 hours reveal a significant up-regulation in desaturase mRNA expression (figure 11). The mean fold change of D5D, D6D and SCD mRNA expression between insulin-treated and untreated THP-1 cells is 2.1, 1.7 and 2.2 respectively. These results are based on four independent experiments.

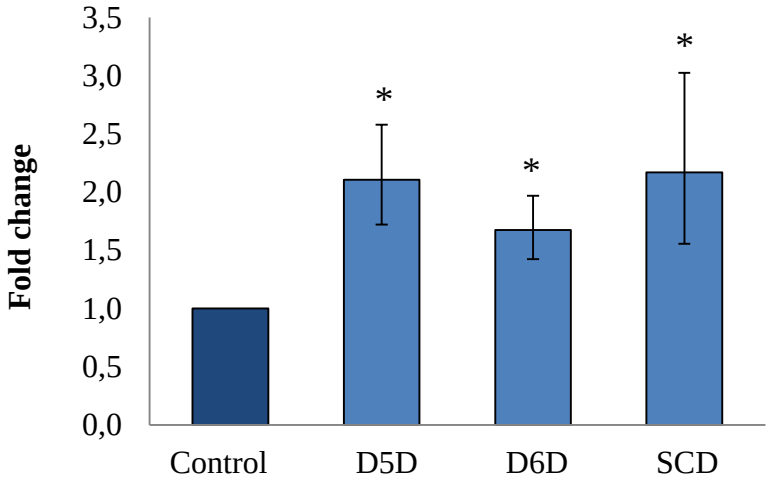

Figure 11: mRNA expression of D5D, D6D and SCD in THP-1 cells is significantly upregulated in response to insulin. THP-1 cells (passage number < 40) were stimulated with insulin (0.01 µM) in 0.5% FBS for 24 hours after serum starvation (0.5% FBS, 16 hours). Expression of D5D, D6D and SCD is normalized with 18s rRNA. The fold change between treated and untreated cells (control) is calculated by the $2^{-\Delta\Delta Cq}$ *method and represents the mean of four independent experiments. * indicates a significant difference (p<0.013) after a Bonferroni correction, based on a two-tailed t-test. Error bars show a 95% confidence interval for the true values of the fold change.*

4.3.3 Desaturases and cytokines

Regulation of the fatty acid desaturases D5D, D6D and SCD is important for a range of cellular functions including the process of inflammation, as they catalyze the metabolism of several pro- and anti-inflammatory agents [11, 12]. As IL-1β and TNF-α have important roles in the inflammatory process, it was of interest to investigate D5D, D6D and SCD mRNA expressions levels in THP-1 cells stimulated with these cytokines. THP-1 cells were also treated with IL-1β and TNF-α in combination with insulin, to reveal any cooperative effect.

THP-1 cells were treated with IL-1β, TNF-α, insulin and insulin combined with IL-1β and TNF-α for 24 hours. Two sets of cDNA were synthesized from total RNA representing each experimental condition and used as template for qPCR with primers for D5D, D6D, SCD and 18s rRNA. Untreated THP-1 cells were used as control.

THP-1 cells treated with IL-1β show a tendency of up-regulation in D5D, D6D and SCD mRNA expression compared to untreated cells (figure 12). Elevated mRNA expression of D5D, D6D and SCD can also be seen in TNF-α-treated cells. However, the clearest induction in D5D and SCD mRNA expression is observed in insulin-treated THP-1 cell, while mRNA expression of D6D shows similar responsiveness to all treatments. Regarding THP-1 cells treated with a combination of the three stimulating factors, an increased mRNA expression of D5D, D6D and SCD can be seen compared to untreated cells. However, cells treated with IL-1β, TNF-α and insulin did not show any cooperative effects for desaturase mRNA expression.

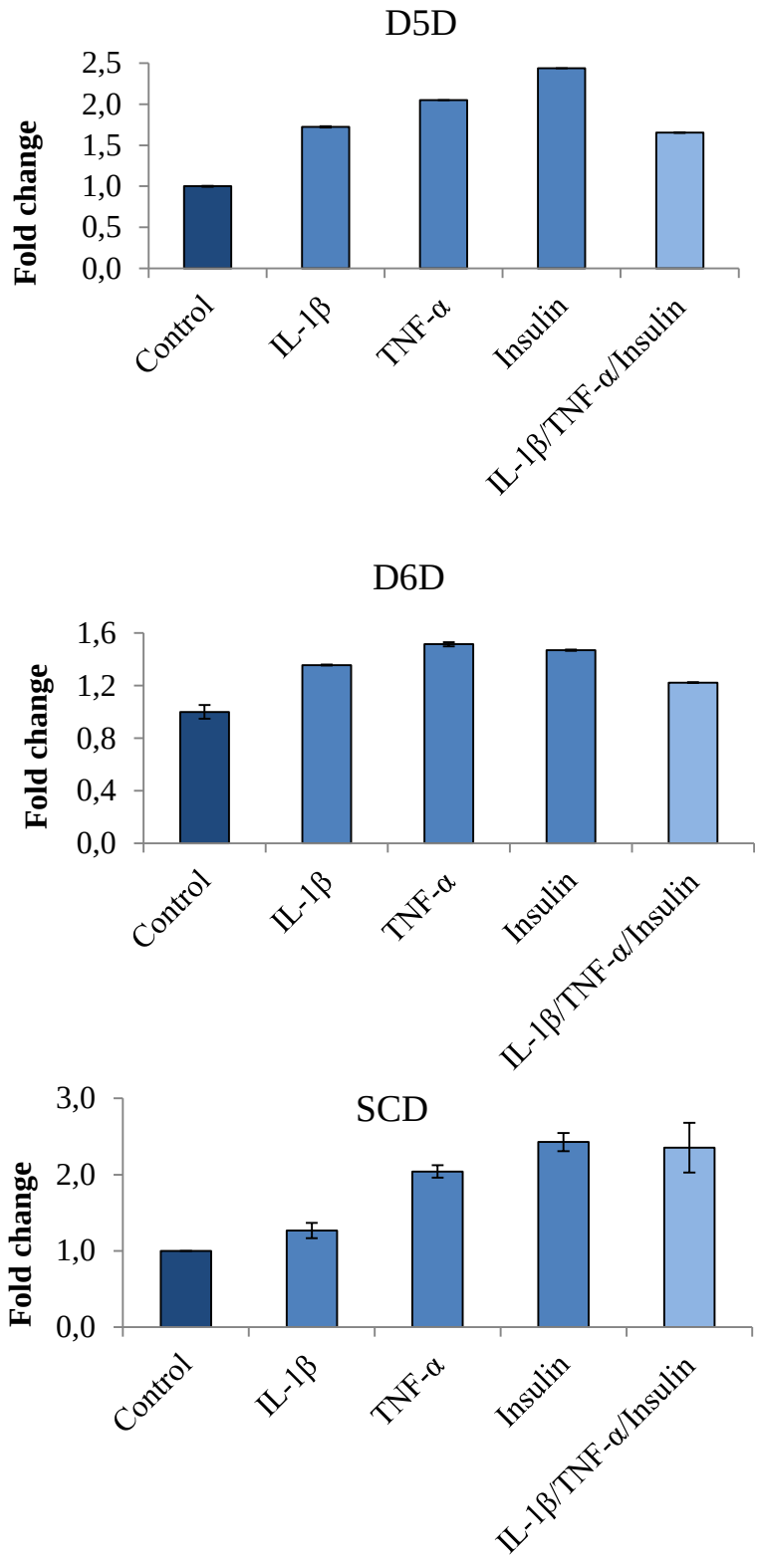

Figure 12: Fold change of desaturases compared to control. THP-1 cells (13 passages) were stimulated with IL-1β (10 ng/mL), TNF-α (10 ng/mL) and insulin *(0.01 µM) and combinations for 24 hours after serum starvation (0.5% FBS, 16 hours). mRNA expression is normalized with 18s rRNA, and the fold change for each gene is calculated by the* $2^{-\Delta\Delta Cq}$ *method. Error bars represent SD for two different cDNA syntheses to illustrate technical variances.*

Furthermore, we wanted to investigate the combination of insulin and cytokines. THP-1 cells were treated with the same stimulation factors as in the previous experiment, but this experiment also included THP-1 cells treated with a combination of IL-1β and TNF-α without insulin, and insulin combined with each one of the cytokines.

Cells stimulated with IL-1β revealed only a slight up-regulation in D5D and D6D mRNA expression compared to untreated cells (figure 13). mRNA expression levels of SCD seemed to be unaffected by IL-1β, as only a negligible increase can be observed. mRNA expression of D5D and D6D in response to TNF-α were elevated compared to untreated cells, while SCD mRNA expression seemed to be slightly down-regulated. Regarding the different combinations, none of the cells that were treated with the various combinations gave a more elevated expression of desaturases than insulin alone. In general, THP-1 cells were less responsive to the treatments in this experiment compared to the previous experiment. A possible explanation for this behavior will be illustrated in section 4.6.

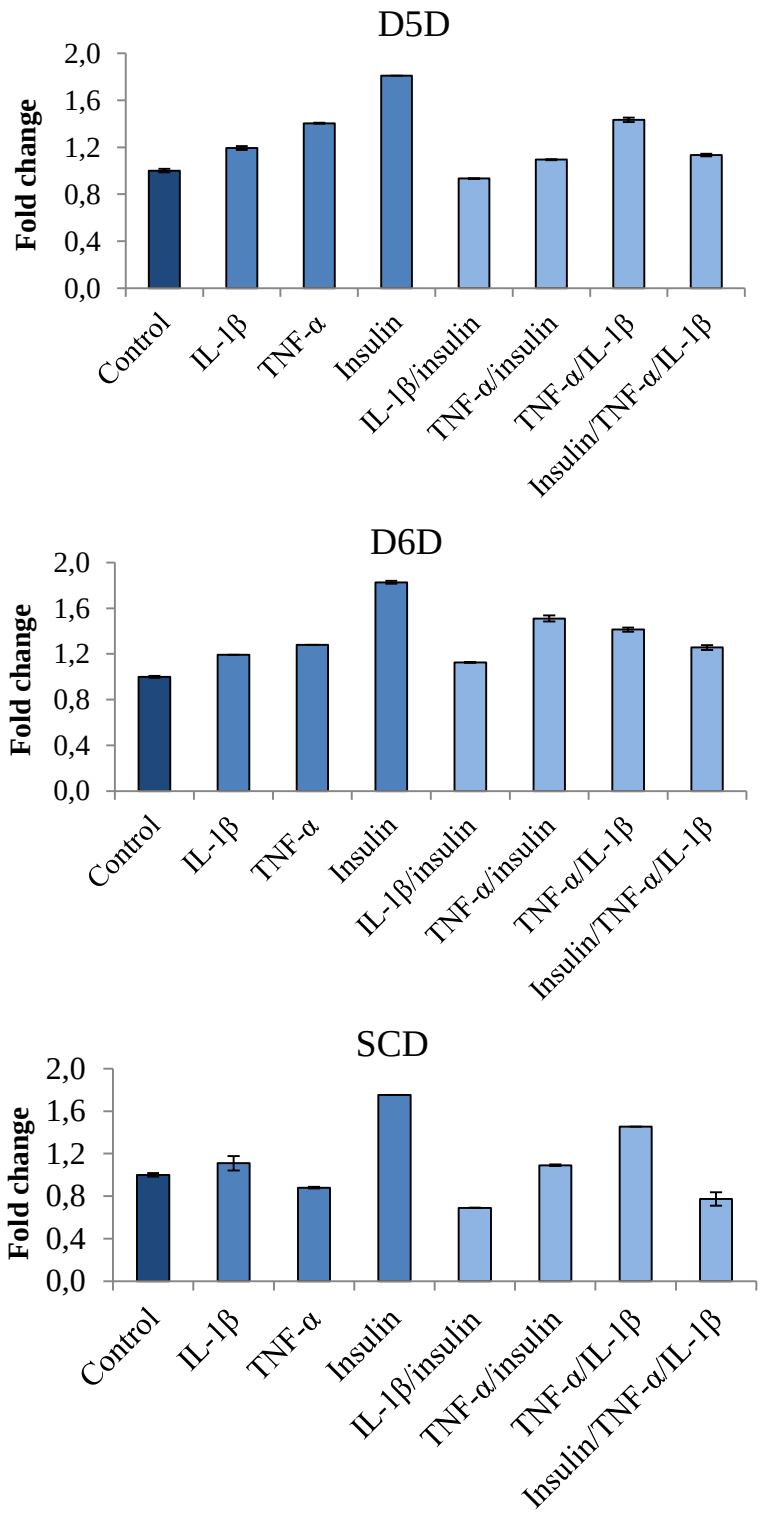

Figure 13: *Fold change of D5D, D6D and SCD mRNA expression compared to control. THP-1 cells (46 passages) were stimulated with IL-1β (10 ng/mL), TNF-α (10 ng/mL), insulin (0.01 µM) and listed combinations in 0.5% FBS for 24 hours after serum starvation (0.5% FBS, 16 hours). Expression of D5D, D6D and SCD is normalized with 18s rRNA, and the fold change for each gene is calculated by the* $2^{-\Delta\Delta Cq}$ *method. The error bars represent the SD for two different cDNA syntheses to illustrate technical variances.*

4.4 FOXO3A

The biological activity of transcription factor FOXO3A is tightly regulated by several modifications, including methylation, acetylation, ubiquitination and phosphorylation [70]. FOXO3A has been reported to be regulated by Akt through the insulin/PI3K/Akt signaling pathway in response to insulin and growth factors [22]. In accordance to this, a suppression of FOXO3A activity has recently been demonstrated in THP-1 cells cultured in high glucose-conditions [106]. The molecular mechanisms regulating the transcription of FOXO3A gene remain mostly unclear, but an increase in transcription has been reported in the liver from rats after 48 hours of fasting [107]. Moreover, investigations of diet-specific changes in leukocyte gene expression reported an upregulation in FOXO3A mRNA in response to a reduction in diet carbohydrate quantity relative to protein and fat [94]. Due to these observations it was of interest to investigate if insulin could be involved in the regulation of FOXO3A mRNA expression.

Next, expression of FOXO3A mRNA is analyzed in THP-1 monocytes treated with insulin and proinflammatory cytokines, in order to reveal any relation to the glucose metabolism and inflammatory-related processes.

4.4.1 Expression of FOXO3A is dose-dependently regulated by insulin in THP-1 monocytes

In order to find the optimal insulin concentration for the investigation of the regulation of FOXO3A mRNA expression, cDNA from the three dose-response experiments described in section 4.3.1 were used as templates for the qPCR.

The gene expression of FOXO3A was unaffected in insulin-treated THP-1 cells in the first (0.1 µM, 1 µM, 10 µM and 100 µM insulin) and the third (0.001 µM, 0.01 µM, 0.1 µM, 1 µM, 10 µM and 100 µM insulin) set of experiments (data not shown). Analyses of the second experiment (0.001 µM, 0.01 µM, 0.1 µM, 1 µM, 10 µM and 100 µM insulin) demonstrated a regulation in FOXO3A mRNA expression in THP-1 cells that were treated with 10 µM and 0.01 µM insulin for 24 hours (figure 14). As FOXO3A mRNA expression seemed slightly

more affected in cells treated with 0.01 µM, and the desaturases seemed to be responsive to this concentration as well, 0.01 µM insulin was used for further experiments.

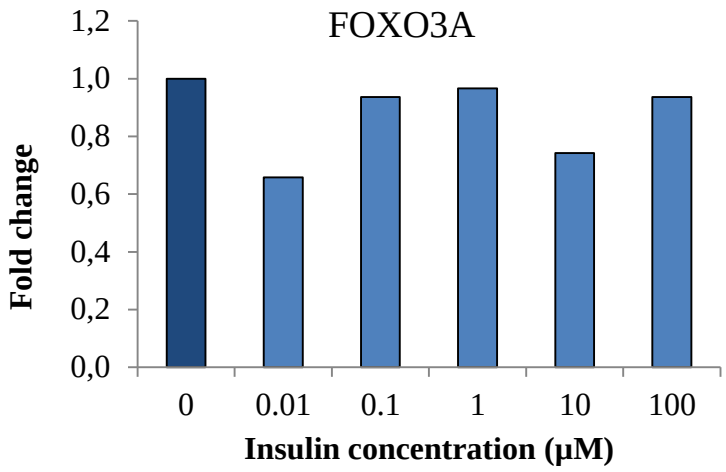

Figure 14: Fold change of FOXO3A mRNA expressions compared to control (0). THP-1 cells (28 passages) were incubated with the listed concentrations of insulin in 0.5% FBS for 24 hours after serum starvation (0.5% FBS, 16 hours). Expression of FOXO3A is normalized with 18s rRNA, and the fold change for each gene is calculated by the $2^{-\Delta\Delta Cq}$ *method.*

4.4.2 FOXO3A expression is regulated by insulin and cytokines at different time points in THP-1 monocytes.

IL-1β and TNF-α are produced and secreted by monocytes and monocyte-derived macrophages and play an important role in the development of inflammatory diseases [108, 109]. Recently, TNF has been shown to induce the translocation of nuclear FOXO3A into cytosol, and thereby decrease its transcriptional activity in human intestinal epithelial cells [110]. Other cytokines, including IL-2, IL-3 and IL-4 have also been reported to induce FOXO3A relocalization through the PI3K/Akt-pathway in mice [111, 112]. FOXO3A transcription factor has important, but cell type specific roles in the regulation of inflammatory processes [59, 60]. It is therefore of interest to study the response on FOXO3A mRNA expression in THP-1 cells treated with IL-1β and TNF-α.

The effect of insulin in FOXO3A gene expression was not very strong and varied to a great extent in the three dose-response experiments from the previous section. The 24 hours was not

necessarily the optimal treatment period to induce regulations of FOXO3A mRNA expression in THP-1 monocytes. Consequently, a time-course experiment with insulin, IL-1β and TNF-α treated cells was performed.

THP-1 monocytes were treated with IL-1β, TNF-α, and insulin for 2, 4, 8, 12 and 24 hours. FOXO3A mRNA expression seemed to be affected in a time-dependent manner for both cytokines and insulin treatments compared to untreated cells (control) (figure 15). A decrease in FOXO3A mRNA expression can be observed already two hours after treatment, but reveals a maximal decrease in THP-1 cells treated with IL-1β, TNF-α or insulin for 4 hours. THP-1 cells stimulated for 8, 12 and 24 hours show the same tendency of reduced FOXO3A mRNA expression, but not with the same response as the 4 hours treatment. The experiment was repeated and resulted in similar observations (figure not shown). Consequently, THP-1 cells were treated with IL-1β, TNF-α and insulin for 4 hours in further experiments.

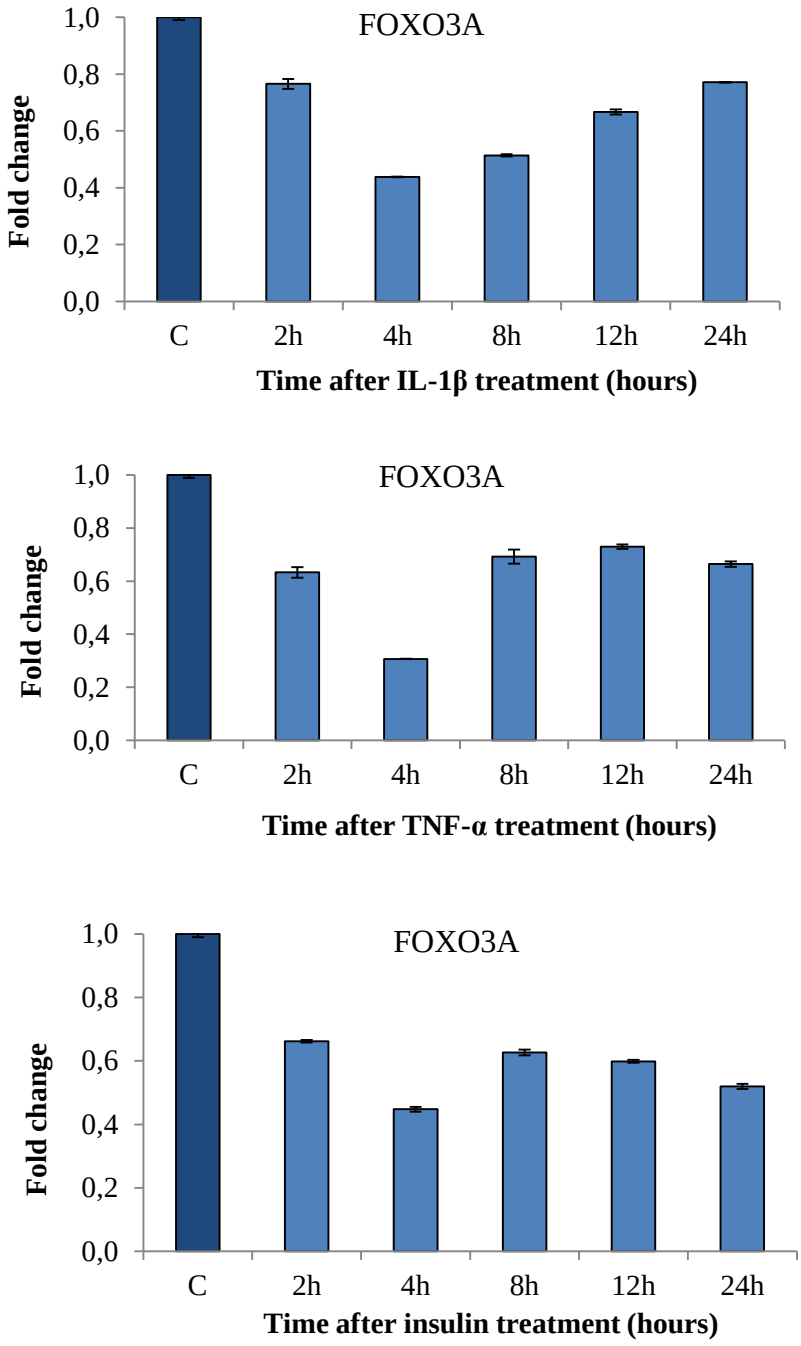

Figure 15: *Fold change of FOXO3A mRNA compared to control (C). THP-1 cells (49 passages) were treated with IL-1β (10 ng/mL), TNF-α (10 ng/mL) and insulin (0.01 µM) in 0.5% FBS for the selected time points after serum starvation (0.5%, 16 hours). Expression of FOXO3A is normalized with 18s rRNA, and the fold change is calculated by the* $2^{-\Delta\Delta Cq}$ *method. Error bars represent the SD for two different cDNA syntheses to illustrate technical variances. The experiment was repeated and resulted in similar observations*

4.4.3 Expression of FOXO3A is significantly down-regulated by insulin, TNF-α and IL-1β in THP-1 monocytes

FOXO3A mRNA expression in THP-1 cells treated with IL-1β, TNF-α or insulin for 4 hours were significantly down-regulated compared to untreated cells (control) (figure 16). A 0.48-fold change can be observed for IL-1β-treated cells, a 0.55-fold change for TNF-α-treated cells and a 0.52-fold change for insulin-treated cells compared to the untreated cells. This demonstrates that FOXO3A is significantly regulated at an early time point in response to insulin and cytokine stimulation in THP-1 monocytes. These results are based on four independent experiments.

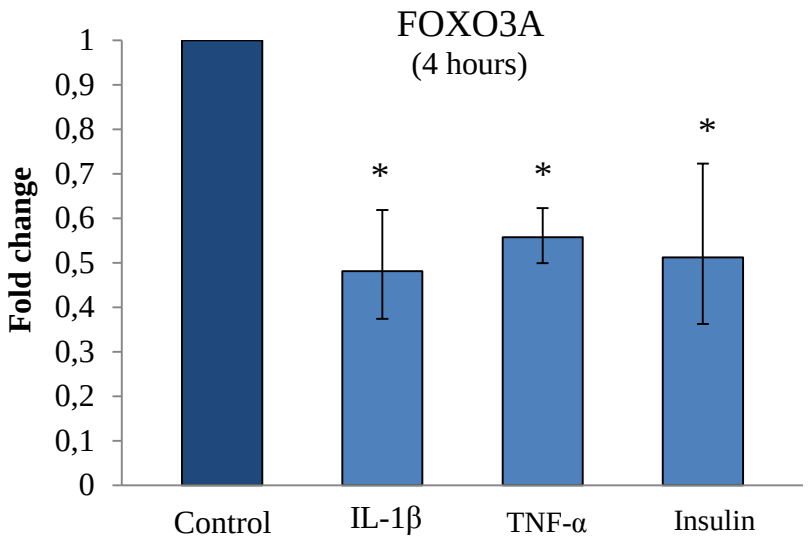

Figure 16: Fold change of FOXO3A mRNA compared to control. THP-1 cells (passage number < 50) were treated with, IL-1β (10 ng/mL), TNF-α (10 ng/mL) and insulin (0.01 µM) in 0.5% FBS for 4 hours, after serum starvation (0.5% FBS, 16 hours. Expression of FOXO3A is normalized with 18s rRNA. The fold change was calculated by the $2^{-\Delta\Delta Cq}$ *method and represents the mean of three independent experiments. * indicates a significant difference (p<0.016) after a Bonferroni correction. Error bars show a 95% confidence interval for the true values of the fold change.*

4.4.4 Regulation of FOXO3A mRNA expression by combinations of insulin and cytokines

THP1-cells treated with IL-1β, TNF-α or insulin for 4 hours did all reveal a significant reduced transcription of FOXO3A compared to untreated cells (see section 4.4.3). Due to these observations, THP-1 cells were treated with different combinations of the stimulating factors in order to investigate the possibilities for any additive, synergetic or even an antagonistic effect.

THP-1 cells were treated with the following combinations of IL-1 β, TNF-α and insulin; IL-1 β in combination with insulin, IL-1β in combination with TNF-α, TNF-α in combination with insulin, and TNF-α in combination with insulin and IL-1β. In the same set of experiments THP-1 cells were also treated with IL-1β, TNF-α and insulin to compare any cooperative effect with the response of the stimulating factors alone.

As in previous experiments, FOXO3A mRNA expression in THP-1 cells treated with IL-1β, TNF-α and insulin seems to be impaired compared to untreated THP-1 cells (control). The fold change of FOXO3A mRNA expression in cells treated with IL-1β, TNF-α and insulin is 0.47, 0.54 and 0.51 respectively (figure 17). Regarding the different combinations, all the listed combinations gave a similar effect, and none of the treatments reported any cooperative effect. The combination of IL-1β and insulin can be seen with a fold change of 0.43 for FOXO3A mRNA expression, while insulin in combination with TNF-α reveals a 0.54-fold change. THP-1 cells treated with a combination of the two cytokines show a 0.51-fold change of FOXO3A mRNA expression compared to untreated cells. Finally, the fold change of FOXO3A in THP-1 cells treated with a combination of insulin with IL-1β and TNF-α is 0.44. This experiment was repeated and revealed similar results (not shown).

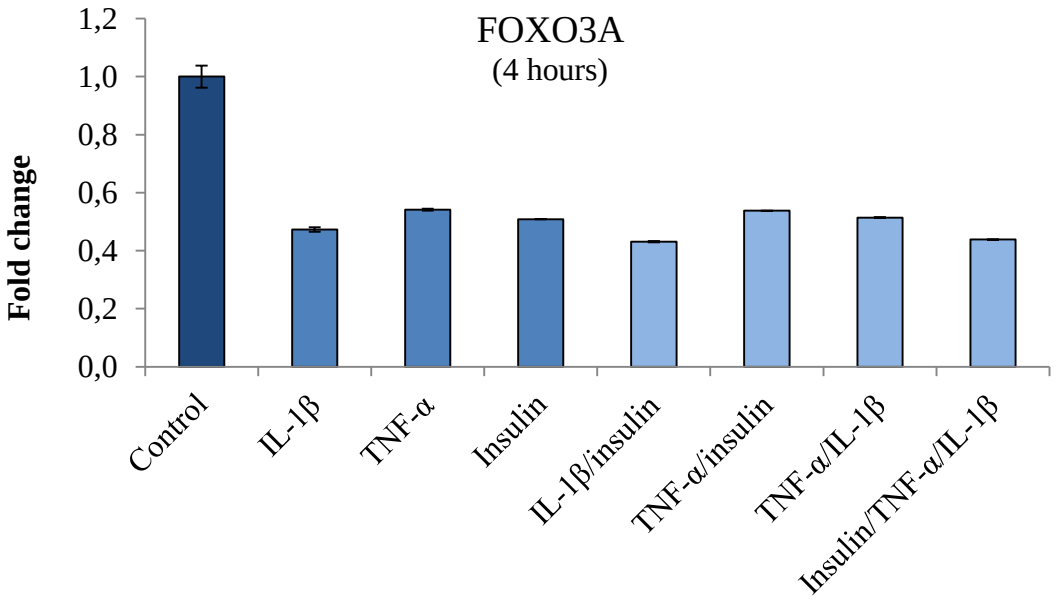

Figure 17: Fold change of FOXO3A compared to control. THP-1 cells (13 passages) were treated with IL-1β (10 ng/mL), TNF-α (10 ng/mL), insulin (0.01 µM) and listed combinations in 0.5% FBS for 4 hours, after serum starvation (0.5% FBS, 16 hours). Expression of FOXO3A is normalized with 18s rRNA. Fold change is calculated by the $2^{-\Delta\Delta Cq}$ method, and error bars represent standard deviation between two cDNA-syntheses to illustrate technical variables. The experiment was repeated and revealed similar results (not shown).

4.5 Expression of the FOXO3A downstream target genes TRAIL, TWIST1 and BTG1 is significantly upregulated in a synergistic manner in response to a combination of insulin, IL-1β and TNF-α

The transcription factor FOXO3A has an important role in a variety of cellular processes through the regulation of several genes involved in development, differentiation, metabolism, cell growth, inflammation and longevity [52-54]. As the transcription of FOXO3A was reduced in response to insulin and cytokines in the current study, we wanted to investigate if this down-regulation is reflected in FOXO3A downstream target genes. As mentioned in section 2.6, expression of TRAIL, TWIST1 and BTG1 has all been demonstrated to be regulated by FOXO3A transcription factor. However, this effect has not yet been reported for THP-1 monocytes.

TRAIL, TWIST1 and BTG1 have also been reported to be regulated by IL-1β, TNF-α and/or insulin; Insulin has been shown to down-regulate the pro-apoptotic ligand TRAIL in vascular

smooth muscle cells [113]. IL-1β and TNF-α on the other hand, induced TRAIL transcription in fetal astrocytes [114], but had no effect on TRAIL mRNA levels in T lymphocytes [115]. Furthermore, it has been shown that insulin and the insulin-like growth factor I (IGF-1) induce mRNA expression of TWIST1 in NIH-3T3 cells (mouse embryo fibroblast cell line) [116]. TWIST1 has also been suggested to be a target gene for the transcription factor NF-κB in the human HeLa cell line, induced by TNF-α [117]. Another pro-apoptotic gene, BTG1, has been reported to be transcriptionally regulated by insulin in human breast cancer cells [118].

THP-1 cells were stimulated with IL-1β, TNF-α and insulin, and with a combination of these stimulation factors for 24 and 12 hours. Two sets of cDNA were synthesized from the RNA representing the experimental conditions and used as template for qPCR with primers for TRAIL, TWIST1, BTG1 and 18s rRNA.

IL-1β, TNF-α and insulin seemed to affect TRAIL and TWIST1 mRNA expression levels, whereas combinations of all three resulted in a synergistic significant up-regulation (figure 18). TRAIL mRNA expression in THP-1 cells treated with TNF-α was significantly up-regulated. The fold change of TRAIL mRNA expression in IL-1β-, TNF-α- and insulin treated cells compared to untreated cells is 1.4, 2.4 and 1.4 respectively, while cells treated with combinations show an 8.2-fold in TRAIL mRNA expression. mRNA expression of TWIST1 shows a 2.1, 1.6 and 3.3-fold change in response to IL-1β, TNF-α and insulin, while combinations of insulin and cytokines resulted in a 7.8-fold change. The synergistic effect from cytokines and insulin was also observed in BTG1, but BTG1 mRNA expression seemed to be unaffected by the stimulation factors alone. Negligible regulations were shown for IL-1β-, TNF-α- and insulin, but a 4.0-fold change in BTG1 mRNA expression can be seen when cells were stimulated with combination of insulin and cytokines. The mean value of the fold change represents data from two (TWIST1) and three (TRAIL and BTG1) biological replicates. THP-1 cells treated with insulin and cytokines for 12 hours did not show the same induction as the 24 hours treatment period, and was therefore not used for further experiments (not shown).

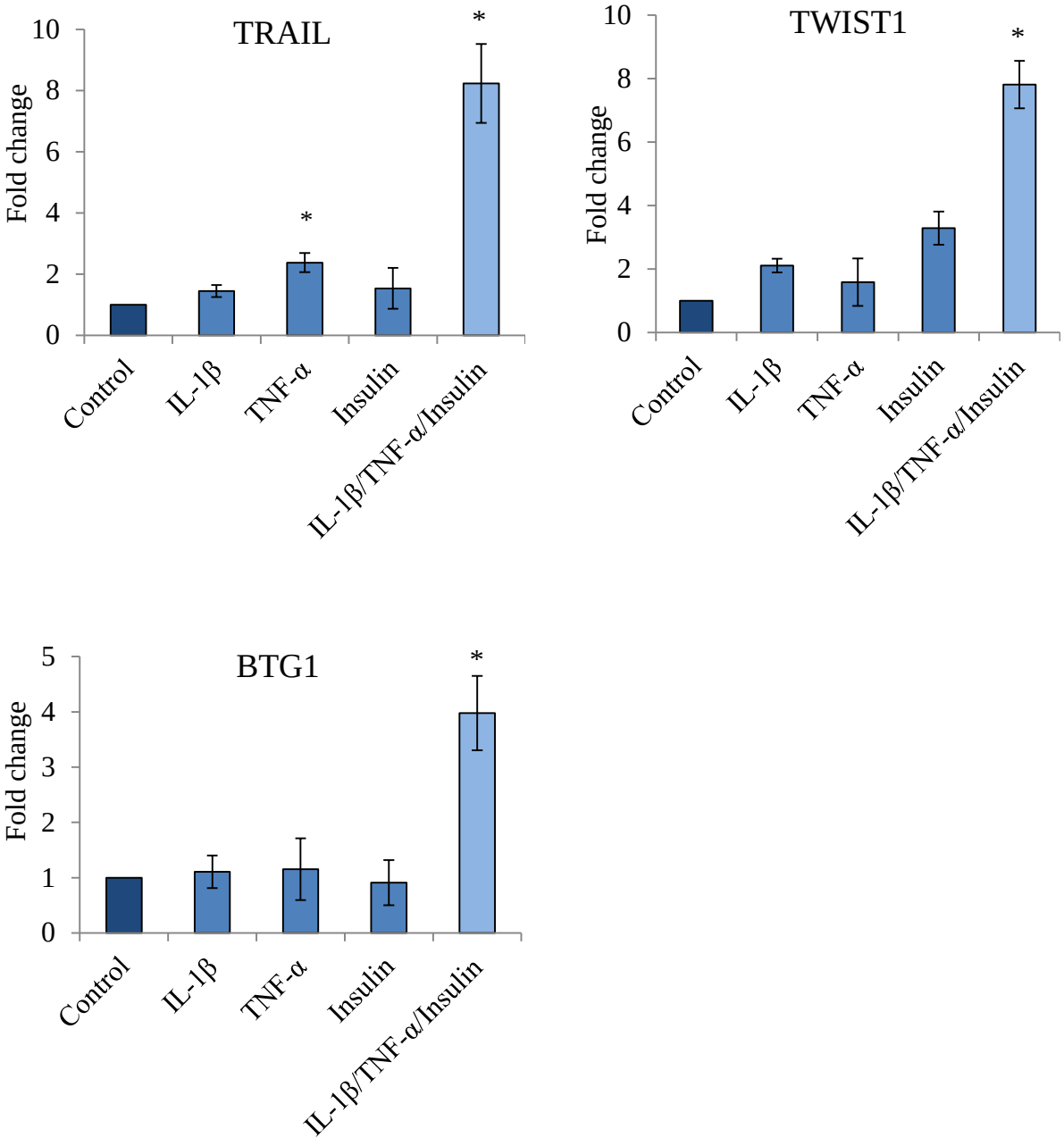

Figure 18: Fold change of TRAIL, TWIST1 and BTG1 compared to control. THP-1 cells (13 passages) were treated with IL-1β (10 ng/mL), TNF-α (10 ng/mL), insulin (0.01 µM) and combinations in 0.5% FBS for 24 hours, after serum starvation (0.5% FBS, 16 hours) Expression of target genes is normalized with 18s rRNA, and the fold change between treated and untreated cells (passage number 13) is calculated by the $2^{-\Delta\Delta Cq}$ *method. * indicates a significant difference (p<0.016/p<0.025) after a Bonferroni correction, based on a two-tailed t-test with 95% confidence interval. Error bars represent SD between three (TRAIL and BTG1) and two (TWIST1) independent experiments.*

4.6 Changes in gene expression due to passage number

At some point during this study, the effects of insulin and cytokines shown in FOXO3A and desaturase mRNA expressions were difficult to reproduce after the THP-1 cells had reached a certain passage number. Consequently, it was of interest to investigate if the response was influenced by changes in phenotypes due to the increasing passage number. Chemokine (C-C motif) ligand 2 (CCL2) and IL-6 (interleukin-6) have been shown to be upregulated in response to TNF-α [119, 120] and were therefore used as "positive controls". cDNA from TNF-α treated (10 ng/mL, 24 hours) and untreated (control) THP-1 cells with different passage number (49 and 59) were used as template for qPCR with primers for IL-6, CCL2 and 18s rRNA.

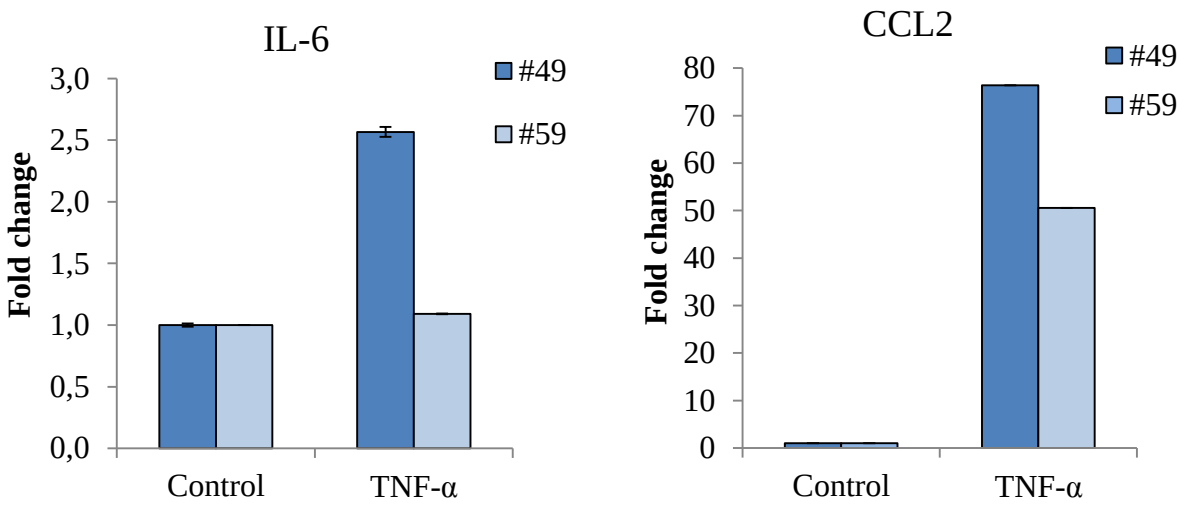

Figure 19: Fold change of IL-6 and CCL2 compared to control. THP-1 cells were treated with TNF-α (10 ng/mL) in 0.5% FBS for 24 hours, after serum starvation (0.5% FBS, 16 hours). Expression of IL-6 and CCL2 is normalized with 18s rRNA. Fold change between treated and untreated cells (passage number 49 and 59) is calculated by the $2^{-\Delta\Delta Cq}$ method. Error bars represents standard deviation between two cDNA-syntheses to illustrate technical variables.

Induction of CCL2 mRNA expression is much more pronounced in THP-1 cells with low passage number; THP-1 cells with passage number 49 show a 76.4-fold increase, while a fold change of 50.6 can be seen in cells with passage number 59 between cells treated with TNF-α and untreated cells (figure 19). This trend was also observed for IL-6 mRNA expression

where THP-1 cells with passage number 49 show a 2.5-fold change, while cells with passage number 59 show a 1.1-fold change.

Due to these observations, a new stock of THP-1 cells with passage number 11 was thawed and cultured for further experiments. A similar experiment was performed on these THP-1 cells in order to investigate the responsiveness of TNF-α treated THP-1 cells with low passage number. Induction of CCL2 mRNA expression was much more pronounced in THP-1 cells with low passage number; Cells with passage number 13 show a 176-fold change, compared to cells with passage number 49 and 59 which show a 76- and a 51-fold change between TNF-α-treated cells and untreated cells, respectively (figure 20).

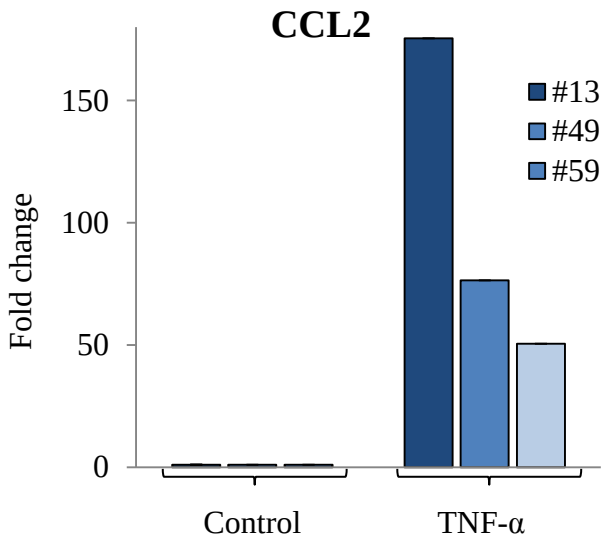

Figure 20: Fold change of CCL2 compared to control. THP-1 cells were treated with TNF-α (10 ng/mL) in 0.5% FBS for 24 hours after serum starvation (0.5% FBS, 16 hours). Fold change between treated and untreated cells (passage number 13, 49 and 59) is calculated by the $2^{-\Delta\Delta Cq}$ method. Expression of FOXO3A is normalized with 18s rRNA.

Due to these observations, qPCR was performed on cDNA from THP-1 cells with passage number 13 treated with IL-1β, TNF-α and insulin, with primers for D5D, D6D, SCD and FOXO3A. The fold change values for FOXO3A and D5D mRNA expressions from insulin and cytokine treated THP-1 cells with various passage numbers reveal similar trends as observed for the CCL2 expression (Figure 21 and 22).

The regulation of D5D mRNA expression in response to the listed stimulation factors is more induced in THP-1 cells with passage 13 and 46, compared to 56 and 58. A similar pattern was also observed for D6D and SCD (not shown). The regulation of FOXO3A mRNA expression is more induced in treated THP-1 cells with a passage number below 50.

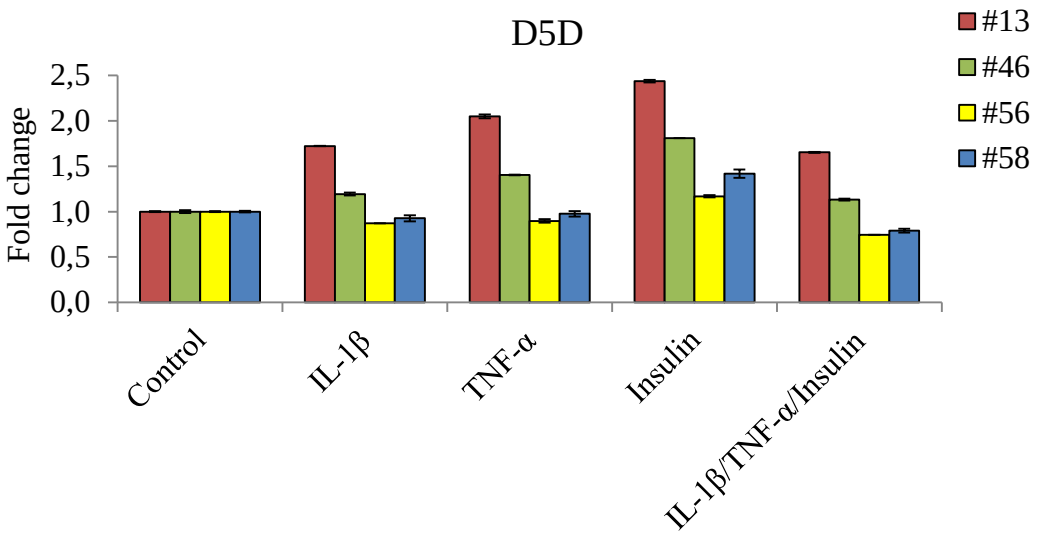

Figure 21: Fold change of D5D compared to control. THP-1 cells were treated with TNF-α (10 ng/mL) in 0.5% FBS for 24 hours, after serum starvation (0.5% FBS, 16 hours). Expression of D5D is normalized with 18s rRNA. Fold change between treated and untreated cells is calculated by the $2^{-\Delta\Delta Cq}$ method for four independent experiments with specified passage number. Error bars represents standard deviation between two cDNA-syntheses to illustrate technical variables.

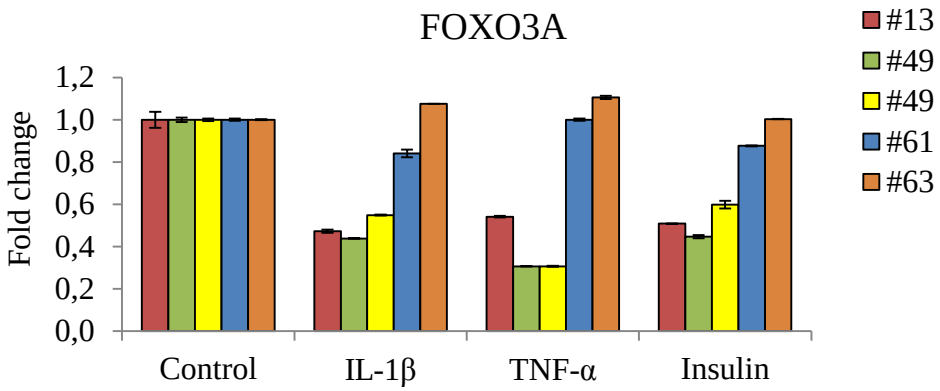

Figure 22: Fold change of FOXO3A compared to control. THP-1 cells were treated with TNF-α (10 ng/mL) in 0.5% FBS for 4 hours, after serum starvation (0.5% FBS, 16 hours). Expression of FOXO3A is normalized with 18s rRNA. Fold change between treated and untreated cells is calculated by the $2^{-\Delta\Delta Cq}$ method for five independent experiments with specified passage number. Error bars represents standard deviation between two cDNA-syntheses to illustrate technical variables.

5 DISCUSSION

THP-1 cells were used as a model system for circulating human monocytes in order to investigate the expression of selected genes were regulated by insulin and cytokines. In this study, gene expression of D5D, D6D and SCD was shown to be significantly up-regulated in THP-1 cells treated with insulin for 24 hours. Moreover, mRNA expression of the transcription factor FOXO3A was significantly down-regulated in THP-1 cells treated with insulin, IL-1β and TNF. However, neither FOXO3A nor the desaturases were cooperatively regulated by these stimulating factors. TRAIL, TWIST and BTG1 on the other hand, were demonstrated to be significantly up-regulated in a synergistic manner when insulin was combined with IL-1β and TNF-α in THP-1 cells treated for 24-hours.

5.1 Validation of primer for FOXO3A

According to BLAST, the FOXO3A primer used in this study is specific for FOXO3A. This primer recognizes sequences located in exon 3 and exon 4 in the FOXO3A gene. According to resent research, FOXO3A and the pseudogene FOXO3B share the entire sequence in exon 2-4 [121]. These findings were published after the current experiments, and were therefore not possible to take into account. The possibility that the current primer might recognize the pseudogene has to be considered. However, the resulting dissociation curve with expected melting point from the qPCR and the pure qPCR product visualized by gel-electrophoresis with expected length, indicate that the primer pair is indeed specific for FOXO3A. Still, more research needs to be done in order to fully exclude the presence of any amplification of mRNA from the pseudogene FOXO3B. There has not been reported any pseudogenes related to the other genes analyzed in this study. Consequently, the primers were considered as specific for its target genes as all primers resulted in single dissociation curves during qPCR, and due to the fact that qPCR products revealed the expected length.

5.2 Evaluation of reference genes

Prior to all gene transcription qPCR studies, it was essential to find one or more appropriate reference gene. The efficiency of amplification of D5D, D6D, SCD and FOXO3A were compared to the potential reference genes GAPDH, B_2M, HPRT and 18s rRNA. Even though GAPDH seemed to be a proper gene for the normalization of the desaturases and FOXO3A, this reference gene was excluded at an early point because it has been reported to be regulated by insulin [122]. B_2M and 18s rRNA were expressed at a higher level inTHP-1 cells compared to the target genes, while HPRT was less expressed. HPRT was therefore not used as a reference gene in the further experiments. Initially, both B_2M and 18s rRNA were used as reference genes in order to ensure a proper normalization. Over time, the Cq-value of B_2M seemed to view minor variations in response to the stimulation factors. To avoid unreliable normalizations in the further experiments only 18s rRNA were used as reference gene. The Cq values for 18s rRNA were stable throughout all qPCR analyses, and was therefore considered to be the most suitable reference gene. All gene-expression changes presented in this thesis is therefore calculated based on 18s rRNA mRNA expressions. Regarding the primers for TRAIL, BTG1 and TWIST1, only the primer pair for TRAIL did actually show similar amplification efficiency as the primer pair for 18s rRNA.

5.3 THP-1 as a model system for circulating monocytes

The THP-1 cell line is one of the most widely used cell lines to investigate the regulation and function of human monocytes and macrophages. Several reports have demonstrated that this cell line is suitable for studying circulating monocytes in inflammation, obesity and diabetes-related studies [83, 84, 123] . Therefore, we chose to utilize THP-1 cells as a model system to study the possible role of insulin and cytokines in the regulation of inflammation related genes in human monocytes.

As the THP-1 monocytes are used to demonstrate features of circulating monocytes, it is appropriate to keep the cells at a low passage number. Circulating monocytes do not divide after leaving the bone marrow [124], and will therefore never obtain a high passage or generation number *in vivo*.

5.4 Passage number affected gene expression in THP-1 monocytes

It has been demonstrated that a high passage number may influence cell morphology, growth rate and gene expression compared to cells with a low passage number [85, 86].

At some point during this study, results from previous experiments were difficult to repeat. The THP-1 cells seemed to be less responsive to the various stimulations, even though the protocols were standardized for all experiments. D5D, D6D and SCD mRNA, which had shown to be significantly induced by insulin previous in this study (see figure 11, section 4.3.2), started to be less responsive at the time when experiments were performed to investigate insulin- and cytokine-treated THP-1 cells. This became an actual challenge during investigations of FOXO3A mRNA expression. In the first two experiments FOXO3A mRNA levels were clearly reduced in THP-1 cells treated with insulin and cytokines for 4 hours compared to untreated cells. In the following sets of experiments, performed with the same protocol, the down-regulation of FOXO3A mRNA levels was barely noticeable. Several experiments were performed in an attempt to achieve the same regulative effect shown in previous experiments. At this point, the number of cell passages was actually above 60. Passage number related phenotypic alterations were suggested to be the reason for the reduced responsiveness.

In order to investigate the responsiveness in THP-1 cells with various passage numbers, CCL2 and IL-6 mRNA expressions were studied in TNF-α-treated THP-1 cells. TNF-α is reported as an inducer for CCL2 and IL-6 transcription [119, 120]. As predicted, cells with low passage number were more responsive to the TNF-α treatment compared to cells with a high passage number. A similar trend was demonstrated for the desaturases and FOXO3A.

These observations demonstrate that the quality of the THP-1 cell line is essential in order to perform successful experiments. There are no tests that directly determine the passage-related effects in a cell line. It is therefore necessary to pay attention to passage numbers in case it might affect the research results. Keeping the cells at a low passage number seems to contribute to reliable and reproducible results.

5.5 D5D, D6D and SCD mRNA expression is significantly induced by 0.01 µM insulin in THP-1 monocytes

D5D, D6D and SCD have been shown to be expressed in THP-1 monocytes in previous studies [27, 125]. Recently, Arbo *et al* [27] demonstrated a significant up-regulation of D5D, D6D and SCD mRNA expression in THP-1 cells treated with 10 µM insulin for 24 hours. However, concentrations from 10 µM and down to 0.001 µM insulin have been used in similar experiments [102, 103, 105], and were therefore all considered to be potential insulin concentrations for this study.

Results from the current study confirmed that D5D, D6D and SCD are expressed in THP-1 monocytes. Here, desaturase mRNA expression was significant up-regulated in THP-1 cells treated with 0.01 µM insulin for 24 hours.

These observations demonstrate that THP-1 cells are sensitive for insulin treatment at low concentration as well. The insulin concentration of 0.01 µM is 100 times lower than the concentration used in Arbo *et al* [27]. The insulin-treatment period of 24 hours used in the current study is consistent with previous similar experiments [27, 102-105].

As insulin levels increase in response to elevated circulating glucose after a meal *in vivo* [17], and insulin has been demonstrated to increase expression levels of desaturases in this study, PUFA metabolism and further inflammation-related eicosanoids might be influenced by nutrient composition in meals. As the mRNA expression of desaturases is positively regulated by insulin in THP-1 cells, a similar response might be true for circulating blood cells. However, investigations of D5D, D6D and SCD need to be performed to understand how the enzyme activity is regulated before such a conclusion can be made.

5.6 FOXO3A mRNA expression is significantly down-regulated by insulin in THP-1 monocytes

Insulin has been reported to regulate the biological activity of FOXO3A through the PI3K/Akt-pathway [22, 71]. Although the post-transcriptional regulations of FOXO3A in response to insulin have been well investigated, the molecular mechanisms that regulate FOXO3A mRNA expression levels remain mostly unclear.

Elevated levels of FOXO3A mRNA expression have been observed in rats after 48 hours of fasting [107]. When the rats were fed for 3 hours after the fasting, the induced FOXO3A mRNA expression went back to levels observed before fasting. In contrast to these findings Imea *et al* [107] also demonstrated a reduced FOXO3A mRNA expression in response to insulin deficiency in rats. Furthermore, Imea *et al* suggest that this suppression of FOXO3A mRNA occurs in order to assuage the excessive actions of FOXO3A transcription factor, as insulin deficiency keeps FOXO3A inside the nucleus. In order to examine the regulative role of insulin, we investigated FOXO3A mRNA expression levels in insulin-treated THP- 1 monocytes.

FOXO3A was expressed in THP-1 monocytes in the current study, which is consistent with previous findings [126, 127]. Here, for the first time a significant down-regulation of FOXO3A expression by insulin in THP-1 monocytes was demonstrated. These observations support previous findings and theory saying that regulation of FOXO3A may be related to the glucose-metabolism. However, it is in contrast to findings by Imae *et al* [107], where a decrease in FOXO3A mRNA expression was reported in the liver from insulin deficiency rats. These contrary findings emphasize that FOXO3A may have cell specific roles.

5.7 FOXO3A mRNA expression is significantly down-regulated by cytokines in THP-1 monocytes

In previous studies, TNF-α has been shown to induce the translocation of nuclear FOXO3 by phosphorylation, and thereby regulate the transcriptional activity in human intestinal epithelial cells [110]. Interleukins, including IL-2, IL-3 and IL-4 have also been reported to induce FOXO3A phosphorylation through the PI3K/Akt-pathway in mice [111, 112]. As FOXO3A transcription factor are active in mediating inflammatory processes [59, 60], it was of interest to investigate FOXO3A mRNA levels in relation to the proinflammatory cytokines IL-1β and TNF-α in THP-1 monocytes.

In this study, a significant down-regulation in FOXO3A gene expression was observed in THP-1 monocytes only four hours after TNF-α and IL-1β were added to the cells.

From these results it appears that the regulation of FOXO3A transcription in THP-1 cells are indeed responsive to cytokines, and this might reflect similar events in circulating monocytes

during inflammation. These observations support previous findings where FOXO3A has been suggested to be an important transcription factor involved in several inflammatory processes [59, 60].

5.8 TRAIL mRNA expression is significantly induced by TNF-α in THP-1 monocytes

Besides being an inducer for apoptosis in tumor- and virally infected cells, previous studies have demonstrated that TRAIL induce transcription of inflammatory related genes [128, 129]. Tang *et al* [128] suggest that TRAIL is able to induce the release of cytokines through activation of NF-κB. The transcription factor NF-κB, which also is induced by TNF-α, regulates a wide range of genes involved in inflammatory processes [51]. TWIST1 has also been related to inflammatory processes, as it has been reported to regulate the expression and secretion of several inflammatory adipokines in human white adipocytes [61]. This gene has also been reported as a target for NF-κB induced by TNF-α [117]. TWIST mRNA expression has also be shown to be up-regulated by insulin and IGF-1 in NIH-3T3 cells [116]. Regulation of the apoptosis-promoting BTG1 gene has not yet been directly related to inflammation, but an increase in gene expression by insulin has been demonstrated in human cancer cells [118].

In this study, TRAIL, BTG1 and TWIST1 were all expressed in THP-1 monocytes. These results support previous findings [130-133]. Furthermore, a significant up-regulation of TRAIL transcription in THP-1 cells treated with TNF-α was demonstrated. These results support the theory from Tang *et al* [128] saying that TRAIL participates in several inflammatory events. These results also suggest that TRAIL is induced by TNF-α in human monocytes *in vivo*.

It is worthwhile to note that TWIST1 seems to be affected by insulin and IL-1β in the current study. As FOXO3A has been shown to inhibit transcription of TWIST1 [63], and FOXO3A mRNA expression was down-regulated by insulin and IL-1β in the current study, there might be a possibility that the observed induction of TWIST1 mRNA expression is linked to FOXO3A inhibition caused by insulin and IL-1β. However, more experiments need to be performed in order to clarify if TWIST1 in fact is regulated by IL-1β or insulin, and to reveal the role of the FOXO3A transcription factor in this potential pathway.

As BTG1 expressions seemed to be unaffected in THP-1 cells treated with separated stimulation factors, the 24 hours-stimulation may not be the optimal treatment period. This also applies to the regulation of TRAIL and TWIST1 expression. A more obvious induction might occur if THP-1 cells were incubated with insulin and cytokines for a longer time period. For instance, 48 or 72 hours have been used to demonstrate cytokine-regulated transcriptional activity [134, 135].

5.9 Insulin and cytokines induce expression of TRAIL, TWIST1 and BTG1 in a synergistic manner in THP-1 monocytes

IL-1β and TNF-α have previously been reported to demonstrate a synergistic effect in the regulation of gene transcription [134]. Moreover, insulin has been shown to enhance cytokine-induced inflammation-related gene transcription in hepatocytes [136]. Okazaki *et al* [136] suggest that this cooperation may be part of the explanation why enhanced production of cytokines and hyperinsulinemia results in inflammation and metabolic syndromes.

In this study, a significant increase in the transcription of TRAIL, TWIST1 and BTG1 in response to insulin combined with IL-1β and TNF-α in a synergistic manner in THP-1 monocytes was demonstrated.

The demonstrated synergistic effect between insulin and cytokines is consistent with previous findings, where insulin and IL-1β cooperatively increased mRNA expressions in a human hepatic cell line [136]. As discussed in the previous section, the induction of TWIST1 mRNA expression might involve the inhibition of FOXO3A activity by insulin and/or cytokines. The enhanced effect that was demonstrated for TRAIL and BTG1 in this study is in contrast to what we expected. As FOXO3A has been reported to induce TRAIL and BTG1 mRNA expression [65-68] and TNF-α and insulin have been shown to inhibit the transcriptional activity of FOXO3A [22, 71, 110], a reduced mRNA expression of TRAIL and BGT1 was expected. Thus, this observed induction of TRAIL and BTG1 expression probably involves other regulatory factors.

Insulin in combination with IL-1β and TNF-α did not demonstrate any cooperative effect in the regulation of FOXO3A mRNA expression. Regarding the regulation of D5D, D6D and SCD mRNA expression, more investigations need to be done to reveal any regulative effect of

IL-1β and TNF-α. However, it is worthwhile to note that IL-1β seemed to inhibit the induction of desaturases caused by insulin as shown in figure 13. This negative regulation of insulin signaling is discussed by Osborne and Olefsky [137], where cytokines including IL-1β is suggested to contribute to induce insulin resistance. Thus, this regulative effect should be further explored in THP-1 cells.

5.10 Conclusion

THP-1 cells were used as a model system for circulating human monocytes in order to reveal possible alterations in expression of inflammatory-related genes in response to insulin and cytokines.

Gene expression of the fatty acids desaturases D5D, D6D and SCD was significantly up-regulated by 0.01 µM insulin in THP-1 cells after a 24-hours treatment. This response has been demonstrated before, but with a higher concentration of insulin. Here it is shown that THP-1 cells are sensitive also to lower concentrations of insulin. FOXO3A mRNA expression was significantly down-regulated by insulin, IL-1β and TNF-α. These observations strengthen the suggested inflammatory role of FOXO3A. A maximum regulation of FOXO3A was observed after a four-hours treatment with insulin and cytokines. This indicates that FOXO3A is transcriptionally regulated at an earlier exposure-time compared to the other genes that were examined in this study. TRAIL, TWIST and BTG1 were significantly up-regulated in THP-1 cells treated with a combination of insulin, IL-1β and TNF-α for 24 hours. The observed induction of TWIST1 mRNA expression might be linked to the demonstrated inhibition of FOXO3A caused by insulin, IL-1β and TNF-α.

The demonstrated insulin- and cytokine-responsiveness in THP-1 cells suggests that this cell line is a suitable model-system for studying changes in gene expression of inflammatory-related genes by cytokines and diet-related hormones.

Moreover, it was demonstrated that high passage THP-1 cells were less responsive to treatment with insulin and cytokines than cells with a low passage number. As previous studies have shown that certain cell lines reveal phenotypic alterations due to a high number of passages, it is likely to believe that the variable responsiveness observed in this study could

be explained by this feature. These observations demonstrate the importance of keeping THP-1 cells at a low passage number during experiments.

The gene-environment interactions in diseases like diabetes, obesity, CVD and other inflammatory-related diseases are still poorly understood. More studies need to be performed in order to understand how nutrition affects these metabolic disorders. The research results presented in this study might be a minor contribution to the bulk of knowledge that is needed to fully understand these issues.

5.11 Proposals for further research

5.11.1 Further studies of the effect of cytokine on desaturase expression

The mRNA expression levels of D5D, D6D and SCD were all significantly up-regulated in response to insulin in THP-1 monocytes. These observations were done in experiments performed on cells with low passage number. During the time when THP-1 cells were treated with cytokines, the passage number was considerably higher. As the passage number seemed to be a critical factor for the responsiveness to insulin and cytokines in THP-1 cells, this probably caused the variable response in expression of mRNA that were observed in the experiments. Consequently, regulation of mRNA expression of D5D, D6D and SCD by IL-1β and TNF-α should be investigated in low passage THP-1 cells to determine any regulative effect.

5.11.2 Further optimization of in vitro conditions

The dose-response experiment for FOXO3A was performed on THP-1 cells treated with insulin for 24 hours. As the 24 hours period was not the optimal treatment period in order to achieve the maximum regulation of FOXO3A expression by insulin, additional experiments should be performed on THP-1 cells treated with various concentrations of insulin for four hours. Various concentrations of cytokines should also be examined in order to find the optimal concentrations for a maximum regulation of desaturases, FOXO3A, TRAIL, TWIST1 and BTG1. Furthermore, a set of dose-response experiments should be performed in order to

find the optimal concentrations of IL-1β and TNF-α for achieving maximum regulation of gene expressions.

During investigations of TRAIL, TWIST1 and BTG1, THP-1 cells were stimulated with insulin and cytokines for 12 and for 24 hours. The cells were more responsive to the 24 hours-treatment. Consequently, this period was selected for further studies. The 24 hours period may not necessarily be the optimal time period in order to achieve maximal regulation of gene expression. Consequently, THP-1 cells should be treated with insulin and cytokines for a longer period to reveal possible higher response in the regulation of TRAIL, TWIST1 and BTG1 mRNA expression.

5.11.3 Combinations of insulin and cytokines in the regulation of TRAIL, TWIST1 and BTG1

mRNA expression of TRAIL, TWIST and BTG1 were only analyzed in THP-1 cells treated with insulin, IL-1β and TNF-α alone or with combination of all three. Different combinations of cytokines and insulin should be studied in the same was as in the current study of FOXO3A and the desaturases. Due to the fact that the combination of insulin with both cytokines induced mRNA expression of TRAIL, TWIST and BTG1 in a synergistic manner, it is of interest to study THP-1 cells treated with insulin in combination with IL-1β, IL-1β in combination with TNF-α, and TNF-α in combination with insulin. In this way it is possible to see how these stimulation factors act in cooperation with each other in the process of stimulating mRNA expression of the relevant genes.

5.11.4 Investigate the regulations at translation and post-translation levels

As mentioned in section 2.3, proteins can be regulated at several levels. In this study, genes were only investigated at the transcriptional level. Even though current observations suggest a potential relation between these genes and the inflammatory processes in our body, more investigations need to be done before a conclusion can be drawn. Consequently, regulation of FOXO3A, desaturases, TRAIL, TWIST1 and BTG1 by insulin and cytokines should be investigated at protein levels in THP-1 cells in further studies. These studies should include

investigating the activity of the transcription factors and the enzymatic activity of the desaturases. The possible involvement of FOXO3A in the up-regulation of TWIST1 should also be investigated.

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Appendix

Paired, two-tailed Student T-test was used to compare the mean of the ΔCq-values for treated cells with the ΔCq-values for untreated cells (control) in relation to the variation in the data. P-values for the T-test performed on relevant genes are listed in table A. p-values that are considered significant after a Bonferroni correction are written in bold.

Table A: p-values for the T-tests performed in this study. p-values that are considered significant are written in bold.

Treatment	Gene	p-value
Insulin	D5D	**0.001**
	D6D	**0.002**
	SCD	**0.005**
	FOXO3A	**0.014**
	TRAIL	0.378
	BTG1	0.623
	TWIST1	0.060
IL-1β	FOXO3A	**0.006**
	TRAIL	0.043
	BTG1	0.711
	TWIST1	0.062
TNF-α	FOXO3A	**0.002**
	TRAIL	**0.008**
	BTG1	0.887
	TWIST1	0.450
Insulin/IL-1β/TNF-α	TRAIL	**0.002**
	BTG1	**0.005**
	TWIST1	**0.020**