# Insulin sensitivity and nutrient utilisation in skeletal muscle

by

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Thesis submitted in fulfilment of the requirement for
the Degree of Doctor of Philosophy

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November 2009

# Chapter 7: Comparing gene expression profile of skeletal muscle cells in response to long-chain saturated and n-3 polyunsaturated fatty acids

## 7.1 Introduction

The inhibitory effect of SFAs on insulin sensitivity has been consistently demonstrated *in vivo* (Vessby et al. 2001) and *in vitro* (Montell et al. 2001). In addition, saturated fat intake has also been associated with an increased risk for cardiovascular disease and some cancers. In a cohort of > 10,000 individuals (≥ 40 yr) followed for 13 yr, SFAs as a percentage of total fat intake was associated with an increased incidence of coronary heart diseases (Goldbourt et al. 1993). Epidemiological data suggest a strong association between SFAs intake and the increased risk and/or incidence of prostate cancer (Kurahashi et al. 2008), postmenopausal invasive breast cancer (Thiebaut et al. 2007) and localised colorectal cancer (Butler et al. 2009).

The data presented in Chapters 5 and 6 suggest that PA promotes intramuscular accumulation of triglyceride and lipid metabolites including ceramide and DAG. Given the effect of PA on activating various signalling and lipid metabolic pathways, it is perhaps not surprising that PA modifies cellular metabolism in aspects other than insulin signalling. For example ceramide is an important bioactive messenger in signal transduction processes mediating cellular differentiation, proliferation, apoptosis and senescence and has been proposed to play a significant role in the pathophysiology of human diseases including cancer, cardiovascular and neurodegenerative disorders in particular Alzheimer's disease (Pandey et al. 2007). *In vitro*, ceramide induce apoptosis in cardiomyocytes (Feuerstein and Young 2000), neuronal cells (Ariga et al. 1998) and

lung endothelial cells (Medler et al. 2008). Ceramide-induced apoptosis involves the activation of the stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) signalling pathway, which induces the translocation of Bax to the mitochondria and subsequently leads to the release of cytochrome c and cell death (Pandey et al. 2007). In addition to activating the inflammatory pathways in skeletal muscle cells (Chapter 5), PA also increased the mRNA and protein content of IL-6 in human coronary artery endothelial cells and smooth muscle cells, which has been shown to induce vascular inflammation and subsequently increased fibrinogen deposition and thrombus formation in the vessel wall (Staiger et al. 2004).

In addition to the effect of n-3 PUFAs on improving insulin sensitivity in skeletal muscle (Chapter 5), the cardiovascular benefits of n-3 PUFAs have also been consistently demonstrated. In individuals free of clinical cardiovascular disease, dietary n-3 PUFAs intake was associated with a reduced prevalence of subclinical atherosclerosis (He et al. 2008). In three large randomised-controlled trials of 32,000 participants, n-3 PUFAs supplementation reduced cardiovascular events by 19 – 45% (reviewed by Lee et al (2008)). The protective effect of n-3 PUFAs against cardiovascular risk may be mediated by alterations in lipid metabolism and inflammatory responses. Dietary n-3 PUFAs supplementation has been shown to reduce circulating levels of triglycerides and low-density lipoprotein cholesterol and increase high-density lipoprotein cholesterol in both healthy and hyperlipidaemic individuals (Grimsgaard et al. 1997; Mori et al. 2000). The hypolipidaemic effect of n-3 PUFAs is mediated by an increase in β-oxidation in liver and to a lesser extent in skeletal muscle (Ukropec et al. 2003) as well as an inhibition of SREBP activation which down-regulates lipogenesis at the transcription level (Schmitz and Ecker 2008). n-3 PUFAs are anti-inflammatory. They suppress the production of

arachidonic acid (AA)-derived eicosanoids, including thrombozane $A_2$ and prostaglandin I2, which promote inflammation and platelet aggregation (Larsson et al. 2004). In contrast, n-3 PUFAs induce the production of thrombosane 3 and prostacyclin 3 which have a less potent effect on platelet aggregation when compared to the AA-derived eicosanoids (Jung et al. 2008). Taken together, n-3 PUFAs induce a shift in the eicosanoid synthesis pathway which creates an anti-thrombotic effect. The anti-inflammatory effect of n-3 PUFAs is also mediated by down-regulating the production of pro-inflammatory cytokines and ROS. In healthy young men, DHA supplementation reduced TNF-α and IL-1β secretion from peripheral blood mononuclear cells *in vitro* by up to 45% (Kelley et al. 1999) which may be a consequence of the effect of n-3 PUFAs on inhibiting NFκB activation (Singer et al. 2008).

n-3 PUFAs are also known for their potential effect on improving cognitive function. A review by Koletzko et al (2008) provides epidemiological evidence for an association between maternal fish consumption during pregnancy and improved developmental outcomes, which include higher novelty preference on visual recognition memory in the short term and higher scores of verbal intelligence quotient as longer term benefits. The effect of n-3 PUFAs supplementation in maternal and term infant nutrition on cognitive development in infants, however, remains equivocal (reviewed by Fleith and Clandinin (2005)). In addition, n-3 PUFAs supplementation has been shown to delay cognitive decline in Alzheimer's disease but the effect is only confined to patients with a very mild form of the condition (Freund-Levi et al. 2006). The potential effect of n-3 PUFAs on improving cognitive function may be mediated by an increase in neurogenesis and/or an increased incorporation of DHA-containing phosphatidylcholine in the neural membrane,

which increases membrane fluidity and in turn may improve cellular function of neural cells (Wurtman 2008).

Given the complex role of fatty acids as signalling molecules in a range of biological processes, assessing their effects on a candidate gene basis is impractical and unlikely to provide mechanistic insight. In contrast, gene expression profiling using microarray analysis provides a global overview of the effects of fatty acids, and thus is more likely to identify mechanisms by which fatty acids modify cellular functions. In the present study, I aimed to determine the effect of long-chain saturated and n-3 PUFAs, specifically PA and DHA, on gene expression in L6 myotubes. This objective was to identify pathways differentially regulated by long-chain saturated and n-3 PUFAs and the connections to and from other known pathways, in particular insulin signal transduction, and to inform future research efforts.

## 7.2 Materials and methods

### 7.2.1 Sample collection

L6 cells were cultured on 6-well plates (Section 2.2.1) and incubated with 400 μM PA or DHA (n = 4/group) for 24 h. α-MEM with 0.08% ethanol (v/v) and 0.53% fatty acid-free BSA (w/v) was used as fatty acid-free control. RNA isolation was performed as described in Section 2.8.2.1.

### 7.2.2 Sample validation

RNA quality was determined by UV spectrophotometry and electrophoresis (Sections 2.8.2.2 and 2.8.2.3). Following reverse transcription of RNA (Section 2.8.1.3) and RT-

PCR (Section 2.8.1.4), the effect of PA and DHA on gene expression in L6 myotubes was verified by comparing their effect on mRNA expression of AMPKα2, SCD1 and PGC-1α with previous studies (Section 6.3.1).

### 7.2.3 Gene expression profiling, data processing and analysis

Global changes in gene expression induced by PA and DHA were determined using Affymetrix GeneChip® Rat Gene 1.0 ST Array as described in Section 2.8.2.3. The raw microarray data was normalised and genes differentially regulated (convergent/divergent) by each of the experimental condition were categorised by GO descriptions to identify biological pathways altered by PA and DHA (Section 2.8.2.4).

## 7.3 Results

### 7.3.1 Sample validation

Distinct bands of 28 S (~5 kb) and 18 S (~2 kb) RNA were visualised in all RNA samples isolated from the controls, PA- and DHA-treated myotubes (Figure 7.1). As shown in Table 7.1, all samples had a rRNA ratio (28 S/18 S) of 1.8 – 2.0 and a RIN of 9.8 – 10 which further confirmed the quality and integrity of the samples (Santella 2006; Schroeder et al. 2006).

While PA had no effect, DHA increased AMPKα2 mRNA content by 61% ($P < 0.001$) as compared to controls (Figure 7.2A). PA increased and DHA decreased SCD1 mRNA by 10% ($P < 0.05$) and 33% ($P < 0.001$) respectively (Figure 7.2B). The mRNA content of PGC-1α was not altered by DHA but decreased by 22% ($P < 0.001$) in response to PA (Figure 7.2C). The differential effects of PA and DHA on the selected genes were similar

to those observed in previous studies (Section 6.3.1) and therefore confirmed the biological relevance of the treatments.

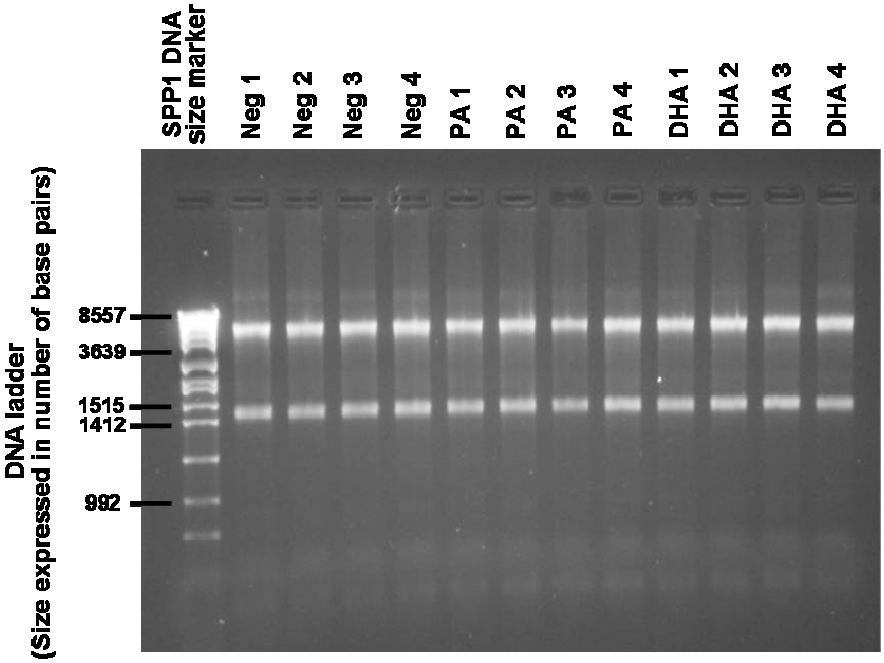



**Figure 7.1. Agarose gel electrophoresis of RNA isolated from L6 myotubes (n = 4/group).** L6 myotubes were cultured with 400 μM of palmitic acid (PA), docosahexaenoic acid (DHA) or fatty acid-free control (Neg) for 24 h. RNA was extracted using the Aurum™ Total RNA Mini Kit. 300 ng RNA was loaded onto a 1.5% agarose gel stained with ethidium bromide and visualised under UV light.

**Table 7.1. Quality of RNA isolated from L6 myotubes (n = 4/group).**

| Samples[6] | rRNA ratio (28 S/18 S) | RNA Integrity Number (RIN) |
|---|---|---|
| Neg1 | 1.9 | 10 |
| Neg2 | 1.9 | 10 |
| Neg3 | 1.9 | 10 |
| Neg4 | 1.9 | 10 |
| PA1 | 1.9 | 9.9 |
| PA2 | 1.9 | 9.9 |
| PA3 | 1.8 | 9.8 |
| PA4 | 1.9 | 9.9 |
| DHA1 | 1.8 | 10 |
| DHA2 | 1.9 | 10 |
| DHA3 | 2.0 | 10 |
| DHA4 | 2.0 | 9.9 |

[6] Neg – Control; PA – Palmitic acid; DHA – Docosahexaeonic acid

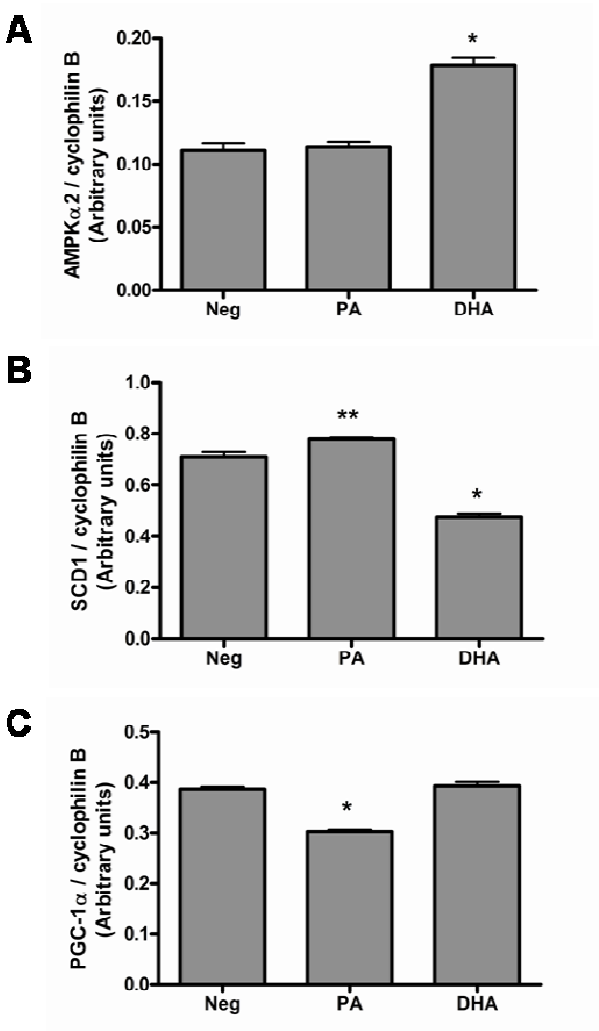



**Figure 7.2. The effect of fatty acids on mRNA content of AMPKα2 (A), SCD1 (B) and PGC-1α (C) in L6 myotubes (n = 4/group).** L6 myotubes were incubated with 400 μM of palmitic acid (PA) and docosahexaenoic acid (DHA) for 24 h. RNA was isolated using the Aurum™ Total RNA Mini Kit and analysed by RT-PCR. Gene expression was normalised using cyclophilin B. Data are shown as mean ± SEM. * $P < 0.001$ and ** $P < 0.05$ compared to fatty acid-free control (Neg).

7.3.2 Initial characterisation of microarray gene expression data

The microarray data were summarised using PCA based on dimensional reduction of gene expression patterns for each of the biological replicates and experimental conditions (Figure 7.3). All four biological replicates treated with PA were grouped in a similar area in the scatter plot. Neg1 deviated from the remaining three replicates of the controls which were grouped closely together. The pattern in the DHA group was more divergent, with only DHA1 and DHA2 were grouped and located closely to the controls. The data indicate considerably more divergence of overall gene expression from control in PA-treated samples than those treated with DHA. Although PCA plots provide justification of outlier samples, the current approach was to undertake unbiased analysis incorporating all replicate samples.

Individual array quality was visualised using MA-plots. As shown in Figure 7.4, the mass of the distribution in the MA-plots across all samples was concentrated along the $M = 0$ axis and no trend in the mean of $M$ as a function of $A$ was observed. These data, which compare the expression values on each array with a synthetic array created using probe set-wise median expression values, indicate no global quality defects in any of the arrays generated.

The distribution of probe intensities across all arrays was summarised using box-plots. As shown in Figure 7.5A, all boxes had similar interquartile range (distance between the $75^{th}$ and $25^{th}$ quartiles which encompasses the middle 50% of the data) and y-position (median of the distribution) which indicate reasonable variability across the biological replicates. The slight deviation of Neg1, DHA3 and DHA4 from the corresponding groups was consistent with the results from the PCA analysis (Figure 7.3). Using RMA,

the data was background-corrected and quantile-normalised, and expression measures for each probe set were generated using a linear model fit to the normalised data (Figure 7.5B).

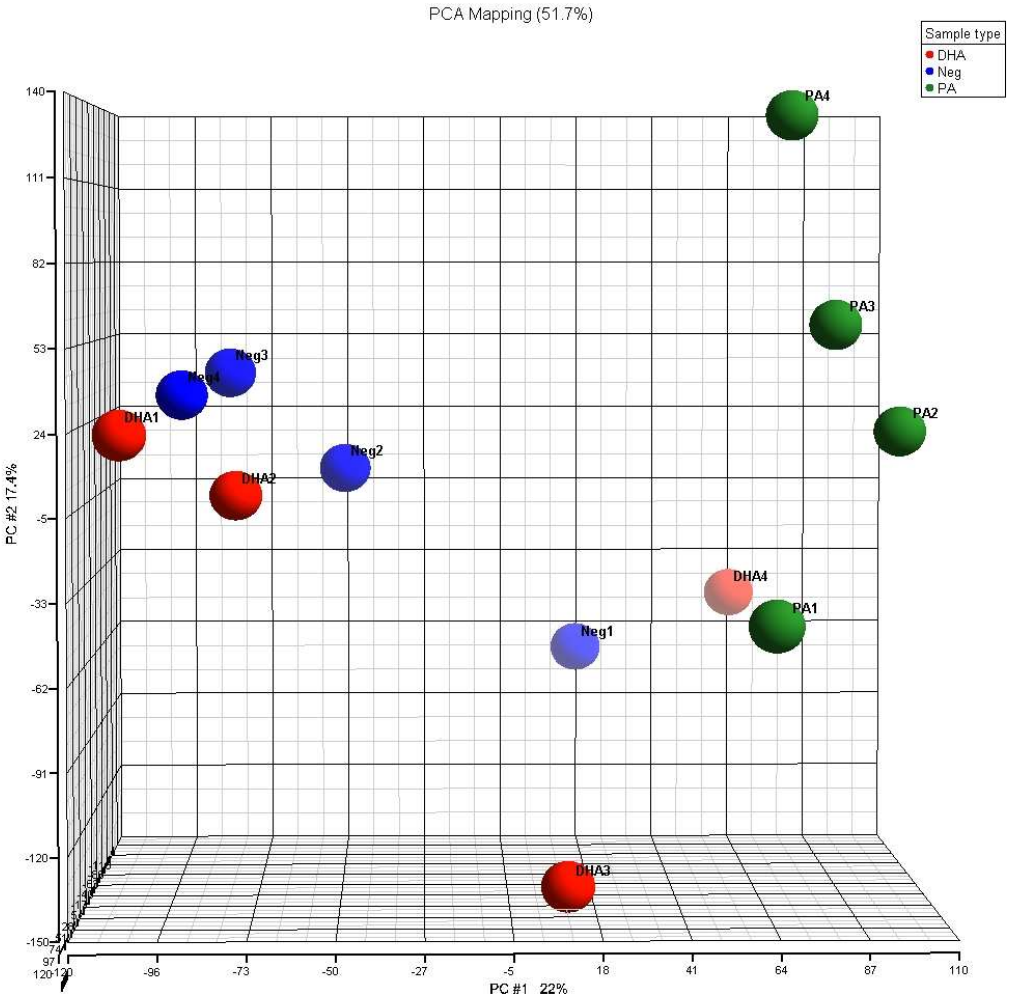



**Figure 7.3. Three-dimensional scatter plot view of biological replicates of L6 myotubes subjected to fatty acid treatments (n = 4/group).** L6 myotubes were cultured with 400 μM of palmitic acid (PA), docosahexaenoic acid (DHA) or fatty acid-free control (Neg) for 24 h. The Whole-Transcript Expression Analysis was performed by hybridising RNA to the GeneChip® Rat Gene 1.0 ST Array. The dataset was visualised using Principal Components Analysis (PCA). Samples were displayed in respect to the first three components and coloured by the treatment parameter: DHA – red; Neg – blue; PA – green.

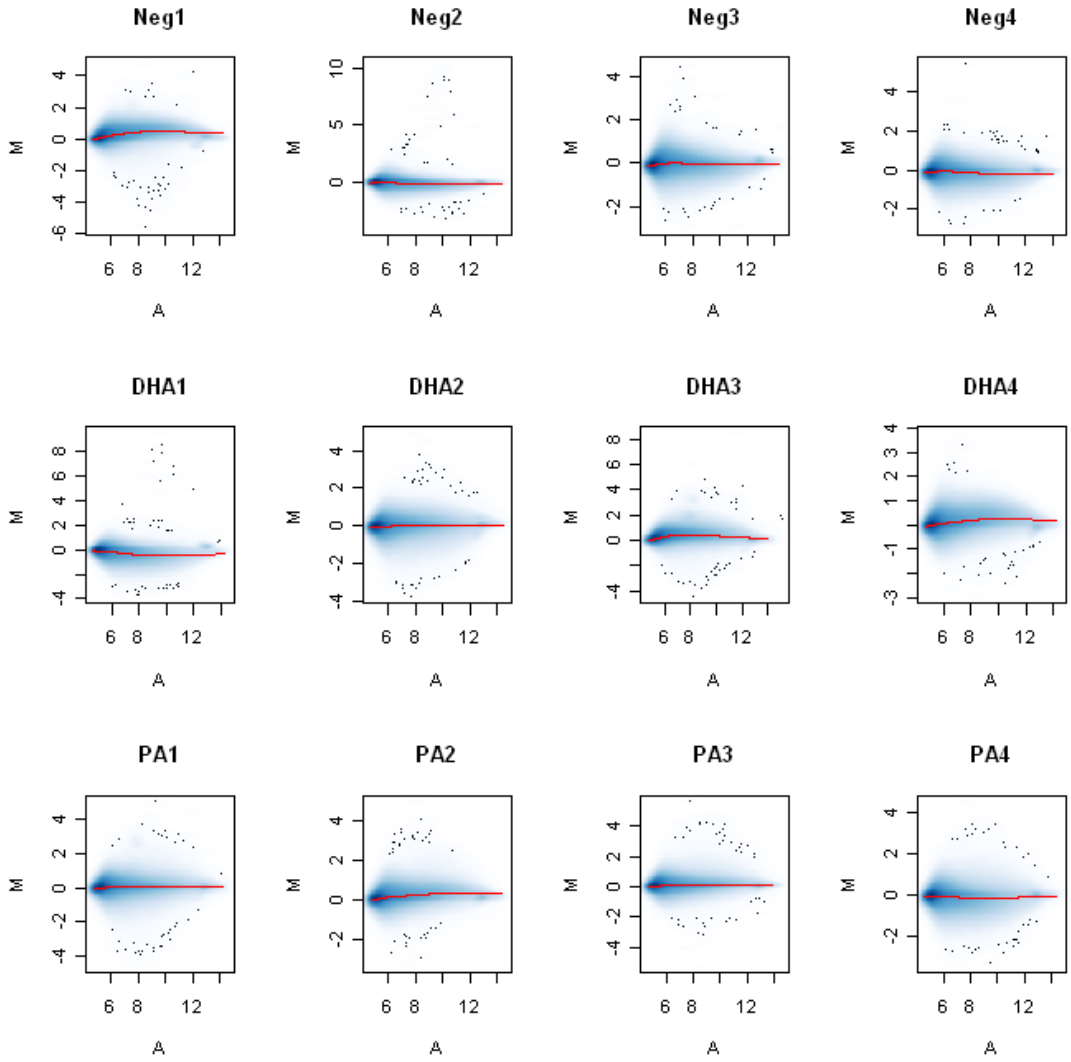



**Figure 7.4. Individual MA-plot of biological replicates of L6 myotubes subjected to fatty acid treatments (n = 4/group).** L6 myotubes were cultured with 400 μM of docosahexaenoic acid (DHA), palmitic acid (PA) or fatty acid-free control (Neg) for 24 h. The Whole-Transcript Expression Analysis was performed by hybridising RNA to the GeneChip® Rat Gene 1.0 ST Array. Mean log intensity $A$ $((\log_2 R + \log_2 G)/2)$ was plotted against the intensity log-ratio $M$ $(\log_2 R - \log_2 G)$ where $R$ and $G$ were the background-corrected red and green intensities for each spot.

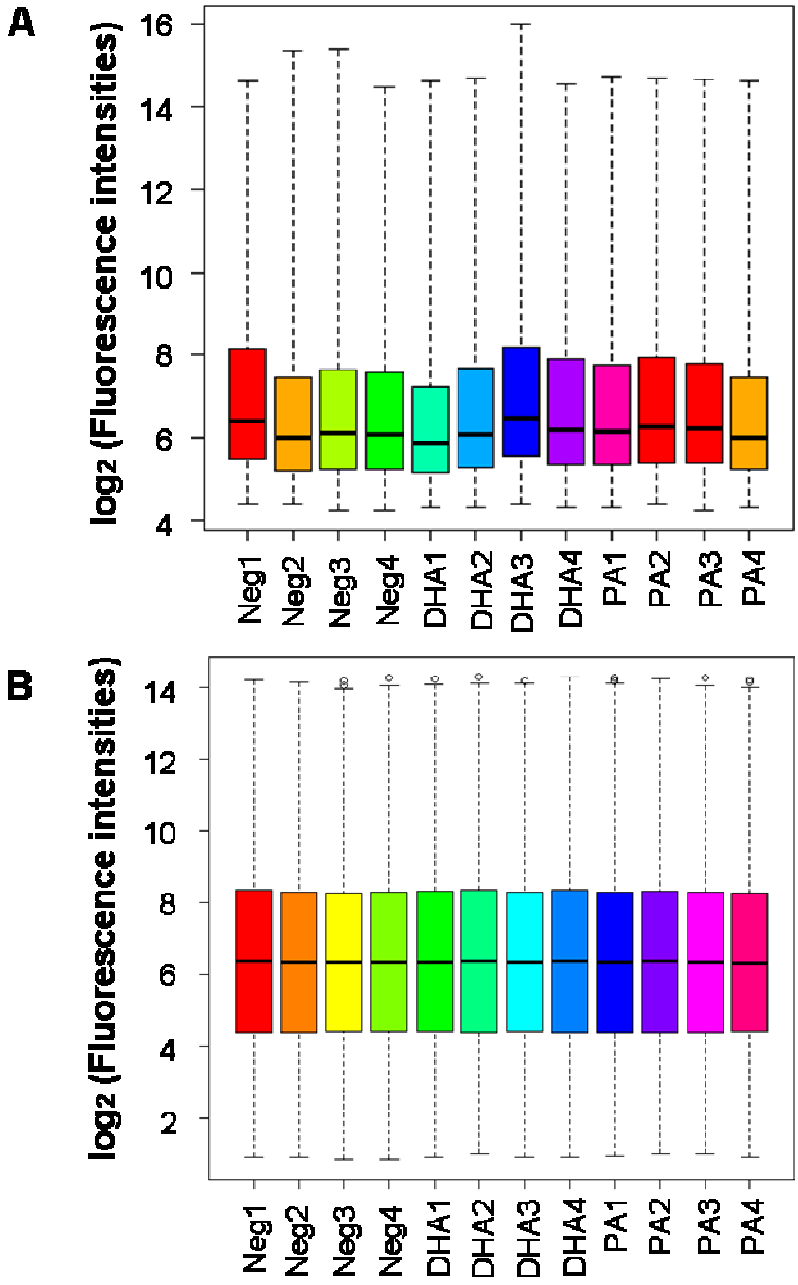



**Figure 7.5. Box-plots of probe intensities before (A) and after (B) normalisation (n = 4/group).** The raw intensity values were background-corrected, quantile-normalised and log (base 2)-transformed at the probe level using the Robust Multi-array Average (RMA). Neg – fatty acid-free control; DHA – docosahexaenoic acid; PA – palmitic acid.

### 7.3.3 Exploratory data analysis

The effect of PA and DHA on repressing or inducing each probe set was determined in order to identify genes up-, down- or non-differentially regulated by each of the treatment using Bayesian statistics and linear model fitting. An overview of the data was generated by hierarchical clustering. As shown in Figure 7.6A, PA1 – 4 were clustered together as would be expected with biological replicates. Neg1, DHA3 and DHA4 were distinct from their individual biological replicates, and therefore possible outliers. Alternatively as discussed above, the DHA treatment may result in gene changes that are significantly less divergent from the controls and PA treatment (Section 7.3.2).

The hierarchical clustering was repeated with Neg1, DHA3 and DHA4 excluded (trimmed dataset). As shown in Figure 7.6B, the grouping of samples was not different from that when the full dataset was used. Importantly, the list of differentially regulated transcripts derived from the analysis of the trimmed dataset (data not shown) was able to discriminate the sample groups completely when applied to the full dataset. Consequently, those genes contributing to variations between replicate samples are less likely to be specific to the treatments themselves. This further validates the use of the full dataset in comprehensive gene expression analyses.

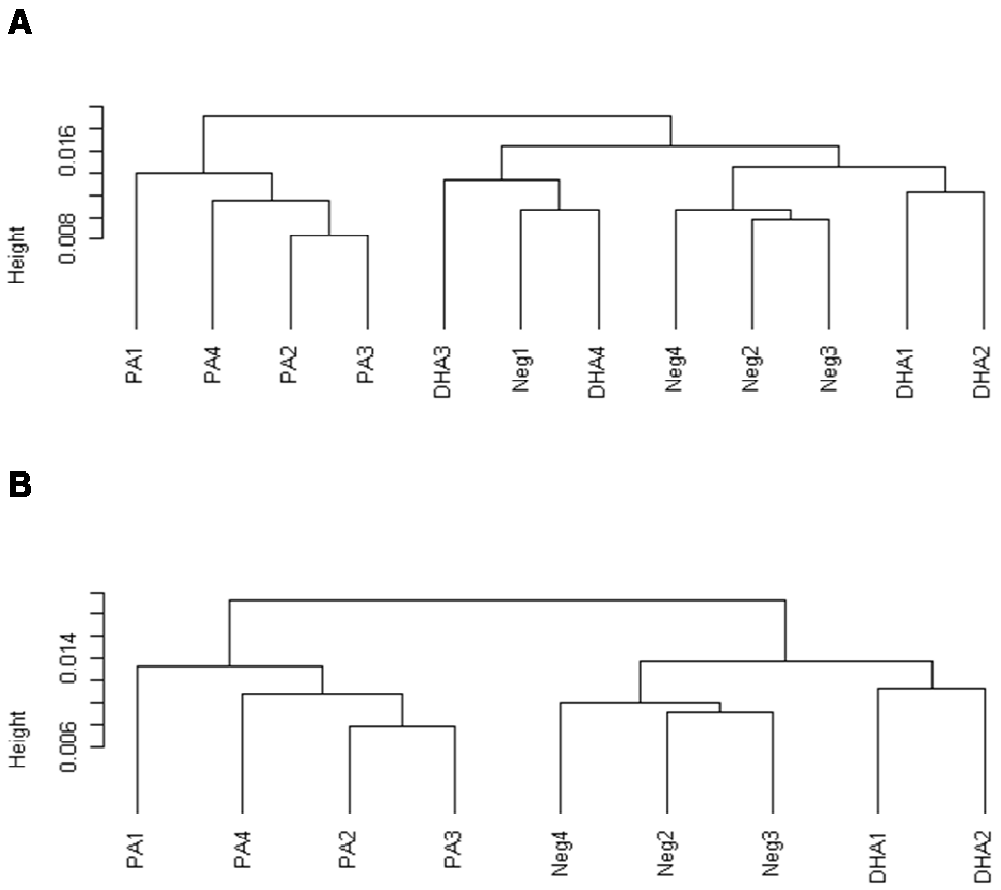



**Figure 7.6. Hierarchical clustering of samples.** Genes which were up-, down- or non-differentially regulated by palmitic acid (PA), docosahexaenoic acid (DHA) and controls (Neg) were determined using Bayesian statistics and linear model fitting with cut-offs at log fold change > 1.5 and p-value < 0.01. Samples from the full dataset (A) and after excluding Neg1, DHA3 and DHA4 (B) were clustered based on similarity of gene expression patterns.

7.3.4 Gene expression profiling

Using very stringent cut-offs of both an adjusted p-value < 0.01 and a log fold-change > 1.5, DHA and PA differentially regulated 11 (Table 7.2; Figure 7.7A) and 63 genes (Table 7.3; Figure 7.7B) respectively. When either of the cut-offs was used, 34 genes were differentially regulated by both DHA and PA (Table 7.4; Figure 7.8).

The differential effect of DHA and PA on gene expression of L6 myotubes was characterised by the regulation of 419 genes which were responsive to either DHA or PA (convergent/divergent), or both. Accordingly, this set of genes discriminate the effect of DHA and PA; among them 79 are known to be involved in cell proliferation/growth/survival, inflammation, lipid, glucose or energy metabolism (Table 7.5).

**Table 7.2. Genes differentially regulated by docosahexaenoic acid (n = 4/group).**

| Annotation | Gene | Gene description |
|---|---|---|
| 10715546 | Scds | stearoyl-CoA desaturase 2 |
| 10895144 | Dusp6 | dual specificity phosphatase 6 |
| 10743199 | Pemt | phosphatidylethanolamine N-methyltransferase |
| 10709093 | Ucp2 | uncoupling protein 2 (mitochondrial, proton carrier) |
| 10712657 | Cpt1a | carnitine palmitoyltransferase 1a (liver) |
| 10722097 | Tph1 | tryptophan hydroxylase 1 |
| 10842500 | RGD1562846 | similar to Docking protein 5 |
| 10839434 | Fgf7 | fibroblast growth factor 7 |
| 10890780 | RGD1562622 | similar to RIKEN cDNA 6330442E10 gene |
| 10730349 | Scd1 | stearoyl-CoA desaturase 1 |
| 10770471 | Disp1 | dispatched homolog 1 (drosophila) |

**Table 7.3. Genes differentially regulated by palmitic acid (n = 4/group).**

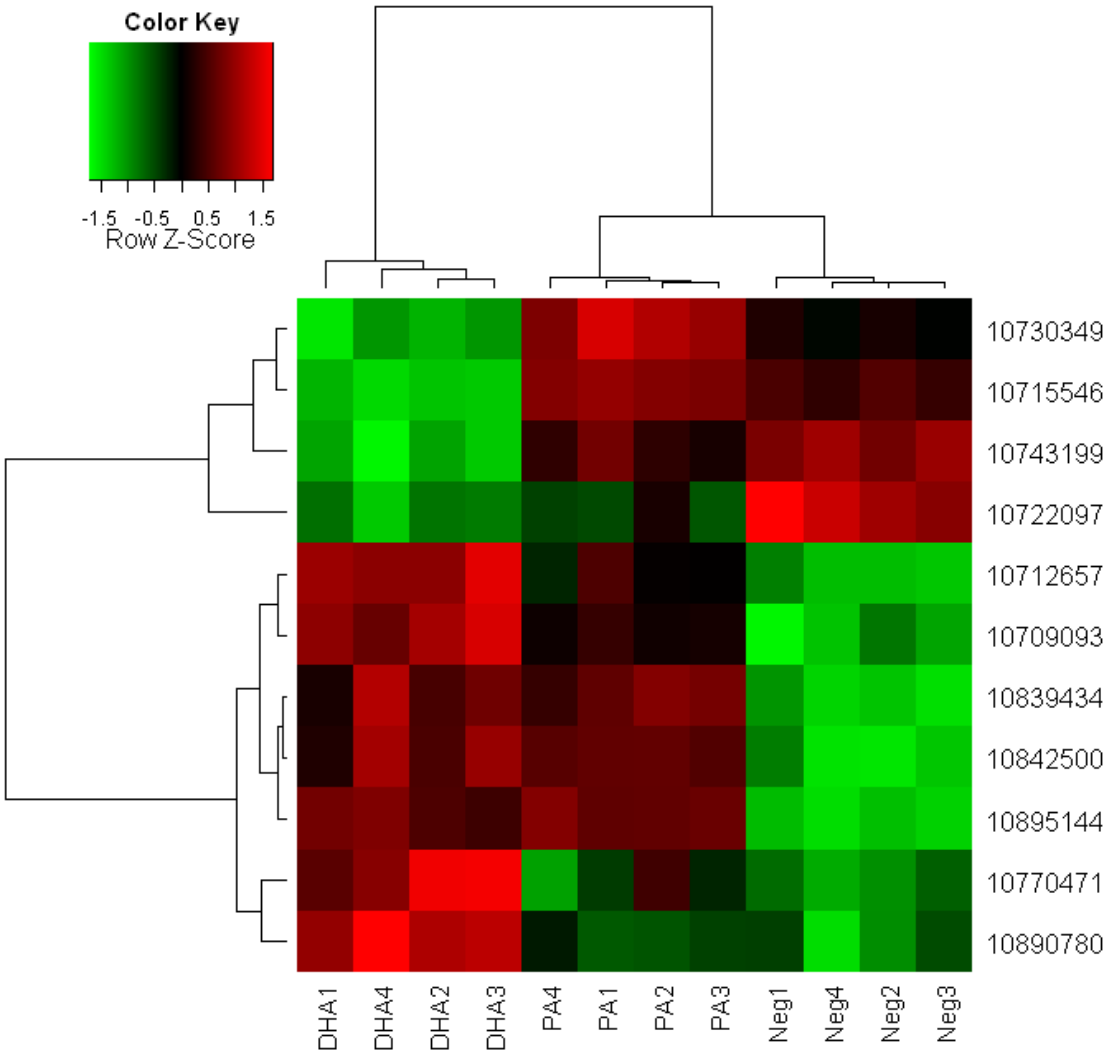
| Annotation | Gene | Gene description |
|---|---|---|
| 10809328 | Herpud1 | homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1 |
| 10876838 | Klf4 | kurppel-like factor 4 (gut) |
| 10850775 | Trib3 | tribbles homolog 3 (drosophila) |
| 10730098 | Pycs | pyrroline-5-carboxylate synthetase |
| 10756393 | Slc7a1 | solute carrier family 7, member 1 |
| 10909482 | Hyou1 | hypoxia up-regulated 1 |
| 10823057 | Slc7a11 | solute carrier family 7, member 11 |
| 10886031 | Fos | FBJ osteosarcoma oncogene |
| 10757082 | Zfand2a | zinc finger |
| 10762893 | Ficd | FIC domain-containing protein |
| 10871444 | Slc6a9 | solute carrier family 6, member 9 |
| 10728631 | Fads2 | fatty acid desaturase 2 |
| 10895144 | Dusp6 | dual specificity phosphatase 6 |
| 10903061 | Shmt2 | serine hydroxymethyltransferase 2 (mitochondrial) |
| 10907825 | Casp4 | caspase 4 apoptosis-related cysteine peptidase |
| 10704505 | Slc1a5 | solute carrier family 1, member 5 |
| 10895861 | Ddit3 | DNA-damage inducible transcript 3 |
| 10782028 | Dnajc3 | DnaJ (Hsp40) homolog subfamily, member 3 |
| 10889766 | Dnajb9 | DnaJ (Hsp40) homolog subfamily, member 3 |
| 10898456 | Creld2 | cysteine-rich with EGF-like domains 2 |
| 10770710 | Atf3 | activating transcription factor 3 |
| 10800387 | Nr4a1 | nuclear receptor subfamily 4 group A, member 1 |
| 10709330 | Art1 | ADP-ribosyltransferase 1 |
| 10761047 | Serpine1 | serine (or cysteine) peptidase inhibitor clade E, member 1 |
| 10919996 | Armet | arginine-rich mutated in early stage tumors |
| 10814142 | LOC365723 | similar to zinc finger protein 458 |
| 10832378 | Lss | lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase) |
| 10807272 | Hsd11b2 | hydroxysteroid 11-β dehydrogenase 2 |
| 10890024 | Nfkbia | nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, α |
| 10865993 | Cd69 | Cd69 molecule |
| 10811560 | Mvd | mevalonate (diphospho) decarboxylase |
| 10702945 | Acat2 | acetyl-CoA acetyltransferase 2 |
| 10931247 | Metrnl | meteorin, glial cell differentiation regulator-like |
| 10859108 | RGD1563148 | similar to osteoclast inhibitory lectin |
| 10907834 | Casp12 | caspase 12 |
| 10778620 | Slc1a4 | solute carrier family 1, member 4 |

| Annotation | Gene | Gene description |
|---|---|---|
| 10868940 | Nr4a3 | nuclear receptor subfamily 4, group A, member 3 |
| 10866819 | Bcat1 | branched chain aminotransferase 1 |
| 10842500 | RGD1562846 | similar to Docking protein 5 |
| 10839434 | Fgf7 | fibroblast growth factor 7 |
| 10894942 | Usp44 | ubiquitin specific protease 44 |
| 10763367 | Serpinb2 | serine (or cysteine) peptidase inhibitor |
| 10712560 | Dhcr7 | 7-dehydrocholesterol reductase |
| 10855676 | Ggct | γ-glutamyl cyclotransferase |
| 10862661 | Ggct | γ-glutamyl cyclotransferase |
| 10913141 | Gmppb | GDP-mannose pyrophosphorylase B |
| 10751498 | Pdia5 | protein disulfide isomerase family A, member 5 |
| 10937719 | Piga | phosphatidylinositol glycan anchor biosynthesis class A |
| 107299241 | Idi1 | isopentenyl-diphosphate δ isomerase 1 |
| 10806250 | Irx3 | iroquois homeobox 3 |
| 10908089 | Taf1d | TATA box-binding protein-associated factor RNA Polymerase 1 subunit D |
| 10717331 | Sgk1 | serum/glucocorticoid regulated kinase 1 |
| 10916534 | Sc5dl | sterol-C5-desaturase |
| 10775900 | Cxcl1 | chemokine (C-X-C motif) ligand 1 |
| 10814151 | Zfp458 | zinc finger protein 458 |
| 10709093 | Ucp2 | uncoupling protein 2 (mitochondrial, proton carrier) |
| 10712997 | Cd248 | CD248 molecule endosialin |
| 10901436 | Eid3 | EP300 interacting inhibitor of differentiation 3 |
| 10850070 | Rnf24 | ring finger protein 24 |
| 10875823 | Manea | mannosidase endo-α |
| 10821924 | Rai14 | retinoic acid induced 14 |
| 10934637 | RGD1560455 | similar to RIKEN cDNA A630033H20 gene |

**Table 7.4. Genes differentially regulated by both docosahexaenoic acid and palmitic acid (n = 4/group).**

| Annotation | Gene | Gene description |
|---|---|---|
| 10894942 | Usp44 | ubiquitin specific protease 44 |
| 10842500 | RGD1562846 | similar to Docking protein 5 |
| 10895144 | Dusp6 | dual specificity phosphatase 6 |
| 10839434 | Fgf7 | fibroblast growth factor 7 |
| 10728631 | Fads2 | fatty acid desaturase 2 |
| 10709093 | Ucp2 | uncoupling protein 2 (mitochondrial, proton carrier) |
| 10752034 | Etv5 | ets variant 5 |
| 10714528 | Dmrt2 | doublesex and mab-3 related transcription factor 2 |
| 10792035 | Dusp4 | dual specificity phosphatase 4 |
| 10888250 | Prepl | prolyl endopeptidase-like |
| 10749716 | Pycr1 | pyrroline-5-carboxylate reductase 1 |
| 10821408 | Fst | follistatin |
| 10843438 | Uap1l1 | UDP-N-acteylglucosamine pyrophosphorylase 1-like 1 |
| 10921117 | Tmem158 | transmembrane protein 158 |
| 10730921 | Gucy2g | guanylate cyclase 2G |
| 10894606 | Slc41a2 | solute carrier family 41, member 2 |
| 10714353 | Tmem2 | transmembrane protein 2 |
| 10751931 | Bcl6 | B-cell CLL/lymphoma 6 |
| 10908776 | Adamts8 | ADAM metallopeptidase with thrombospondin type 1 motif, 8 |
| 10722097 | Tph1 | tryptophan hydroxylase 1 |
| 10902280 | Nav3 | neuron navigator 3 |
| 10931252 | Tbcd | tubulin-specific chaperone d |
| 10730349 | Scd1 | stearoyl-CoA desaturase 1 |
| 10795673 | Fzd8 | frizzled homolog 8 (drosophila) |
| 10712657 | Cpt1a | carnitine palmitoyltransferase 1a liver |
| 10717141 | Arhgap18 | Rho GTPase activating protein 18 |
| 10857314 | Slc6a6 | solute carrier family 6, member 6 |
| 10832934 | Chst3 | carbohydrate sulfotransferase 3 |
| 10722241 | Csrp3 | cysteine and glycine-rich protein 3 |
| 10744324 | Slc2a4 | solute carrier family 2 (facilitated glucose transporter) |
| 10855449 | Gpnmb | glycoprotein (transmembrane) nmb |
| 10928602 | Acadl | acyl-CoA dehydrogenase |
| 10838155 | Cat | catalase |
| 10863430 | Hk2 | hexokinase 2 |

**A**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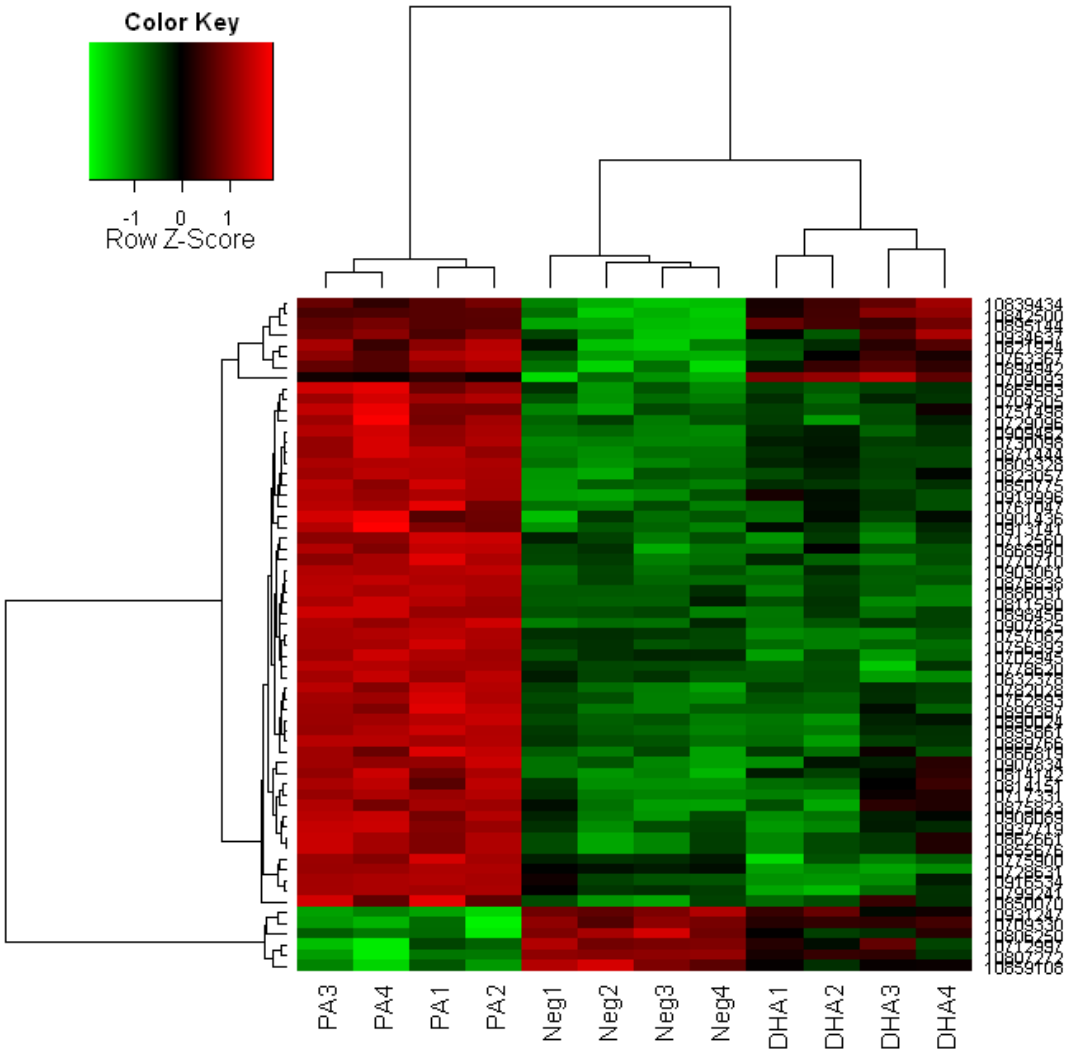


**Figure 7.7. Heatmaps of genes differentially regulated by docosahexaenoic acid (DHA; A) and palmitic acid (PA; B) as compared to controls (Neg) (n = 4/group).** Genes regulated (convergent/divergent) by the fatty acid treatments were determined using Bayesian statistics with a log fold-change cut-off of 1.5 and Benjamini & Hochberg adjusted p-value of < 0.01. The genes differentially regulated by the fatty acid treatments were listed as their annotations for the GeneChip® Rat Exon 1.0 ST Array.

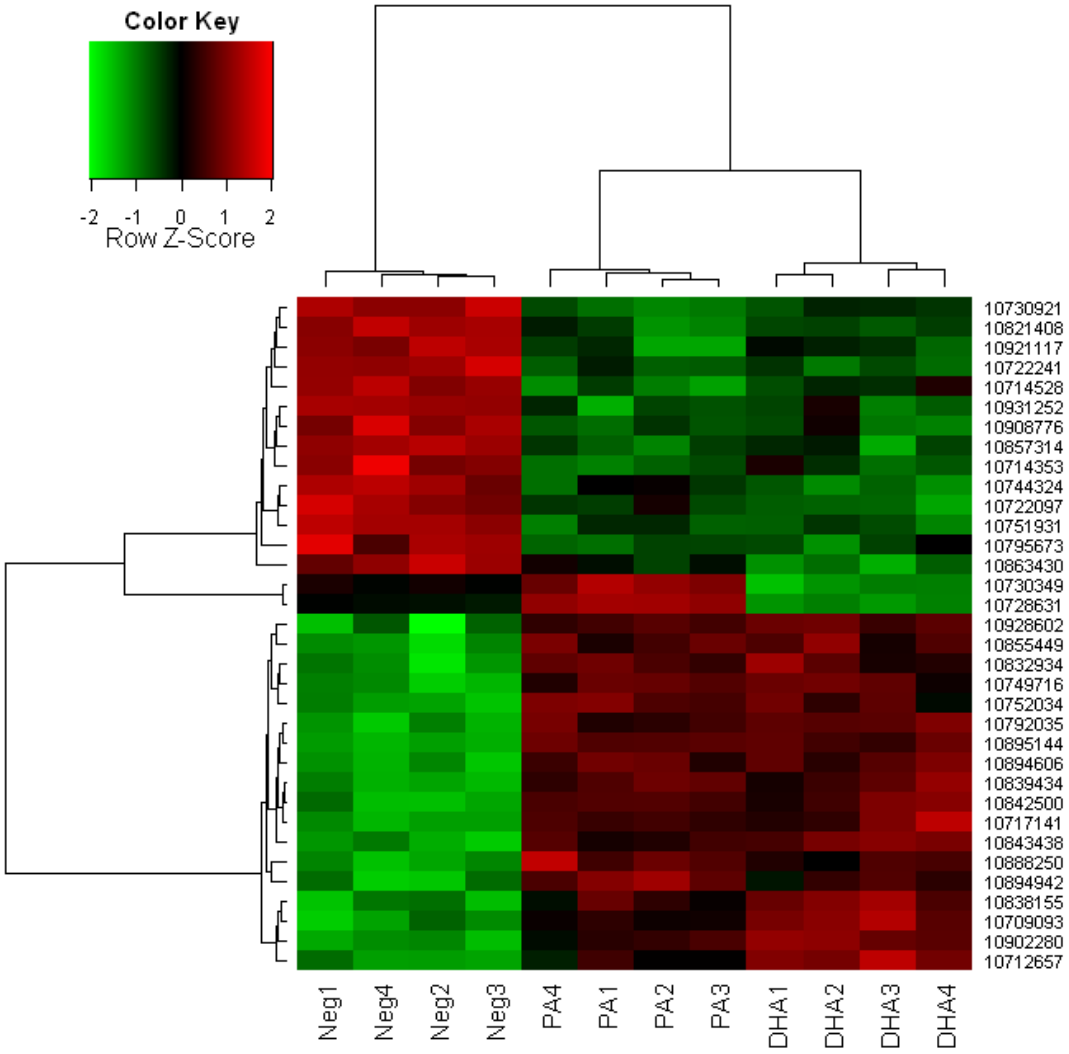


**Figure 7.8. Heatmap of genes differentially regulated by both docosahexaenoic acid (DHA) and palmitic acid (PA) as compared to controls (Neg) (n = 4/group).** Genes regulated (convergent/divergent) by both DHA and PA were determined using Bayesian statistics with a log fold-change cut-off of 1.5 or Benjamini & Hochberg adjusted p-value of < 0.01. The 20 genes significantly regulated by both PA and DHA were listed as their annotations for the GeneChip® Rat Exon 1.0 ST Array.

**Table 7.5. A selection of genes differentially regulated by either docosahexaenoic acid or palmitic acid (n = 4/group).**

*Cell proliferation/growth/survival*

| Annotation | Gene | Gene description |
|---|---|---|
| 10770471 | Disp1 | dispatched homolog 1 (drosophila) |
| 108339434 | Fgf7 | fibroblast growth factor 7 |
| 10895144 | Dusp6 | dual specificity phosphatase 6 |
| 10792035 | Dusp4 | dual specificity phosphatase 4 |
| 10885299 | Rhoj | ras homolog gene family, member J |
| 10722241 | Csrp3 | cysteine and glycine-rich protein 3 |
| 10808563 | Cdh15 | cadherin 15 |
| 10739796 | Sphk1 | sphingosine kinase 1 |
| 10758930 | Hspb8 | heat shock protein 8 |
| 10886031 | Fos | FBJ osteosarcoma oncogene |
| 10899387 | Nr4a1 | nuclear receptor subfamily 4, group A, member 1 |
| 10855676 | Ggct | γ-glutamyl cyclotransferase |
| 10862661 | Ggct | γ-glutamyl cyclotransferase |
| 10907825 | Casp4 | caspase 4 apoptosis-related cysteine peptidase |
| 10717331 | Sgk1 | serum/glucocorticoid regulated kinase 1 |
| 10866819 | Bcat1 | branched-chain aminotransferase 1, cytosolic |
| 1091996 | Armet | arginine-rich mutated in early stage tumors |
| 10889177 | Rhob | ras homolog gene family, member B |
| 10732652 | Dusp1 | dual specificity phosphatase 1 |
| 10783583 | Jub | jub aujba homolog (xenopus laevis) |
| 10794576 | Tbc1d7 | TBC1 domain family, member 7 |
| 10857610 | Bhlhe40 | basic helix-loop-helix family, member e40 |
| 10878938 | Plk3 | polo-like kinase 3 (drosophila) |
| 10791011 | Lass1 | LAG1 homolog ceramide synthase 1 |
| 10879726 | Zc3h12a | zinc finger CCCH type containing 12A |
| 10913003 | Hyal2 | hyaluronoglucosaminidase 2 |
| 10769695 | Ddr2 | discoidin domain receptor tyrosine kinase 2 |
| 10904169 | Ndrg1 | N-myc downstream regulated gene 1 |
| 10923580 | Cflar | CASP8 and FADD-like apoptosis regulator |
| 10733417 | PPP2ca | protein phosphatase 2 catalytic subunit α isoform |
| 10726929 | Dusp8 | dual specificity phosphatase 8 |
| 10903459 | Klf10 | Kruppel-like factor 10 |

*Inflammation*

| Annotation | Gene | Gene description |
|---|---|---|
| 10857783 | Il17re | interleukin 17 receptor E |
| 10907834 | Casp12 | caspase 12 |
| 10850775 | Trib3 | tribbles homolog 3 (drosophila) |
| 10865993 | Cd69 | Cd69 molecule |
| 10890024 | Nfkbia | nuclear factor kappa light polypeptide gene enhancer in B-cells inhibitor |
| 10763367 | Serpinb2 | serine (or cysteine) peptidase inhibitor, clade B, member 2 |
| 10775900 | Cxcl1 | chemokine (C-X-C motif) ligand 1 |
| 10764551 | Ptgs2 | prostaglandin-endoperoxide synthase 2 |
| 10872330 | Stk40 | serine/threonine kinase 40 |
| 10736257 | Traf4 | TNF receptor associated factor 4 |
| 10794225 | Nfil3 | nuclear factor interleukin 3 regulated |
| 10915726 | Ecsit | ECSIT homolog (drosophila) |
| 10703075 | Map3k4 | mitogen activated protein kinase kinase kinase 4 |

*Lipid metabolism*

| Annotation | Gene | Gene description |
|---|---|---|
| 10712657 | Cpt1a | carnitine palmitoyltransferase 1a liver |
| 10715546 | Scd | stearoyl-CoA desaturase |
| 10730349 | Scd1 | stearoyl-CoA desaturase 1 |
| 10728631 | Fads2 | fatty acid desaturase 2 |
| 10749716 | Pycr1 | pyrroline-5-carboxylate reductase 1 |
| 10870481 | Ppap2b | phosphatidic acid phosphatase type 2B |
| 10908521 | Ldlr | low density lipoprotein receptor |
| 10932795 | Acsl4 | acyl-CoA synthetase long-chain family, member 4 |
| 10928602 | Acadl | acyl-CoA dehydrogenase |
| 10802691 | Acaa2 | acetyl-CoA acyltransferase 2 |
| 10712317 | Pnpla2 | patatin-like phospholipase domain containing 2 |
| 10832378 | Lss | lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase) |
| 10712560 | Dhcr7 | 7-dehydrocholesterol reductase |
| 10702945 | Acat2 | acetyl-CoA acetyltransferase 2 |
| 10811560 | Mvd | mevalonate (diphospho) decarboxylase |
| 10916534 | Sc5dl | sterol-C5-desaturase |
| 10861986 | Insig1 | insulin induced gene 1 |
| 10841348 | Acss2 | acyl-CoA synthetase short-chain family, member 2 |
| 10896772 | Sqle | squalene epoxidase |
| 10820666 | Hmgcr | 3-hydroxy-3-methylglutaryl-CoA reductase |
| 10728123 | Tm7sf2 | transmembrane 7 superfamily, member 2 |

| | | |
|---|---|---|
| 10928963 | Prkag3 | protein kinase AMP-activated γ 3 non-catalytic subunit |
| 10738274 | Coasy | Coenzyme A synthase |
| 10859880 | Insig1 | insulin induced gene 1 |
| 10813214 | Hmgcs1 | 3-hydroxy-3-methylglutaryl-CoA synthase 1 |

*Glucose metabolism*

| Annotation | Gene | Gene description |
|---|---|---|
| 10863430 | Hk2 | hexokinase 2 |
| 10744324 | Slc2a4 | solute carrier family 2 (facilitated glucose transporter) |
| 10809001 | Pdp2 | pyruvate dehydrogenase phosphatase isoenzyme 2 |
| 10780360 | Pck2 | phosphoenolpyruvate carboxykinase 2 (mitochondrial) |

*Energy metabolism*

| Annotation | Gene | Gene description |
|---|---|---|
| 10709093 | Ucp2 | uncoupling protein 2 (mitochondrial, proton carrier) |
| 10709083 | Ucp3 | uncoupling protein 3(mitochondrial proton carrier) |
| 10815442 | Spg20 | spastic paraplegia 20 (Troyer syndrome) homolog |
| 10723020 | Idh2 | isocitrate dehydrogenase 2 (NADP), mitochondrial precursor |
| 10734822 | Slc25a35 | solute carrier family 25, member 35 |

7.3.5 Pathway analysis

The baseline GO analysis for the array was set up by excluding the GO categories that were enriched purely on the basis of array constituents; I chose to tolerate false-negatives in order to eliminate any misleading false-positives. Based on a hypergeometric distribution p-value of $P < 0.001$, 181 of ~17,000 GO categories were excluded based on under/over-representation of the gene constituents of those categories on the Affymetrix 1.0 ST rat arrays.

GO analysis was performed to investigate the biological processes, molecular functions and cellular components affected by gene expression changes induced by DHA and PA compared to vehicle (Ashburner et al. 2000). Using a cut-off of either an adjusted p-value < 0.01 or a log fold-change > 1.5, 70 and 383 genes were differentially regulated by DHA and PA respectively and entered the GO analysis. When DHA and PA were compared with controls, 9 and 15 GO categories with a p-value enrichment of < 0.05 were generated respectively. An additional direct comparison of genes differentially expressed in PA- and DHA-treated myotubes generated 19 enriched GO categories. The significant GO annotations and the genes that contributed to each category were shown in Tables 7.6 – 7.8.

**Table 7.6. Gene Ontology (GO) categories enriched by docosahexaenoic acid as compared to controls (n = 4/group).**

| GO category[1] | Term | Genes regulated by DHA in category |
|---|---|---|
| 0004768 (MF) | stearoyl-CoA 9-desaturase activity | Fads2, Scd, Scd1 |
| 0006629 (BP) | lipid metabolic process | Acadl, Cpt1a, Acsl4, Acaa2, Ldlr |
| 0000303 (BP) | response to superoxide | Ucp3, Ucp2 |
| 0017017 (MF) | MAP kinase tyrosine/serine/threonine phosphatase activity | Dusp4, Dusp6 |
| 0006839 (BP) | mitochondrial transport | Ucp3, Ucp2 |
| 0030140 (CC) | trans-Golgi network transport vesicle | Slc2a4, Spg21 |
| 0051272 (BP) | positive regulation of cell motion | Chst3, Bcl6 |
| 0006656 (BP) | phosphatidylcholine biosynthetic process | Pemt, Fgf7 |
| 0014068 (BP) | positive regulation of phosphoinositide 3-kinase cascade | Cat, Jak2 |

[1] BP – biological process; CC – cellular component; MF – molecular function

**Table 7.7. Gene Ontology (GO) categories enriched by palmitic acid as compared to controls (n = 4/group).**

| GO category[1] | Term | Genes regulated by PA in category |
|---|---|---|
| 0006695 (BP) | cholesterol biosynthetic process | Hmgcr, Hsd17b7, Hmgcs1, Dhcr7, Lss, Mvd, Idi1, Ebp |
| 0030968 (BP) | endoplasmic reticulum unfolded protein response | Ddit3, Eif2ak3, Serp1, Herpud1, Casp12, Ppp1r15a, Ero1l, Aars |
| 0005788 (CC) | endoplasmic reticulum lumen | Hspa5, Serpinh1, Pdia3, Ptgs2, Pdia4, Ugcgl1, Hyou1, Pdia5, Dnajb11 |
| 0017017 (MF) | MAP kinase tyrosine/serine/threonine phosphatase activity | Dusp4, Dusp1, Dusp6, Dusp8 |
| 0006457 (BP) | protein folding | Dnajb9, Ugcgl1, Ero1l, Hspa9, Aars, Sgtb, Hspa4l, Dnajb4, Dnajb11, Fkbp14 |
| 0005793 (CC) | ER-Golgi intermediate compartment | Hspa5, Serpinh1, Lman1, Ugcgl1, Golga3 |
| 0042127 (BP) | regulation of cell proliferation | Serpine1, Nfkbia, Ptgs2, Mafg, Dhcr7, Klf10, Klf4, Sat1, Bcl6 |
| 0003756 (MF) | protein disulfide isomerase activity | Pdia3, Pdia4, Pdia5 |
| 0006986 (BP) | response to unfolded protein | Eif2ak3, Dnajc3, Herpud1, Hspa4l |
| 0043154 (BP) | negative regulation of caspase activity | Hspa5, Nr4a1, Herpud1, Cflar |
| 0032764 (BP) | negative regulation of mast cell cytokine production | Hmox1, Bcl6 |
| 0006564 (BP) | L-serine biosynthetic process | Psat1, Shmt2, Psph |
| 0006983 (BP) | ER overload response | Hspa5, Ddit3, Eif2ak3 |
| 0015175 (MF) | neutral amino acid transmembrane transporter activity | Slc3a2, Slc1a5, Slc1a4 |
| 0005518 (MF) | collagen binding | Smad3, Serpinh1, Smad7, Ddr2 |

[1] BP – biological process; CC – cellular component; MF – molecular function

**Table 7.8. Gene Ontology (GO) categories enriched by palmitic acid (PA) as compared to docosahexaenoic acid (DHA) (n = 4/group).**

| GO category[1] | Term | Genes differentially regulated by PA and DHA in category |
|---|---|---|
| 0006695 (BP) | cholesterol biosynthetic process | G6pd, Hmgcr, Hsd17b7, Fdft1, Dhcr7, Lss, Mvd, Idi1, Ebp, Dhcr24, Nsdhl |
| 0016491 (MF) | oxidoreductase activity | G6pd, Hmox1, Lox, Hsd11b2, Hmgcr, Ftl, Ptgs2, Hsd17b7, Fdft1, Txnrd1, Dhcr7, Fads2, Scd, Fads1, Sc5dl, Gmpr, Sc4mol, Ero1l, Scd1, Dhcr24, RGD1306939, Nsdhl, Dhrs3 |
| 0005788 (CC) | endoplasmic reticulum lumen | Ptgs2, Ppib, Pdia4, Ugcgl1, Hyou1, Txndc4, Pdia5, Dnajb11 |
| 0006633 (BP) | fatty acid biosynthesis process | Ptgs2, Scd, Acsl3, Sc5dl, Sc4mol, Elovl5, Scd1 |
| 0005173 (MF) | stem cell factor receptor binding | Kitlg, Spred1, Spred2 |
| 0042127 (BP) | regulation of cell proliferation | Serpine1, Nfkbia, Ptgs2, Mafg, Dhcr7, Klf10, Klf4, Gnl3, Sat1, Chst11 |
| 0030968 (BP) | endoplasmic reticulum unfolded protein response | Ddit3, Herpud1, Casp12, Ppp1r15a, Ero1l |
| 0004768 (MF) | stearoyl-CoA 9-desaturase activity | Fads2, Scd, Scd1 |
| 0003756 (MF) | protein disulfide isomerase activity | Pdia4, Txndc4, Pdia5 |
| 0016126 (BP) | sterol biosynthetic process | Dhcr7, Ebp, Sc4mol |
| 0005899 (CC) | insulin receptor complex | Insr, Irs1 |
| 0032288 (BP) | myelin formation | Pmp22, Ilk |
| 0043405 (BP) | regulation of MAP kinase activity | Trib1, Trib3 |
| 0051425 (MF) | PTB domain binding | Insr, Nup62 |
| 0006564 (BP) | L-serine biosynthetic process | Psat1, Shmt2, Psph |
| 0006457 (BP) | protein folding | Dnajb9, Ppib, Ugcgl1, Ero1l, Hspa9, Dnajb4, Txndc4, Hsp90aa1, Dnajb11 |
| 0042169 (MF) | SH2 domain binding | Insr, Irs1, Nup62, Lax1 |
| 0003690 (MF) | double-stranded DNA binding | Junb, Myc, Cebpg, Smad3, Hmgb2, Klf4, Fos |
| 0000188 (BP) | inactivation of MAPK activity | Spred1, Spred2, Lax1 |

[1] BP – biological process; CC – cellular component; MF – molecular function

## 7.4 Discussion

The present findings demonstrate the effects of PA and DHA on global gene expression in L6 myotubes. PA and DHA differentially regulate pathways which are involved in lipid metabolism, inflammation and cell proliferation/growth/survival. The data highlight some key metabolic pathways which determine the specific effects of long-chain SFAs and n-3 PUFAs on metabolic health.

### 7.4.1 Differential effects of PA and DHA on gene expression profile

The most striking difference between PA and DHA is their opposing effect on pathways regulating lipid utilisation. PA up-regulates the expression of genes which are involved in fatty acid (e.g. Fads2) and cholesterol (e.g. Hmgcr and Dhcr7) synthesis. In contrast, DHA down-regulates SCD activity, a key enzyme which promotes triglyceride synthesis, and other pathways involved in lipogenesis. DHA also increases the capacity and rate of fatty acid oxidation by up-regulating the expression of Ucp2, Ucp3 and Cpt1 and therefore prevents intracellular lipid accumulation.

PA and DHA differentially regulate pathways which modulate ER functions and MAPK activity, both involved in the initiation of inflammatory responses. The formation of disulphide bonds as part of the protein-folding process requires molecular oxygen as the terminal electron acceptor and therefore an increased protein-folding load in the ER increases ROS production, generates a state of oxidative stress, and subsequently initiates an inflammatory response (Zhang and Kaufman 2008). The effect of PA on inducing ER stress is evident by an up-regulation of genes which are involved in ER unfolded protein response (e.g. Serp1 and Herpud1) and ER overload response (e.g. Hspa5 and Ddit3), which may be a consequence of an increase in lipid synthesis and the subsequent

increased demand on ER metabolism (Boden 2009). Similarly, a chronic exposure to PA (500 μM; 20 h) has been shown to increase the mRNA content of ER stress markers (e.g. ATF3, CHOP and XBP1) in primary human myotubes (Peter et al. 2009). The effect of PA on inducing ER stress has also been reported in pancreatic β-cells (Karaskov et al. 2006), HepG2 cells (Das et al. 2008) and in mouse 3T3-L1 and primary rat adipocytes (Guo et al. 2007). In contrast, DHA has no effect on ER functions but down-regulates genes which mediate ER stress when compared to PA. The present data also showed that PA up-regulates whereas DHA down-regulates MAPK activity, an important inflammation mediator which is activated by inflammatory cytokines via MEKs (Kyriakis and Avruch 2001). Taken together, PA promotes, while DHA reduces, the initiation of inflammatory responses at the level of gene transcription.

In addition to the involvement of MAPK in cell death and differentiation by coordinating gene transcription, protein synthesis and cell cycle machinery (Kyriakis and Avruch 2001), PA and DHA have other specific effects on cellular functions and survival. PA up-regulates genes involved in cell proliferation (e.g. Klf4 and klf10) and down-regulates caspase activity which mediates apoptosis. DHA up-regulates the synthesis of phosphatidylcholine. The subsequent increase in membrane fluidity, together with the effect of DHA on pathways which increase cell motion, improve overall cellular function.

### 7.4.2 Mechanisms for the effect of SFAs and n-3 PUFAs on muscle insulin sensitivity

Saturated fat intake is associated with insulin resistance and is a risk factor for type 2 diabetes (Marshall et al. 1991; Riccardi et al. 2004). The data in Chapter 5 provide direct evidence that PA inhibits insulin-stimulated glucose uptake in skeletal muscle and for the

involvement of inflammatory pathways in mediating PA-induced insulin resistance. The gene expression profile in response to PA implicates the roles of ER stress and MAPK activity in mediating the relationship between over-nutrition, inflammation and insulin resistance. The mRNA content of key ER stress markers in subcutaneous fat of obese individuals is > 60% higher as compared to healthy controls and is positively correlated with the percentage of body fat (Sharma et al. 2008). High-fat feeding induces an increase in ER stress in mouse liver (Delibegovic et al. 2009). In Fao liver cells, inducing ER stress using a pharmacological agent reduced insulin-stimulated tyrosine phosphorylation of IRS-1 (Ozcan et al. 2004). Alleviating ER stress by chemical chaperones normalised blood glucose levels and improved systemic insulin sensitivity in a mouse model of type 2 diabetes (Ozcan et al. 2006). ER stress has been shown to activate NFκB in primary cultures of human muscle fibres (Nogalska et al. 2007), suggesting that ER stress may mediate insulin resistance in skeletal muscle upstream of the IKK/IκB/NFκB pathway. Although the mechanisms remain largely unclear, the ER-associated NFκB activation requires both calcium ions and ROS released from the overloaded ER which leads to the subsequent degradation of IκB (Pahl and Baeuerle 1997). Interestingly, ER stress has also been implicated in the development of diabetes mellitus as a result of β-cell loss from ER stress-induced apoptosis (van der Kallen et al. 2009).

The MAPK pathway appears to have a role in mediating the effect of PA on insulin sensitivity. In C2C12 myotubes, p38 MAPK phosphorylation increases in response to PA (Crunkhorn et al. 2007). In endothelial cells, either silencing p38 MAPK or inhibiting its activation prevented the inhibitory effect of resistin and TNF-α on insulin signal transduction (Shen et al. 2006; Li et al. 2007). Similarly, TNF-α inhibited insulin-

stimulated glucose transport in primary neonatal rat myotubes in a p38 MAPK-dependent manner (de Alvaro et al. 2004). Inhibiting MEK1/2 prevents the degradation of IkBα and the subsequent activation of NFκB in response to PA (Coll et al. 2006). Taken together, these data suggest the ERK/MAPK signalling pathway may function upstream of the IKK/IκB/NFκB pathway which mediates PA-induced insulin resistance in skeletal muscle.

The microarray data suggest that in contrast to PA, DHA may increase insulin sensitivity. The GO analysis indicates that DHA up-regulates the PI3K pathway which is essential for insulin signalling. DHA also promotes phosphatidylcholine synthesis, which may increase membrane fluidity and thus enhances insulin receptor activation (Storlien et al. 1991; Liu et al. 1994) (Section 1.5.4). There was, however, no effect of DHA on insulin-stimulated glucose uptake in L6 myotubes (Section 5.3.2). DHA partially reversed PA-induced insulin resistance in skeletal muscle (Chapter 5). Although this has been attributed to an increase in fatty acid oxidation which reduces substrate availability for the synthesis of ceramide of DAG, a concept further supported by the effect of DHA on up-regulating Cpt1, Ucp2 and Ucp3 gene expression, other effects of DHA may also play a role. These include the above mentioned effects on membrane fluidity, PI3K pathway, and ER stress and MAPK activity where the deleterious effect of PA is partially reversed. Taken together, these data highlight the importance of overall dietary fatty acid composition, which has to be low in SFAs as well as higher in n-3 PUFAs, for diabetes prevention and management. The addition of n-3 PUFAs to a diet already high in SFAs and calories is without benefits (Kaushik et al. 2009).

7.4.3 Implications of the effect of SFAs and n-3 PUFAs on other aspects of health

Although the effect of fatty acids on cellular metabolism may be tissue-specific, the differential effect of PA and DHA on metabolic pathways, in particular those which regulate lipid metabolism, inflammation and cell proliferation, have important implications on the molecular mechanisms involved in the relationships between long-chain SFAs, n-3 PUFAs and aspects of health beyond skeletal muscle energy metabolism.

Fatty acid composition of the diet is a major determinant of cardiovascular health. Dietary SFA intake has been associated with increased incidence of coronary heart disease (Goldbourt et al. 1993) whereas n-3 PUFAs are proposed to be cardioprotective (Jung et al. 2008). An energy-restricted diet low in saturated fat (6% of total energy) improved cardiovascular risk factors in obese individuals (Noakes and Clifton 2000). In patients surviving a recent myocardial infarction, n-3 PUFAs supplementation (1 g/day) reduced the risk of sudden cardiac death after 4 months by 45% (Marchioli et al. 2002). Traditionally the differential effect of SFAs and n-3 PUFAs on heart health has been attributed to their opposing effect on lipid metabolism, in particular cholesterol synthesis, but the simplicity of the concept has recently been challenged (Volk 2007). The effects of the fatty acids on inflammation, vascular smooth muscle cell proliferation and myocardial cellular energy metabolism may be equally important.

There is mounting evidence for the role of fatty acids in cognitive function. Beydoun et al (2007) studied the relationship between plasma fatty acids and cognitive function in adults aged 50 – 65 yr. Elevated circulating levels of PA were positively correlated with an increased risk of global cognitive decline, whereas higher plasma concentrations of n-

3 PUFAs were associated with less of a decline in verbal fluency but no effect on global cognitive decline. Aged rats fed with a diet containing 10% saturated fat and 2% cholesterol showed an impaired memory and hippocampal morphology (Granholm et al. 2008). In contrast, maternal supplementation with 10 ml of cod liver oil (1,183 mg DHA + 803 mg EPA) daily during pregnancy and lactation has been associated with higher mental processing scores in 4-year-old children (Helland et al. 2003). Daily n-3 PUFAs supplementation (1.7g DHA + 0.6 g EPA) for 6 months also conferred a slower cognitive decline in patients with a very mild form of Alzheimer's disease (Freund-Levi et al. 2006). It has been proposed that n-3 PUFAs may improve cognitive functions via several mechanisms. These include the increase in the proportion of DHA-containing phosphatidylcholine which, in turn increases membrane fluidity and cellular functions of neuronal membranes, and the effect of DHA on promoting neurogenesis by inducing neuronal stem cell differentiation (Wurtman 2008). In addition, DHA-derived metabolites (e.g. neuroprotectin D1) counteract the pro-inflammatory and cell-damaging events induced by cytokines and amyloid peptide factors and therefore improve neural cell survival in the case of Alzheimer's disease (Lukiw et al. 2005). In the present study, the effect of DHA on up-regulating genes which are involved in cell motion (e.g. Chst3 ad Bcl6) and phosphatidylcholine synthesis (e.g. Pemt and Fgf7) provide evidence for the proposed molecular mechanisms by which n-3 PUFAs improve neural functions. More importantly, the opposing effect of DHA and PA on inflammation and cell proliferation/growth/survival may suggest the protective role of n-3 PUFAs against the detrimental effect of SFAs on cognitive performance.

### 7.4.4 Conclusions

Taken together, PA and DHA differentially regulate the expression of genes which are involved in pathways regulating lipid metabolism, inflammation and cell proliferation/growth/survival. The present findings reveal the potential role of ER stress and the MAPK signalling pathway in mediating PA-induced inflammatory response and insulin resistance in skeletal muscle. The effect of DHA on up-regulating fatty acid oxidation and down-regulating ER metabolism and MAPK pathway support the potential role of DHA on reversing PA-induced insulin resistance and improves muscle energy metabolism. More importantly, the contrasting effect of PA and DHA on global gene expression implicates the importance of dietary fatty acid composition not only as an important part of the nutritional management for diabetes but also to achieve overall health and disease prevention. Although there is some evidence for DHA to reverse the detrimental effect of PA on some metabolic pathways, increasing the proportion of dietary fat from n-3 PUFAs while at the same time reducing SFAs intake is most likely to achieve maximal health benefits.

# Chapter 8: Conclusions, implications and future studies

## 8.1 Summary of findings

Insulin resistance is integral to the pathophysiology of a range of metabolic complications including type 2 diabetes and cardiovascular disease. Central adiposity and skeletal muscle mass and function determine insulin sensitivity and metabolic risk. A low ratio of visceral fat to skeletal muscle mass is one of the key determinants of a favourable metabolic profile. While *in vivo* studies suggest an association between dietary saturated fat intake and insulin resistance, unsaturated fatty acids, in particular n-3 PUFAs, may improve metabolic health. Despite being recognised as important regulators of insulin sensitivity, the molecular mechanisms by which adipose tissue and fatty acids modify insulin sensitivity of skeletal muscle remain largely unclear. This is likely to be a consequence of the limitations of the existing *in vitro* systems, which often fail to demonstrate the *in vivo* interactions between various factors which affect muscle metabolism simultaneously via both common and distinct mechanisms under physiological conditions.

In the present study, a novel *in vitro* system using adipose tissue-conditioned media was developed to study the *in vivo* crosstalk between adipose tissue, fatty acids and skeletal muscle. This model has been validated and successfully demonstrates the *in vivo* differential effects of fat depots and fatty acids on energy metabolism in skeletal muscle *in vitro*. Secretory factors from IAB but not SC fat inhibited insulin-stimulated glucose uptake in L6 myotubes via a NFκB/mTORC1-dependent pathway, which was predominantly mediated by IL-6. PA reduced insulin-stimulated glucose uptake in L6 myotubes, which may be a consequence of intramuscular accumulation of ceramide and

the activation of NFκB and mTORC1. The effects of fatty acids were similar in the presence of CM from either fat depot, where the effect of PA was partially reversed by DHA and completely by LA. DHA and LA may improve insulin sensitivity by promoting fatty acid oxidation and therefore reducing substrate availability for the synthesis of ceramide and DAG.

Gene expression profiling in L6 myotubes in response to PA and DHA revealed the potential role of ER stress and the MAPK signalling pathway in mediating PA-induced inflammatory response and insulin resistance in skeletal muscle. The differential effects of PA and DHA on pathways regulating lipid metabolism, inflammation and cell proliferation/growth/survival implicate the importance of dietary fatty acid composition not only as an important part of the nutritional management for diabetes but also to achieve overall health and disease prevention.

### 8.2 Implications of findings

The findings presented in this thesis further our understanding of the molecular mechanisms by which adipokines and fatty acids interact and modify insulin sensitivity and nutrient utilisation in skeletal muscle. As shown in Figure 8.1, both SFAs and pro-inflammatory cytokines inhibit insulin-stimulated tyrosine phosphorylation of IRS-1 via the activation of mTORC1 and subsequently reduce the effect of insulin on increasing glucose uptake. These data suggest that mTORC1 is the common mediator for nutrients and inflammation to regulate insulin sensitivity of skeletal muscle. This concept further supports the role of mTORC1 in nutrient-hormonal signalling in energy metabolism as mTORC1 is also known to be activated by increased intracellular amino acid concentrations (Dann et al. 2007; Flati et al. 2008). Given insulin resistance is

fundamentally a protective mechanism against nutrient overload, it is perhaps not surprising to find the concomitant effects of pro-inflammatory cytokines (from excessive energy storage), SFAs and amino acids (from over-nutrition) on inhibiting insulin action to avoid excess nutrient influx and maintain energy homoeostasis of skeletal muscle. Although mTORC1 has been identified as an important regulator of insulin sensitivity in skeletal muscle, direct interventions to suppress mTORC1 activity do not appear to be an option to prevent insulin resistance. In addition to nutrient sensing, mTORC1 is also involved in essential cellular functions including protein synthesis and cell proliferation and mTOR-deletion is lethal to animal models (Dann et al. 2007).

Nevertheless, the comprehensive mapping of metabolic pathways regulating insulin sensitivity in skeletal muscle provides novel insights into effective strategies for prevention of insulin resistance and treatment of obesity. In general, limiting energy intake and increasing energy expenditure remain the keys to prevent and possibly reverse skeletal muscle and adipose tissue dysfunctions which subsequently induce insulin resistance. In addition, interventions targeting adipose tissue (e.g. reducing inflammation) and skeletal muscle (e.g. increasing the capacity and rate of fatty acid oxidation) metabolism are of potential importance to manage pre-existing insulin resistance. The possible specific interventions including nutritional management, physical activity and pharmacological treatments are detailed below.

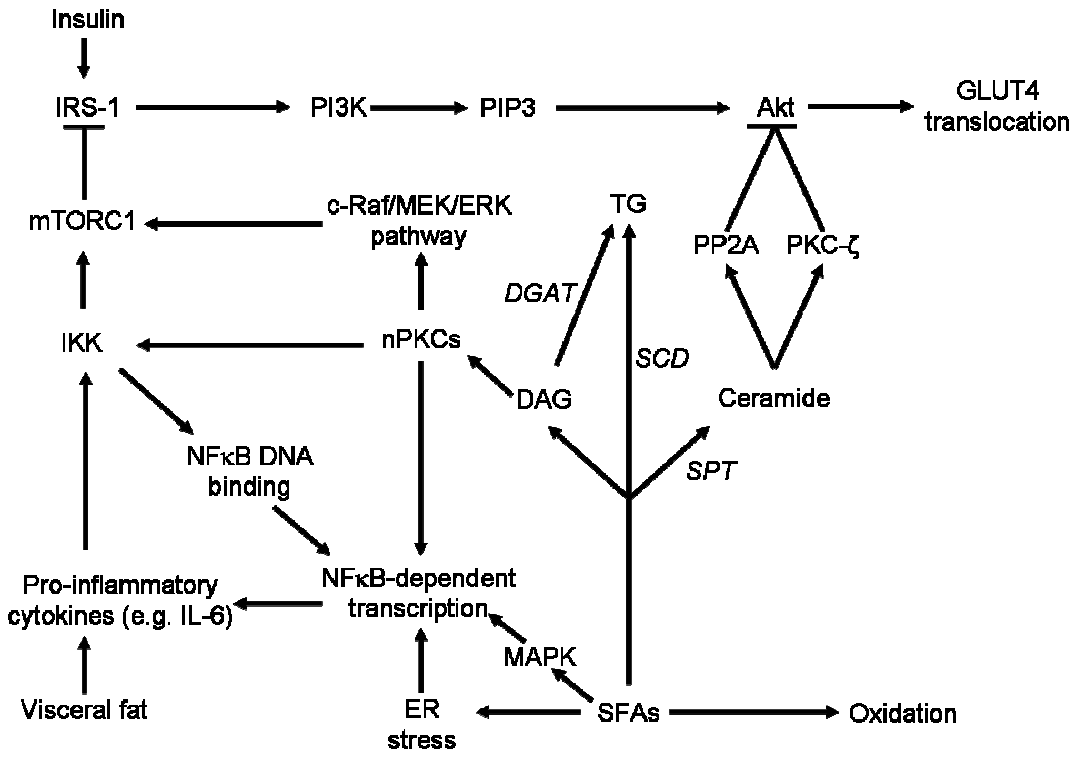


**Figure 8.1. Schematic model showing the relationship between long-chain saturated fatty acids (SFAs), visceral fat and insulin-stimulated glucose uptake in skeletal muscle.** Ceramide inhibits insulin-stimulated Akt activation. Diacylglycerol (DAG), endoplasmic reticulum (ER) stress and mitogen-activated protein kinase (MAPK) and pro-inflammatory cytokines from visceral fat all activate the inhibitor kappa B kinase/mammalian target of rapamycin complex 1 (IKK/mTORC1) pathway which subsequently inhibits insulin-stimulated insulin receptor substrate (IRS)-1 activation.

8.2.1 Nutritional management for obesity

The findings presented in this thesis provide evidence that long-chain SFAs induce insulin resistance in skeletal muscle. The partitioning of long-chain SFAs towards oxidation, the synthesis of ceramide, DAG or triglycerides, appears to be pivotal in determining the responsiveness of the cell to insulin (Figure 8.1). For example one may expect suppressing the *de novo* synthesis of ceramide would at least partially prevent SFA-induced insulin resistance in skeletal muscle, which however has been shown to partition fatty acids towards DAG accumulation which subsequently inhibited insulin-stimulated IRS-1 activation (Watson et al. 2009). Similarly, suppressing SCD expression, and therefore lowered triglyceride synthesis, increased intramuscular ceramide and DAG content and reduced insulin-stimulated glucose uptake in L6 myotubes (Pinnamaneni et al. 2006). Conversely, muscle-specific over-expression of DGAT1, the gene encoding DGAT which catalysed the final conversion of DAG to triglyceride, reduced intramuscular ceramide accumulation and improved insulin sensitivity in mouse skeletal muscle *ex vivo* (Liu et al. 2007). In response to PA, over-expressing SCD reduced intramuscular ceramide and DAG content and improved insulin-stimulated glucose uptake in L6 myotubes (Pinnamaneni et al. 2006). Taken together, the key to prevent SFA-induced insulin resistance is to reduce substrate availability for both ceramide and DAG synthesis.

Partitioning SFAs towards triglyceride accumulation may improve insulin sensitivity in skeletal muscle. It also needs to be considered that the capacity of skeletal muscle to store triglyceride is limited and therefore while this mechanism is effective as a buffer for intermittent or very short-term over-feeding, it cannot compensate for chronic nutrient overload. The adverse effect of SFAs include the induction of ER stress (Chapter 7) and

the generation of lipid peroxidation products as a result of oxidative stress (Moro et al. 2008); these in turn may inhibit insulin signalling via the activation of inflammatory responses.

Increasing fatty acid oxidation overcomes the problem of limited triglyceride storage and therefore abrogates lipid-induced insulin resistance. Over-expressing CPT-I in rat skeletal muscle increased fatty acid oxidation and prevented the effect of high-fat diet on reducing insulin-stimulated tyrosine phosphorylation of IRS-1 and glucose uptake (Bruce et al. 2009). In the present study, the effect of DHA and LA on promoting fatty acid oxidation in skeletal muscle (Chapter 6) supports the role of increasing dietary intake of PUFAs in improving metabolic health.

Some cautions should be exercised in considering the long-term effect of high-PUFA consumption on energy metabolism. PUFAs promote a shift of energy substrate from glucose to fat without increasing the overall utilisation of energy, which subsequently may spare glucose for storage in the form of glycogen. This concept is supported by a study by Harrington et al (2007), in which pharmacologically activating fat oxidation increased liver weight in diet-induced obese mice, which is likely to be a consequence of increased glycogen storage. Therefore long-term over-consumption of PUFAs may also result in nutrient overload and insulin resistance similar to the effect of a diet high in SFAs. A similar phenomenon is seen in rats fed with a high-fat diet consisting primarily of MUFAs (Buettner et al. 2006). Accordingly, the beneficial effects of PUFAs for energy metabolism and insulin sensitivity require a state of energy balance or deficit to have maximal benefits. This can be achieved either with caloric restriction or increased physical activity. Taken together, the traditional hypocaloric and low-fat diet remains an

important intervention to achieve energy deficit for weight loss (Schoeller and Buchholz 2005) but the present findings highlight the potential importance of dietary fatty acid composition. SFAs, in particular PA, should be markedly restricted with n-3 and n-6 PUFAs replacing some of the fats in the diet rather than being 'add-ons'. Therefore, incorporating PUFAs into an energy-restricted diet, which is also low in saturated fat may offer maximal benefits for weight management and metabolic health.

### 8.2.2 Physical activity

Physical activity is the most variable determinant of energy expenditure (Jakicic and Otto 2005) and therefore has been considered as an integral part of the treatment of obesity. Exercise regimen for weight management often focuses on endurance training, which increases energy expenditure and the utilisation of fat stores (Hainer et al. 2008). Endurance training reduces the percentage of body fat in lean, overweight (Wilmore et al. 1999) and obese (De Glisezinski et al. 1998) individuals. The effect of endurance training on fat loss is mediated by mobilising the energy store and increasing fatty acid oxidation in skeletal muscle. In obese individuals, endurance training increased lipolysis in adipose tissue by enhancing the β-adrenergic response and reducing the anti-lipolytic effects of $\alpha_2$-adrenoceptor and insulin (De Glisezinski et al. 1998). Similar training also increased mitochondrial fatty acid oxidation in skeletal muscle of obese individuals, which may be a consequence of an improved capacity for mitochondrial fatty acid uptake associated with an increase in CPT-I activity (Bruce et al. 2006). In healthy subjects, the oxidative phosphorylation capacity of skeletal muscle increased with the level of physical performance, an effect associated with an increase in the mRNA content of PGC-1α and transcriptional factors regulating the expression of mitochondrial proteins (Garnier et al. 2005). Taken together, these data suggest that endurance training improves

the oxidative function of skeletal muscle, which subsequently increase whole-body energy expenditure and facilitates the mobilisation of fat stores.

Diet-induced weight loss is often associated with a reduction of fat-free mass and subsequently decreases resting metabolic rate (Stiegler and Cunliffe 2006). The effect of resistance training on increasing muscle mass and strength is important to maintain fat-free mass and achieve long-term weight maintenance. Compared to isoenergetic aerobic exercise, strength training induced similar weight loss but retained more fat-free mass and increased flexed arm muscle mass and grip strength in moderately obese individuals (Geliebter et al. 1997). Resistance training increases muscle mass and strength as a consequence of modifying muscle structure and improving neuromuscular functions. Resistance training increases the synthesis of sarcoplasmic proteins (Kim et al. 2005) and the number of capillaries per fibre (Thompson 1994), which subsequently induces muscle fibre hypertrophy and increases the aerobic capacity respectively of skeletal muscle. Compared to aerobic training alone, the combined effect of aerobic and resistance training induced a greater loss of both subcutaneous and visceral fat and increased lean body mass (Park et al. 2003). Taken together, these data strongly suggest that both endurance and resistance training are important to promote fat loss and at the same time maintain and even improve skeletal muscle mass and function as part of the treatment for obesity.

### 8.2.3 Pharmacological treatments for obesity

#### *8.2.3.1 Anti-obesity agents*

The present study demonstrates the deleterious effect of excess visceral fat on insulin sensitivity in skeletal muscle and highlights the clinical significance of reducing fat mass

to ameliorate obesity-related complications. Mobilising the energy store by pharmacologically increasing lipolysis and fatty acid oxidation has been proposed as potential treatment for obesity. Indeed, a short-term (2-week) administration of agents which stimulated both lipolysis and CPT-I reduced total body weight and fat mass in obese non-insulin-dependent diabetic KK mice, an effect associated with reduced triglyceride content in the circulation and liver (Murosaki et al. 2007). Although the short-term effect of promoting lipid catabolism on weight loss appears to be promising, the effectiveness of these interventions is unlikely to sustain and may even be detrimental to metabolic health in the long-term. Increasing lipolysis releases free fatty acids, which if not metabolised accordingly, subsequently induce insulin resistance in peripheral tissues. Furthermore, an up-regulation of the β-oxidation pathway is not necessarily supported by a coordinated increase in downstream metabolic pathways including the TCA cycle and electron transport chain (Muoio and Koves 2007). Substrate availability modifies the relative proportion of energy fuel to be oxidised but does not increase the overall substrate oxidation above that is required to supply energy for ATP repletion (Hancock et al. 2008). When the influx of fatty acids exceeds ATP production in mitochondria, the increased NADH/NAD and ATP/ADP ratios create a high-energy redox state which inhibits the TCA cycle and electron transport chain and subsequently leads to incomplete fat oxidation, a condition in which fatty acids are only partially degraded (Muoio and Koves 2007; Taube et al. 2009). The mitochondrial accumulation of lipid intermediates including acyl-CoAs, acylcarnitines promotes ROS production which further compromises mitochondrial functions (Taube et al. 2009).

A pharmacological increase in energy expenditure is potentially a very attractive treatment option for obesity. Rimonabant, a selective antagonist of cannabinoid receptor

1, has been shown to reduce body weight and fat mass in animal models, an effect associated with an increase in energy expenditure independent of food intake and physical activity (Herling et al. 2007; Herling et al. 2008; Flamment et al. 2009). Rimonabant reduces oxidative phosphorylation efficiency and therefore increases the ratio of energy substrate consumption to ATP production (Flamment et al. 2009).

Similarly, energy expenditure may also be increased by promoting thermogenesis. $\beta_3$-adrenergic receptors are highly responsive in brown adipose tissue and when activated by pharmacological agonists, increased resting metabolic rate and thermogenic response in obese-diabetic mice (Yoshida et al. 1994). A highly selective human $\beta_3$-adrenergic agonist, however, failed to increase energy expenditure and had no major lipolytic or thermogenic effect in overweight/obese men (Larsen et al. 2002). It has been proposed that the small abundance and questionable functional capacity of brown adipose tissue in humans may limit the effect of thermogenic agonists on increasing energy expenditure (Larsen et al. 2002). Recent advances in methodologies assessing the mass and activity of brown adipose tissue in humans provide novel insight into the potential physiological importance of the tissue, for example the inverse relationship between the prevalence of detectable brown adipose tissue and BMI suggests that the fat depot may protect against obesity (Cypess et al. 2009). Accordingly, pharmacological interventions which stimulate the generation and activation of brown adipose tissue may increase energy expenditure and have potential importance in obesity treatment. It may also be possible to increase thermogenesis in skeletal muscle by increasing the expression of uncoupling protein (UCP)-3. Highly expressed in skeletal muscle, UCP3 is a mitochondrial anion carrier protein which has been proposed to cause proton leak and therefore increases

thermogenesis (Costford et al. 2007). The molecular mechanisms and physiological function of UCP3, however, are largely unclear.

*8.2.3.2 Anti-inflammatory agents*

The activation of the inflammatory pathway is an important mechanism mediating the inhibitory effect of both the IAB fat (Chapter 4) and SFAs (Chapter 5) on insulin sensitivity in skeletal muscle cells. The effect of anti-inflammatory drugs on improving glycaemic response and insulin sensitivity in obese individuals has been consistently demonstrated (Hundal et al. 2002; Fleischman et al. 2008). In addition, anti-inflammatory agents improve cellular functions of adipocytes including an improved capacity to release adiponectin (Fleischman et al. 2008) and reduced responsiveness to TNF-α stimulated lipolysis (Zu et al. 2008). Although the direct effect on weight management may be limited, the potential importance of anti-inflammatory agents as part of the obesity management to alleviate obesity-related metabolic complications and improve metabolic health should not be underestimated.

Already commonly recommended in patients suffering from active inflammation including rheumatoid arthritis (Rennie et al. 2003) and Crohn's disease (Akobeng 2008) as anti-inflammatory agents, n-3 PUFAs may also alleviate the chronic inflammatory state and therefore improves adipose tissue function in obesity. Dietary n-3 PUFAs supplementation reduces the production of inflammatory mediators including eicosanoids (Kelley et al. 1999), pro-inflammatory cytokines (Batetta et al. 2009) and ROS (Fisher et al. 1990). The association between EPA supplementation (1.8 g/day) and increased plasma concentration of adiponectin in obese individuals suggests the effect of n-3 PUFAs on improving adipose tissue function, which has been validated *in vitro* where

the effect of macrophage on reducing adiponectin secretion in 3T3-L1 adipocytes was partially reversed in the presence of EPA (Itoh et al. 2007). While the habitual dietary intake of n-3 PUFAs remains very low in the western diet (~ 0.2 – 0.7 g/day) (Katan et al. 1994; Pischon et al. 2003), supplementation at a pharmacological dose may be required to achieve the anti-inflammatory effect in obese individuals and that again raises the concerns about over-consumption of PUFAs.

### 8.3 Future studies

In the present study, a novel system has been developed to determine adipose tissue function *in vitro*, in which CM preserves the *in vivo* secretory profile of adipose tissue. This system facilitates the understanding of the physiological roles of adipokines in the context of the complex interactions between a multitude of secretory factors from specific adipose tissue depots. For example the effect of newly discovered adipokines on a tissue of interest may be elucidated in the context of other secretory factors which may not have previously been identified.

Although CM is a more physiologically relevant way to characterise the secretory function of adipose tissue, it still does not reproduce the complex endocrine interactions of the tissue. Adipokine and fatty acid profiles of CM from an isolated fat explant only reflect the secretory function of adipose tissue in a non-stimulated state. In reality, adipokine secretion depends on hormonal regulation, plasma nutrient levels, neural signals and crosstalk between organs and between the brain and adipose tissue. While it is unlikely that the complex *in vivo* physiology can be reproduced, some sophistication may be added to the system, for example, by culturing whole adipose tissue explants in controlled experimental conditions such as varied glucose and/or fatty acid

concentrations, insulin and other hormones involved in intermediary metabolism. The use of exposure to CM of various durations, including perfusion of cultured cells on columns, may further mimic the *in vivo* system. Finally, primary cultures of tissue from humans with pre-existing disease states, e.g. insulin resistance and diabetes mellitus, may further inform the role of adipose tissue in determining the overall metabolic health.

Given the extensive interactions between adipose tissue and other tissue types including skeletal muscle, liver and pancreas as well as the complex intermediary metabolism involved, the potential use of adipose tissue co-culture system in elucidating molecular mechanisms of tissue interactions is enormous. In the tissue of interest the pathways that mediate specific effects may be further elucidated using a range of techniques including over-expressing/silencing genes of interest by transient transfections and suppressing protein activity using specific inhibitors or antibodies.

Taken together, using CM to demonstrate the adipose tissue-skeletal muscle interactions *in vitro* is a small but very important step towards the establishment of more sophisticated co-culture systems to study the molecular mechanisms involved in the interactions between adipose tissue and other tissue types. Although developing *in vitro* systems to mimic physiological conditions is a daunting challenge, *in vitro* models allow easy manipulation and study of isolated variables, which is undoubtedly an indispensable advantage over *in vivo* studies.