Doctoral Thesis for the degree of Doctor of Philosophy, Faculty of Medicine

Cardiovascular and metabolic control in obese children and adolescents

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The Road goes ever on and on

Down from the door where it began.

Now far ahead the Road has gone,

And I must follow, if I can,

Pursuing it with eager feet,

Until it joins some larger way

Where many paths and errands meet.

And whither then? I cannot say.

"The Fellowship of the Ring" J R R Tolkien 1954

To my beloved family

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Gothenburg, Sweden. Thesis defended 13 June, 2008**

Abstract

Childhood obesity is an emerging risk factor for disease and mortality worldwide. The cardiovascular consequences and prevention thereof need to be further investigated. Exercise and weight loss are well examined and effective in the prevention of cardiovascular risk, but warrant well motivated patients with strong social support. The benefits of a diet rich in marine essential (n-3) fatty acids on cardiovascular risk in adults such as prevention of arrhythmias, lowering blood pressure and heart rate, decreasing platelet aggregation and lowering triglyceride levels are well known.

The aims of this thesis were to characterize the vascular changes and cardiac autonomic function in obese children compared to lean subjects and to test whether supplementation with n-3 fatty acids may improve the vascular and metabolic risk profile in obese adolescents.

Very high resolution ultrasound, pulse wave velocity measurements, baroreceptor sensitivity measurements and exercise tests were performed in order to characterize vascular changes and autonomic control in obese compared to lean children and adolescents. Supplementation with 1,2 g/day of n-3 fatty acids was tested in a randomized, placebo-controlled trial with a double-blind, cross-over design. Blood samples and anthropometric measurements were taken before the start of treatment and after each 3 month treatment period. At the end of each treatment period, muscle and adipose tissue biopsies were obtained; insulin sensitivity and vascular function were tested.

Obese children show increased intimal wall thickness in radial artery, increased vascular diameter in peripheral arteries and decreased pulse wave velocity compared to lean subjects. Obese children and adolescents also show cardiac autonomic dysfunction in terms of decreased baroreceptor sensitivity, decreased maximal exercise heart rate and greater heart rate increase during the first minute of exercise, indicating moderate cardiac autonomic dysfunction. After 3 months supplementation with marine fatty acids, n-3 fatty acid content of phospholipids in serum, skeletal muscle and adipose tissue increased. Vascular function measured as vasodilatory response to hyperaemia was improved, and the number of lymphocytes and monocytes was lowered. In females, insulin sensitivity and glucose tolerance improved after n-3 fatty acid supplementation.

In conclusion, obese children show signs of increased risk for cardiovascular disease in terms of increased intimal wall thickness and cardiac autonomic dysfunction. It is possible to modify this increased risk in obese adolescents by supplementing with n-3 fatty acids, which improves vascular function, decreases subclinical inflammation and improves insulin sensitivity.

Key words: Obesity, children, omega-3 fatty acids, atherosclerosis, ultrasound, insulin

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Populärvetenskaplig sammanfattning

Då andelen feta och fetmarelaterade sjukdomar ökar kraftigt i samhället och grundläggs tidigt är det av stor betydelse att hitta mekanismer som påverkar utvecklingen av t ex hjärt-kärlsjukdomar. Vi vet att barn med fetma uppvisar förändringar i hjärtmuskelstorlek, leverförfettning, höga insulinnivåer och blodfetter samt ett förändrat fettsyramönster i blodet med låga nivåer av omega 3 fettsyror. Vi har, med en helt ny ultraljudsmetod även kunnat undersöka blodkärlens väggar i detalj, och hos barnen med fetma fann vi tidiga tecken på begynnande åderförkalkning. Rubbningar i nervstyrningen av hjärtat har också diskuterats som en viktig sjukdomsfaktor vid framför allt vuxenfetma, och tecken på detta har vi i vår studie även funnit hos barnen med fetma. Kost och motion är faktorer som påverkar utvecklingen av fetma, hjärt-kärlsjukdom och åldersdiabetes. Världen över diskuteras idag betydelsen av ändrad fettsammansättning och lågt innehåll av omega 3 fettsyror i kosten för uppkomsten av dessa livsstilssjukdomar. Studier på vuxna visar att man löper mindre risk att drabbas av hjärt-kärlsjukdom om man äter mycket fisk, som är den huvudsakliga källan till omega 3 fettsyror. Då det är svårt att göra stora livsstilsförändringar har vi istället valt att undersöka effekterna av ett kosttillskott av omega 3 fettsyror på blodkärlsfunktion, blodfetter, inflammation och blodsockeromsättning hos ungdomar med fetma. Vi fann att vi med tre månaders kosttillskott av omega 3 fettsyror, i en dos som motsvarar 70 g sill om dagen, kunde förbättra blodkärlens funktion, minska inflammationen och förbättra blodsockeromsättningen. Slutsatserna man kan dra av denna studie är att barn och ungdomar med fetma tidigt uppvisar riskfaktorer för framtida hjärt-kärlsjukdom, men att dessa går att mildra med kosttillskott av omega 3 fettsyror.

List of papers

This thesis is based on the following papers, which will be referred to in the text by their roman numerals:

I: **Dangardt F**, Osika W, Volkmann R, Gan LM, Friberg P. Obese Children Show Increased Intimal Wall Thickness and Decreased Pulse Wave Velocity. *Clinical Physiology and Functional Imaging.* doi: 10.1111/j.1475-097X.2008.00806.x

II: **Dangardt F**, Volkmann R, Osika W, Zafar M, Nilén K, Marild S, Friberg P. Cardiac Autonomic Function in Obese Children. *Submitted.*

III: **Dangardt F**, Osika W, Chen Y, Nilsson U, Gan LM, Gronowitz E, Strandvik B, Friberg P. Supplement with Omega-3 Fatty Acids Improves Endothelial Function in Obese Adolescents. *Manuscript.*

IV: **Dangardt F**, Chen Y, Gronowitz E, Dahlgren J, Friberg P, Strandvik B. Improved Insulin Sensitivity with Omega-3 Fatty Acid Supplementation in Obese Adolescent Girls. *Manuscript.*

List of contents

Abbreviations

PUFA	Poly- unsaturated fatty acids
SFA	Saturated fatty acids
LC-PUFA	Long- chain poly- unsaturated fatty acids
MUFA	Mono- unsaturated fatty acids
EFA	Essential fatty acids
EET	Epoxyeicosatrienoic acid
AA	Arachidonic acid
EPA	Eicosapentaenoic acid
DHA	Docosahexaenoic acid
GLA	Gamma linoleic acid
FFA	Free fatty acids
PWV	Pulse wave velocity
BMI	Body mass index
z-score	Standard deviation score
ECG	Electrocardiogram
SBP	Systolic blood pressure
DBP	Diastolic blood pressure
HR	Heart rate
BRS	Baroreflex sensitivity
RA	Radial artery
DPA	Dorsal pedal artery
IT	Intima thickness
IMT	Intima- media thickness
MT	Media thickness
RH-PAT	Reactive hyperaemia peripheral arterial tonometry
NO	Nitric oxide
AUC	Area under curve
IVGTT	Intravenous glucose tolerance test
GDR	Glucose disposal rate
ISI	Insulin sensitivity index
HRR	Heart rate recovery

Introductory remarks

Obesity is increasing rapidly worldwide, and could be considered the world's next "big killer" after smoking[1]. Obesity starts already in childhood, and obese children often become obese adults[2], which leads to an increased risk of cancer[3] and cardiovascular disease[4]. The prevailing increase in childhood obesity is likely to lead to an increase in cardiovascular deaths[5-7]. To prevent this, we need to further explore the mechanisms behind the development of childhood obesity. The cardiovascular risk factors could be divided into different groups. There are modifiable life style risk factors such as smoking, sedentary life style and dietary habits. In the other end we find the consequences of life style habits, such as impaired cardio-respiratory fitness, decreased insulin-sensitivity, obesity, hypertension and other features of the metabolic syndrome.

Apparently, the change in dietary fat may contribute to the increase in childhood obesity[8-10]. It is known that Western diet contains a high amount of omega-6 (n-6) polyunsaturated fatty acids (PUFAs), which has been recommended to replace saturated fatty acids (SFAs) over the last 50 years. Unfortunately, the relative dominance of n-6 intake has contributed to the displacement of the n-6/n-3 ratio towards a much higher value (10-30:1) than the one in the Palaeolithic diet (1-2:1)[11]. Increased n-6/n-3 ratio rather than the amount of dietary fat per se could be related to risk factors for cardiovascular disease, diabetes and obesity[10, 12-14].

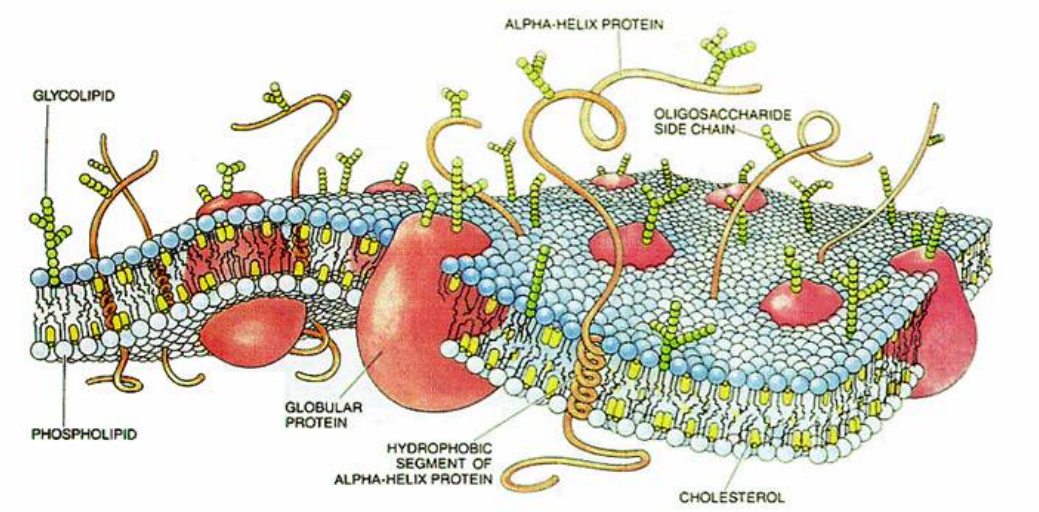
The present study explores the early neurogenic, metabolic and vascular consequences of childhood obesity and whether they are modifiable by a change in dietary fat composition.

Background

Plasma membrane

The plasma membrane surrounding the cells of the human body is a dynamic phospholipid bilayer, which separates the internal components of the cell from the extracellular milieu. The receptors and other membrane proteins and glycoproteins are integrated in the plasma membrane, and are dependent on its composition for optimal function. Similarly, the organelles of the cell are also separated by membrane structures with functional adjustments of its permeability properties. This allows for a fine regulation of ionic gradients and potential differences, as well as the passage of hormones, substrates, nutrients and intracellular signals across membranes, which is crucial to maintain normal cell function.

Fig 1 Plasma membrane



The phospholipids consist of a hydrophilic phosphate end coupled to two hydrophobic fatty acids. If the plasma membrane phospholipids have a large amount of saturated fatty acids and cholesterol, it will be rigid and non-permeable. With increasing amount of long-chain poly-unsaturated fatty acids (LC-PUFA), the plasma membrane becomes more fluent and permeable, which affects transport systems, receptor function and enzymatic activities[15-17]. Cellular membrane phospholipid fatty acid composition is dependent on dietary fatty acid composition, which has changed markedly towards less saturated fatty acids (SFA) and more n-6 polyunsaturated fatty acids (PUFAs) in the past 40 years, and is closely connected to development of obesity and cardiovascular risk factors[12, 15, 18, 19].

Fatty acids

There are three main groups of fatty acids in human tissue, SFAs, without double bonds, monounsaturated fatty acids (MUFAs), with one double bond, and PUFAs, with more than one double bond. Most of these fatty acids can be synthesized by the human body, but some are *essential* and needs to be provided in the diet. The essential fatty acids (EFAs) are also called omega-3 and omega-6 fatty acids (n-3 and n-6), and are derived from the precursors linoleic and α-linoleic acid, or directly from the diet. In the body, EFAs serve multiple functions, which are strongly affected by the balance between dietary n-3 and n-6 PUFAs. The EFAs are synthesized to eicosanoids and epoxyeicosatrienoic acids (EETs) affecting inflammation, macrophage chemotaxis and vascular tone. They are also involved in cell signalling and transcripition[20], directly activating or inhibiting transcription factors such as NFκB, linked to cytokine-production. The n-6 EFA arachidonic acid (AA) can be further synthesized to endocannabinoids, which are involved in different aspects of obesity and the metabolic syndrome such as regulation of appetite, energy balance, adipogenesis, lipoprotein metabolism, insulin sensitivity, glucose homeostasis, and possibly even the development of atherosclerosis[21-24].

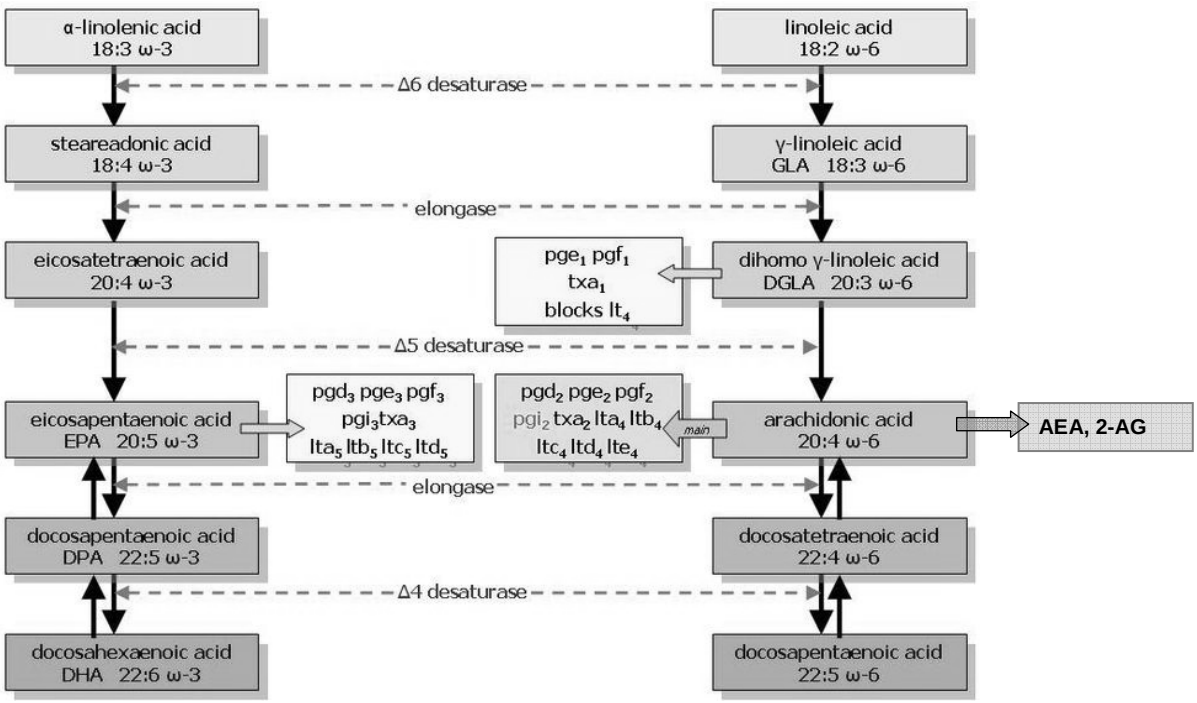


Figure 2 Metabolism of EFAs to eicosanoids and endocannabinoids, pg = prostaglandin, pgi = prostacyclin, tx = thromboxane, lt = leukotriene, AEA = anandamide, 2-AG = 2-arachidonoylglycerol

Dietary lipids have been found to affect obesity, inflammation and the risk of cardiovascular disease[13, 18, 19, 25-42]. Already in early development, the dietary fatty acid composition is proposed to be an important factor for the increase in childhood obesity[8, 9, 32], since it is suggested to influence the adipose tissue development, and thereby affect the number of adipocytes as well as promote adipogenesis and adipocyte growth[20, 43].

Adipose tissue

Adipose tissue involvement in obesity and insulin resistance was first recognized in the 1960´s by Rabinowitz and coworkers[44]. The adipose tissue is an endocrine organ, which produces and secretes different hormones and inflammatory compounds such as leptin, adiponectin, tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6)[45, 46]. The adipose tissue works as an energy storage, and consists of large amounts of triacylglycerol. The lipolysis is stimulated by β-adrenergic receptors, which regulates the outflow of free fatty acids (FFAs) into the circulation, which in combination with the inflammatory state affects the development of atherosclerosis in obesity. In type 2 diabetes and obesity, alterations in the insulin-glucose transport of adipocyte membrane insulin receptors were shown already in the 1980´s[47, 48], and has later been found to involve regulation of translocation of glucose-transporter GLUT4 to the cell surface[49].

Vascular biology and atherosclerosis

The artery wall consists of three layers, the tunicae intima, media and adventitia. The intima with its endothelial cells and underlying connective tissue is the layer adjacent to the blood. The media consists of smooth muscle cells, and the adventitia of connective tissue.

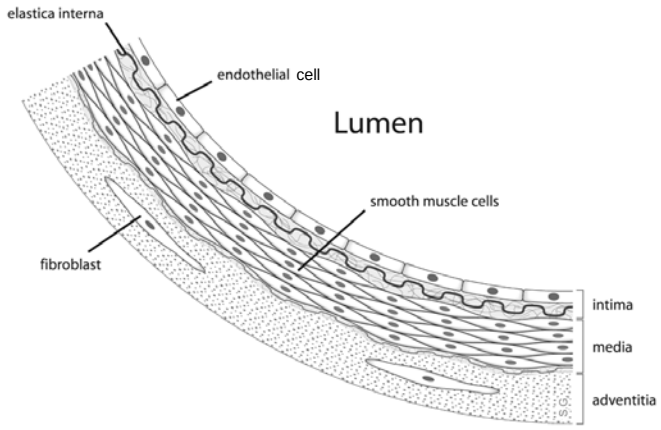


Figure 3 The arterial wall consisting of tunicae intima, media and adventitia.

The endothelium is involved in the regulation of vascular tone by formation of nitric oxide, endothelin, prostaglandins and leukotriens. These compounds affect the smooth muscle cells, and cause the media to constrict or dilate. One of the earliest signs of atherosclerosis, and closely related to obesity, is impaired endothelial function[50-55]. This is followed by intimal thickening due to lipoprotein and lipid retention, and inflammatory changes of the intima[56]. The process of atherosclerosis starts already in early childhood[57, 58]. According to the Pathobiological Determinants of Atherosclerosis in Youth (PDAY) study[59-61], the prevalence of fatty streaks and atherosclerotic lesions in the coronary arteries of humans aged 15-34 years is higher in males and increases with BMI and panniculus thickness. The major links between obesity and increased vascular changes are low-grade, chronic systemic inflammation and the increased amount of circulating non-esterified fatty acids[32, 45, 62-65]. This, in turn, affects the development of insulin resistance, also a major risk factor for cardiovascular disease[45, 57, 66-69]. Apart from inflammation and insulin, oxidative stress also plays a part in the development of endothelial dysfunction and in the pathogenesis of atherosclerosis[70].

Skeletal muscle

Skeletal muscle tissue consists mainly of two different types of muscle fibres. Type I, the slow oxidative, red muscle fibre, is dense with capillaries and rich in mitochondria and myoglobin. Type II is the fast, white muscle fibres, which contains less mitochondriae and myoglobin, but more glycogen and intramyocellular fat depots, and thereby serve as energy storage. In a recently published work, we have shown a heterogeneous lipid distribution in muscle tissue of obese children, and that type I muscle fibres contained more lipids than type II[71]. The effectiveness and function of the glucose transport and insulin receptor signalling may be modulated by intracellular lipids and membrane fatty acid composition, which might explain the relation between insulin sensitivity and the n-6/n-3 ratio in skeletal muscle phospholipids[72, 73].

Insulin resistance

Insulin resistance and mitochondrial function

Intramyocellular lipid content has emerged as a critical modulator of insulin resistance. Obese youth with impaired glucose tolerance demonstrates defects in non-oxidative glucose metabolism, and visceral fat and intramyocellular lipid accumulation is coupled to insulin resistance[74]. A number of different metabolic abnormalities may increase intramyocellular fatty acid metabolites; these include increased fat delivery to muscle as a consequence of either excess energy intake or defects in adipocyte fat metabolism[75]. Another possible mechanism behind intramyocellular lipid accumulation could be reduced mitochondrial oxidative phosphorylation, since mitochondria convert fatty acid and glucose into energy via oxidation. Indeed, muscular mitochondrial dysfunction does cause build-up of fats and fatty acid inside muscle producing insulin resistance and leading to diabetes[76, 77]. Moreover, mitochondrial dysfunction has also been reported in white adipocytes from obese mice[77]. The mechanisms involved in mitochondrial dysfunction, are less clear. However, treatment of adipocytes with the n-3 FA eicosapentaenoic acid (EPA) has been shown to increase the percentage of this FA in the mitochondrial membrane lipids, possibly increasing the beta-oxidation in adipocytes by altering the structure or dynamics of the mitochondrial membranes[78].

Insulin resistance and fatty acid composition

Decreased insulin sensitivity has been shown to be associated with decreased concentration of polyunsaturated fatty acids (PUFAs) in skeletal muscle phospholipids[79], raising the possibility that changes in the fatty acid composition of muscles modulate the action of insulin. In line with this, epidemiologic studies conducted in adults show that dietary fat and plasma fatty acid composition are related to insulin sensitivity and several features of the metabolic syndrome[80]. Interestingly, intake of n-3 PUFAs in adults has been shown to reduce adipose tissue mass and improve insulin sensitivity[81]. Generally, one mechanism of action of PUFAs is altering membrane lipid composition, cellular metabolism, signal transduction, and regulation of gene expression. Furthermore, n-3 PUFAs serve as peroxisomal proliferator-activated receptor (PPAR) ligands, leading to PPAR activation and subsequent transcriptional up regulation of an array of genes encoding enzymes involved in mitochondrial and peroxisomal and microsomal fatty acid oxidation[82]. In this context, a recent study shows that n-3 PUFAs of marine origin up-regulates mitochondrial biogenesis and induces beta-oxidation in white fat[83].

Autonomic nervous system

The autonomic nervous system regulates and co-ordinates cardiovascular, digestive and respiratory functions. Insulin signalling and lipolysis as well as cardiac and vascular control is dependent on optimal autonomic function. Major risk factors, predicting mortality, found in both healthy adults and adults with prevailing cardiovascular disease, are decreased vagal function and increased sympathetic function, reflecting a disturbance in the autonomic nervous system[84-87]. Studies have shown that autonomic dysfunction in obese children[88] as well as adults[89], is a consequence of obesity. In addition, sympathetic neural activity to the gut leads to increased secretion of ghrelin assessed in portal venous blood[90], adding support to the contention that gastro- intestinal sympathetic nerves are involved in preprandial responses. An imbalance in this system might signal to the central nervous system to continue eating, and therefore also contribute to the development of obesity. However, also other factors, such as hypertension and coronary heart disease, in adjunct to obesity, may cause autonomic dysfunction[91]. Cardiac autonomic function measured as heart rate variability is improved by increased levels of dietary n-3 fatty acids[92-94].

Fish oil history

The essential fatty acids were discovered by the American researchers Evans and Burr in 1929[95]. Their research rouse the interest of Hugh Sinclair, who visited the Eskimo's of Greenland in 1944, aimed to study the beneficial effects of their marine fatty diet on their cardiovascular health. This resulted in the letter "Deficiency of essential fatty acids and atherosclerosis, etcetera" published in Lancet in 1956[96]. The hypothesis of the beneficial cardiovascular effects of the Eskimo's marine diet was further investigated by the Danish scientists Bang and Dyerberg, who found in the 1970's that EPA protects against thrombosis[97] and atherosclerosis[98]. Epidemiological studies by Kromhout and co-workers in the 1980's supported these data further by showing an inverse relation between fish-consumption and coronary heart disease[99]. In the early 1990's, Thelle et al presented one of the first randomized trials proving that dietary EPA supplementation reduced blood pressure in essential hypertension[41]. Other studies followed with evidence of beneficial effects of fish oil or marine fatty acids on not only hypertension, but also on arrhythmias, insulin resistance and many other cardiovascular risk factors.

Current status and unsolved issues

Despite the increasing knowledge about the adulthood metabolic syndrome, little is known of the mechanisms behind childhood obesity and insulin resistance. We and others have shown recently that overweight/obese adolescents have higher saturated fatty acid and lower n-3 PUFAs in plasma compared with normal-weight adolescents[100, 101]. However, whether dietary supplementation of n-3 PUFAs would slow down the progression of childhood obesity and insulin resistance is unknown.

Aims

The aims of the study were

to characterize the vascular changes and cardiac autonomic function in obese children compared to lean subjects

to test whether supplementation with omega-3 fatty acids may improve the vascular and metabolic risk profile in obese adolescents

Methodological considerations

Ethics

In all studies, informed consent and written protocols, approved by the ethics committee at Sahlgrenska Academy in Göteborg, were presented to the children or adolescents and their parents. Written consent was obtained from both the children or adolescents and their parents.

Study populations and design

We recruited children and adolescents with obesity, as defined by the IOTF[102], but otherwise healthy, who were referred to the hospital for obesity treatment. Matched lean controls were recruited from schools in the Gothenburg area.

Study I

Ultrasound and pulse wave velocity (PWV) measurements were performed in 33 children and adolescents with obesity (13.9 ± 1.6 years) and in 18 matched lean controls (14.3 ± 2.2 years).

Study II

The exercise tests of 101 children and adolescents (48 females and 53 males) with obesity and 31 lean controls (19 females and 12 males) were analyzed. Cardiac baroreflex sensitivity was measured in 315 patients, also including 21 of the controls and 45 of the obese subjects described in the exercise test. Of these 315, 129 (65 females and 64 males) were obese (BMI z-score >2.5), 35 (21 females and 14 males) were overweight (BMI z-score 1.5-2.49) and 151 (78 females and 73 males) were lean (BMI z-score <1.5).

Study III and IV

Thirty- one adolescents with obesity agreed to participate in the study. One subject was excluded before the study due to smoking, one moved abroad, three dropped out during the study and one was excluded due to poor compliance. Twenty-five subjects completed the study. The study was performed as a randomized, placebo-controlled trial with a double-blind,

cross-over design. The subjects received 2x5 capsules/day containing either 93 mg EPA, 29 mg DHA, 10 mg GLA and 1,8 mg Vitamine E /capsule or medium chain triglycerides (MCT) capsules as placebo. Blood samples and anthropometric measurements were taken before the start of treatment and after each 3 month treatment period. At the end of each treatment period, muscle and adipose tissue biopsies were obtained, insulin sensitivity and vascular function and characteristics were tested.

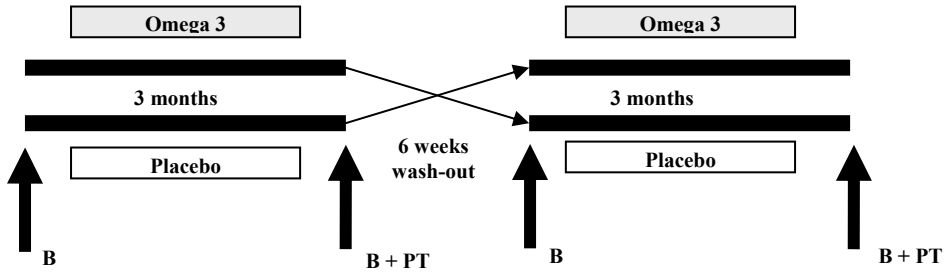


Figure 4 Study design of study III and IV
Baseline (B): Anthropometry and blood sampling (fasting), Post treatment (PT): Pulse wave velocity measurement, endothelial function measurement, high-resolution ultrasound, hyperinsulinemic-euglycemic clamp, fat and muscle biopsies.

Autonomic function (paper II)

Exercise test

In brief, blood pressure and 12-lead ECG were registered at rest in supine position, before start of the exercise test.

For the bicycle exercise test an electrically braked SensorMedics 800 Ergometer was used (Yorba Linda, CA, USA). Baseline data were recorded having the subjects sitting on the bike before the exercise test. Exercise to exhaustion was performed with a continuous, progressively increasing load protocol and a pedalling rate of 60-65 revolutions per minute. The starting workload was 1 watt (W)/kg up to maximum 100 W and was then increased at a rate of 10 W per minute. For ergospirometry, a mouthpiece and a nose clip were fitted on the subject at least two minutes before start of the cycling and were kept on throughout the test, also for 2 minutes post exercise.

Every minute during exercise either a 30-second tidal breathing registration or a vital capacity manoeuvre for intrabreath analyses of gases were made. Breath-by-breath gas analyses were made on samples taken at the mouth during late expiration. Oxygen gas analysis was based on

high sensitivity paramagnetic technology (SensorMedics Corp., Yorba Linda, CA, USA), whereas a rapid response infrared analyzer was used for carbon dioxide measurements.

The anaerobic threshold (AT) point was determined using two slopes, the aerobic and the anaerobic slope, determined by the test system. This point was analyzed in 13 of the controls and 21 of the obese subjects. Data at this point are presented as 20-second average. Baseline was defined as mean values of heart rate, blood pressure and oxygen uptake during the resting period on the bike before starting the test.

Cardiac baroreflex sensitivity and QT variability index

In brief, after 10 minutes of rest, ECG and beat-to-beat blood pressure was registered over 20 minutes by Portapres® equipment (TNO Biomedical, Amsterdam, Netherlands), with the subject in supine position. Registrations were recorded at a sampling frequency of 1000 Hz and stored on a computer. The recordings were inspected off-line for removal of artefactual segments and sequences containing non-sinus beats. Ectopic beats were corrected by interpolation.

The time series of SBP and RR interval from the entire period of recording (20 minutes) were scanned to identify baroreflex sequences, which were defined as three or more consecutive beats in which successive SBP and RR intervals concordantly increased or decreased, according to the classical criteria suggested by Bertinieri et al.[103]. Linear regression was applied to each sequence and only those for which the square of the correlation coefficient (r^2) was greater than 0.85 were accepted for further analysis. The spontaneous BRS was calculated, reflecting the average regression slope for all the linear regressions.

A stationary period of 5 minutes was chosen for the temporal QT interval variability analysis using a computer algorithm[104]. The examiner defined a template QT interval for one beat, which was used for finding the QT intervals of all other beats. RR interval mean (RRm) and variance (RRv) and QT interval mean (QTm) and variance (QTv) were derived from the respective time series. QT variability index, which represents the log ratio between normalized QT and RR interval variability, was calculated.

Time-domain heart rate variability

RR data from a 20 minute recording section was used to derive standard deviation of normal-to-normal RR intervals (SDNN)[105].

Vascular measurements (papers I and III)

Ultrasound measurements (papers I and III)

We used high-resolution ultrasound of 55 MHz (VisualSonics Inc**.** Toronto, Ontario, Canada), validated for use in human peripheral arteries[106]. Subjects were resting in a supine position and radial artery (RA) and dorsal pedal artery (DPA) were scanned. Four consecutive beats were saved and 2-D images were subsequently analyzed off-line.

Intimal thickness (IT) was defined as the total thickness measured with callipers within a higher resolution zoom. Three measurements of the IT were performed in systole at the artery's largest diameter. The medial thickness (MT) was calculated as the difference between intimal-medial thickness (IMT) and IT (MT=IMT-IT), according to a previously established protocol[106]. IMT was defined as the distance from the leading edge of the lumenal-intimal interface to the leading edge of the medial-adventitial interface. Lumen diameter was defined as the distance between the leading edges of the intimal-lumenal interface of the near wall and the lumenal-intimal interface of the far wall[107].

The coefficient of variation of repeated measurements by the same operator (i.e. the intra individual variation) was studied in a separate group of 10 obese subjects, and was 8.1 %, 4.0 % and 1.5 % in RA IT, RA IMT and RA diameter, respectively. In DPA IT, DPA IMT and DPA diameter, intra individual variation was 9.2 %, 8.2 % and 1.7 %, respectively. Reproducibility was studied, and intra-observer variability expressed as coefficients of variation were for RA IT and IMT 7 % and 5 %, respectively, and for DPA IT and IMT 8 % and 7 % respectively[108].

Pulse wave velocity measurements (papers I and III)

The velocity of the blood pressure pulse waveform is dependent on the stiffness of the artery. In brief, a pressure tonometer was used to record transcutaneously the pressure pulse waveform in the underlying artery (SphygmoCor® system, AtCor Medical, Australia). Records were made simultaneously with an ECG signal, which provided an *R*-timing reference[109]. Pressure pulse recordings were performed consecutively at two superficial artery sites (carotid-radial segment or radial-dorsal pedal segment). Each set of pressure-pulse and ECG waveform data was used to calculate the mean time difference between *R*-wave and pressure wave on a beat-to-beat basis, with an average of 10 consecutive cardiac cycles. The

pulse wave velocity (PWV) was then calculated using the mean time difference and distance between the two recording points. Quality indices, included in the software, were set to ensure uniformity of data. In a separate group of healthy children and adolescents (n=10), we found that the reproducibility (calculated as coefficient of variations) for the radial-carotid PWV was 9%.

Endothelial function measurements (paper III)

Endothelial function as measured as vasodilatory response to hyperaemia was assessed non-invasively using reactive hyperaemia peripheral arterial tonometry (RH-PAT) method with Endo-PAT® device (Itamar Medical Ltd, Caesarea, Israel). In brief, pulse wave amplitude is recorded from finger-tip probes on both index fingers with the subject at rest in supine position for the duration of the study. After five minutes of continuous baseline measurements, arterial flow to the arm is occluded for five minutes using a blood pressure cuff inflated to 200 mmHg, or at least 50 mmHg, whichever is highest, above systolic pressure. After the five minute occlusion, the cuff is rapidly deflated to allow for reactive or flow-mediated hyperemia. Pulse wave amplitude is recorded for at least five minutes after the cuff is deflated (Fig 5). An integrated software program compares the ratio of arterial pressure in the two fingers before and after occlusion to calculate the RH-PAT score in an operator-independent manner. The RH-PAT index (RHI) is calculated as the ratio of the average pulse wave amplitude measured over 60 seconds starting one minute after cuff deflation divided by the average pulse wave amplitude measured at baseline and normalized to the concurrent signal from the contra-lateral finger to correct for changes in systemic vascular tone [52]. The F-RHI is the natural logarithm of the RHI.

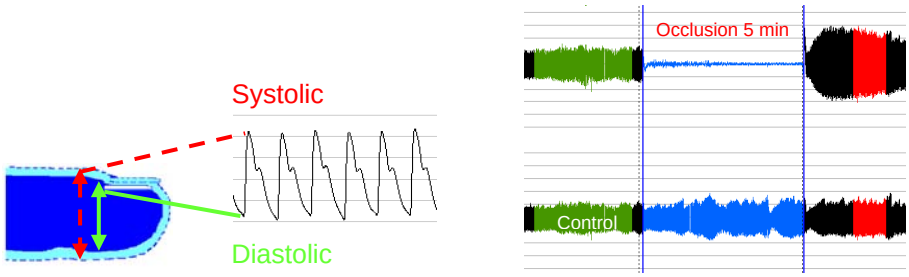


Figure 5 RH-PAT measurement with finger probes and parallel amplitude registration

Finger probe measures volume-changes during systolic and diastolic phase showing as a pulse wave. Amplitudes registered during 5 min rest, 5 min occlusion and 5 min post occlusion in both index fingers.

The choice to use the average one-minute PAT signal starting one minute after cuff deflation to describe the magnitude of reactive hyperaemia was based on the observation that this time interval provided the best information regarding detection of coronary endothelial dysfunction as determined by receiver operating characteristic curve analysis as well as the best correlation with coronary blood flow response to acetylcholine; and an attenuated RH-PAT score is predictive of coronary heart disease in adults[110, 111].

The ratio between average baseline amplitude and post-occlusion amplitude was calculated as an average of each 30-second interval from 0 to 5 minutes post occlusion. The post-occlusion period was analyzed in terms of area under curve (AUC) for 0-1 minutes post occlusion, 0-3 minutes post occlusion and 0-5 minutes post occlusion. The maximum flow-mediated dilation (FMD) was extracted manually and analyzed. These different measures are commonly used in earlier studies of endothelial function in children and adults[51, 53, 112, 113].

In another study, the reproducibility of RH-PAT measurements was investigated. In a separate group of healthy children (n=33), each subject was studied twice with a 10-week interval. The coefficient of variation was 11%.

Biochemical analyses

White blood cells, red blood cells, and platelets were analyzed by fluorescence-activated cell sorting. Fasting total cholesterol, HDL cholesterol, and triacylglycerol were analyzed by using enzymatic methods (Roche Diagnostics, Mannheim, Germany). LDL-cholesterol concentrations were calculated by using Friedewald's equation. Fasting serum insulin was analyzed with a radio immunochemical method (Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden), and fasting blood glucose was analyzed using an enzymatic approach. The intra-assay coefficient of variation for insulin in the range of our measurements was 6.4 %. In study IV, plasma leptin was analyzed by ELISA from Mercodia (Uppsala, Sweden) and high-molecular weight-adiponectin (HMW-Adiponectin) was analyzed by Linco (St. Charles, Missouri, USA).

Fatty acid analyses

Fatty acid analyses were performed in phospholipids of serum, and biopsies from M. vastus lateralis and abdominal subcutaneous adipose tissue. After intra and subcutaneous administration of local anaesthesia, muscle and adipose tissue biopsies were obtained from 18

of the subjects after both treatment periods. The muscle biopsy was obtained from M. vastus lateralis with a Bergmann needle, following an incision with scalpel of the skin, subcutaneous fat and muscle fascia. Adipose tissue was aspirated by use of a 20 ml syringe and 14 gauge needle obliquely horizontally inserted in subcutaneous adipose tissue of the abdomen. Biopsy was immediately placed in a test tube and frozen in liquid nitrogen, and then stored at -70 ºC until analysis.

All organic solvents were of HPLC grade, water of analytical grade Milli-RX™ (MILLIPORE), reagents and standards were of documented purity.

In brief, the serum phospholipid fatty acids were analysed after lipid extraction according to Folch et al.[114], fractionated and eluated after washing. The fraction of lipids was transmethylated and the FA methyl esters (FAME) were extracted with hexane, washed with water, dried over $MgSO_4$ and resolved in hexane (grade for spectroscopy), and separated by capillary gas-liquid chromatography (GLC) in a Hewlett-Packard 6890 gas chromatograph. Helium at 1.4 ml/min was used as carrier gas. The injector and detector temperatures were 250°C. The column oven temperature was sequentially programmed from 60°C to 230°C where it was run for 10 minutes. The separation was recorded with HP GC Chem Station software (HP GC, Wilmington, DE). Heneicosanoic acid (21:0) was used as internal standard and the FAME identified by comparison with retention times of pure reference substances (Sigma Aldrich Sweden AB, Stockholm, Sweden).

Muscle biopsy samples were weighed, minced into pieces and then homogenized. Lipids were extracted by the procedure of Rose and Oklander[115] using chloroform and 2-propanol and sonication. The phospholipid HPLC method described by Silversand and Haux [116] was slightly modified for use of an internal standard and to collect lipid fractions from split post column flow. The system consisted of two delivery pumps (Bischoff 2250), an injector of 20 µL, a gradient mixing chamber 1.8 mL (SPARK) and a detector ELSD Varex MKIII (Alltech). The column was a LiCrospher 100 Diol 5 µm 250 x 4 mm with Si guard column. The column temperature was 55°C. The software for pump control and evaluation of detector signals was Clarity (DataApex LTD, Prague).
Standards were composed of mixtures of phospholipids from Larodan Fine Chemicals (Malmö, Sweden). Fractions corresponding to phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl inositol, phosphatidyl glycerol and

diphosphatidyl glycerol and sphingomyeline were collected. The fractions were in this study combined and dried under nitrogen and FAME prepared and analysed by capillary GLC as described above.

Adipose tissue specimen was homogenized, and after centrifugation, 50 µl of the extract was used for determination of total fatty acids. The fatty acids were transmethylated, internal standard was added, and the extract resolved in hexane and total FAME were analysed on GLC. Aliquots, 2 x 1 ml of the lipid extract, were evaporated, resolved in chloroform and phospholipids separated as described for plasma phospholipids and after transmetylation the FAME were separated and identified on GLC as described above.

The levels of individual fatty acids were expressed as percentage of total fatty acids identified (molar %). These values were used to calculate indexes and sums presented.

Ascorbyl radical measurements (paper III)

To assess oxidative stress, we measured ascorbyl radicals at the end of each treatment period. In brief, the intensity of the ascorbyl radical in plasma sample was measured with a Bruker ECS 106 EPR spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany). The amount of ascorbyl radical was expressed as relative change in ESR signal intensity compared with control values before the induction of asphyxia. The values were multiplied with a factor of 0,0042 for the concentration in µM.

Insulin sensitivity measurements (paper IV)

Intravenous glucose tolerance test (IVGTT)

In brief, after an overnight fast, catheters were inserted for infusions of glucose and withdrawal of arterialised venous blood. After baseline blood collection, 300 mg glucose /kg body weight (30% glucose solution) was given within 2 minutes to acutely increase the blood glucose level. Blood samples were drawn at 2, 4, 6, 8, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120 minutes after glucose infusion. Plasma glucose was analyzed with a glucose analyzer (HemoCue Glucose 201 DM Analyzer, HemoCue AB, Sweden) and serum insulin was analyzed by Enzyme-Linked Immunosorbent Assay (ELISA, Mercodia, Uppsala, Sweden). The magnitude of insulin response to glucose was quantified as the incremental area

under the curve (ΔAUC). Glucose disappearance constant (Kg) was calculated as the slope of the logarithm of glucose values between 10 and 30 minutes after glucose infusion.

Euglycemic hyperinsulinemic clamp

Insulin sensitivity was determined using the euglycemic hyperinsulinemic clamp technique, as described earlier[117, 118]. Briefly, 30 minutes after the IVGTT, human insulin (Actrapid, Novo Nordisk, Copenhagen, Denmark) was infused as a priming dose for the first 10 minutes, followed by a continuous infusion (80 mU/m^2 body surface/min) for 120 minutes. Glucose infusion was started simultaneously and the infusion rate adjusted to clamp the blood glucose at 5.0 mmol/L, assessed at 5-minute intervals with a glucose analyzer (HemoCue Glucose 201 DM Analyzer, HemoCue AB, Sweden). The glucose infusion rate during the last 60 minutes served as a measure of the subject's insulin sensitivity and was expressed as glucose disposal rate (GDR) (mg/kg body weight/min). The GDR, also known as M-value, was calculated as the mean value of the amount of glucose infused for each 20 minute interval during the last 60 minutes of the clamp. The insulin sensitivity index (ISI) was calculated by dividing the M-value by the steady-state insulin concentration during the last 60 minutes of the clamp [mg glucose/kg body weight/min/insulin (mU/L)].

Statistical analyses

Statistical analyses were performed with the statistical software SPSS 15.0 for Windows (SPSS Inc., Chicago, Illinois, USA). All results are expressed as mean ± SD. Association between variables were analysed using simple correlation. GraphPad Prism 4.03 (GraphPad Software Inc, San Diego, California, USA) was used for all curve analysis. P–values below 0.05 were considered statistically significant.

In paper III, to compare the RH response curves after n-3 and placebo treatment pair- wise for each subject, global fitting was used. This is a non-linear regression method in which one curve (placebo) for each subject is used as model (or baseline), allowing evaluations of discrepancy of the other curve. This method works in analogy to the paired samples t-test, using a pair of curves instead of a pair of values.

In paper I, 2-way ANOVA was used when comparing HRR, and HR. Age dependency of SBP and DBP was assessed by univariate analyses, and adjusted accordingly in a general linear model.

Results and discussion

Cardiovascular changes in obese children

In papers I and II, we found that obese children are exposed to several cardiovascular risk factors already at an early age, such as altered vascular characteristics and cardiac autonomic dysfunction.

Previously, IMT studies in children and adolescents were performed in the carotid artery, except only one study assessing aortic IMT[119]. These investigations were not focused on components of the IMT complex. In paper I, by using new ultrasound technique with 30 μm resolution[106] , we assessed IT and MT separately in superficial peripheral arteries. The possibility to discriminate different parts of the vascular wall may help increasing the understanding of the origins of atherosclerosis in vivo. The fact that we also are able to examine peripheral arteries further increases the knowledge of the atherosclerotic process, and the mechanisms by which might differ between arterial sites. Increased IT of the RA (from 0.049 mm) by 10 % was found in obese compared to lean subjects (p=0.02), but no differences in RA IMT or MT could be observed. There was no difference in DPA IT between groups, whereas the DPA MT was increased (from 0.148 mm) by 17% (p=0.02) and the DPA IMT was increased (from 0.202 mm) by 13% in the obese compared to lean, p=0.01. In contrast to what we expected, the difference between groups in RA IT was not found in DPA IT, indicating that various arterial locations and their respective arterial wall structures may respond differently to different factors. It is conceivable that the increased blood pressure load (including the hydrostatic pressure) in the foot contributes to the increased media thickness of the dorsal pedal artery in obese children we have shown in this study and, further, to the development of atherosclerosis.

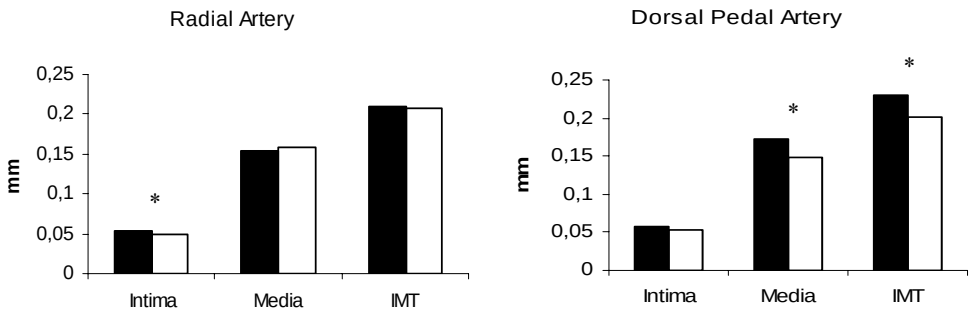


Figure 6 Arterial wall components in obese (■) and lean (□) children. * p<0.05

According to Tounian et al., obesity (adjusted BMI >30) in children is directly associated with increased arterial wall stiffness and endothelial dysfunction but not with carotid IMT[50]. Others have demonstrated that increased carotid IMT is directly related to inflammatory markers, elevated blood pressure, and left ventricular hypertrophy[51, 62, 120-122]. The present study underscores the effects of childhood obesity on early structural atherosclerotic markers and extends these effects to peripheral arteries such as RA and DPA.

Obese females had an 18% larger IT than lean females, and accounted for the entire difference in RA IT between the obese and lean groups (p=0.04, Fig 7). This finding could indicate that girls are more susceptible to the influence of obesity or that the relative advantage of the female sex, in terms of postponing cardiovascular risk and events, is obliterated by obesity[123]. In line with research in adults, where women present with later development of atherosclerosis[124], the lean girls present with thinner intima than the lean boys. We can only speculate that the obese girls, at this early stage and without any other cardiovascular risk factors such as smoking and hypertension, are more susceptible to intimal thickening than obese boys. There are studies showing that insulin resistance in obese females is associated with increased rigidity of the aortic wall, suggesting different sensitivity to insulin resistance in male and female obese adolescents[125] . Such aortic changes may also involve smaller sized arteries as in the present study. Another factor may be the presence of undiagnosed polycystic ovary syndrome in the obese girls. This disease is far more common in obese than lean children, and may contribute to an earlier development of atherosclerosis[126, 127].

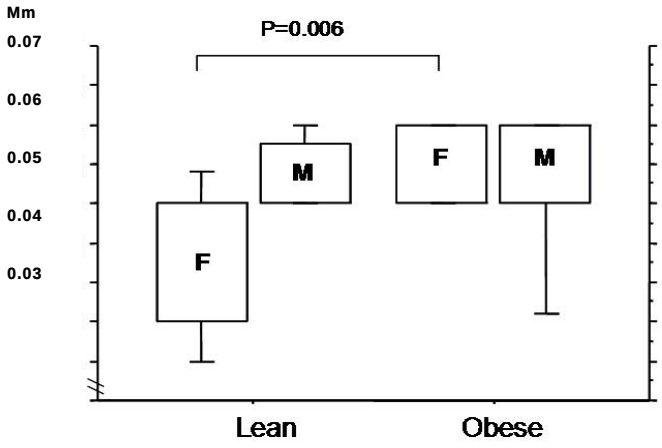


Figure 7 Radial intimal thicknesses in obese and lean adolescents divided by gender.

Arterial stiffness, as reflected by increased PWV, is determined by the arterial wall structure in relation to the arterial blood pressure and has been shown to be a predictor of cardiovascular mortality in certain patient groups[128, 129]. A few studies have used various methods and arterial sites to assess arterial stiffness in obese children and adolescents. Tounian et al.[50] found increased carotid artery stiffness in obese children by using ultrasound parameters for vessel wall distensibility calculations. In contrast, our approach to assessing PWV using the carotid and radial arteries as measuring sites showed that the obese children had decreased PWV (6.2 ± 0.8 vs. 7.0 ± 0.9 m/s in obese and lean, respectively, p=0.001) and lower diastolic blood pressure (58 ± 9 vs. 66 ± 6 mmHg in obese and lean respectively, p=0.001) compared to lean subjects, suggesting normal to decreased arterial stiffness and lower peripheral resistance. The RA and DPA of obese children had consistently increased lumenal diameters (RA: 1.8 ± 0.3 vs. 1.5 ± 0.4 mm in obese and lean, p=0.006 and DPA: 1.5 ± 0.5 vs. 1.0 ± 0.3 mm in obese and lean, p=0.0006) and thus reduced IT-to-lumen ratios compared with lean subjects. To our knowledge, this is a novel finding in young obese subjects and may reflect a "physiological structural adaptation" to altered metabolic and/or hemodynamic demands, such as increased overall vasodilatation, blood volume, and cardiac output, which, together with decreased total peripheral resistance, have been documented in adult obesity[130]. In other words, arterial vasodilatation may be a functional consequence of the hyperinsulinemic state and not a pathological process *per se*, and it might be reversible if the individual loses weight. Over the long term, a condition of dilated vessels with increased blood flow in which the structural component does not match the increased vessel diameter may lead to augmented wall tension. This in turn could result in impairment of vascular features and dysfunctional regulation, such as hampering of endothelial responses.

In paper II, we found that obese children show a different heart rate response to exercise compared to lean controls. They have a greater increase in heart rate during the first minute of exercise (ΔHR_{1min}) than lean subjects, being 36 ± 12 vs. 27 ± 12 beats in controls, p=0.001. Increased $\Delta HR_{1\ min}$, is an autonomic measure mirroring the increase in sympathetic activation and concurrent vagal withdrawal[131]. Since there were no differences in resting heart rate (88 ± 14 and 86 ± 13 bpm, in obese and lean subjects, respectively, ns), i.e. the starting point, this would be a reliable variable to use in our cohort. Described by Leeper et al, ΔHR at 1 minute of exercise is associated with both cardiovascular- and all-cause mortality in adults[131]. This pattern may depend upon the obese state *per se*, which at least in adults is associated with increased sympathetic tone[89, 132], but it could also be attributed to lower degree of fitness

level. The obese children also show lower maximum heart rate (HR_{max}) at peak exercise compared to lean controls (186 ± 13 beats/min vs. 195 ± 9 in the lean controls, p=0.0003). In adults, an impaired heart rate response to exercise test is a predictor of mortality[133], and may therefore be of importance to consider also for the risk assessment of adolescents with obesity. The lower HR_{max} in the obese children might be explained by the fact that that they didn't perform as much work. Investigating this more closely, we found that the groups did not differ, neither in maximal workload (153 ± 10 and 162 ± 12 Watts for the obese and controls, ns), nor in maximal oxygen uptake (2.3 ± 0.4 l/min in both groups), which would be expected if this assumption was correct. Increased work load (108 ± 12 Watts vs. 81 ± 8 Watts for the controls, p= 0.002) and oxygen uptake (1.4 ± 0.1 l /min vs. 1.1 ± 0.2 for the controls, p=0.005) in children with obesity at the same heart rate as lean controls (143 ± 7 vs. 140 ± 9 bpm for the controls, ns) at anaerobic threshold suggests that children with obesity have increased leg strength and oxygen uptake compared with the lean controls, possibly due to the increased body weight. Moreover, obese subjects had 35 % shorter duration of exercise tests than controls (p<0.0001), indicating faster exhaustion and impaired cardio-respiratory fitness. This may be due to the lower heart rates, or to decreased mitochondrial function and increased intramyocellular fat, as shown by Caprio et al[74]. Poor mental stimulation to perform maximal exercise is less probable since they have overall lower heart rates. Given the cardio-respiratory data at anaerobic threshold, we would anticipate a higher work load and oxygen uptake at peak exercise in subjects with obesity vs. lean subjects.

Slow heart rate recovery after exercise is a simple way of determining vagal dysfunction[91]. When evaluating heart rate recovery in adults, a decrease of at least 12 beats during the first minute after exercise is considered normal[87]. To our knowledge there are limited data of heart rate recovery in healthy children and adolescents. In our dataset of children, the decrease during the first minute in controls was 32 beats, and in children with obesity 37 beats, reflecting a more rapid vagal response compared to adults, although difficult to interpret because of the difference in max HR between obese and controls. However, a similar response pattern was seen between groups after exercise when heart rate was expressed as percentage lowering (being 88% of max HR at 30 seconds post exercise compared to 92 % in the lean controls, p= 0.03 and 79 % of max HR compared to 84 % for the lean controls, p= 0.03 at 1 min post exercise), suggesting a rapid return of vagal activity in both groups.

Because of relatively similar patterns of aerobic capacity between groups, HRR assessment and interference with "metabolic recovery", may not be sensitive enough to detect differences in cardiac autonomic modulation among different groups[134], particularly not in the young where differences may be subtle. An exercise test involves activation of several systems, like the neurohormonal system and the circulation, with increased blood-flow and circulating metabolites such as lactate. One of the key factors influencing the HR response after exercise is an altered metabolic status, which is impaired in obese[135]. Following exercise, there is a total body recovery, which both depends on neurogenic and metabolic factors. HRR depending on pure vagal reactivation is present only the first 30 seconds post exercise[91]. One has to consider signalling from metaboreceptors rather soon after commencing the exercise test strongly stimulate the sympathetic nervous system and suppresses the activity in the cardiac parasympathetic division of the autonomic nervous system, that might be different in different subjects. HR is a direct reflection of metabolic need, and remains elevated up to 30 minutes after exercise[136].

Therefore, cardiac baroreceptor sensitivity may represent a more sensitive and reliable method to detect autonomic dysfunction, also for children and adolescents[137-139]. Cardiac baroreflex sensitivity was 24 % lower in subjects with obesity compared to lean controls, indicating a reduced cardiac vagal function. No difference was found between subjects with overweight and lean controls. When taking pubertal status into consideration, we found similar results. These findings thus indicate that children with obesity, already at this young age, show several signs of autonomic dysfunction, although to a mild degree.

The lower BRS that we observed in children and adolescents with obesity may be attributed to several mechanisms. Firstly, a functional change in BRS may prevail in these young subjects having obesity, inasmuch that they demonstrate decreased cardio respiratory fitness, as indicated by the blunted maximal heart rate response and the rapid increase in heart rate to exercise. If this is the case, it might be possible to improve the autonomic function in adolescents with obesity by increased physical activity, as suggested by Nagai et al[138]. Secondly, the autonomic dysfunction might also be explained by the obese state per se and its possible hampering effects on vascular structure and distensibility[50]. Thirdly, a deranged metabolic situation due to the obese state may affect autonomic output. BRS have been shown to increase in subjects with obesity after dietary weight loss[140], which could be explained in part by increased insulin sensitivity. Insulin has been shown in obese humans to increase

muscle sympathetic nerve activity during euglycemic insulin clamp[141, 142], possibly influencing the central nervous system to modify the autonomic nervous system, which is indicated in the study by Pricher et al, where insulin in the brain increases gain of baroreflex control[143]. Free fatty acids (FFA) have been shown to increase blood pressure in rats by stimulation of excitatory hepatic afferent vagal nerves[144], and should also be of interest in studies of subjects with obesity, considering their increased FFA outflow from adipose tissue. Adipose tissue also produces cytokines and other inflammatory components affecting autonomic function[145].

Intervention

The common treatment for childhood obesity such as diet and exercise have proved to be less successful in terms of long-time compliance and effectiveness, and requires highly motivated participants[146, 147]. Although diet and exercise programs can be effective in reducing cardiovascular risk in obese children[120, 148, 149], we need to reach also those without strong social support and motivation, and therefore possibly at a greater risk of developing future cardiovascular disease[150].

In our study on omega 3 supplementation, which to our knowledge is the first randomized, placebo-controlled study in children, we could show a reduction of cardiovascular risk factors such as endothelial dysfunction and insulin resistance without any modification of diet or exercise habits.

In paper III, we found a significantly improved endothelial function, measured as vasodilatory response to post-ischemic hyperaemia, after three months n-3 treatment in obese adolescents (pair- wise global fitting, $p<0.01$). Endothelial function is closely related to the dietary FA composition[151-153]. N-3 treatment, but not placebo, has been shown to improve both forearm vasoconstrictive responses and endothelial function in adults[154, 155]. These results could be explained by an increased level of endogenous nitric oxide (NO), as shown by Harris et al[156], where n-3 supplementation was given for three weeks. In another study, McVeigh et al showed improved endothelium-dependent responses, mirroring stimulated NO release, after six-weeks n-3 supplementation in subjects with type-2 diabetes[157]. In line with these studies, we demonstrated that n-3 supplementation improved endothelial function in obese adolescents. As both NO-dependent and -independent vasodilatation might be affected

by n-3 treatment[158] , we analyzed the entire vasodilatory response. In order to best illustrate all components of the improvement in endothelial function, we performed pair- wise comparison for the entire post- occlusion hyperaemic response curve.

Other measures of vascular function were also improved, such as the augmentation index (AI). AI assessed by PAT was decreased by 24 % with n-3 treatment compared to placebo (p=0.05), and was inversely correlated to the changes in 20:5n-3 (EPA) (r= -0.47, p=0.025) and sum of n-3 (r= -0.47, p=0.02) in serum phospholipids.

Obese children have elevated inflammation, which can affect the endothelium and vascular wall[65]. We found that the number of lymphocytes were decreased by 7 %, from 2.7 10^9/L (p=0.037), and the monocytes by 11 %, from 0.61 10^9/L (p=0.021) after n-3 treatment, but remained unaffected by placebo. There was a statistically significant correlation between the change in lymphocytes and the change in n-6/n-3 ratio of serum phospholipids(r=0.4, p=0.05). The change in lymphocytes statistically significantly correlated to the change in PWV measured at radial-dorsal pedal sites (r=0.59, p=0.003), as well as with the change in DPA IT (r=0.48, p=0.03), suggesting a relationship between reduced inflammation and arterial stiffness and also intimal thickness after n-3 treatment. The collective improvement of pulse wave velocity, augmentation index and endothelial function in these obese children may constitute a moderate anti-hypertensive effect by the n-3 treatment, effects that may not be detectable by sphygmanometry. The beneficial effects of n-3 PUFAs on hypertension has been recognized for decades[41], and it is possible that n-3 reduces the obesity-induced hypertensive effects on the vascularity already at this early age.

We also found an inverse correlation between the change in PWV, measured at carotid-radial sites, and the change in insulin sensitivity index (ISI) found in paper IV, as assessed by hyperinsulinemic-euglycemic clamp (r= - 0.46, p= 0.047). Furthermore, the change in PWV was correlated with the change in restoration of insulin concentration at IVGTT, measured as $AUC_{60\text{-}80}$ (r=0.52, p=0.019). Insulin resistance has been shown to be associated with vascular abnormalities in obese children, inasmuch that HOMA-IR was positively correlated with IMT and inflammatory markers[69]. Lee and co-workers have shown associations between decreased insulin sensitivity and circulating endothelial biomarkers in youth[55].

Oxidative stress is also considered of importance in the development of atherosclerosis[70], and interestingly, we found that the change in ascorbyl radicals correlated to the change in dorsal

pedal IT (r=0.61, p=0.003). Although there are some anti-oxidative effects of n-3 PUFAs[159], there are recent studies showing that there are anti-oxidative properties also in the aqueous part of the fish[160, 161], which might be of importance when analyzing epidemiological data of beneficial effects of fish consumption on obesity and cardiovascular risk[162].

In paper IV, we demonstrated that n-3 treatment improves glucose tolerance and insulin sensitivity in obese girls, but not boys.

In girls, the glucose tolerance, determined as Kg, was improved by 39 % after n-3 treatment ($p<0.05$). In line with this, ISI obtained from euglycemic-hyperinsulinemic clamp was increased by 20 % after n-3 treatment, but borderline significantly so ($p=0.07$). The restoration of insulin concentration ($\Delta AUC_{60\text{-}80min}$) was improved by 34 % after n-3 treatment ($p=0.02$). Thus, our data indicate that n-3 PUFAs, or the balance between n-6/n-3 fatty acids, have an important role in the insulin and glucose metabolism and that this association is more readily influenced in girls than boys at this young age. Peripheral insulin sensitivity is mostly regulated by the skeletal muscle tissue, but in obese patients with a large fat mass, the adipose tissue and liver are also of importance[163, 164]. After n-3 treatment the n-6/n-3 ratio in skeletal muscle phospholipids was decreased by 37 % ($p<0.0001$) and 38 % ($p=0.002$) in females and males, respectively, mostly due to an increase of the total percentage of n-3 PUFAs, and mainly EPA. Similar pattern was found in adipose tissue, although only EPA was statistically significantly altered by n-3 supplementation.

Obese children with impaired glucose tolerance demonstrate defects in non-oxidative glucose metabolism, and visceral fat deposits and intramyocellular lipid accumulation are related to insulin resistance[117]. As n-3 PUFAs prevents fatty acid accumulation in liver and skeletal muscle, and thereby improve defects in insulin signalling and prevent alterations in glucose homeostasis[165, 166], they might be important in the effort of preventing the development of type 2 diabetes in obese children. A number of different metabolic abnormalities may increase intramyocellular fatty acid metabolites, such as increased fat delivery to muscle as a consequence of either excess energy intake or defects in adipocyte fat metabolism[75]. Another possible mechanism behind intramyocellular lipid accumulation could be reduced mitochondrial oxidative phosphorylation, since mitochondria convert fatty acid and glucose into energy via oxidation. Indeed, muscular mitochondrial dysfunction does cause deposits of lipids inside the muscle, producing insulin resistance and leading to diabetes[76, 77, 167]. Since

mitochondriae are rich in EPA and DHA it would be of interest to investigate the different species of phospholipids in the skeletal muscle whether an improvement would be associated with enrichment of the n-3 fatty acids in phosphatidyl ethanolamine and phosphatidyl serine, especially concentrated in the inner membrane of the mitochondria.

Our data suggest that changes in the fatty acid composition of skeletal muscles modulate the action of insulin, which might be associated with increased oxidative capacity[168]. Insulin action and resistance are also associated to the regulation of exercise capacity, heart rate recovery and autonomic nervous system function[135, 143, 145, 169, 170], as well as in the development of atherosclerosis, measured as endothelial function, arterial stiffness and IMT[55, 125, 171]. This means that insulin resistance is involved in the development of the cardiovascular risk factors we examined in our studies, and may be modulated by the membrane fatty acid composition. The membrane fatty acid composition in itself might be important not only in muscle tissue, regulating insulin action and oxidation, but also in the nerve cell, enhancing signalling. N-3 PUFAs are important not only in regulating cardiac autonomic function[92], but also in cognitive function and development[93, 172, 173], suggesting effects on different parts of the nervous system. In our study of n-3 supplementation, we did not examine the subjects regarding autonomic function.
Fatty acid composition of the endothelium, where n-3 PUFAs regulate formation of leukotriens, prostaglandins, thromboxanes and adhesion molecules[98, 152, 174], is also important in the development of atherosclerosis, besides the inflammation and insulin action on this tissue, and we were able to show that supplementation of n-3 PUFAs improves endothelial function in obese adolescents.

The subjects received 1,2 g pure n-3 fatty acids per day, which is relatively low dose for healthy adults[175, 176]. It corresponds to a daily consumption of 70 g herring, which could be considered reasonable to incorporate into the diet. Considering the influence of the large amount of adipose tissue in obese children, this dose might be too low for optimal results. In obese adults, 4 g/day seems to be an adequate dosage[175-177], which is almost four times as high as the dose we used. Since there are no studies of n-3 supplementation in obese children, we can only speculate that a higher dose might have influenced the results.

Taken together, the insulin resistance is an important factor in the development of the metabolic syndrome and cardiovascular disease. In obese children, we have shown that it

might be possible to improve insulin resistance, at least to some extent, with supplementation of n-3 LC-PUFAs. The reduced insulin resistance and the direct vascular effect of n-3 LC-PUFAs in terms of improved vascular function, reduced inflammation and a possible improvement of autonomic function, makes this treatment of cardiovascular effects of childhood obesity appealing; of course as a complement to the optimal diet and exercise programmes.

What was known?

- Obese children show increased carotid IMT, impaired endothelial function and arterial stiffness.
- Obese children show a deranged FA pattern in plasma, with low n-3 FAs.
- Supplementation with n-3 FAs in adults is beneficial for cardiovascular risk factors.

What this study adds*?*

- Increased understanding of the cardiac vagal function in obese children. Obese children show a reduced cardiac vagal function compared to both overweight and lean children.
- Deeper knowledge of the vascular changes in obese children, and possible gender effects. Obese children show increased vascular diameter in peripheral arteries and decreased pulse wave velocity compared to lean subjects, and obese girls also show increased intimal wall thickness in radial artery compared to lean girls.
- This unique placebo-controlled study of n-3 supplementation shows, for the first time in obese children:

- large changes in muscle FAs consisting of increased concentrations of EPA, DHA, total n-3 and decreased n-6/n-3 ratio
- improved endothelial function as determined by increased vasodilatory capacity in response to hyperaemia
- improved insulin resistance in girls

Clinical relevance and perspectives

Obese children and adolescents are a growing population of patients in strong need for effective treatment to prevent future morbidity. Omega 3 PUFAs present with a wide range of positive effects on different aspects of the metabolic syndrome (Fig 8), and are probably more effective in preventing disease the earlier they are used. Considering the negligible side effects and positive effects we have shown in this study, n-3 PUFA supplementation should be used as a complement to the ordinary diet and exercise programmes in the treatment of children and adolescents with obesity.

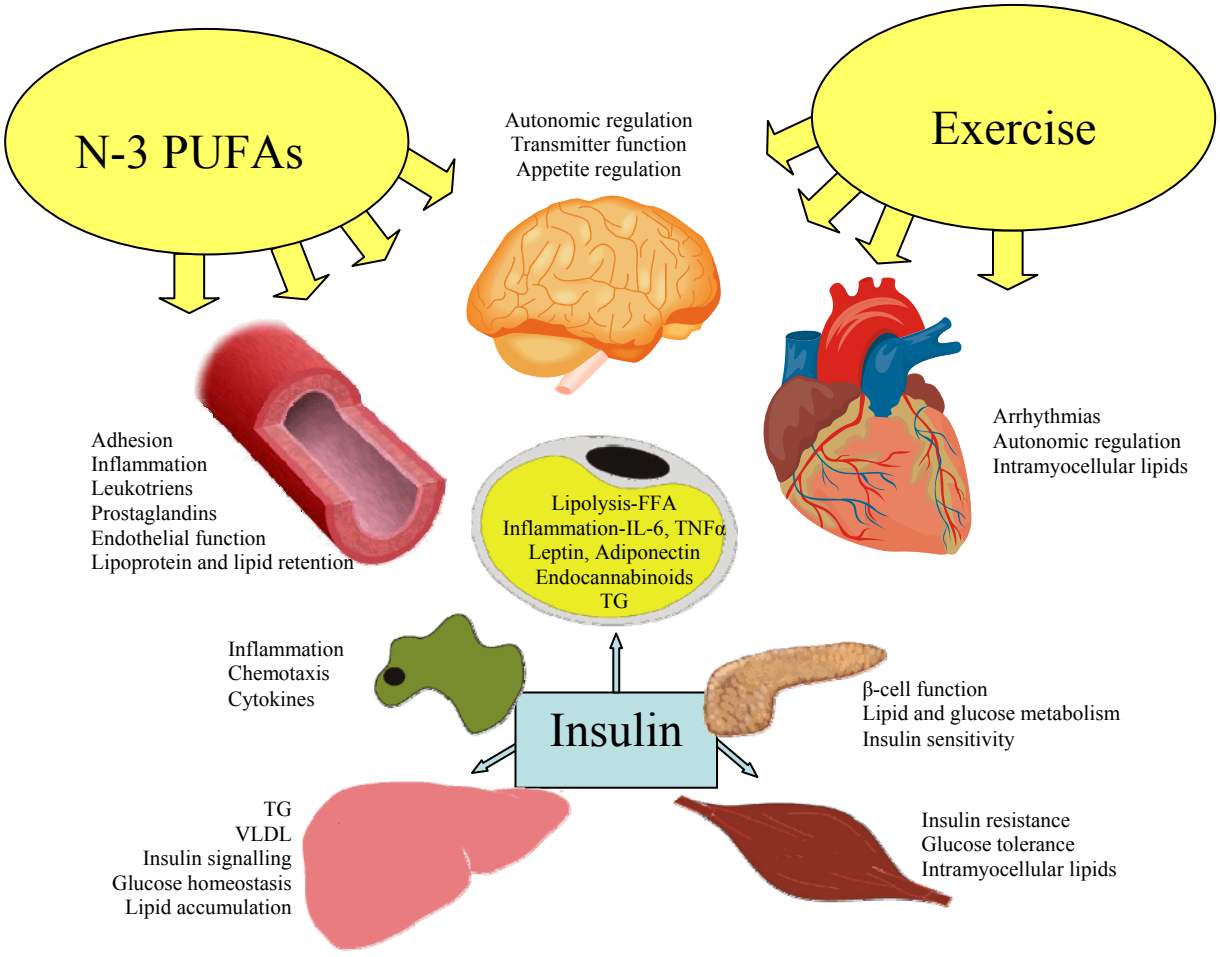


Figure 8 Integrative illustrations of the consequences of obesity in different organs and possible antagonists

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