Content

List of papers

The dissertation is based on the following original research papers, which are referred to in the text by their Roman numerals:

I. Anna Bugge, Bianca El-Naaman, Magnus Dencker , Karsten Froberg, Ingar Morten K. Holme, Robert G. McMurray and Lars Bo Andersen. Effects of a 3-year intervention: The Copenhagen School Child Intervention Study. MSSE

II. Anna Bugge, Bianca El-Naaman, Karsten Froberg, Robert G. McMurray and Lars Bo Andersen. Tracking of cardiovascular risk factors clustering in youth

III. Anna Bugge, Bianca El-Naaman, Robert G. McMurray, Karsten Froberg, Claus Henrik Nielsen, Klaus Müller and L. B. Andersen. Inflammatory markers and clustered cardiovascular disease risk factors in Danish Adolescents

IV. Anna Bugge, Bianca El-Naaman, Robert G. McMurray, Karsten Froberg, Claus Henrik Nielsen, Klaus Müller and L. B. Andersen. Sex differences in the association between level of childhood interleukin-6 and insulin resistance in adolescence

Dansk resume

Baggrund

Hjertekarsygdom (HKS) er den førende årsag til død i Danmark og i den vestlige verden. Selvom HKS ikke er manifest hos børn og unge, så har flere studier vist, at hos nogle individer starter den patologiske atherosclerotiske proces i barndommen og videreudvikles gennem livet. Som følge heraf er der behov for at skaffe viden om de mekanismer, der er underliggende for udviklingen af HKS, ligesom det er af stor betydning for samfundet at finde effektive forebyggelses strategier. Imidlertid er det vanskeligt at måle HKS risikoen hos tilsyneladende raske børn. Det er blevet foreslået at en beregning af graden af "cluster", det vil sige ophobning, af de individuelle HKS risikofaktorer, er en god metode til dette. Denne fremgangsmåde er dog kun af interesse, hvis denne *clustering* har en rimelig stabil karakteristik gennem barndommen. En forøgelse af det fysiske aktivitets niveau er blevet foreslået som en måde at reducere HKS risikoen. Skolen som arena er ideel til populations-baserede fysisk aktivitets interventioner, da skolen er det bedste sted at nå et stort antal børn med forskellig socioøkonomisk baggrund. En af de foreslåede mekanismer involveret i udviklingen af HKS er *low grade inflammation*, karakteriseret ved en forhøjet udskillelse af inflammatoriske markører, der inkludere både cytokiner og hormoner. Disse er desuden under påvirkning af både kropsfedt og fysisk aktivitet. Studier af børn og unge har, ligesom studier af voksne, fundet korrelationer mellem *clustered* HKS risikofaktorer og nogle, men ikke alle, undersøgte inflammatoriske markører. Ingen studier har inkluderet de anti-inflammatoriske naturlige blokkere af pro-inflammatoriske cytokiner, sTNFRI and IL-1Ra, i denne type af analyser i normale pædiatriske populationer. Også longitudinelle data på inflammatoriske markører og HKS risiko i pædiatriske populationer er sparsomme.

Fokus og mål

Hovedfokus for denne afhandling var derfor *clustering* af HKS risikofaktorer og målene var:

- At beskrive effekten af en skolebaseret fysisk aktivitets intervention på både individuelle og *clustered* HKS risikofaktorer.
- At evaluere graden af *tracking* af *clustered* HKS risikofaktorer fra barndom til tidlig ungdom, ved brug af tre måletidspunkter; 6 år, 9 år og 13 år.
- At undersøge sammenhængen mellem en række inflammatoriske markører og *clustering* af HKS risikofaktorer hos børn og unge, både tværsnitsligt og longitudinelt.

Metode

Denne afhandling er baseret på data fra Copenhagen School Child Intervention Study, et quasi-eksperimentelt longitudinelt studie. Studiet involverede 18 skoler (10 intervention og 8 kontrol) og inkluderede en opfølgning 4 år efter interventionen var afsluttet. 696 børn deltog ved baseline (gennemsnitsalder 6,7 år), 613 efter intervention, (gennemsnitsalder 9,5 år) og 513 ved opfølgningen (gennemsnitsalder 13,4 år). Interventionen bestod af en fordobling af mængden af idrætsundervisning (fra 90 til 180 min/uge), efteruddannelse af idrætslærere og en opgradering af idræts- og legefaciliteter. Antropometri og blodtryk blev målt. VO_{2peak} blev målt direkte, og fysisk aktivitet blev bestemt ved accelerometri. Faste blodprøver blev analyseret for HKS risikofaktorer og inflammatoriske markører.

Hovedresultater

Små, men signifikante effekter af interventionen blev fundet for drengene; de forøgede deres HOMA score mindre fra baseline til efter interventionen i forhold til kontrolgruppe drengene (P = 0.004) og deres systoliske blodtryk blev forøget mindre fra baseline til follow-up (P = 0.010). Der var ingen andre signifikante forskelle mellem grupperne.

Der blev fundet signifikant *tracking* af *clustering* af HKS risikofaktorer i mellem alle måletidspunkter (r = 0,514, 0,559 og 0,381 mellem 6 til 9, 9 til 13 og 6 til 13 år, respektivt, alle P< 0.0001). *Clustering* af HKS risikofaktorer var negativt associeret med adiponektin og positivt associeret med CRP, IL-6 og TNFα niveauer (P alle < 0.05). Ingen associationer blev fundet for sTNFR1 or IL-1Ra. IL-6 niveauet i 9-års alderen var korreleret til IL-6 i 13-års alderen (r = 0.360, P = 0.0001) og til HOMA-IR i 13-års alderen (r = 0.223, P = 0.008).

Konklusioner

Vi fandt at *clustering* af HKS risikofaktorer har en rimelig stabil karakteristik gennem barndommen, og forebyggende strategier bør derfor starte i tidlig skolealder. Dog fandt vi kun moderate interventionseffekter, hvilket indikerer at en fordobling af idrætstimerne, efteruddannelse af lærere of forbedring af udstyr ikke er tilstrækkeligt til at inducere markante forbedringer i HKS risikofaktorerne hos normale børn. Mere intensive interventioner er tilsyneladende påkrævet hvis forebyggelsesstrategier skal være effektive.

HKS risikoen var associeret med adiponectin, TNFα, CRP og IL-6, men ikke med de anti-inflammatoriske markører sTNFR1 or IL-1Ra hos 13-årige. IL-6 niveauet i 9-års alderen var associeret til både IL-6 og HOMA-IR i 13-års alderen hos piger. Dette betyder at *low grade inflammation* allerede

er til stede hos unge og kan være involveret i udviklingen af forringet metabolisme. Der er dog stadig behov for flere studier på dette område.

English summary

Background

Cardiovascular disease (CVD) is the leading cause of death in Denmark and in the western world. Even though CVD is not present in youth, several studies have shown that the pathological processes of atherosclerosis begin in childhood and progress throughout life. Consequently, there is a need to gain knowledge of underlying mechanisms for the development of CVD, as well as the finding of effective prevention strategies are of great public importance. However, measuring CVD risk in apparently healthy children is difficult. Assessing the level of clustering of individual CVD risk factors has been suggested as a good method. This approach is, however, only of interest if the clustering can be considered a fairly stable characteristic throughout childhood. Increasing physical activity has been suggested as a tool to reduce CVD risk. The school setting is an ideal environment for population-based physical activity interventions, as the school setting may be the easiest way to reach a large number of children with different socio-economic backgrounds. One of the proposed mechanisms involved in the development of CVD is low grade inflammation characterized by excess release of inflammatory markers, including both cytokines and hormones, which are influenced by both fatness and exercise. Studies in youth have, like in adults, found correlations between clustered CVD and some, but not all, inflammatory markers. No study has, however, included the anti-inflammatory natural blockers of pro-inflammatory cytokines, sTNFRI and IL-1Ra, in this type of analysis in normal pediatric populations. Also, longitudinal data on inflammatory markers and CVD risk in pediatric populations are sparse.

Focus and aim

The main focus of this thesis was therefore the clustering of CVD risk factors and the aims were;

- To describe the effect of a school-based physical activity intervention on individual and clustered CVD risk factors.
- To evaluate tracking of clustered CVD risk in youth using three time-points; age 6, age 9 and age 13 years.

- To examine the relationship between a wide range of markers of inflammation and clustering of CVD risk factors in youth both cross-sectionally and longitudinally.

Methods

This thesis is based on data from the Copenhagen School Child Intervention Study, a quasi-experimental longitudinal study. The study involved 18 schools (10 intervention and 8 controls) and included a follow-up 4 years after the end of intervention. 696 children participated at baseline (mean age 6.7 years), 613 at post-intervention (mean age 9.5 year) and 513 at follow-up (mean age 13.4 year). The intervention consisted of a doubling of the amount of physical education (from 90 to 180 min/week), training of PE teachers, and upgrading of PE and playing facilities. Anthropometrics and systolic blood pressure were measured. VO_{2peak} was directly measured, and PA was assessed using accelerometry. Fasting blood samples were analyzed for CVD risk factors and inflammatory markers.

Main results

Small, but significant effects of the intervention was found for boys; their HOMA score increased less from baseline to post-intervention compared with control boys ($P = 0.004$) and their systolic blood pressure increased less from baseline to follow-up ($P = 0.010$). There were no other significant differences between groups.

Significant tracking coefficients were found between clustered z-score at all time intervals ($r = 0.514$, 0.559 and 0.381 between ages 6 to 9, 9 to 13 and 6 to 13 years, respectively, all $P< 0.0001$).

The clustered z-score was negatively associated with adiponectin and positively associated with CRP, IL-6 and TNFα levels (P-values all < 0.05). No associations were found for sTNFR1 or IL-1Ra. For girls but not boys, levels of IL-6 at age 9 years correlated with IL-6 at age 13 years ($r = 0.360$, $P = 0.0001$) and with HOMA-IR at age 13 years ($r = 0.223$, $P = 0.008$).

Conclusions

The clustered z-score was found to be a fairly stable characteristic through childhood and preventive strategies should therefore start at early school-age. However, our results on the effect of the intervention on CVD risk factors are only modest and this might indicate that a doubling of PE and providing training and equipment may not be sufficient to induce major improvements in CVD risk factors in a normal population. More intensive interventions are warranted in order to make effective preventive strategies.

In adolescents, CVD risk was associated with the level of adiponectin, TNFα, CRP and IL-6, but not with the anti-inflammatory markers sTNFR1 or IL-1Ra. The level of IL-6 at age 9 years was associated with the level of IL-6 at age 13 years and HOMA-IR at age 13 years. This means that low grade inflammation is already present at young ages and could be involved in the development of impaired metabolism. However, there is still a need for further studies in this area.

Abbreviations

BMI:	Body mass index
CHD:	Coronary heart disease
CG:	Control group
CI:	Confidence interval
CoSCIS:	Copenhagen School Child Intervention Study
CRP:	C-reactive protein
CVD:	Cardiovascular disease
DNA:	Deoxyribonucleic acid
EYHS:	European Youth Heart Study
HDLc:	High density lipoprotein cholesterol
HOMA-IR:	Homeostasis model assessment for insulin resistance
HR:	Heart rate
IDF:	International Diabetes Federation
IG:	Intervention group
IL-1:	Interleukin 1
IL-1Ra:	Interleukin-1 receptor antagonist
IL-6:	Interleukin-6
IL-8:	Interleukin-8
IL-10:	Interleukin-10
LC-CoA:	Long-chain fatty acyl-CoA
LDLc:	Low density lipoprotein cholesterol
LPL:	Lipo-protein lipase
MetS:	Metabolic syndrome
MVPA:	Moderate-to-vigorous physical activity
NCEP:	ATP III: National Cholesterol Education Program's Adult Treatment Panel III
NHANES:	National health and Examination Survey
PA:	Physical activity
PE:	Physical education
RCT:	Randomized controlled trials
RER:	Respiratory exchange ratio
ROS:	Reactive oxygen species
S4SF:	Sum of four skinfolds
sTNFR:	Soluble tumor necrosis factor-alpha receptors
TC:	Total cholesterol
TG:	Triglycerides
TNFα:	Tumor necrosis factor-alpha
TNFR1:	Tumor necrosis factor receptor 1
VLDLc :	Very low density lipoprotein cholesterol
VO_2:	Oxygen uptake
VO_{2peak}:	Peak oxygen uptake
WHO:	World Health Organization
zBMI:	Body mass index z-scores

1. Introduction

The main focus of this thesis is the development of cardiovascular disease (CVD) risk factors in children and adolescents. This focal point is being explored from different angles; how stable the CVD risk factors are over the course of childhood (tracking), how they relate to inflammatory markers and how a school-based PA intervention can affect the CVD risk factors.

CVD is one of the main causes of global mortality and disease related morbidity (1). Therefore, gaining a better understanding of the underlying mechanisms for the development of CVD as well as findings of effective prevention strategies are of great public importance. CVD comprises a variety of diseases that involve the heart or blood vessels, but is in this thesis referred to as a category of diseases related to atherosclerosis. This introduction will briefly describe the atherosclerotic process and the traditional physiologic risk factors for CVD constituting the metabolic syndrome (MetS). A relatively new area of interest in CVD research is the relation to low-grade inflammation. Selected markers of low grade inflammation and their relation to CVD are described.

As CVD is not present in youth, methods to assess CVD risk in this population are presented, as well as the relation of CVD risk to physical activity, physical fitness and low-grade inflammation in children and adolescents. Also, the current knowledge regarding tracking of CVD risk from childhood and adolescence to adulthood will be shortly described. Finally, this section contains a brief introduction to intervention studies aiming at reducing CVD risk factors in children and adolescence.

The Copenhagen School Child Intervention Study (CoSCIS) is a school-based study with the aim of measuring the effect of extra physical education (PE) lessons, teacher education and upgrading of physical education and outdoor facilities on a wide range of health parameters in children and adolescents (see section *2.1. Study design*). Data for this thesis are from baseline, post-intervention and follow-up measurements.

1.1 The atherosclerotic process

Atherosclerosis is degenerative changes in the arterial wall which decreases the elasticity and narrows the lumen of the vessel. Eventually it may result in heart attack, stroke or gangrene in the extremities,

depending on the site of the atherosclerosis. The atherosclerotic process is slow; it develops over a period of several years (decades) through a complex series of cellular events occurring within the arterial wall (2-4). The atherosclerotic process can for simplicity be divided into four stages; fatty streak development, early fibroatheroma, vulnerable plaque formation and complex lesion development (5).

The earliest recognizable lesion is the "fatty streaks", which is the first visible lesion in the development of atherosclerosis. For some individuals fatty streaks start to develop in childhood or adolescence (6). One theory suggests that low density lipoprotein cholesterol (LDLc) particles enter the inner lining of the arteries, the arterial intima, which triggers a series of events. The LDLc particles are oxidized into pro-inflammatory particles activating an innate immune-response. At this time smooth muscle cells start to accumulate fat and secrete chemokines, endothelial cells become activated and secrete adhesion molecules, and all this draw monocytes, mast cells, neutrophils and T lymphocytes into the intima of the arterial wall (5). The smooth muscle cells furthermore secrete connective tissue to the extracellular matrix.

Early fibroatheroma is the second step, where the monocytes migrate into the sub-endothelial space, differentiate into macrophages, take up lipids and become foam cells (2). When foam cells, other inflammatory cells and the arteries own cells start to accumulate, inflammation increases and macrophage and smooth muscle cell death occurs, which further provoke inflammation. Smooth muscle cells start to migrate into the damaged area, and all this, in turn, develops into fibrous plaques. These plaques usually contain a lipid core consisting of debris, cholesterol and foam cells, which are covered by connective tissue with embedded smooth muscle cells. These lesions gradually increase in size resulting in a narrowing of the arterial lumen, which may impede the blood flow (5).

Vulnerable plaque formation is the third step where the fibrous cap in some sites becomes thin and susceptible to rupture.

Finally, if the plaques continue to aggregate, rupture might occur and cause thrombosis, eventually resulting in a complete occlusion (2-4). The increased mass of some plaques may, without rupture, become sufficiently large to form significant occlusion of the vessel, which may cause lethal ischemia simply through flow restriction (5).

1.2 Traditional CVD risk factors

The concept of risk factors for CVD was introduced by researchers from the Framingham Heart Study (7). The traditional risk factors for CVD can be categorized in 4 categories; 1) inherited factors including sex, age and family history of CVD (genes), 2) behavioral or lifestyle related factors including diet, physical (in)activity, cardiorespiratory fitness and tobacco exposure, 3) physiological factors including elevated blood pressure, central overweight/obesity, dyslipidemia and impaired glucose metabolism and 4) medical diagnoses such as diabetes mellitus type 1 and 2, kidney disease and more (7;8). In this introduction focus will be on the physiological risk factors and how they interact, but a section on physical (in)activity and cardiorespiratory fitness is also included.

1.2.1 Physiological risk factors

Dyslipidaemia

Dyslipedaemia indicates an unfavorable lipid and lipoprotein profile usually characterized by high plasma concentrations of LDLc and very low density lipoprotein cholesterol (VLDLc), with depressed concentrations of high density lipoprotein cholesterol (HDLc) (9). Also the size of the cholesterol particles is an important factor in dyslipidemia; with smaller LDLc and HDLc particles being more atherogenic independent on the concentration of the lipoproteins (10-12). As described above, the level of LDLc, particular small LDLc particles, in the blood plays a crucial role for both the initial lesion development and the subsequent building of plaques and many of the other CVD risk factors actually impact the development of CVD by their influence on the lipid metabolism (5). Studies have found a strong graded association between CVD and total cholesterol (TC) (13), LDLc (14) and a negative association to HDLc (15;16). Moreover, the level of free fatty acids in the blood has also been shown to contribute to insulin resistance by inhibiting insulin signaling (17;18). Also the intramyocellular accumulation of triglycerides (TG) and long-chain fatty acyl-CoA (LC-CoA) esters has, in different lines of research, been shown to impair insulin signaling (18).

Insulin resistance

Insulin sensitivity can be defined as the ability of insulin to stimulate its target tissues (most important muscle, fat and liver). Insulin resistance on the other hand, can be defined as the situation where a normal concentration of insulin produces a less than normal response (19). Insulin resistance is highly

related to the development of both CVD and type 2 diabetes (20;21). Insulin resistance affects the other CVD risk factors in several ways; blood pressure is affected directly because of an impairment of the insulin-mediated vasodilation and indirectly as hyperinsulinemia increases the sympathetic nervous system activity. In the insulin resistant and hyperinsulinemic state the lipid profile is impacted by increased synthesis of VLDL in the liver, decreased concentrations of lipoprotein lipase and promotion of lipid synthesis within the arterial wall (22). Furthermore, hyperinsulinemia is directly causing obesity as insulin inhibits adipocyte lipase and up-regulates adipocyte lipo-protein lipase (LPL), thereby increasing transportation and storage of fat in adipocytes (23). Finally, different studies indicate that hyperinsulinemia may contribute to the sympathetic overdrive in obesity (24).

Hypertension

Hypertension is strongly related to CVD in general and in particular to stroke and heart failure (25;26). Because of increased workload on the heart in hypertension left ventricular hypertrophy is often seen, which is associated with increased incidence of heart failure, ventricular arrhythmias and myocardial infarction. Hypertension is also part of the atherosclerotic process as it is related to the reduced elasticity of the vessels, a factor contributing to plaque formations, which, as described above, again results in reduced lumen size, impaired blood flow and increased total peripheral resistance (4).

Central obesity

Obesity is a powerful predictor of CVD (27;28) and significantly increases the risk of hypertension, hyperglycemia and dyslipidemia. The distribution of body fat is highly important as abdominal obesity is a better predictor of CVD, than overall adiposity (29). Adipose tissue was earlier regarded a passive energy reserve, but is now recognized as very metabolically active, especially the visceral adipose tissue. Excess adiposity is associated with increased free fatty acid flux to the liver which may impair liver metabolism, leading to increased gluconeogensis and VLDL production (29). Additionally, the excess visceral adipose tissue acts as a secretory gland releasing hormones and adipokines creating a state of low grade inflammation, which will be described further in section *1.2.3. Inflammatory markers*. All of this influence the regulation of biological functions such as appetite and energy balance, insulin sensitivity, lipid metabolism and blood pressure (8;28;30;31). Finally, central obesity has been associated with chronic sympathetic over-activity with an elevated sympathetic outflow to

organs such as the heart, kidneys, and blood vessels. Chronic sympathetic nervous system over-activity can, among other things, contribute to the development of insulin resistance and dyslipidemia by stimulating lipolysis to increase non-esterified free fatty acids (NEFA) and thereby contribute to the creation of a vicious cycle (24).

Physical (in)activity and (low) cardiorespiratory fitness as risk factors for CVD

Even though physical activity (PA) and cardiorespiratory fitness level are not included in the traditional physiologic risk factor group, both factors are highly important physiologically and interact with the other physiologic CVD risk factors in several ways.

PA has both immediate and long term effects on health outcomes. In adults, low level of PA is known to contribute to the early onset and development of MetS and CVD (32-37). The mechanisms whereby PA influences the CVD risk factor level are multiple and include the reduction of dyslipidemia, insulin resistance, hypertension and low grade inflammation, enhancement of glycemic control and endothelial function and regulation of body weight (36).

Regular aerobic exercise training is positively associated with muscle fiber mitochondrial volume and aerobic enzyme activity (38), as well as enhanced capilarization of muscle fibers (39). This leads to an improved metabolic capacity in muscles resulting in broad beneficial effects, including improvements in nutrient metabolism. PA enhances glucose uptake in myocytes by two different mechanisms; an insulin-dependent mechanism and a contraction induced mechanism (40-42). The underlying mechanisms are not fully elucidated yet, but insulin sensitivity might be enhanced by exercise training because of the positive effect on some of the inhibitors of insulin signaling (inflammatory cytokines, intramuscular lipids etc.). The contraction-induced glucose transport is mediated by adenosin mono phosphat-activated protein kinase regulated by the cells energy status (43) and has been found to be independent of insulin sensitivity in the muscles (42).

Despite the well-established benefits of PA in CVD protection and the well-established relationship between lipid profile and risk of CVD, evidence regarding the effect of regular exercise on the atherogenic lipid profile is sparse (44). In fact, most studies have found no effect of regular exercise independent of weight loss on TC or LDLc (for review see (44)). However, in a longitudinal study by Kraus and colleagues (45) exercise training for 8 months increased LDLc particle size, independent of training intensity and weight loss, and without a concomitant reduction in LDLc concentration.

Furthermore, increased capilarization enhances LPL concentration, thereby improving fat metabolism and it has been proposed that exercise training enhances fat oxidation in the cell and reduces intra-myocellular lipid accumulation, which might decrease insulin resistance (18). Physical activity can also affect blood pressure, probably by increasing adrenalin sensitivity. Finally, increased level of PA is also associated with a decreased level of markers of low-grade inflammation (46).
Besides protecting against disease, regular PA is also important with regard to achieving a sufficient level of health-related cardiorespiratory fitness. In adults, low level of cardiorespiratory fitness has been found to contribute to the early onset and progression of CVD, the overall risk of premature death (47;48) and has been related to the metabolic syndrome (32;49). In general, there seems to be a dose-response relationship between cardiorespiratory fitness, total mortality and the level of CVD risk factors.

1.2.2 Underlying mechanisms behind the development of adverse CVD risk factor level

As described above the CVD risk factors are interconnected and affect each other in several ways. The underlying mechanisms linking the CVD risk factors to each other and to CVD have been the subject for intense investigation in recent decades (8). In the scientific community there are ongoing discussions of which factors are most important in initiating the simultaneous rise in many CVD risk factors, and central obesity, insulin resistance and physical inactivity have been proposed. Other proposed mechanisms not considered classic physiological CVD risk factors include; adipocyte dysfunction, mitochondrial dysfunction and oxidative stress and low-grade inflammation.
1) Adipocyte dysfunction is triggered by excessive levels of circulating glucose and triglycerides, which causes adipocyte hypertrophy and hyperplasia. Adipocyte dysfunction is characterized by infiltration of inflammatory cells in adipocytes, elevation of pro-inflammatory cytokines both locally and systematic, and endoplasmatic reticulum stress resulting in a state of insulin resistance and impaired lipid metabolism (50-53). However, even though adipose dysfunction is important in the understanding of the development of CVD, it is very difficult to measure in humans (8) which limits the relevance in population-based studies.
2) Mitochondrial dysfunction in adipocytes and oxidative stress are conditions also caused by the excess circulating nutrients and resulting in a high production of reactive oxygen species (ROS). When this production can no longer be controlled by compensatory antioxidants, it can have detrimental

effects throughout the body. Excess ROS production can lead to mitochondrial deoxyribonucleic acid (DNA) damage, not only in adipocytes, but also in myocytes and hepatocytes. Malfunctioning mitochondria may cause lipid accumulation in these tissues, which again causes insulin resistance contributing to the vicious cycle of high circulating levels of nutrients, ultimately resulting in the formation of atherosclerotic plaques (53-55). No sensitive measureable marker(s) of mitochondrial dysfunction and oxidative stress has been found (8), so the relevance in epidemiologic research is limited.

3) Low grade inflammation is characterized by an excess release of inflammatory markers, including cytokines and hormones, from the visceral adipose tissue and other tissues, at least partly as a result of both adipocyte dysfunction and oxidative stress (8;28;30;31). Different lines of research have led to an understanding of the importance of low grade inflammation in all stages of the atherosclerotic process, including plaque development, disruption, and thrombosis (2;8;56). In the following section selected markers of low grade inflammation are described and related to CVD and CVD risk factors.

1.2.3 Inflammatory markers

Adiponectin

Adiponectin is a protein exclusively secreted from adipocytes and is considered anti-inflammatory (57). Circulating adiponectin level is negatively associated with the level of body fatness (58), especially visceral fat (59). In population studies adiponectin has been found negatively related to the development of type 2 diabetes (60;61), MetS (62) and CVD (63;64). The protective mechanisms of adiponectin have not been completely revealed yet. It is known, however, that the actions are multiple; in vitro experiments have shown that adiponectin reduces the expression of adhesion molecules in endothelial cells (65) and suppresses proliferation and migration of smooth muscle cells into the intima media (66). Furthermore, animal models have shown that adiponectin ameliorates insulin resistance and increases fatty acid oxidation (67;68).

C-reactive protein (CRP)

CRP is produced in the liver and is considered a strong inflammatory marker. It is secreted from hepatocytes in response to interleukin-6 (IL-6) (69). CRP is correlated to obesity in a dose dependent fashion, with no preferential expression from subcutaneous or visceral fat (24). In adults the level of

CRP has been found related to the number of MetS components present (70) and also to predict development of CVD (69-72) and type 2 diabetes (73). *In vitro* and *in vivo* studies have found that CRP induces endothelial cell activation and dysfunction (69;74;75), impairs endothelial vasoreactivity (75), may induce oxidative stress and the secretion of other cytokines (69;76) and is directly involved in hepatic insulin resistance (77). However, as CRP is significantly associated with various established CVD risk factors including obesity, hypertension and dyslipidemia, it is difficult to determine whether CRP is a cause of CVD risk or a bystander to the other risk factors (78). Furthermore, recent studies doing Mendelian randomization analyses of important genes coding for CRP, found no indications of CRP concentration *per se* as being a causal factor in CVD (79-81). Therefore, the precise role of CRP in the development of CVD risk factors in humans remains unknown.

Tumor necrosis factor-alpha (TNFα) and soluble TNFα receptors (sTNFR)

TNFα is a cytokine produced primarily by macrophages, but also by a broad variety of other cell types including endothelial cells, cardiac myocytes, adipose tissue, lymphoid cells, mast cells, fibroblasts, and neuronal tissue. It is involved in systemic inflammation and is a member of a group of cytokines that stimulate the acute phase reaction in inflammation. Chronic elevation has been found in obesity and type 2 diabetes (82). The specific role of TNFα in the development of CVD is still not completely discovered, but high systemic levels of TNFα are consistently found related to CVD risk. In rodent models, biochemically or genetically blocking of TNFα in obesity results in improved insulin sensitivity (82). Studies in humans have come to conflicting results; in one study TNFα neutralization did not improve whole body insulin sensitivity (83), whereas another study found that TNFα infusion in healthy humans induced insulin resistance in skeletal muscles (84). TNFα has also been found involved in impaired lipid metabolism (85). Furthermore, it has been found that TNFα induces the expression of intercellular adhesion molecule (ICAM)-1, an inflammatory molecule involved in the early process of atherosclerosis (8).

TNF-receptor 1 (TNFR1) initiates the majority of TNF's biological responses. The binding of TNFα to TNFR1 triggers a series of intracellular events including inflammatory responses (86). sTNFR is secreted in response to TNFα as well as to interleukin 1 (IL-1)β, IL-6, and interleukin-8 (IL-8) and sTNFRI is therefore considered a marker of cytokine-induced inflammation. sTNFRI binds TNFα, thereby preventing TNFα binding to cellular TNFR's, thus reducing inflammation. TNFα is difficult to

measure partly because of the very low levels in normal individuals, in particular in children, and partly because of its unstable nature. Furthermore, TNFα exerts its greatest impact locally and is metabolized quite rapidly. In contrast, sTNFR1 is considered a more robust marker of cytokine induced inflammation as sTNFRI has a much longer half-life and is present at higher concentrations in the blood compared to TNFα (87).

IL-6

IL-6 is secreted by T cells and macrophages in order to stimulate an immune response, and by adipocytes relative to the level of obesity. However, IL-6 is also secreted by myocytes in response to exercise (88). The role of IL-6 in insulin resistance and development of CVD is highly controversial. On one hand IL-6 has consistently been observed in patients with type 2 diabetes mellitus, and as a consequence it has been related to insulin resistance and/or impaired glucose disposal (89). On the other hand administration of IL-6 to healthy humans did not impair muscle glucose uptake or whole-body glucose disposal (90). Some in-vitro studies have demonstrated that IL-6 causes insulin resistance in mice and human adipocytes and hepatocytes (91-93), whereas this was not found in skeletal muscle cells (94). One study found that IL-6 infusion in healthy humans increased insulin-stimulated glucose uptake in skeletal muscles (95). Furthermore, it has been shown that IL-6 knockout mice develop mature-onset obesity and impaired glucose tolerance, which is reversed by the administration of exogenous IL-6 (96). These last results could imply a positive effect of IL-6 on glucose metabolism at least in skeletal muscles, mediated by increased insulin sensitivity and glucose uptake.
Reports have suggested both pro- and anti-inflammatory effects of IL-6 (88;97). Due to its direct stimulatory effect on the production of acute-phase reactants, including CRP, and its correlation with disease activity in some autoimmune conditions, it is generally considered pro-inflammatory. The anti-inflammatory effects of IL-6 include inhibition of TNFα and stimulation of the production of the anti-inflammatory interleukin-1 receptor antagonist (IL-1Ra) and interleukin-10 (IL-10) (88). In observational studies IL-6 and its down-stream inflammatory biomarkers (e.g. CRP and fibrinogen) has been related to all stages of the pathogenesis of CVD (98), but causality in the development of CVD has not been established. However, recent large-scale studies on human genetic and biomarker data have found evidence for a causal relationship between IL-6 and the occurrence of CVD (99;100).

IL-1Ra

IL-1Ra is an agent that binds non-productively to the cell surface interleukin-1 receptors (IL-1R). IL-1R is the receptor that binds IL-1 α and β, both cytokines considered strongly pro-inflammatory. Hence IL-1Ra prevents IL-1 from signaling to its target cells (101). The ratio between IL-1Ra/IL-1 in the plasma is close to 1 in a healthy population, whereas higher levels of IL-1 are seen in diseases such as type 2 diabetes, atherosclerosis and some cancers (102). Paradoxically, IL-1Ra is, at least in rodent models, found to cause obesity and probably to be involved in insulin resistance (101). However, the mechanisms behind this have not been revealed yet.

From the above it can be concluded that much is known about low-grade inflammation, but also that much has still to be learned. Both mechanistic studies and observational/epidemiologic studies are needed.

1.3 Clustering of CVD risk factors and the metabolic syndrome (MetS)

As described above the physiological CVD risk factors interact and affect each other (as well as the underlying mechanisms). Therefore, a clustering of CVD risk factors is seen in some individuals and this cluster constitute a state of increased risk for several metabolic diseases including type 2 diabetes and CVD. The MetS is one definition of risk factor clustering. However, no consensus exists regarding the best way to define this syndrome. The three most commonly used definitions are from the World Health Organization (WHO) (103), the National Cholesterol Education Program's Adult Treatment Panel III (NCEP: ATP III) (104) and the International Diabetes Federation (IDF) (105). All groups agree on the components of the syndrome, however, they provide different clinical criteria (see table 1.1).

Regardless of applied definition studies have shown that the prevalence of MetS worldwide is increasing. E.g. recent estimates indicate that 34% and 35% of North American men and woman, respectively, have MetS as defined by NCEP: ATP III (106).

A study with data from 1982-84 of 2493 Danish adults using the same definition found a prevalence of 18.6% and 14.3% for men and woman, respectively (20). These numbers would probably be higher today.

Table 1.1. Criteria for the diagnosis of metabolic syndrome according to three commonly used definitions.

IDF	NCEP ATP III	WHO
Diagnosed if glycemia is abnormal and 2 further criteria are present	Diagnosed if 3 out of 5 criteria are present	Diagnosed if glycemia is abnormal and 2 further criteria are present
Fasting glycemia 100-125 mg/dL or type 2 diabetes	Fasting glycemia 100-125 mg/dL	Glucose intolerance, type 2 diabetes or insulin resistance measured by HOMA-IR
WC ≥ 94 cm (males) ≥ 80 cm (females)	WC ≥ 102 cm (males) ≥ 88 cm (females)	BMI > 30 and waist-to-hip > 0.9 (males) > 0.85 (females)
TG ≥ 150 mg/dL or HDL < 40 (males) < 50 (females)	TG ≥ 150 mg/dL or HDL < 40 (males) < 50 (females)	TG ≥ 150 mg/dL or HDL < 35 (males) < 39 (females)
BP ≥ 130/85 mm HG or on hypertensive treatment	BP ≥ 130/85 mm HG	BP ≥ 160/90 mm HG or on hypertensive treatment
		Microalbuminuria ≥ 20 mcg/min

BMI: body mass index, BP: blood pressure, HOMA-IR: homeostasis model assessment, IDF: international Diabetes Foundation, NCEP ATP III: National Cholesterol Education Program's Adult Treatment Panel III, TG: triglycerides, WC: waist circumference, WHO: World Health Organization.

1.4 CVD risk in youth

"High risk" for CVD is difficult to define in children, as no hard endpoints such as manifest disease or death have yet occurred. Even though clinical manifestations of CVD do not normally appear before middle-age, studies have shown that atherosclerosis originates during childhood and adolescence (107;108). Results from the Pathological Determinants of Atherosclerosis in Youth Study showed that the degree of atherosclerosis found in youth is related to the level of physiologic CVD risk factors measured post-mortem (108;109).

Several large-scale cohort studies have investigated the level of individual risk factors in children and adolescents (110-115). Results from these studies revealed large differences in the level of risk factors among 'normal' children. However, as the level of some individual risk factors tend to fluctuate on a day-by-day basis, a high level does not necessarily indicate an increased risk for future disease (116;117). However, when several risk factors are high in the same individual, it might indicate that some underlying mechanism(s) has been turned on, and that the child is at increased risk for future CVD. Different methods to examine this have been proposed and will be presented in the following.

1.4.1 Metabolic syndrome in youth

As described above, the MetS is one way to classify clustering of risk factors. Several studies have attempted to make a MetS definition suitable for children and adolescents (118-122), but no commonly accepted definition exists regarding cut-offs of risk factors or the number of risk factors that should be present for diagnosis (123). Most studies on prevalence of MetS in pediatric populations use age- and gender-specific percentiles either from national reference data or study-specific. Jolliffe and Janssen used growth curve modeling to develop age- and sex-specific criteria for MetS in youth linked to adult cut-off points from the NCEP ATP III and the IDF definitions (121). About the same time IDF published a consensus statement, where they recommend using the same cut-off points as for adults (105), except for waist circumference, where age-, race- and sex-specific percentiles should be used instead of absolute values. Further, they recommended only assessing waist circumference until the age of 10 years (120).

By applying the age- and sex-specific criteria for MetS in youth, Joliffe and Janssen found a MetS prevalence in adolescents (age 12-19 years) from the National health and Examination Survey (NHANSES) (1999 to 2002) of 7.6% and 9.6% according to cut-off points linked to the NCEP ATP III or IDF definitions, respectively. A much lower prevalence was found in children from European Youth Heart Study (EYHS) using the cut-off points by IDF (124). In this cohort the researchers found a prevalence of 0.2% and 1.4% in 9- and 15-y-olds, respectively.

1.4.2 Clustering of CVD risk factors in youth

The dichotomization of the risk factors in any definition of MetS is, however, a reduction of the available data and does not provide the full spectrum of knowledge available. Clustering of individual risk factors in the same individual has been suggested as a good method to assess CVD risk level in

apparently healthy children (117). This approach sum standardized values (z-scores) of selected CVD risk factors (e.g. blood pressure, insulin or homeostasis model assessment for insulin resistance (HOMA-IR), TC, TG, a measure of fatness and the negative values of fitness and HDLc). However, no consensus exists in regard to which risk factors to include in the cluster score, and no reference data have been developed. That means that studies are not easy to compare and that the level of risk is based on the study population.

By using a clustered score consisting of HOMA, TC:HDLc ratio, TG, systolic blood pressure and sum of four skinfolds a previous study from our group found no clustering of CVD risk factors in 6 year old children, whereas clustering was found at age 9 years. Being within the upper quartile of three or more risk factors was found in 3.33 (95% confidence intervals (CI): 1.41 – 7.87) times as many participants as expected if risk factors had been independently distributed (125).

Results from EYHS have shown that CVD risk factors tend to cluster in overweight and inactive children and in children with low peak oxygen uptake (VO_{2peak}) (117). This is alarming, as the prevalence of obesity and overweight is increasing in child populations throughout the world (126). At the same time it has been shown that PA and VO_{2peak} have decreased particularly in the low fit pediatric population (127;128).

1.4.3 Physical activity, cardiorespiratory fitness and CVD risk factors in youth

As in adults, studies in children have shown a range of beneficial effects of PA on health. Results from observational studies suggest a weak beneficial effect of PA on HDLc and TG, blood pressure and insulin resistance levels, but no consistent effect on TC or LDLc levels (129;130). Several explanations exist for the inconsistent and weak relationships. First thing is the large variation in the individual risk factors caused by fluctuation, measurement error etc. Maybe even more important is that the majority of studies assess PA by self-report, which inaccurately measures the PA of children and adolescents (131). When objective measures of PA are used more consistent findings are seen. For example, an inverse correlation between PA measured by accelerometry and insulin resistance was found in some (116;132;133), but not in all studies (134). Using data from EYHS, Andersen and colleagues found significant inverse correlations between PA and almost all single risk factors, including waist circumference, sum of 4 skinfolds, blood pressure, HOMA-IR, TC and TG level, but no correlations to BMI and HDLc (116). The same study also investigated the relationship between PA and clustered

CVD risk factors and found a significant and graded inverse relationship over quintiles of PA. This inverse relationship between objectively measured PA and clustered CVD risk factors has also been found in other studies (125;135-138).

Associations between cardiorespiratory fitness and single CVD risk factors are generally stronger, compared to the association to PA, mainly because the error variation in cardiorespiratory fitness is much smaller. Previous population studies in youth have shown weak independent relationship between cardiorespiratory fitness and single CVD risk factors (139-142). Several studies have investigated the relationship between clustered CVD risk factors and fitness (114;117;136;137;142-146). Some studies have found that the relationship between clustered CVD risk factors and fitness disappear after adjusting for body fatness (114;137;143), while others have found that fitness are independently associated with clustered CVD risk factors in children (117;136). This type of adjustments will be discussed in section *4. General discussion*.

1.4.4 Low grade inflammation in youth

As in adults, studies in youth populations have linked markers of low-grade inflammation, like CRP, TNFα, adiponectin and IL-6, to all of the individual CVD risk factors (147-152); as well as to the MetS (118;153;154). Four studies in youth have examined the relation between low-grade inflammation and a clustering of CVD risk factors (148;151;155;156). These studies found a relation between the clustering of CVD risk factors and CRP (148;151;156) and adiponectin (155), but not IL-6 and TNFα (148;156). No study has, however, included the anti-inflammatory natural blockers of pro-inflammatory cytokines, sTNFRI and IL-1Ra, in this type of analysis in normal pediatric populations. Also, longitudinal data on inflammatory markers and CVD risk in pediatric populations are sparse. Studies in youth have given convincing evidence of a relationship between the level of inflammatory markers and lifestyle related factors such as level of body fatness (148-151) and cardiorespiratory fitness (157;158).

Pediatric populations are interesting in this type of research because, as described earlier, for some individuals this is the time when clustering of CVD risk factors are initiated. Studying children and adolescents therefore might add significantly to the picture of how low-grade inflammation affects the development of an adverse risk profile and how lifestyle affects low-grade inflammation.

1.5 Tracking of clustered CVD risk factors

The clustering of CVD risk factors seen in some children is, however, only of interest if the clustering could be considered a stable characteristic. One study has linked MetS to CVD morbidity in adulthood (159) and most studies, despite differences in methodology, find significant tracking of clustered CVD risk factors from childhood to adulthood (160-163). Likewise, tracking is also found for the individual risk factors; blood pressure (108;164;165), overweight/obesity (161;165-173) and insulin/HOMA(163). However, the shorter-term stability of clustered risk factors over the course of childhood and adolescence has yet to be elucidated.

1.6 Interventions aiming at improving metabolic health in children and adolescents

Considering the world-wide rise in pediatric obesity and the increase in children with impaired metabolism and early signs of life-style related diseases, effective intervention strategies are warranted. PA has been suggested as an important area in prevention of both obesity and lifestyle diseases (136;174). The school setting has been proposed as an ideal environment for population-based PA interventions, as schools provides an advantageous setting for encouraging a healthy lifestyle among children and enhancing PA (175-177). The compulsory physical education (PE) in schools is the only setting that ensures PA for all children, including overweight, inactive and unfit children, who are difficult to target by other means (177).

1.6.1 School-based interventions

Several school-based intervention studies have been conducted with a large diversity of results on fatness (178), PA (175;179;180), fitness (175;180) and CVD risk factors (181). These will be discussed in greater detail in section *4.1. Effects of the intervention*. Many intervention studies suffer from methodological weaknesses, e.g. low sample size, inadequate duration and volume of the PA intervention or are not suitable for long-term implementation in large populations (178;180). The challenge is to find interventions, which both provide effective means to improve the health of children and, at the same time, are possible to implement on large scale populations.

1.7 Aims of the thesis

1. To describe the effect of a school-based physical activity intervention providing a doubling of time for PE, new didactic tools to the PE teachers, and an upgrading of sports and playing facilities in intervention schools on CVD risk factors (**Paper I**).

2. To evaluate tracking of clustered CVD risk in youth using three time-points; age 6, age 9 and age 13 yrs. Secondly, to analyze the effects of overweight/obesity and cardiorespiratory fitness on the level of tracking (**Paper II**).

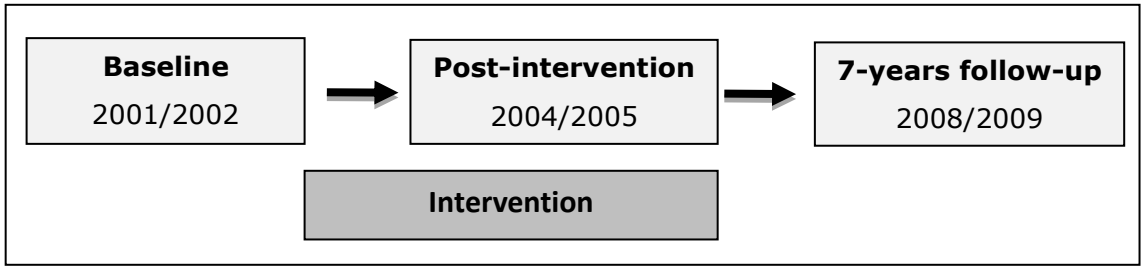
3. To examine the relationship between markers of inflammation and CVD risk factors in youth cross-sectionally (**Paper III**) and longitudinally (**Paper IV**).

2. Methods

2.1 Study design; Copenhagen School Child Intervention Study

CoSCIS is a controlled longitudinal intervention study that took place in 18 public schools (10 interventions schools (IG) and 8 controls schools (CG)) in two suburbs of Copenhagen. Baseline measurements were carried out in 2001/2002, post-intervention measurements in 2004/2005 and follow-up measurements in 2008 (see figure 1). All tests were performed at the participating schools in a gym or a classroom except for the fitness test which was performed using permanently installed equipment in a camper trailer.

Figure 1. Study design – Copenhagen School Child Intervention Study



Intervention

The intervention and control schools were not randomly selected. The local Authority of Ballerup had decided to upgrade the physical activity opportunities for their youngest school-children and contacted the research group in order to quantify and measure the effect of such an intervention. The intervention was hereafter planned in cooperation between the intervention local Authority and the researchers. In 2000 the intervention was started and involved all 1st to 3rd grade classes. The program consisted of four constituent parts.

1. An increase in the amount of PE lessons from 90 to 180 minutes per week, given as two double sessions each week.
2. Extra lessons in health education, focusing on the importance of PA and healthy eating.

3. 3-4 full days a year of supplementary training for the PE teachers focused on didactic tools to enhance the children's motivation for and enjoyment of PA and, at the same time, keeping the intensity in PE lessons moderate to vigorous.
4. Fourth, indoor and outdoor PE and playing facilities were upgraded in all intervention schools.

Schools within Tårnby local Authority were chosen as control group as this local area resembles the socio-demographics of the intervention Authority. Control schools followed the normal curriculum of one double PE session per week (90 min/week) and had no teacher training or facility upgrading.

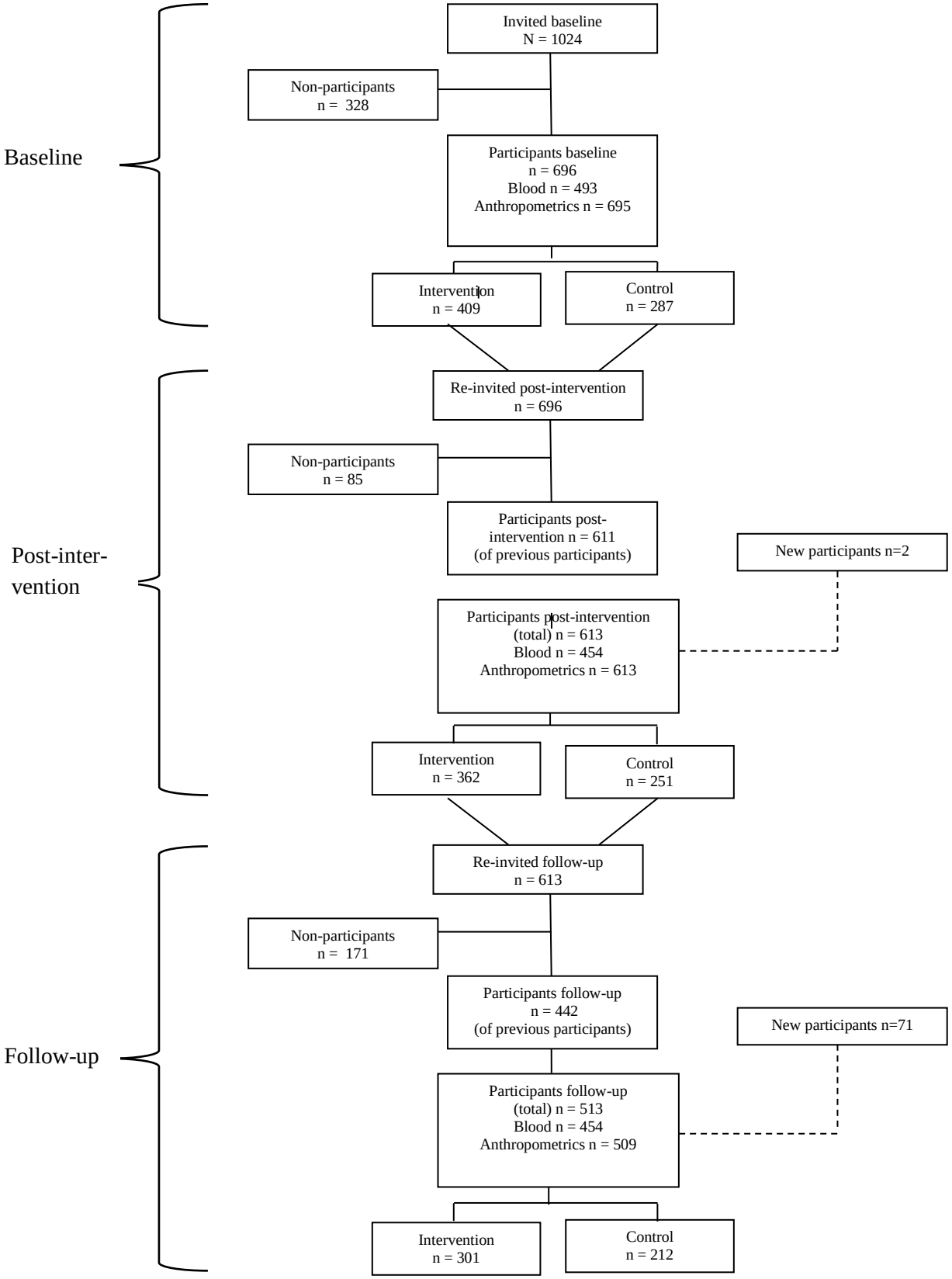
Participants

All children from all preschool classes (6-7 years of age) in public schools in the two local areas were invited to participate in the study (n = 1024). Figure 2 displays an overview of study design and participants.

A total of 706 children (69 % of the population) volunteered to participate. Three children failed to attend any test, five only had a blood sample taken and two carried out only the physical activity assessment, giving a final number of 696 children (364 boys and 332 girls, 409 from intervention and 287 from control) at baseline with a mean age of 6.75 ± 0.37 years. In first grade, one year after the baseline measurements, all children were assessed by a school doctor. At this time-point there were no differences between participants and non-participants with respect to age, height, weight and body mass index (BMI) for either sex (182).

Post intervention a total of 613 children participated in the study of which 2 children did not participate at baseline. From baseline to post-intervention 85 children dropped out of the study (12.21 %). At follow-up 442 of previous participants volunteered to participate again. 171 of participants dropped out from post-intervention to follow-up (a drop-out of 36.5 % from baseline to follow-up and a dropout from post intervention to follow-up of 27.9 %). At follow-up 71 children who did not participate at any of the other time points were included in the study.

Figure 2


Baseline
Invited baseline
N = 1024
Non-participants
n = 328
Participants baseline
n = 696
Blood n = 493
Anthropometrics n = 695
Intervention
n = 409
Control
n = 287
Post-inter-
vention
Re-invited post-intervention
n = 696
Non-participants
n = 85
Participants post-
intervention n = 611
(of previous participants)
New participants n=2
Participants post-intervention
(total) n = 613
Blood n = 454
Anthropometrics n = 613
Intervention
n = 362
Control
n = 251
Follow-up
Re-invited follow-up
n = 613
Non-participants
n = 171
Participants follow-up
n = 442
(of previous participants)
New participants n=71
Participants follow-up
(total) n = 513
Blood n = 454
Anthropometrics n = 509
Intervention
n = 301
Control
n = 212

Ethics

Written, informed consent was obtained from the child's parent or legal guardian after being given a detailed written explanation of the aims of the study, any possible hazards, discomfort, and inconvenience, and the option to withdraw at any time. The study was approved by the ethics committee at the University of Copenhagen.

Quality control

To reduce measurement error, which can result in bias, quality control was performed at different levels. A detailed manual was written for all measurements. The manual included all practical procedures related to the data collection and manual notation of results. All central variables were tested for reproducibility before baseline measurements. All test personal were trained before testing. The same trained bio-technician performed all blood draws, three skilled researchers performed all skinfolds measurements and three experienced researchers performed all fitness testing. Manually entering of data was done continuously and data was double checked by a second person. Each subject was given a unique ID-number and treated as anonymous. Outliers were checked.

Sample size and power calculations

Power and sample size calculations were done *a priori* before the study was started to ensure sufficient power to detect physiologic meaningful differences between IG and CG on main parameters. However, these calculations were based on assumptions on dropout and variation. Furthermore, analyses done on subgroups with a smaller number of subjects than in the entire cohort might suffer from a lack of power. Therefore, all results should be interpreted both by the statistical significance level, but also by observing the absolute values of eventual differences between groups.

2.2 Measurements

All measurements were performed between 0800 and 1400 h. Blood draws were completed before 0930 h. Accelerometer data was normally collected the week after the other measurements. Since the data collection took several months, testing was done alternating weeks in intervention schools and control schools to account for growth, maturation and seasonality in PA.

Age and anthropometry

Age was calculated from day of birth and day of testing. Body height was measured without shoes to the nearest 1 mm using a Harpenden stadiometer (West Sussex, UK). Body mass was measured in light clothing to the nearest 0.1 kg using an electronic scale (Seca 882, Brooklyn, NY). BMI was calculated ($kg \bullet m^{-2}$) and BMI z-scores (zBMI) were computed based on WHO recommendations (183). Biceps, triceps, subscapular and suprailiac skinfolds were measured to the nearest mm, in triplicate, with Harpenden calipers (Harpenden, West Sussex, UK). The mean of 3 measurements was used for analysis. All skinfold measurements were taken on the self-reported non-dominant side of the body by the same three skilled researchers. The sum of four skinfolds (S4SF) was calculated according to the method presented by Durnin and Rahaman (184) and used as an estimate of body fatness. Waist circumference was measured to the nearest mm with an anthropometric tape, midway between the lower rib margin and the iliac of the trunk. Sexual maturation was assessed at post-intervention and at follow-up by self-report using a scale of pictures of breast and genital development for girls and boys, respectively (185).

Blood pressure

Blood pressure was measured after 15 minutes of rest with a Dinamap XL vital signs blood pressure monitor (Critikron, Inc., Tampa, FL) using appropriate sized cuffs. Five measurements were taken over 10 minutes and the mean of the last three measurements was recorded.

Blood sampling

A band-aid with an analgesic cream (EMLA) was sent to all participants prior to the day of the blood sample. At the start of the test day the children were asked what they had been eating and drinking from the night before. Only water and sugar-free chewing gum were allowed for a child to be accepted as fasting. Non-fasted children were asked to come back one of the following days to have their blood drawn. Blood samples were collected from the antecubital vein. After sampling the children were offered breakfast. Glucose was analyzed immediately after sampling (Hemocue). The remainders of the samples were centrifuged; plasma aliquoted within 30 min, kept at -20^{o}C, and later stored at -80^{o}C. Insulin was analyzed spectrophotometrically using an enzyme linked immunosorbent assay (DAKO Insulin, Code no. K6219). TC, HDLc and TG were analyzed on a COBAS FARA (Roche, Switzerland)

using spectrophotometry (ABX Diagnostics, Montpellier, France). Inflammatory markers were analyzed with high-sensitivity immunoassays or multi-plex kits. For IL-6, Quantikine High-Sensitivity ELISA with a detection limit of 0.5 pg·mL−1 (R&D, Minneapolis, MN) was used. For TNFα, Quantikine High-Sensitivity ELISA with a detection limit of 0.1 $pg \cdot mL^{-1}$ (R&D, Minneapolis, MN) was used. sTNFRI (the total amount of free receptor plus the total amount of receptor bound to TNFα), IL-1Ra and adiponectin was measured on a Luminex 100 (Luminex Corporation, Austin, TX, USA) using Invitrogen kits (Invitrogen Corporation, California, USA), with detection limits of 15 $pg \cdot mL^{-1}$, 30 $pg \cdot mL^{-1}$ and 0.058 $ng \cdot mL^{-1}$ for sTNFRI, IL-1Ra and adiponectin, respectively. C-reactive protein was determined using particle-enhanced turbidimetric immunoassay: Tina-quant CRP (latex) high-sensitivity assay (Cobas, Roche Diagnostics, Mannheim, Germany) with a detection limit of 0.1 $mg \cdot L^{-1}$. All essays were used according to the manufacturer's instructions. Insulin resistance was estimated according to homeostasis model assessment (HOMA-IR) as glucose ($mmol \cdot 1^{-1}$) multiplied by insulin ($mU \cdot 1^{-1}$) divided by 22.5 (186).

Physical activity measurements

PA was assessed using a combination of a questionnaire and the Actigraph 7164 activity monitor (Actigraph Inc., Pensacola, Florida, USA). This accelerometer has been validated and has shown a high degree of mechanical reproducibility (187) and good validity with respect to free living energy expenditure assessed by doubled labeled water in children (188). The accelerometer was secured directly to the skin at the lower back using an elastic belt. The children were instructed to wear the accelerometer for five consecutive days, three weekdays and two weekend days. They were also instructed to wear the accelerometer during the entire day and only removed it e.g. during water activities. The accelerometer was programmed to start monitoring on the second day in order to familiarize the children with the device and avoid artificially elevated measurements in the beginning of recording caused by excitement.

After the sampling the accelerometers were collected and memory downloaded. A recording epoch of 10 seconds was selected. All continuous sequences of 60 consecutive epochs (i.e. 10 minutes) or more with zero counts were considered as non-wearing and were subsequently deleted (189). Only children providing a minimum of three days with eight hours of valid recording, after removal of missing data, were included in the analyses. At post-intervention and follow-up a PA questionnaire was distributed

and returned with the accelerometers. Children and parents were, among other things, asked to note time and reason for any period of "non-wear". Accelerometer data was subsequently adjusted for water activities information obtained by the questionnaires. All non-wear periods at post-intervention and follow-up, which could be identified as water activity, were substituted with blocks of activity corresponding to the mean counts per minute in a normal PE lesson. In this thesis and in **Paper I** the overall amount of PA (mean counts per minute) and minutes per day spent on moderate to vigorous (MV) PA (≥ 1500 counts/min)(190) is reported.

Cardiorespiratory fitness (VO_{2peak}) testing

VO_{2peak} was assessed using a continuous running protocol on a treadmill. VO_{2peak} was measured directly on an AMIS 2001 Cardiopulmonary Function Test System (Innovision, DK 5260 Odense) at baseline and at post-intervention and using the COSMED $K4b^2$ portable metabolic system (COSMED, Rome, IT) at follow-up. Both systems were calibrated immediately before each trial. We were unfortunately unable to cross validate the two systems, but both have been validated against the Douglas bag method and were found to obtain valid measures of VO_2 (191;192). At baseline and post-intervention the test protocol was as described in Table 2.1. No children completed 15 minutes.

Table 2.1. VO_{2peak} test protocol.

Minutes	0	3	5	7	9	11	13	15
Velocity	4 km/h	8 km/h	8 km/h	8 km/h	8 km/h	9 km/h	13 km/h	15 km/h
Grade	0 %	0 %	3 %	6 %	9 %	9 %	9 %	9 %

At follow-up a pilot-study was conducted prior to the test period. It was found that by using the same protocol as at baseline and post-intervention, some children would be running for too long before reaching maximal effort. We therefore modified the protocol and increased intensity every minute (incline or speed) until near max (by observing the child and following both heart rate and respiratory exchange-ratio (RER)). In time to exhaustion the minutes taken out of the protocol were added to the final running time.

The children were instructed to run until exhaustion. To determine if a test was performed satisfactorily at least one of three objective physiological criteria should be fulfilled; heart rate (HR) > 200 beat/min,

RER ≥ 0.99, or a defined plateau of oxygen uptake ($VO_{2)}$ (an increase of less than 2.1 $ml \cdot min^{-1} \cdot kg^{-1}$) together with a subjective criteria for exercise intolerance (193). For this thesis and all papers VO_{2peak} is expressed in mL/kg/min. For children who ran to exhaustion but did not attain a valid measurement because of equipment failure, VO_{2peak} was estimated from a regression equation calculated from all the valid tests using running time to exhaustion and sex (~10% of participants). At baseline 57 children ran to exhaustion without having a VO_2 recording due to unwillingness to wear the necessary equipment or not wearing the equipment satisfactorily. At baseline, analysis of covariance showed no difference neither in intercept nor slope between sexes, and a common equation was calculated: VO_2 = 29.4 + 0.037 * time to exhaustion (sec).

At post-intervention and follow-up analysis of covariance showed differences between sexes and the equations were calculated for each sex separately. At post-intervention VO_{2peak} for boys was calculated as follows: 25.4 + (2.299 * time to exhaustion (sec)) and for girls: 21 + (2.451 * time to exhaustion (sec)), for boys and girls, respectively. At follow-up VO_{2peak} was calculated as follows: 24.7 + (1.9 * time to exhaustion (sec)) and 20.9 + (1.9 * time to exhaustion (sec)), for boys and girls, respectively.

2.3 Participants and Statistics

Differences in baseline values of BMI, waist, S4SF and VO_{2peak} between children who participated at post-intervention and follow-up compared to children who dropped out of the study was calculated using paired t-tests adjusted for sex. Differences between children who gave vs. not gave blood in BMI, waist, S4SF and VO_{2peak} were calculated using paired t-tests adjusted for sex. Number and percentages of children meeting the criteria for MetS were calculated.

Paper I

In **Paper I** all children participating in CoSCIS were included except for children only participating at follow-up and one child, who moved from the intervention to the control community. The number of children in different analyses differs considerably between different variables and time-intervals (see Table 1 and 2, **Paper I**), because of children choosing not to participate in all tests (e.g. blood sampling or fitness test).

Means and standard deviations for physical characteristics and CVD risk factors at baseline, post-intervention and follow-up were calculated by sex and group (Table 1, **Paper I**). A composite risk score (clustered z-score) was computed from the sex-specific sum of z-scores for systolic blood pressure, TG, ratio of TC to HDL, HOMA-IR, S4SF, and the negative value of VO_{2peak} z-score. BMI, waist circumference, S4SF, HOMA-IR, TG, TC to HDL-ratio and sum of z-scores were positively skewed and therefore transformed (natural log) for the analyses. All statistical analysis is described in the Methods of **Paper I**.

Furthermore, to investigate the intervention effect on the children most likely to benefit from the intervention two subgroups were constructed. One subgroup; the overweight subgroup, was based on baseline S4SF. Children with S4SF z-scores (by sex) above 1 were included (n = 69, 36 from IG and 33 from CG) corresponding to ~ 10% of the total population. The other subgroup; the unfit subgroup, was based on baseline $VO_{2\text{-}peak}$. Children with baseline $VO_{2\text{-}peak}$ z-scores at -1 or below were included (n =104, 61 from IG and 43 from CG) corresponding to ~ 16%. These results are presented in the *Appendix*.

Paper II

In **paper II** all children with complete measures of CVD risk factors at least at two of the three time-points were included (n = 434). A composite CVD risk score (clustered z-score) was constructed by adding the sex-specific z-scores for systolic blood pressure, HOMA-IR, TG, S4SF and the negative z-scores for HDLc and VO_{2peak}. In analysis with stratification for S4SF, S4SF was removed from the clustered z-score and in analysis with stratification for VO_{2peak}, this variable was removed from the clustered z-score. As we did not find any differences in the clustered z-score between intervention and control group in **Paper I**, the two groups were pooled for all analyses in this paper. All statistical analyses are described in the Methods of **Paper II**.

For this thesis, children were grouped in tertiles based on fitness and fatness. Clustered z-score (without fitness or fatness, respectively) tracking coefficients were calculated within each tertile using Pearson correlations.

Paper III

Data for **Paper III** was from the follow-up measurement in 2008 (mean age 13.4 yrs ± 0.3). A composite CVD risk factor score (clustered z-score) was computed by summing z-scores "by sex" of following six variables; the ratio of TC to HDLc, TG, HOMA-IR, systolic blood pressure, S4SF, and inverse of VO_{2peak}. Complete data for the clustered z-score and the inflammatory markers were available for 413 subjects. IG and CG were pooled for all analysis. Means and standard deviations for all variables were computed by groups based on median split for fitness and S4SF (Table 1, **Paper III**). All statistical analysis is described in the Methods section of **Paper III**.

Paper IV

Data used in this paper is from post-intervention and follow-up measurements. Complete data on IL-6 from both assessments were available for 292 subjects. Since no differences between IL-6 levels in the intervention and control group were found, data from the two groups were pooled for all analyses. Means and standard deviations for all variables were computed by sex (Table 1, **Paper IV**). All statistical analysis is described in the Methods section of **Paper IV**.

Furthermore, for this thesis correlations between IL-6 at age 9 years and the individual risk factors, systolic blood pressure, total cholesterol, HDLc and TG, are presented.

1. Results

3.1 Characteristics of the participants in CoSCIS

Drop out analysis

There were no significant differences in baseline levels of S4SF, BMI, waist circumference or VO_{2peak} between the children who participated at post-intervention and the children who dropped out of the study, with or without adjusting for sex. There was no difference between IG and CG in drop-out rate. Children who participated at follow-up had significantly lower baseline BMI, waist and S4SF (17.0 vs. 17.86, 55.2 cm. vs. 56.5 cm, and 25.7 mm. vs. 28.2 mm. respectively) and a higher baseline VO_{2peak} (47.4 vs. 45.7 ml kg^{-1} min^{-1}) compared to children who dropped out (p=0.002, 0.001, 0.001, and

0.0001; respectively), with and without adjusting for sex. There was no difference between IG and CG in drop-out rate.

Missing blood

Of the 696 children participating at baseline blood was obtained from 493 children (70.8 %). 203 did not have blood taken. At baseline children without blood samples had a significantly higher BMI (16.3 vs. 15.9), S4SF (13.2 vs. 8.0) and waist circumference (56.4 vs. 55.4 cm) compared to children who gave blood (p = 0.022, 0.000 and 0.022, respectively). There was no difference between children who gave vs. not gave blood on VO_{2peak}. More children in IG had blood drawn compared to CG (73% vs. 63%, p=0.004).

Of the 613 children participating in the study at post-intervention blood was obtained from 454 children (74.0 %) and there was no difference between IG and CG in the percentage of children with and without blood samples. Children without blood samples had a significantly higher S4SF (36.1 mm vs. 32.6 mm) and a lower VO_{2peak} (47.1 ml kg^{-1} min^{-1} vs. 49.7 ml kg^{-1} min^{-1}) compared to children who gave blood (p = 0.022 and p = 0.000 respectively), whereas there were no differences in waist circumference or BMI between children who gave vs. not gave blood. Of the 513 children participating at follow-up blood was obtained from 454 children (88.5 %).

At follow-up children without blood samples had a significantly lower $VO_{2\text{-}peak}$ (46.7 ml kg^{-1} min^{-1} vs. 49.4 ml kg^{-1} min^{-1}) compared to children who gave blood (p = 0.039), but this was no longer significant after adjusting for sex. There were no significant differences in waist circumference, S4SF or BMI between children who gave vs. not gave blood at follow-up. Adjusting for sex did not change any of these findings.

Participants

Each paper contains a table with characteristics of included participants (Table 1, **Paper I, II, III** and **IV**). For this thesis, to further assess the health status of the children the total number of children meeting the criteria of the MetS for youth was assessed. At baseline and post-intervention the criterion was being at or above 90^{th} percentile for waist circumference using the references from Fernandez and colleagues (194) and from McCarthy and colleagues (195) (Table 3.1).

Table 3.1. Numbers and percentages of participants at or above 90th percentile for waist circumference for age and sex at baseline and post-intervention using two different references.

Variable	Sex	N waist total	N above 90th percentile	% of total	% in intervention/ control group
Baseline waist circumference by	Boys	364	13	3.2	
Fernandez	Girls	329	18	5.5	3.9/5.3
Baseline waist circumference by	Boys	364	85	23.4	
McCarthy	Girls	329	104	31.6	28.2/26.0
Post-intervention waist	Boys	317	21	5.2	
circumference by Fernandez	Girls	295	26	8.8	8.6/6.4
Post-intervention waist	Boys	317	140	44.2	
circumference by McCarthy	Girls	295	123	41.7	43.1/42.8

Age- and sex-specific waist circumference cut-points for being at or above the 90th percentile according to Fernandez and co-workers (194) and McCarthy and co-workers (195).

At 7-years follow-up the number of children meeting the criteria for adolescent MetS cutpoints presented by Jolliffe and Janssen (121) were assessed (Table 3.2). A total of 8 or 9 children (>2%) met the criteria for having MetS depending on which criteria were being used. The percentage of adolescents meeting the cut-points of the individual risk factors varied considerable with most adolescents meeting the cut-point for high glucose concentration and fewest meeting the cut-points for waist circumference as defined by ATP and TG.

Sexual maturation

At baseline all children were Tanner stage 1. At post-intervention 15 boys (4.9 %) and 118 girls (40.4 %) were Tanner stage 2, no boys and 14 girls (4.8 %) were Tanner stage 3 and the rest of the children were Tanner stage 1. At 7-years follow-up 3 boys (1.2%) and 1 girl (0.4%) were Tanner stage 1, 16 boys (6.3 %) and 13 girls (5.2 %) were Tanner stage 2, 120 boys (47.2 %) and 54 girls (21.6 %) were Tanner stage 3, 101 boys (39.8 %) and 136 girls (54.4 %) were Tanner stage 4 and 14 boys (3.4 %) and 46 girls (18.4 %) were Tanner stage 5.

Table 3.2. Numbers and percentages of participants at or above adolescent metabolic syndrome (MetS) cut-points for age and sex at 7-years follow-up according to criteria by Jolliffe and Janssen (121).

Variable	Sex	N total	N meeting criteria	% of total	% in intervention/ control group
	Boys	258	0	0	
Waist ATP	Girls	250	5	2.0	1.7/0.0
	Boys	258	6	2.3	
Waist IDF	Girls	250	26	10.4	7.4/4.8
	Boys	258	24	9.3	
Systolic BP	Girls	251	16	6.4	9.1/6.2
	Boys	235	36	15.3	
HDLc	Girls	215	44	20.5	18.2/17.2
	Boys	235	9	3.8	
TG	Girls	211	4	1.9	3.3/2.2
	Boys	236	149	63.1	
Glucose	Girls	216	113	52.3	59.6/56.0
Meeting ATP MetS	Boys	233	4	1.7	
criteria[a]	Girls	212	5	2.4	2.6/1.1
Meeting IDF MetS	Boys	233	4	1.7	
criteria[b]	Girls	212	4	1.9	1.9/1.7

ATP: National Cholesterol Education Program Adult Treatment Panel III

IDF: International Diabetes Federation

[a]; ATP MetS definition: at least 3 of the following;: high waist circumference, high systolic or diastolic blood pressure, low HDLc, high TG and high blood glucose.

[b]; IDF MetS definition: high waist circumference and at least 2 of the following; high systolic or diastolic blood pressure, low HDLc, high TG and high blood glucose.

3.2 Effects of the intervention (Paper I)

Table 2, **Paper I** presents the immediate and long-term effect of the intervention. The immediate effect is the difference between IG and CG change-scores from baseline to post-intervention. The long-term effects are the difference between IG and CG change-scores from baseline to follow-up.

Anthropometrics

No significant difference between IG and CG were found for any anthropometric measure.

Physical activity and cardiorespiratory fitness

No significant difference between IG and CG were found for mean PA (counts/min), MVPA (minutes spent above 1500 counts/min) measure or cardiorespiratory fitness. However, the difference in MVPA (corresponding to a ~ 12 minutes/day less decrease in MVPA in IG compared to CG) became statistically significant when results were not adjusted for school-cluster (data not shown).

CVD risk factors

From baseline to post-intervention IG had a borderline smaller increase in systolic blood pressure compared to CG ($p = 0.092$) and IG boys had a significantly smaller increase in HOMA-IR compared to CG boys ($p = 0.004$); whereas no difference between groups was found for girls (Figure 1, **Paper I**). Long-term effects of the intervention were measured comparing IG and CG change-scores from baseline to follow-up. The change-scores of systolic blood-pressure were significantly lower for IG boys compared to CG boys ($p = 0.010$), but this difference was not found for girls (Figure 2, **Paper I**). There were no other significant differences in change-scores. After Bonferroni correction no differences were found between groups.

Overweight subgroup

To investigate the intervention effect in the children most likely to benefit from the intervention two subgroups were constructed. Intervention effects in the most overweight subjects were investigated by comparing the children in IG with a baseline S4SF z-score at or above 1 with the same group in CG (Table 1, *Appendix*). The only significant difference found was for change-scores in zBMI from baseline to post-intervention, where IG children had a greater increase compared to CG. There was a borderline significant difference in MVPA with IG children increasing their value, whereas CG children decreased their value. No other differences between groups were found in the overweight subgroup. Post hoc analyses with a subgroup based on McCarthy waist cut-points (195) at baseline (n =

189), showed the same tendencies, however both the difference in BMI and in MVPA became statistical significant (data not shown).

From baseline to post-intervention the group with a S4SF z-score below 1 SD (the normal-weight group) in IG had a significant less decrease in MVPA compared to the same group in CG. Furthermore, as in the entire cohort IG boys had a lesser increase in HOMA-IR compared to CG boys (P = 0.045). From baseline to follow-up normal-weight IG children had a significantly lesser increase in systolic blood pressure compared to the same children in CG (P = 0.038) (data not shown).

Unfit subgroup

Intervention effects in the most unfit subjects were investigated by comparing the children in IG with a baseline $VO_{2\text{-peak}}$ z-score at or below -1 with the same group in CG (Table 2, *Appendix*). From baseline to post-intervention the only significant difference found was for change-scores in zBMI, where IG children had a greater increase compared to CG. IG had a significant greater increase in BMI and HOMA-IR from baseline to follow-up. There were no other differences between IG and CG in the unfit subgroup.

Comparing the children in IG with a baseline $VO_{2\text{-peak}}$ z-score above -1 (normal fitness group) with the same group in CG it was found that IG children increased their S4SF more compared to CG (P = 0.039). Furthermore, the boys in the IG had a less increase in HOMA-IR compared to boys in CG and decreased their sum of z-scores, whereas boys in CG increased their sum of z-scores (P = 0.004 and 0.042, respectively) (data not shown).

3.3 Tracking of CVD risk factor clustering (Paper II)

BMI, S4SF, systolic blood pressure and HOMA-IR increased with age. VO_{2peak} increased from age 6 to age 9 years, TG increased from age 9 to age 13 years and HDLc increased from age 6 to age 9 years and then decreased from age 9 to age 13 years (Table 1, **Paper II**). Moderate correlations were found between clustered z-score at age 6 to 9 and 9 to 13 years, whereas the coefficient between age 6 and 13 years was somewhat weaker (Table 2, **Paper II**). These coefficients did not change substantially when analyses were done for each sex separately (data not shown). For the single risk factors highest tracking coefficients were seen for S4SF, followed by systolic blood pressure and HDLc. Tracking coefficients

for HOMA-IR and TG were low to moderate. The tracking coefficients for VO_{2peak} were high from 6 to 9 years and 9 to 13 years, but low from 6 to 13 years (Table 2, **Paper II**).

Logistic regression showed that children with moderate and high risk respectively, in clustered z-score at first time-point had a 6.1 (95% CI: 2.37-7.93) and 21.2 (95% CI: 7.93 – 56.80) times greater risk, respectively, of having a clustered z-score above 1 SD at the second time-point between the ages 6 to 9 years, 4.51 (95% CI: 1.71 – 11.89) and 30.8 (95% CI: 9.73 – 97.50) times greater risk between the ages 9 to 13 years, and 2.3 (95% CI: 1.04 – 5.19) and 4.9 (95% CI: 1.92 – 12.34) times greater risk between the ages 6 to 13 years, compared to children with low risk in clustered z-score (Table 3, **Paper II**).

Tracking coefficients for groups based on level of VO_{2peak} are presented in Table 3.3.

Table 3.3. Tracking coefficients between clustered z-score (without VO_{2peak}) at different ages stratified for VO_{2peak}

	6 to 9 years	9 to 13 years	6 to 13 years
Grouping (mean (SD))	*r*	*r*	*r*
By VO_{2peak} at age 6 years			
1st tertile (40.8±3.3 ml kg^{-1} min^{-1})	0.590		0.501
2nd tertile (46.9±2.4 ml kg^{-1} min^{-1})	0.489		0.425
3rd tertile (53.3(±3.5 ml kg^{-1} min^{-1})	0.396		0.253*
By VO_{2peak} at age 9 years			
1st tertile (42.8±4.5 ml kg^{-1} min^{-1})		0.670	
2nd tertile (50.0±3.3 ml kg^{-1} min^{-1}2)		0.497	
3rd tertile (56.5±4.7 ml kg^{-1} min^{-1})		0.290**	

Pearsons correlations. All *P*-value < 0.0001, unless marked. *: *P*-value = 0.01, **: *P*-value = 0.002

Tracking coefficients were highest in the 1st tertile (least fit) at all three time intervals (*r*-values 0.501-0.670). The coefficients were strong in the 1st and 2nd tertiles and low in the 3rd tertiles.

Tracking coefficients for groups based on S4SF are presented in Table 3.4. Lowest tracking coefficients were found in the 1st tertile (least fat) at all three time intervals. However, the picture was not as clear as for VO_{2peak}. Between age 6 and 9 years there was no significant coefficient in the 1st tertile, a strong coefficient in the 2nd tertile and moderate coefficient in the 3rd. Between age 6 and 13 years the coefficients were weak in all tertiles. Between age 9 and 13 the coefficients were weak in the 1st and 2nd tertile and stronger in the 3rd.

Table 3.4. Tracking coefficients. Pearson coefficients between clustered z-scores at different ages stratified for S4SF

Grouping (mean (SD))	6 to 9 years *r*	9 to 13 years *r*	6 to 13 years *r*
By S4SF at age 6 years			
1st tertile (19.5±2.4 mm)	0.068*		0.201*
2nd tertile (23.9±2.4 mm)	0.499		0.314**
3rd tertile (33.9±9.1 mm)	0.340		0.233†
By S4SF at age 9 years			
1st tertile (20.5±3.0 mm)		0.317**	
2nd tertile (27.4±4.1 mm)		0.297**	
3rd tertile (47.0±14.6 mm)		0.492	

All *P*-value < 0.0001, unless marked. *: non-significant, **: *P*-value ≤ 0.01, †: *P*-value = 0.042

3.4 Low grade inflammation (Paper III and IV)

Cross-sectional analysis

The general characteristics of the included subjects are presented by fitness/fatness groups in Table 1, **Paper III**. After adjusting for sex and pubertal status, the high-fitness group had lower BMI (only in the high fat group) and S4SF, higher VO_{2peak}, systolic blood pressure and HDLc and lower clustered z-score, adiponectin and IL-6 levels than the low fitness group. Adolescents in the high fat group were younger, had higher BMI, S4SF, systolic blood pressure, HOMA-IR, clustered z-score and CRP level, but lower fitness, HDLc concentration and adiponectin level compared to the low fat group.

Partial correlations, adjusted for sex and pubertal status, between clustered z-score and the inflammatory markers are presented in Table 2, **Paper III**. Significant, but weak, correlations were found between clustered z-score and circulating levels of adiponectin, CRP, IL-6 and TNFα. When analyzed in groups based on median split of S4SF by sex, correlations between the clustered z-score and adiponectin and CRP were only significant for adolescents with S4SF values in the upper half. This group also exhibited a greater correlation coefficient for IL-6 and lower correlation coefficient for TNFα compared to adolescents with S4SF values in the lower half, but these differences were non-significant.

Table 3, **Paper III** shows the odds ratios for high clustered z-score for quartiles based on the level of the inflammatory markers. Table 4, **Paper III** shows the correlations between the inflammatory

markers. The anti-inflammatory markers adiponectin, sTNFR1 and IL-1Ra were correlated, as were the pro-inflammatory markers CRP, IL-6 and TNFα. Furthermore, adiponectin was inversely correlated to CRP.

Longitudinal analysis

We did not find any significant cross-sectional correlations between IL-6 levels and HOMA-IR at age 9 years or at age 13 years, irrespective of adjustment for fatness and fitness. HOMA-IR age 9 years was correlated with HOMA-IR age 13 years for girls (r = 0.242, P = 0.004), but not for boys (r = 0.143, P = 0.083). A significant correlation between levels of IL-6 at age 9 years and levels of IL-6 at age 13 years was found for girls. Furthermore, the girls also displayed correlations between IL-6 levels at age 9 years and HOMA-IR four years later. None of these correlations were found for boys (Table 2, **Paper IV**). Adjusting for TNFα at age 13 did not change these results substantially (data not shown). The correlations were weakened after adjusting for fatness, and fitness and fatness combined at age 13 years. When the children were stratified by Tanner stages, only the most mature girls (Tanner stages 4-5) displayed a significant correlation between IL-6 levels at age 9 years and HOMA-IR at age 13 years (Table 3, **Paper IV**).

Girls with IL-6 levels within the upper quartile at age 9 years had an odds ratio of 2.55 (95% CI: 1.12 – 5.80) for having IL-6 levels within the upper quartile at age 13 years and an odds ratio of 3.86 (95% CI: 1.58 – 8.57) for being in the upper quartile of HOMA-IR, compared with the rest of the girls. There were no other significant increased risks (P > 0.05) (Table 4, **Paper IV**).

The correlations between IL-6 and the other individual risk factors are presented in Table 3.5. The girls displayed correlations between IL-6 levels at age 9 years and TG levels four years later. For boys, levels of IL-6 at age 9 years correlated with level of TC at age 13 years. There were no significant cross-sectional correlations between IL-6 levels and any of the risk factors at baseline, irrespective of adjustment for fatness and fitness (data not shown).

Table 3.5. Pearson correlations between IL-6 at age 9 and cardiovascular risk factors at age 13; 1) unadjusted, 2) adjusted for sum of 4 skinfolds (S4SF), 3) VO_{2peak} (ml/kg), and 4) both S4SF and VO_{2peak} at age 13.**

	IL-6 age 9			
Risk factors age 13	Unadjusted	Adjusted for S4SF	Adjusted for VO_{2peak}	Adjusted for S4SF & VO_{2peak}
Systolic Blood Pressure				
Boys	0.023	0.023	0.049	0.050
Girls	0.042	0.072	0.057	0.074
Total Cholesterol				
Boys	**0.172***	**0.177***	**0.172***	**0.177***
Girls	-0.051	-0.031	-0.049	-0.042
HDLc				
Boys	0.134	0.130	0.128	0.125
Girls	-0.129	-0.101	-0.147	-0.129
Triglycerides				
Boys	0.130	0.140	0.131	0.137
Girls	**0.190***	**0.176***	**0.185***	**0.179***

*: *P*-value < 0.04. Significant correlations in bold face.

** All correlations adjusted for risk factor value at age 9 years.

2. General Discussion

This thesis presents data and results from Copenhagen School Child Intervention Study, a 3-years controlled intervention study with a follow-up four years after the end of the intervention. The intervention was proposed as a way to enhance and improve physical activity and thereby health in Danish children in public schools.

4.1 Effects of the intervention (Paper I)

Effects of the intervention were assessed as differences between intervention and control group in change-scores from baseline to post-intervention and from baseline to follow-up. In the following the results will be compared to results from other school-based studies and discussed.

Body composition

Most obesity treatment studies have showed limited long-lasting effects and primary prevention is therefore essential (196). However, extensive reviews on studies addressing all kinds of obesity prevention programs in children concluded that most studies show limited success in preventing childhood obesity (178;197). In the present study no positive intervention effects were found for BMI, zBMI, waist circumference or S4SF. The results of other school-based intervention studies are conflicting. Some did find a significantly better development in BMI in intervention groups (198-202), while others did not (139;203-206). BMI is commonly used, but probably not the best measure of body fatness in studies of growing children, primarily because BMI is not linearly proportional with age. Also, theoretically, it is possible that intervention studies only measuring BMI could be biased by increases in lean body mass as a result of the increased PA. However, it is unlikely that most school-based interventions substantially influence the amount of lean body mass, as more intense strength training is needed in order to induce muscle hypertrophy in children (207;208). Finally, increases in BMI do not affect body composition in the same way in all children. A study by Demerath and colleagues found that a similar increase in BMI z-scores resulted in a greater increase in body fat for girls and younger boys, whereas older boys (13-18 years) increased their lean body mass proportionally more (209). Other measures of body fatness, however, also provide conflicting results of different

interventions. Some school-based intervention studies have found positive intervention effects on obesity prevalence or body fatness assessed by a composite score of both BMI and triceps skinfolds (210)(only girls), skinfolds (211), percentage body fat by bio-impedance (200), waist circumference (199) and percentage of participants with a waist circumference at or above the 90th percentile (201). Other studies have not found any group differences in body fatness by under-water weighting (205), by waist circumference (139), by skinfold and bio-impedance (212) or by skin fold alone (204;213). It is of great importance to gain an understanding of the reasons why some school-based intervention studies succeed in preventing overweight/lowering body fat, while others do not. Several possible explanations for the differences in results between studies exist; one example being the diversity in intervention components. In the review by Brown & Summerbell (178) the authors conclude that there is not enough evidence to conclude on the effectiveness of diet vs. PA interventions, or the combination of the two. It is, however, likely that multi-component interventions have a greater chance of reducing overweight. All above mentioned studies finding a better development in intervention group BMI compared to control, had both a diet and a PA component (198-201), whereas 3 out of 5 studies without an effect on BMI were PA only interventions (139;203;206). Speculation suggests that combining the PA intervention with focus on diet could reduce the chance that intervention school children increase their energy intake in order to compensate for the increased energy expenditure caused by the extra PA. Furthermore, focus on PA also outside school could reduce the possibility for a compensatory decrease in PA after school (see below). Also the amount of extra PA delivered in the intervention differs between studies, but it is not consistently found that studies with the highest amount of PA have the greatest effect on body fatness.

Finally, it is possible that the baseline level in fatness parameters can influence the intervention effect on these parameters. The majority of children in CoSCIS were of normal weight, thus they had a limited potential for change, at least in regard to BMI. In a study by Marcus and colleagues, evaluating the effect of a 1-4 year school-based multi-component intervention, no difference was found in change-scores of BMI standard deviations, whereas the proportion of children who were initially overweight or obese and reached normal body weight was greater in the intervention schools compared to the control schools (206). This suggests that it can be important to analyze data apart from the mean, to evaluate the effect of the intervention on the children who would actually benefit from a change in body composition. A subgroup based on baseline S4SF (at or above 1 SD) was made, but the effect of the

intervention on body composition was not greater in this group compared with the total population. On the contrary, IG children in this subgroup actually had a greater increase in zBMI compared to the subgroup in CG and the same was also found in the unfit subgroup. Thus, in our study, the intervention was not more effective in altering body composition in the most fat or most unfit group of children. However, a physically active lifestyle in a long-term perspective might ultimately turn out to be a solid approach to regulating BMI regardless of initial BMI status.

Physical activity

Surprisingly, doubling the amount of PE had no significant effect on overall physical activity levels in IG compared to CG. One possible explanation is that IG children compensated for the increased school-time PA during the remainder of the day, a phenomenon found in the study by Donnelly and colleagues (205). However, these data were obtained from questionnaires, which is an inaccurate measure of PA in children and adolescents (131). Even though we used objective measures of PA and combined them with data from a questionnaire, it is possible that the method was not sensitive enough to capture small differences between groups. However, contrary to our result, a recent review summarizing previous reviews and newly published school-based intervention studies on PA-outcome found that 9 out of 10 newer studies assessing overall PA were effective in proving a positive intervention effect (180). This shows that it is actually possible to induce and measure intervention effects on overall PA in this type of studies, both by objective and subjective means.

In the present study, although not statistically significant, the IG did decrease their MVPA less compared to CG from baseline to post-intervention; the difference amounted to ~12 min/day. This difference was actually significant when analyses were done without adjusting for school-cluster (data not shown). Two other studies have reported similar results for MVPA (214;215). As MVPA is related to CVD risk factors (116;136), this modest, 12-min difference could explain some of the small positive metabolic changes found in this study. This difference in MVPA was also found for the overweight subgroup, which suggests that the intervention actually also reached these children. The applied method of assessing PA in this study is discussed in Section *4.5 Study strength and limitations*.

Cardiorespiratory fitness (VO_{2peak})

We did not find any positive intervention effect on VO_{2peak}. Researchers have suggested that to improve VO_{2peak} of children, the focus has to be on high intensity exercise with a minimum of 3-5 training sessions per week (129;193). Many population-based long-scale intervention studies with PE intervention comparable to CoSCIS fail to achieve improvements in cardiorespiratory fitness (204;205;214;216), which emphasizes the difficulty in implementing large-scale interventions sufficiently intense to significantly alter cardiorespiratory fitness level in normal populations. Other studies have, however, found an effect on fitness (211;217-219). Obvious differences between the interventions in CoSCIS and in the studies of Kriemler and co-workers and Resaland and co-workers are the number of minutes of PE/PA lessons (180 vs. 225 and 300 min) and the distribution throughout the week (two times/week vs. 5 times/week). The intervention in the study by Hansen and colleagues consisted of 50 min extra PE 3 days a week on top of the normal curriculum (219). The authors do not state what the normal amount of PE was, but it was probably 45-50 min 2 times per week, which is standard in Denmark. This gives a total of 240-250 min per week. The study by Trevino and colleagues had a 45 minute lesson 4 days a week, which corresponded to the amount in the present study, but with a more frequent delivery (217). Results from these studies show that it is possible to induce positive changes in cardiorespiratory fitness in normal pediatric populations, but that amount and frequency are important factors to consider. It should be noted, though, that only the study by Resaland and co-workers measured peak oxygen consumption directly in a max test (218). In the study by Hansen and colleagues VO_{2peak} was estimated from maximum mechanical power in a maximum progressive test and the other two studies used performance tests (shuttle run test and modified step test).

When analyzing the effect of an intervention on VO_{2peak} it is important to consider that the distribution might be uneven. In the study by Resaland and colleagues the greatest improvements in VO_{2peak} were found in the two quartiles with lowest baseline VO_{2peak} (218). Therefore, examining changes in sub-groups are necessary. However, in the present study there were no differences between IG and CG in change-scores of VO_{2peak} in either the overweight or the unfit subgroups, even though these groups might have had the greatest room for improvements.

Blood pressure

We found a borderline smaller increase in systolic blood pressure from baseline to post-intervention compared to controls. This difference in systolic blood pressure became significant at follow-up, but only for boys. A meta-analysis of randomized controlled trials (RCT) concluded that short-term exercise does not appear to reduce blood pressure in children and adolescents (220). Another review of more than 850 studies on exercise in youth found four studies showing an effect on blood pressure in children with elevated blood pressure, but no effect on children with normal blood pressure (129). However, some studies have found improvements in blood pressure following a short-term exercise intervention in children (221), a 2-year school-based PA intervention (139) and an 8-month school-based intervention (219). These results, together with our results, suggest that it is possible to generate positive alterations on blood pressure in both normo- and hypertensive children. A study by Leary and colleagues investigating the relationship between objectively measured PA using accelerometers and blood pressure concluded that the volume of PA (mean counts/min) was more closely related to blood pressure compared to MVPA (min/day above 3600 counts/min) (222). Our results seem to contradict this, as we did not find any differences between groups in mean PA, but did find a tendency for a lesser decrease in MVPA in the intervention group.

Apart for the possible effects of the increased amount of exercise, it is plausible that the improvements in HOMA-IR positively affected the blood pressure of IG children (see section *1.2.1 Physiological risk factors*). Finally, increased PA has also been found to lower activity in the sympathetic nervous system resulting in a reduction in total peripheral resistance and to lower plasma rennin and aldosterone (221). These changes can positively influence BP, but none of these factors were measured in the present study.

HOMA-IR

A smaller increase in HOMA-IR from baseline to post-intervention was observed for the boys in the IG compared to CG, but this difference did not persist to follow-up. Previous studies have likewise reported positive intervention effects on glucose and/or insulin levels (200;201;217), while others have not found any effects on these variables (139;205). Two studies found that the improvements in glucose and/or insulin levels were accompanied by improvements in measures of fatness (200;201), whereas one study, like CoSCIS, found that the improvements were independent of changes in body

composition (217). The independency of improvements in weight status has also been found in studies on overweight and obese children (223;224). These results, together with our results, suggest that it is possible to make favorable changes in insulin sensitivity by targeting PA, even without changes in fatness. McMurray and colleagues (225) investigated the effect of an exercise intervention on insulin levels in children and concluded that only children improving VO_{2peak} obtained positive effects on insulin levels. We did not find any improvements in VO_{2peak} and must conclude that it is possible to make changes in HOMA-IR without changes in fitness. It is possible that the better maintenance of PA and MVPA in IG compared to CG, even though not statistically significant, could have mediated these changes. No positive intervention effects were noted for the overweight or the unfit subgroups, whereas in both 'normal' groups the boys increased their HOMA-IR less in the IG compared to the CG.

Lipids

We did not find any intervention effects on blood lipids, similar to the results from some (204;226), but not all other intervention studies in children (139;227). The review by Strong and co-workers found evidence for a weak relationship between PA and blood lipids, especially for HDL-C and TG. They concluded that there appears to be a minimum threshold of 40 minutes of activity per day, 5 days a week (total 200 min/week), to achieve an improvement in blood lipid profile (129). The PE intervention of CoSCIS (180 min/week given in two sessions) did not meet these thresholds and this may be one reason why we do not find any effect on blood lipids. However, these criteria were not met in one of the two mentioned interventions, which did find an effect (227). In that study the physical activity component consisted of two weekly sessions of 45 min each, indicating that results on blood lipids might not be limited to very intense interventions.

Clustered z-score

To our best knowledge, only one other school-based intervention study, the KISS-study by Kriemler and colleagues, has analyzed the intervention effect on a clustered risk score. In that study the clustered risk score consisted of the standardized values of waist circumference, mean blood pressure, glucose, TG and inverted HDLc (211). The researchers found a significantly larger decrease in the cardiovascular risk score in the intervention group compared to the control group; which is in contrast to our findings. Previous results from EYHS and the present study show that both low cardiorespiratory

fitness and high levels of body fat are strong predictors for clustering of CVD factors in children (117;125;228). The fact that we do not find an effect of the intervention on sum of z-scores may be caused by the lack of intervention results on these variables. In support of this, the study by Kriemler and colleges also found positive intervention results in measures of fatness and VO_{2peak} (211). When looking at the most fit group, boys in IG group actually increased their clustered z-score less compared to CG boys, which again show that the intervention effect in this study was not greater in subgroups of the initially unhealthiest children.

Implementation of intervention

The extra PE session was administered by the normal PE teachers and the content was not controlled or supervised by the researchers. Approximately one third of the extra PE lessons were swimming lessons. The goal in the PE sessions was to make fun activities with a high level of intensity and incorporating both strength and cardiovascular training. The final planning and execution of the lessons was done by the PE teachers. The teachers were asked to keep a monthly record of the content of the lessons, but the drop-out in these data was too great for them to be used. Therefore, there was no strict control with the content of the PE classes (normal + extra) in CoSCIS. Thus, the amount and intensity of PA in classes is unknown, which could be a weakness of the study. However, any positive intervention effect found is caused by this "real world scenario" and therefore maybe possible to reproduce on larger scale populations.

All PE teachers were obliged to participate in the teacher training program and a record was kept, to ensure participation. There were of course teachers who stopped working in the intervention schools and new teachers starting without the training. A regular teacher training program would, in a long-term perspective, overcome this problem. Also, the upgrading of PE and playgrounds at intervention schools was done gradually and was not terminated when the intervention was started. This could possibly influence the measured effect of the intervention as the number of permanent play facilities has been found to affect the PA of children (229).

This is the first Danish large-scale school-based PA intervention using the best available methods in measuring PA, VO_{2peak} and CVD risk factors. Other school-based intervention studies providing more substantial effects on CVD risk factors and VO_{2peak} have conducted intervention more focused on high intensity, had a greater overall amount of PA and a higher frequency of PA (e.g. (201;211;218)).

However, these interventions could be difficult to implement on a population basis because of the large time and resource expenditure.

4.2 Tracking of CVD risk factor clustering (Paper II)

In **Paper II** the short-term tracking of a clustering of CVD risk in the CoSCIS population was examined. We found moderate to high level of tracking between all the measured time points (Table 2, **Paper II**), similar to the results of the only other study examining the stability of clustered CVD risk factors from childhood (age 9 years) to adolescence (age 15 years) (230). Tracking of clustered CVD risk factors has also been found from childhood or adolescence to adulthood (160-163). Interestingly, clustering of CVD risk factors was not evident in this cohort at age 6 years (228), whereas a cluster was found in some individuals from the age of 9 years (125). Therefore, it was unexpected to find a high degree of tracking between the ages 6 to 9 years and 6 to 13 years. The results of **Paper II** indicate that a relative high level of several CVD risk factors at this low age is predictive for high future metabolic risk.

When tracking coefficients of the summed CVD risk factors were calculated within each tertile of VO_{2peak}, a lower baseline VO_{2peak} level was associated with a higher tracking coefficient of clustered z-score and this was consistent for all time intervals (Table 3.3). This implies that within the least fit group, the stability of metabolic health is greater compared to within the most fit group.

When the children were grouped based on their S4SF no clear picture was found between groups in tracking of clustered CVD risk factors. In cross-sectional studies overweight and obesity have consistently been found related to clustering of CVD risk factors in children and adolescents (117;137;231;232) and convincing evidence suggests that overweight and obesity tracks from childhood into adulthood (166). Furthermore, some studies have found that overweight or obesity in childhood is associated with increased risk of later development of CVD risk factor clustering (233;234). The results from these studies show that the level of body fatness is important for the development of CVD risk factor clustering, but our results suggest that fatness might not be pivotal for the stability of CVD risk factor clustering over time.

Our results and the results from the above mentioned studies show that clustering of CVD risk factors is a relatively stable characteristic from early school-age and to the start of adolescence, and this

knowledge has potentially important clinical implications, as it points to implementation of preventive strategies starting in early childhood.

4.3 Inflammation in youth (Paper III and IV)

In adults convincing evidence suggest that low-grade inflammation has a fundamental role in all stages of the atherosclerotic process (2;8;50;56). Also in youth relationships between markers of low-grade inflammation and the level of individual CVD risk factors have been found (147-152).

Cross-sectional analysis on inflammatory markers and clustered z-score (Paper III)

We evaluated the associations between a clustered z-score and six different markers of low-grade inflammation. The clustered z-score was positively correlated with circulating CRP, TNFα and IL-6 levels and inversely correlated with adiponectin. No correlations were found for IL-1Ra and sTNFR1. Other studies have found similar results for adiponectin and CRP (148;151;153-156), whereas our results on TNFα and IL-6 are novel, as other studies by Steene-Johannessen and colleagues (148) and Andersen and colleagues (156) did not find these relationships. Subjects in our study had a mean age of 13.4 years ± 0.3, whereas the subjects in the earlier study by Andersen and colleagues were ~ 9.5 years (156) and in the study by Steene-Johannessen and colleagues half of the subjects were ~ 9.5 years and the other half ~ 15.5 years (148). It is possible that a measureable relationship between IL-6, TNFα and CVD risk factors takes time to develop and is not yet present at age 9 years. This is supported by observational studies in adults reporting findings of a relationship between IL-6, TNFα and different CVD outcomes (98). Furthermore, also in support of our results regarding IL-6, one study in youth did find that the level of IL-6 increased with increasing number of MetS components present (153). Recently, two studies on human genetic and biomarker data have found evidence for a causal relationship between IL-6 and CVD (99;100). In a meta-analysis of 82 studies it was found that the Asp358A1a gene variant, previously found to impair IL-6 receptor signaling, was associated with low concentrations of CRP and fibrinogen and high concentrations of IL-6 and soluble IL-6 receptors (100). Results from a Mendelian randomization analysis of 133.000 individuals found independent effects of IL-6 on CVD risk, and blocking the IL-6 receptor in patients with rheumatoid arthritis in clinical trials showed similar results (99).

We did not find any correlation between the clustered z-score and sTNFR1 in this cohort, which might imply that any inflammation in these adolescents is still insufficient to induce an accompanying release of anti-inflammatory mediators from leukocytes and other tissues. This is also supported by the fact that we did not find any correlation between clustered z-score and the anti-inflammatory protein IL-1Ra. Our finding is of potential interest, since elevation in pro- inflammatory cytokines both in children and adults with inflammatory diseases is usually accompanied by a release of sTNFR1 and IL-1Ra (e.g. (235;236)). The anti-inflammatory mediators in **Paper III** were all inter-correlated, as were the pro-inflammatory mediators, whereas the only correlation between pro- and anti-inflammatory mediators was an inverse correlation between adiponectin and CRP. It may therefore be speculated that cytokine release from fat-tissue, as opposed to cytokine release during leukocyte activation, is insufficiently counter-balanced by anti-inflammatory molecules with a risk of continuous low-grade inflammation, which could ultimately result in CVD. However, it is also possible that this relatively normal-weight and healthy population had not yet developed metabolic disturbances and low-grade inflammation to an extent sufficient to activate the anti-inflammatory response.

Longitudinal analysis of the relationship between IL-6 and HOMA-IR (Paper IV)

Longitudinal data on the inflammatory markers of the entire cohort were unfortunately only available for IL-6. Because of economic consideration only half of the sample of 9-years olds was analyzed immediately post-intervention (156). These children were selected based on their clustered risk score. Children in the lower and upper quartile of clustered z-score were analyzed first. The other half (second and third quartile) was analyzed with the follow-up samples in 2009 and unfortunately results on TNFα and CRP in the two halves were not comparable. It was therefore not possible to determine the correlations between the other inflammatory markers and IL-6 or their influence on the correlation between IL-6 and HOMA-IR.

In **Paper IV** a correlation was observed between circulating IL-6 levels at age 9 years and HOMA-IR at age 13 years for girls, but not for boys. Roth and colleagues found a weak but significant correlation between change-scores of IL-6 and HOMA-IR over one year in an intervention study on obese children (237). Taken together, these results could suggest a role of IL-6 in the longitudinal development of insulin resistance. However, we did not find any correlations between IL-6 and HOMA-IR in the cross-sectional analysis, supporting the results of other cross-sectional studies (148;149;152;156), which

might imply that IL-6 is not closely related to HOMA-IR in these age-groups. Also, the specific role of IL-6 in the pathogenesis of insulin resistance is still controversial. On the one hand, IL-6 has consistently been observed in patients with type 2 diabetes mellitus, and as a consequence has been related to insulin resistance and/or impaired glucose disposal (88), but on the other hand administration of IL-6 to healthy humans did not impair muscle glucose uptake or whole-body glucose disposal (90). It has been shown that IL-6 suppresses the production of TNFα (97), but concomitantly, TNFα causes IL-6 production and also the release of IL-6 to the circulation (238). Therefore, hypothetically it is possible that TNFα actually induces impaired glucose metabolism, while high systemic levels of IL-6 reflect a high local production of TNFα, and are not directly involved in the pathogenesis of insulin resistance (238). However, adjusting for TNFα at age 13 years did not modify the correlation found in girls between IL-6 at age 9 years and HOMA-IR at age 13 years significantly (data not shown). Positive correlations between IL-6 at age 9 years and total cholesterol and TG at age 13 years were found for boys and girls, respectively (Table 3.5). In contrast, a study by Gobel and colleagues found no correlation between IL-6 and any measure of blood lipids in overweight and normal weight adolescents (239). No correlation between HDLc and IL-6 was found replicating the findings of Nemet and colleagues (158). The complete relationship between IL-6 and the lipid profile in adults has not been revealed yet. Studies addressing the effect of IL-6 on fat metabolism show that IL-6 has diverging effect depending on the target tissue. Mechanistic studies have not found an effect of IL-6 on lipolysis and fatty acid kinetics in human adipocytes (92;240). However, systemic fatty acid oxidation and lipolysis was increased after human recombinant IL-6 infusion in healthy males. This increase was found to be caused by an increase in skeletal muscle lipolysis causing fatty acid and glycerol release to the circulation (240). Results from these studies suggest that IL-6 can induce whole body lipolysis and fat oxidation without causing hypertriacylglycerolemia (238). Interestingly, blocking of IL-6 in diseases like rheumatoid arthritis and Castleman's disease have been found to result in dyslipidemia (241-243), but how the relation is in a normal population is not known (244).

Inflammatory markers, fatness and fitness

In **Paper III** adiponectin was negatively and CRP positively related to S4SF, replicating the results of others (118;118;148-150;150;151;156;158;245). Interestingly, we only found a significant correlation between adiponectin and CRP and the clustered z-score in the group with S4SF values above the

median, which emphasizes that fatness is important for these associations. This observation, however, does still not tell whether adiposity is the cause, a mediator or a confounder in the mechanistic chain. Paradoxically, fitness was also negatively correlated to adiponectin after adjusting for fatness, which was also found in a study by Nemet and colleagues (158). In contrast, Rubin and colleagues found a positive correlation (152). CRP was not significantly correlated to VO_{2peak} after adjusting for S4SF. Other studies have found negative correlations between VO_{2peak} and CRP in youth (156;157;245;246). Two of these studies find, as we do, that the correlation was no longer significant after adjusting for fatness (157;246). However, this does not necessarily eliminate fitness as an important factor, because fatness could be an intermediate between fitness and low-grade inflammation, and adjusting for fatness could therefore be misleading.

No correlations between fatness and IL-6 or TNFα were found. The correlation between the clustered z-score and TNFα was strongest in the group with S4SF values below the median, but did not reach statistical significance in either group. These results indicate that the relationship between CVD risk and TNFα and possibly also IL-6, might be independent of adiposity in youth.

While no correlation was found for TNFα, IL-6 was negatively related to fitness, whereas previously studies in youth did not find any relationship (156;158). The circulating level of IL-6 can be affected both chronically by release from the adipose tissue in overweight, but also acutely by release from muscle cells during and after bouts of exercise (88). In CoSCIS blood samples were taken first thing in the morning, which means that they were probably not affected by any acute exercise bouts, but more likely reflected the level of body fatness. Body fatness is also strongly correlated to cardiorespiratory fitness expressed in milliliters VO_2 per kilogram (247).

Neither of the anti-inflammatory markers IL-1Ra and sTNFR1 were correlated to fatness or fitness.

In **Paper IV** adjusting for fitness, fatness or fitness and fatness combined did not change the results substantially.

Final remarks on inflammatory markers

The complex mechanisms leading to impaired metabolism and the possible roles of the inflammatory markers are still under investigation. Cross-sectional, our results in **Paper III** showed a significant correlation between the clustered z-score and circulating levels of CRP, TNFα and IL-6, and an inverse correlation with adiponectin, whereas no correlations were found for IL-1Ra and sTNFR1. For CRP,

adiponectin and IL-6 the correlations to clustered z-score were strongest in adolescents above the median for fatness, whereas the opposite was found for TNFα. However, as previously mentioned, it should be emphasized that this does not tell us anything about causality, as fatness theoretically could be the cause, a mediator or a confounder in the association between CVD risk and low grade inflammation. This is the first study to examine the relation between clustered z-score and the anti-inflammatory markers IL-1Ra and sTNFR in youth, and interestingly, in contrast to what is seen in a number of inflammatory diseases, up-regulation of pro-inflammatory signals was not accompanied by an increase in these anti-inflammatory molecules.

VO_{2peak} was negatively correlated to clustered z-score, adiponectin and IL-6 levels. S4SF was positively correlated to clustered z-score and CRP levels and negatively to adiponectin levels, which implies that life style modifications that alter fitness level and body composition can positively influence the level of at least some markers of low grade inflammation and maybe, thereby, the metabolic risk profile in adolescents.

Longitudinally, the results in **Paper IV** show that IL-6 levels in childhood tracked into adolescence, especially for girls. Further, IL-6 levels in childhood were related to HOMA-IR and TG four years later for girls, but not for boys, whereas IL-6 levels in childhood were related to total cholesterol level for boys, but not girls. These relationships remained significant after adjusting for fatness and fitness. To our knowledge this is the first study to examine the longitudinal relation between IL-6 and HOMA-IR in a normal pediatric population.

4.4 Study strengths and limitations

Study strengths

The main strengths of CoSCIS were the length of the school-based intervention, the relatively large study population and the inclusion of both a post-intervention and a 4-year follow-up measurement. Also the use of accurate methods of measuring PA, VO_{2peak} and CVD risk factors is a strength. In **Papers I-III**, a continuous composite risk score for assessing CVD risk was used. As stated earlier, this type of outcome may be a better indicator of health in apparently healthy children and adolescents, compared to the individual risk factors. Furthermore, a clustered score can to some extent compensate for the day-to-day fluctuations in the single risk factors. **Paper I** included PA variables consisting of

accelerometer data adjusted for water-based activities. Even though this is not an optimal measure, it is probably at the time being the best objective measure of PA in epidemiological studies. Limitations of this approach will be discussed below.

In **Paper III** several markers of low-grade inflammation were included; both the highly studied CRP and adiponectin, the primarily pro-inflammatory cytokine TNFα, the pro- and anti-inflammatory cytokine IL-6 and finally the anti-inflammatory natural blockers of pro-inflammatory cytokines, sTNFRI and IL-1Ra. The two last mentioned have not been included in this type of analysis beforehand.

Study limitations

Study design and participants

One limitation of the study was the quasi-experimental study design (**Paper 1**). A randomized controlled study design would have been stronger, but was, because of practical issues, not a possibility at the time the study was started. However, the IG and CG were matched on socio-demographic factors, which eliminate some of the potential confounders. In addition, we did not have a stringent control with the content of the extra PE sessions. Even though lack of stringent control over intervention components is a limitation in our study, it could be stated that our results reflects the real-life school system. The results are therefore more generalizable and easy to apply on a population basis than a researcher led program.

Another factor influencing our results in **Paper I** was the drop-out. We found that the children who dropped out were heavier and had poorer VO_{2peak} than those retained. This was found in both IG and CG. Since the fattest and most unfit children are the ones most expected to gain from an intervention like CoSCIS, this could theoretically cause an underestimation of the true intervention effect. Also, a dropout rate similar to this study is very common and difficult to avoid in long-term, large-scale school-based interventions. Furthermore, the amount of missing data was larger for some variables, namely blood variables, cardio respiratory fitness and PA due to the unpleasant and comprehensive nature of these measurements. This could have limited our power to detect differences between groups in these variables. The same is the case for the subgroup analyses where the number of subjects became very small. However, by looking at the absolute values of differences between groups the conclusions are the same as for the statistical significance level.

A general 'weakness' of all included papers is the relatively lean and healthy population in CoSCIS. This could potentially limit the generalizability of the results, especially to populations with a higher prevalence of overweight and impaired metabolism. However, as mentioned, it has previously been found that CVD risk factors cluster in some children already from the age of 9 years in this cohort (125), which implies that some children have an impaired metabolic risk profile. Finally, although many of the analyses were adjusted for several confounding factors including age, sex and sexual maturity, unmeasured confounding factors such as genetic variation, energy intake and patterns, smoking, family history of CVD and other socio-cultural factors could possibly explain some of our observations. A weakness of **paper III** is the cross-sectional study design, which does not allow for any conclusions on causality.

Methods and measurements

The use of accelerometers in **Paper I** is limited by the inability to capture cycling and load-bearing activities. However, this potential bias is probably equal in intervention and control group and should therefore not influence the results. The accelerometer data do not capture swimming either, but were adjusted for water-based activity information collected by questionnaires. The reason for this adjustment was the fact that part of the intervention was delivered as swimming lessons. It is, however, not very accurate to consider all water-based activities equal in intensity. Furthermore, the choice of substituting these blocks of non-wear with blocks of an average intensity of a normal PE lesson is conjecture, but our best guess. This approach is conservative, as the physical activity level during water activities probably is higher, but this could at worst underestimate the PA level in the intervention group. Finally, the accumulation of physical activity over three to four days may not be fully representative of this complex behavior.

A weakness with the cardiorespiratory exercise test **(Papers I–IV)** is the use of arbitrary criteria to define maximum effort. However, mean maximal HR during the VO_{2peak} tests were 196, 203 and 201 beats per minute at baseline, post-intervention and follow-up, respectively. We are therefore confident that the subjects in the present study were exhausted and that 'true' VO_{2peak} values are reported. Furthermore, two different VO_2-analysers were used; the AMIS 2001 Cardiopulmonary Function Test System (Innovision, DK 5260 Odense) at baseline and at post-intervention and the COSMED K4b^2 portable metabolic system (COSMED, Rome, IT) at follow-up. We were unfortunately unable to cross

validate the two systems, but both have been validated against the Douglas bag method and were found to obtain valid measures of VO_2 (191;192). Moreover, eventual bias caused by the use of two different systems, is equal in IG and CG (**Paper I**) and absolute values are not essential for the results of **Paper II** and **IV**. Other measures of fitness than the cardiorespiratory could have been included. Muscle fitness has been increasingly recognized in the prevention of chronic disease in adults (248) and features of the metabolic syndrome. Consequently, inclusion of resistance training as part of an exercise program for promoting health in adults has been endorsed by several organizations (249). Therefore, new school-based intervention studies could with advantage include measures of muscle fitness in the test battery.

All papers include HOMA-IR as a measure of insulin resistance. The 'gold standard' for measuring insulin resistance is the euglycaemic, hyperinsulinaemic clamp technique (250). However, this method is time-consuming, more invasive than only one blood sample, costly and labour intensive, which makes this method more difficult to use in large-scale studies and normally not used in studies of children. HOMA-IR has been validated against the clamp method in youth and was found to function well as a surrogate measure (251). Furthermore, a study by Andersen and colleagues showed that HOMA-IR actually was a better predictor of clustered CVD risk factors than the 2-hours oral glucose tolerance test and fasting levels of insulin and glucose (252).

In **paper III** a wide range of inflammatory markers were analyzed, but other markers such as retinol binding protein 4, resistin, fibrinogen, plasminogen activator inhibitor-1 and brain-derived neurotrophic factor, could have been included. At the time of the analyses, with limited funding, the most promising markers found in the literature were chosen after discussion in the study group. **Paper IV** seriously lacks the inclusion of other inflammatory markers, especially TNFα and TNF modifying mediators. We cannot exclude that these markers, which are related to both IL-6 and insulin resistance, are confounding the investigated associations in **Paper IV**. Therefore, no conclusions regarding the mechanisms by which low-grade inflammation affects insulin resistance can be drawn. Furthermore, a possible bias in both **Papers III** and **IV** is that although we excluded children with any signs of infections or fever, undetected subclinical infection could have caused an increase in the level of inflammatory markers in some children. We did, however, try to run all analyses omitting outliers, and that did not change our results substantially.

3. Conclusions

This study found that a three year school-based exercise intervention resulted in positive changes in systolic blood pressure and HOMA-IR in IG compared to CG, especially for the boys. We found an intervention effect on MVPA corresponding to a difference of ~ 12 min/day less decrease in IG compared to CG. This difference was no longer significant after adjusting for school-cluster. We did not find any effects on VO_{2peak} and measures of fatness and no differences were statistically significant after Bonferroni corrections. Therefore, our results indicate that interventions inducing small changes in PA, even without changes in fitness and fatness, can positively influence the health of children and adolescents. However, we mostly saw positive effects in boys and the improvements were relatively modest. Therefore, our data also suggest that a doubling of PE exposure and providing training and equipment may not be sufficient to induce major changes in CVD risk factors in normal populations, at least not when administered as two double lessons per week. Furthermore, if positive changes should be sustained, the increased amount of PE exposure should probably continue throughout the children's school-life.

In this population moderate to strong tracking was found for the clustered z-score from age 6 to age 9 years, 9 to 13 years and 6 to 13 years. This suggests that a high level in several CVD risk factors in early school-age is already predictive for the development of the clustering of CVD risk factors seen in the older age-groups. Tracking of clustered z-score differed between tertiles of fitness, with children in the lowest fitness group displaying the highest tracking coefficients. The picture was not as clear for tertiles of fatness.

Our results showed a significant correlation between the clustered z-score and circulating levels of CRP, TNFα and IL-6, and an inverse correlation with adiponectin, whereas no correlations were found for IL-1Ra and sTNFR1. For CRP, adiponectin and IL-6 the correlations to clustered z-score were strongest in adolescents with S4SF above the median, whereas the opposite was found for TNFα. IL-1Ra and sTNFR1 was not up-regulated by the pro-inflammatory markers as shown by a lack of inter-correlation between inflammatory and anti-inflammatory markers. VO_{2peak} was negatively correlated to

clustered z-score, adiponectin and IL-6 levels. S4SF was positively correlated to clustered z-score and CRP levels and negatively to adiponectin levels.

Finally, it was found that IL-6 levels in childhood track into adolescence, especially for girls. Further, IL-6 levels in childhood were related to HOMA-IR four years later for girls, but not for boys. These relationships remained significant after adjusting for fatness and fitness. However, we did not find any cross-sectional association between IL-6 and HOMA-IR at either age 9 or age 13 years, suggesting that in these age groups the level of IL-6 is not directly related to HOMA-IR.

4. Practical implications and future research

The intervention of Copenhagen School Child Intervention Study was proposed as a way to enhance and improve physical activity and thereby health in Danish children in public schools. The plan was to test the largest possible change in physical activity in schools, which was considered politically realistic to implement nationwide. Also, the magnitude and costs of the intervention elements in CoSCIS were realistic, and did not exceed what could be implemented on large scale populations. Positive results could therefore provide politicians with an idea of a relative simple way to improve health in school-aged children. Thus the results from CoSCIS are potentially important in future planning and implementing of public health strategies.

As the positive intervention effects in CoSCIS were modest, further research is needed in order to investigate how interventions should be conducted to obtain greater positive intervention results in rather healthy youth populations. Future PA intervention studies should specifically focus on the influence of duration, frequency, and intensity of the programs on the development in health in children. For this purpose studies with a stronger design and more comprehensive interventions compared to CoSCIS have now been started in Scandinavia. It should also be noted that in CoSCIS the intervention had no greater effect on subgroups of initially overweight and unfit children. How to target these groups should also be a focus in future studies.

The results regarding stability of clustering of CVD risk factors have important clinical implications, as they show stability of clustering of CVD risk factors across school-age and points to implementation of preventive strategies starting in early childhood. Furthermore, there is no reason to wait to take action, as it is probably easier to teach good habits in early childhood compared to in adolescence or adulthood.

Our results on inflammatory markers add significantly to the current knowledge regarding low-grade inflammation in youth. The correlations between the clustered z-score and IL-6 and TNFα had not been found in youth before. The same is the case for the lack of an up-regulation of the anti-inflammatory cytokines IL-1Ra and sTNFR1 with increased levels of pro-inflammatory markers. These results should therefore be verified on other cohorts. Also, the use of our longitudinal data on inflammatory markers is limited because only one marker was included. Data on the longitudinal relationships between pro-inflammatory and anti-inflammatory markers and the development of CVD risk factors are warranted.

Finally, the finding of relationships between inflammatory markers and VO_{2peak} and level of body fatness implies that life style modifications that alter fitness level and body composition can positively influence the level of markers of low grade inflammation and maybe, thereby, the metabolic risk profile in adolescents. However, well-designed intervention studies should verify this.

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Anna

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Paper I

Effects of a Three-Year Intervention: The Copenhagen School Child Intervention Study

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[1]Centre of Research in Childhood Health, Institute of Sport Science and Clinical Biomechanics, University of Southern Denmark, Odense M, DENMARK; [2]Unit of Clinical Physiology and Nuclear Medicine, Department of Clinical Sciences, Skåne University Hospital, Lund University, Lund, SWEDEN; [3]Department of Sports Medicine, Norwegian School of Sport Sciences, Oslo, NORWAY; and [4]Department of Exercise and Sport Science, University of North Carolina, Fetzer Gym, Chapel Hill, NC, USA

ABSTRACT

BUGGE, A., B. EL-NAAMAN, M. DENCKER, K. FROBERG, I. M. HOLME, R. G. MCMURRAY, and L. B. ANDERSEN. Effects of a Three-Year Intervention: The Copenhagen School Child Intervention Study. *Med. Sci. Sports Exerc.*, Vol. 44, No. 7, pp. 1310–1317, 2012. **Introduction**: This study assessed short-term and long-term effects of a 3-yr controlled school-based physical activity (PA) intervention on fatness, cardiorespiratory fitness ($\dot{V}O_{2peak}$) and CVD risk factors in children. **Methods**: The study involved 18 schools (10 intervention and 8 controls) and included a follow-up 4 yr after the end of intervention. The analyses included 696, 6- to 7-yr-old children at baseline, 612 postintervention (age 9.5 yr) and 441 at follow-up (age 13.4 yr). The intervention consisted of a doubling of the amount of physical education (PE; from 90 to 180 min·wk^{-1}), training of PE teachers, and upgrading of PE and playing facilities. Anthropometrics and systolic blood pressure (SBP) were measured. $\dot{V}O_{2peak}$ was directly measured, and PA was assessed using accelerometry. Fasting blood samples were analyzed for CVD risk factors. A composite risk score was computed from *z*-scores of SBP, triglycerides, total cholesterol-to-HDL cholesterol ratio, homeostatic model assessment (HOMA score), skinfolds, and inverse $\dot{V}O_{2peak}$. **Results**: The HOMA score of the intervention group boys had a smaller increase from baseline to postintervention compared with control boys ($P = 0.004$). From baseline to follow-up intervention group boys had a smaller increase in SBP compared with control boys ($P = 0.010$). There were no other significant differences between groups. **Conclusions**: This 3-yr school-based PA intervention caused positive changes in SBP and HOMA score in boys but not in girls, and no effects were seen in PA, $\dot{V}O_{2peak}$, fatness, and the other measured CVD risk factors. Our results indicate that a doubling of PE and providing training and equipment may not be sufficient to induce major improvements in CVD risk factors in a normal population. **Key Words:** SCHOOL BASED, PHYSICAL ACTIVITY, FITNESS, CVD RISK FACTORS, CHILDREN, ADOLESCENTS

EPIDEMIOLOGY

The prevalence of obesity and overweight is increasing in child populations throughout the world, affecting short- and long-term health (33). At the same time, it has been shown that physical activity (PA) and cardiorespiratory fitness ($\dot{V}O_{2peak}$) have decreased particularly in the low-fit pediatric population (25,36). Results from the European Youth Heart Study shows that CVD risk factors tend to cluster in overweight and inactive children and in children with low $\dot{V}O_{2peak}$ as young as the age of 9 yr (2). Although clinical manifestations of CVD do not normally appear before middle age, studies have shown that atherosclerosis in some individuals originates during childhood and adolescence (4). Taken together, the health consequences of these trends will be an upcoming adult population with a high prevalence of obesity and metabolic diseases, which will cause an enormous burden both at the individual and at the societal level.

PA has been suggested as an important area in prevention of both obesity and lifestyle diseases (10,15). Schools provide an advantageous setting for encouraging a healthy lifestyle among children and enhancing PA (13,16). The compulsory physical education (PE) in schools is the only setting that ensures PA for all children, including overweight, inactive, and unfit children, who are difficult to target by other means (16). Several school-based intervention studies addressing different health-related outcomes have been conducted with a large diversity of results on fatness, fitness, glucose metabolism, and lipids (5,7,12–14,18–21,28,30,32, 37–39). The challenge is to find an intervention that will provide effective means to improve the health of children and that will be possible to implement on large-scale populations.

The overall objective of the Copenhagen School Child Intervention Study (CoSCIS) was to evaluate the effect of

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a school-based PA intervention providing a doubling of time for PE, new didactic tools to the PE teachers, and an upgrading of sports and playing facilities in intervention schools on CVD risk factors. CoSCIS was the first large school-based PA intervention in Denmark and assessed health using both single risk factors and a composite risk score, which have been suggested as a good method to assess CVD risk level in apparently healthy children (2).

METHODS

CoSCIS is a controlled longitudinal intervention study that took place in 18 public schools (10 interventions and 8 controls) in two suburbs of Copenhagen. The local Authority of Ballerup had decided to upgrade the PA opportunities for their youngest schoolchildren and contacted the research group to quantify and measure the effect of such an intervention. The intervention was hereafter planned in cooperation between the intervention local authority and the researchers. Baseline measurements were carried out in 2001/2002, postintervention measurements were carried out in 2004/2005, and follow-up measurements were carried out in 2008. All tests were performed at the participating schools in a gym or a classroom except for the fitness test, which was performed using permanently installed equipment in a camper trailer outside the schools.

Participants. All children from 46 preschool classes (age 6–7 yr) in the schools in the two local authorities were invited to participate in the study. A total of 706 children (69%) volunteered to participate and 696 children actually participated at baseline. Written informed consent was obtained from the child's parent or legal guardian after being given a detailed written explanation of the aims of the study, any possible hazards, discomfort, and inconvenience, and the option to withdraw at any time. The study was approved by the ethics committee of the University of Copenhagen. There were no differences between participants and nonparticipants with respect to age, height, weight, and body mass index (BMI) for either sex measured 1 yr after the baseline measurements (9).

Intervention. The intervention and control schools were not randomly selected. In 2000, the intervention group (IG) was started in the local authority of Ballerup and involved all first- to third-grade classes. The program consisted of four constituent parts. First, an increase in the amount of PE lessons from 90 to 180 min·wk^{-1}, given as two double sessions each week. The extra PE session was administered by the normal PE teachers, and the content was not controlled or supervised by the researchers. Approximately one-third of the extra PE lessons were swim lessons. The goal of the PE sessions was to make fun activities with a high level of intensity and incorporating both strength and cardiovascular training. The final planning and execution of the lessons were done by the PE teachers; thus, the situation resembles "a real-world scenario." The teachers were asked to keep a monthly record of the content of the lessons, but the dropout was too big for the data to be used. Second, the children were given lessons in health education, focusing on the importance of PA and healthy eating. Third, the PE teachers received three to four full days a year of supplementary training focused on didactic tools to enhance the children's motivation for and enjoyment of PA and, at the same time, keeping the intensity in PE lessons moderate to vigorous. All PE teachers were obliged to participate in the teacher training program, and a record was kept to ensure participation. Fourth, indoor and outdoor PE and playing facilities were upgraded in all intervention schools.

Schools within Tårnby local authority were chosen as control group (CG) because this local area resembles the sociodemographic characteristics of the intervention authority. Control schools followed the normal curriculum of one double PE session per week (90 min·wk^{-1}) and had no teacher training or facility upgrading.

Measurements. All measurements were performed between 8:00 a.m. and 2:00 p.m. Blood draws were completed before 9:30 a.m. followed by breakfast. Accelerometer data were collected the week after the other measurements. Because the data collection took several months, testing was done in alternating weeks in intervention schools and control schools to account for growth, maturation, and seasonality in PA. Age was computed from date of birth, which was obtained from the national registry and verified by the parents. Height was measured without shoes to the nearest 1 mm using Harpenden stadiometer (West Sussex, UK). Body mass was measured in light clothing to the nearest 0.1 kg using an electronic scale (Seca 882, Brooklyn, NY). BMI was calculated (kg·m^{-2}), and BMI *z*-scores were computed based on World Health Organization recommendations (40). Biceps, triceps, subscapular, and suprailiac skinfolds were measured to the nearest millimeter, in triplicate, with Harpenden calipers (Baty International, West Sussex, UK). The mean of three measurements was used for the analysis. All skinfold measurements were taken on the self-reported nondominant side of the body by the same two skilled researchers. The sum of four skinfolds (S4SF) was calculated according to the method presented by Durnin and Rahaman (8) and used as an estimate of body fatness. Waist circumference was measured to the nearest millimeter with an anthropometric tape, midway between the lower rib margin and the iliac of the trunk. Sexual maturation was assessed postintervention and at follow-up by self-report using a scale of pictures of breast and genital development for girls and boys, respectively (35). Blood pressure was measured after 15 min of rest with a Dinamap XL vital signs blood pressure monitor (Critikron, Inc., Tampa, FL) using appropriately sized cuffs. Five measurements were taken during 10 min, and the mean of the last three measurements was recorded.

Cardiorespiratory fitness ($\dot{V}O_{2peak}$) was assessed using a protocol of continuous running on a treadmill. $\dot{V}O_{2peak}$ was measured directly on an AMIS 2001 Cardiopulmonary Function Test System (DK 5260; Innovision, Odense, Denmark) at baseline and postintervention and using the

COSMED K4b^2 portable metabolic system (COSMED, Rome, Italy) at follow-up. Both systems were calibrated immediately before each trial. We were unable to cross-validate the two systems, but both have been validated against the Douglas bag method and were found to obtain valid measures of $\dot{V}O_2$ (17,23). The children were instructed to run until exhaustion. To determine whether a test was performed satisfactorily, at least one of three objective physiological criteria should be fulfilled; HR > 200 bpm, RER ≥ 0.99, or a defined plateau of $\dot{V}O_2$ (an increase of <2.1 mL·min^{-1}·kg^{-1}) together with a subjective criteria for exercise intolerance (31). For children who ran to exhaustion but did not attain a valid measurement because of equipment failure, $\dot{V}O_{2peak}$ was estimated from a regression equation calculated from all the valid tests using running time to exhaustion and sex (~10% of participants). This method has been reported earlier (9).

PA was assessed using a combination of a questionnaire and the ActiGraph 7164 activity monitor at baseline and postintervention and the GTIM activity monitor at follow-up (ActiGraph, Inc., Pensacola, FL). The accelerometer was secured directly to the skin at the lower back using an elastic belt. The children were instructed to use the accelerometer for four consecutive days, two weekdays, and two weekend days. They wore the accelerometer during the entire day and only removed it, e.g., during water activities. A recording epoch of 10 s was selected for this study. All continuous sequences of 60 consecutive epochs (i.e., 10 min) or more with zero counts were considered as nonwearing and were subsequently deleted (29). Only children providing a minimum of 3 d with 8 h of valid recording, after removal of missing data, were included in this analysis. The PA questionnaire was distributed and returned with the accelerometers. Children and parents were, among other things, asked to note time and reason for any period of "nonwear." Accelerometer data were subsequently adjusted for water activities information obtained by the questionnaires. This was done by inserting blocks of activity corresponding to the mean counts per minute in normal PE lessons in all nonwear periods that could be identified as water activity. This was done because part of the intervention was delivered as swim lessons and would not be captured by accelerometers alone. For this article, we report the overall amount of PA (mean counts per minute) and minutes per day spent on moderate-to-vigorous PA (MVPA) (min·d^{-1} ≥ 1500 counts per minute) (27).

Blood samples were collected from the antecubital vein after a verified overnight fast. The children were asked what they had been eating and drinking from the night before, and only water and sugar-free chewing gum were allowed for a child to be accepted as fasting. Glucose was analyzed immediately after sampling (Hemocue). The remainder of the samples was centrifuged; plasma aliquoted within 30 min, kept at −20°C, and later stored at −80°C. Insulin was analyzed spectrophotometrically using an enzyme-linked immunosorbent assay (code no. K6219; DAKO Insulin). Total cholesterol (TC), HDL cholesterol, and triglyceride (TG) were analyzed on a COBAS FARA (Roche, Switzerland) using a spectrophotometer (ABX Diagnostics, Montpellier, France). Insulin resistance was estimated according to homeostasis model assessment (HOMA-IR) as glucose (mmol·L^{-1}) multiplied by insulin (mU·L^{-1}) divided by 22.5 (22).

Statistical analysis. SPSS version 15 was used for all analyses. Dropout analyses and analyses of missing data were done using independent-samples *t*-tests adjusting for sex comparing baseline BMI *z*-scores, waist circumference and $\dot{V}O_{2peak}$ between participants, and dropouts between children who gave blood versus those who did not.

Means and SD for physical characteristics and CVD risk factors at baseline, postintervention and at follow-up were calculated by sex and group. BMI, waist circumference, S4SF, HOMA-IR, TG, TC-to-HDL ratio, and sum of *z*-scores were positively skewed and therefore transformed (natural log) for the analyses. Because single risk factors tend to fluctuate on a day-to-day basis, we included a composite CVD risk score, which may provide a more valid overall risk assessment (1,2). We constructed the composite score from the sex-specific sum of *z*-scores for systolic blood pressure (SBP), TG, TC-to-HDL ratio, HOMA-IR, S4SF, and the negative value of $\dot{V}O_{2peak}$ *z*-score.

Differences between groups at baseline were analyzed using a general linear model adjusted for sex. Change scores were calculated by subtracting baseline values from postintervention and follow-up values, respectively. Differences between IG and CG in change scores from baseline to postintervention and from baseline to follow-up were analyzed using a general linear model adjusted for sex, maturation, and baseline level, if this differed between groups. The child's school at baseline was included in the analyses as a cluster option to account for any between-school variation. Group-by-sex interaction was tested, but where no interaction was found, the interaction term was removed from the final model. In case of significance on sex × group interaction, only the interaction *P* value was presented in the table. Then, follow-up analyses were done with split for sex and presented in the figures. A significance level of $P < 0.05$ was chosen. Because of multiple testing, *P* values were subsequently Bonferroni adjusted.

RESULTS

Characteristics of participants in IG and CG are presented by sex in Table 1. At baseline, children in IG were significantly older and had a significantly higher SBP compared with children in CG ($P = 0.022$ and 0.016, respectively). There were no other significant group differences at baseline.

Table 2 presents the immediate and long-term effect of the intervention. The immediate effect is the difference between IG and CG change scores from baseline to postintervention. IG had a borderline smaller increase in SBP compared with CG ($P = 0.092$). There was a significant sex × group interaction for change scores of HOMA-IR (Table 2). IG boys had a significantly smaller increase in

TABLE 1. Physical characteristics of participants by sex and group (IG and CG): at baseline, Postintervention at follow-up.

			Baseline		Postintervention		Follow-Up	
Variables	**Group**	**Sex**	***n***	**Mean ± SD**	**n**	**Mean ± SD**	**n**	**Mean ± SD**
Age (yr)*	IG	Boys	222	6.8 ± 0.4	198	9.6 ± 0.4	143	13.4 ± 0.4
		Girls	186	6.7 ± 0.4	164	9.5 ± 0.4	117	13.3 ± 0.3
	CG	Boys	142	6.8 ± 0.3	121	9.6 ± 0.3	84	13.3 ± 0.3
		Girls	144	6.6 ± 0.4	130	9.4 ± 0.4	97	13.3 ± 0.3
BMI ($kg \cdot m^{-2}$)	IG	Boys	222	16.0 ± 1.7	197	17.2 ± 2.3	143	19.1 ± 2.6
		Girls	184	16.1 ± 1.9	164	17.5 ± 2.7	117	19.4 ± 2.9
	CG	Boys	141	16.1 ± 1.6	121	17.3 ± 2.4	83	18.9 ± 2.7
		Girls	144	16.0 ± 1.9	130	17.0 ± 2.4	96	18.9 ± 2.5
zBMI for age	IG	Boys	222	0.24 ± 0.98	197	0.31 ± 1.03	143	0.04 ± 0.99
		Girls	184	0.30 ± 0.96	164	0.31 ± 1.11	117	−0.03 ± 1.07
	CG	Boys	141	0.34 ± 0.96	121	0.38 ± 1.09	83	0.00 ± 1.03
		Girls	144	0.26 ± 1.02	130	0.18 ± 1.02	96	−0.19 ± 1.05
Sum of four skinfolds (mm)	IG	Boys	220	24.3 ± 9.6	197	29.9 ± 14.7	143	30.3 ± 14.5
		Girls	186	28.4 ± 9.6	163	37.9 ± 17.3	116	36.5 ± 17.9
	CG	Boys	140	24.5 ± 8.1	121	30.3 ± 14.5	82	31.9 ± 19.0
		Girls	139	30.0 ± 11.4	130	36.5 ± 17.9	96	36.6 ± 14.6
Waist (cm)	IG	Boys	222	55.8 ± 5.3	197	62.6 ± 6.6	143	68.5 ± 6.5
		Girls	185	56.0 ± 5.5	164	62.2 ± 7.5	116	66.3 ± 6.0
	CG	Boys	142	55.5 ± 4.5	120	62.7 ± 7.3	83	68.2 ± 7.1
		Girls	142	55.2 ± 5.5	130	61.4 ± 6.7	96	65.2 ± 5.2
Mean PA (counts per minute)	IG	Boys	186	777.6 ± 210.5	157	733.6 ± 193.4	92	519.5 ± 143.1
		Girls	148	692.4 ± 169.7	132	655.3 ± 171.9	83	443.4 ± 125.0
	CG	Boys	106	775.3 ± 237.9	87	706.1 ± 172.2	57	533.8 ± 168.7
		Girls	119	721.4 ± 155.0	99	650.5 ± 162.7	68	460.3 ± 178.3
MVPA ($min \cdot d^{-1}$ >1500 counts per min)	IG	Boys	186	123.1 ± 34.8	157	123.9 ± 32.4	92	90.5 ± 26.7
		Girls	148	108.8 ± 28.0	161	108.8 ± 32.2	83	72.2 ± 23.1
	CG	Boys	119	125.0 ± 37.9	87	112.3 ± 26.7	57	91.0 ± 33.0
		Girls	106	111.0 ± 27.9	99	101.3 ± 26.2	68	73.3 ± 28.7
Cardiorespiratory fitness ($\dot{V}O_{2peak}$) ($mL \cdot kg^{-1} \cdot min^{-1}$)	IG	Boys	208	48.6 ± 6.0	192	52.3 ± 6.6	135	53.2 ± 8.1
		Girls	176	44.6 ± 5.3	161	45.9 ± 5.5	111	44.6 ± 6.3
	CG	Boys	129	48.2 ± 5.8	117	51.7 ± 6.6	73	52.5 ± 8.4
		Girls	133	45.2 ± 5.4	124	46.2 ± 6.5	91	45.5 ± 7.3
SBP (mm Hg)*	IG	Boys	220	99.2 ± 0.4	197	105.2 ± 8.7	143	111.3 ± 9.4
		Girls	186	97.9 ± 0.4	162	103.0 ± 9.3	116	109.8 ± 8.7
	CG	Boys	142	97.3 ± 7.0	121	104.4 ± 8.4	83	112.3 ± 8.3
		Girls	143	96.9 ± 7.5	130	102.6 ± 8.3	97	107.8 ± 7.0
Insulin resistance (HOMA-IR)	IG	Boys	163	0.77 ± 0.82	146	1.15 ± 0.78	134	2.74 ± 1.49
		Girls	134	0.77 ± 0.46	118	1.44 ± 0.93	98	3.05 ± 1.86
	CG	Boys	95	0.78 ± 0.53	86	1.40 ± 0.83	70	2.43 ± 1.59
		Girls	87	0.83 ± 0.56	88	1.33 ± 0.67	85	2.86 ± 1.17
TC/HDL ratio	IG	Boys	164	3.07 ± 0.60	149	2.51 ± 0.59	135	2.94 ± 0.65
		Girls	136	3.12 ± 0.64	122	2.69 ± 0.66	98	2.92 ± 0.68
	CG	Boys	94	3.02 ± 0.54	87	2.51 ± 0.44	70	2.91 ± 0.62
		Girls	88	3.19 ± 0.56	90	2.76 ± 1.74	85	3.09 ± 1.58
TG ($mmol \cdot L^{-1}$)	IG	Boys	162	0.57 ± 0.20	149	0.53 ± 0.26	135	0.81 ± 0.40
		Girls	134	0.62 ± 0.31	122	0.56 ± 0.23	98	0.78 ± 0.26
	CG	Boys	93	0.57 ± 0.24	87	0.56 ± 0.29	70	0.71 ± 0.27
		Girls	88	0.60 ± 0.26	90	0.57 ± 0.24	85	0.78 ± 0.36
Composite *z*-scores	IG	Boys	149	−0.13 ± 2.81	142	0.12 ± 4.76	127	5.14 ± 5.51
		Girls	124	−0.18 ± 3.26	115	1.36 ± 5.23	92	7.31 ± 5.61
	CG	Boys	84	−0.22 ± 2.79	82	0.72 ± 4.83	60	4.83 ± 6.87
		Girls	78	−0.43 ± 2.83	86	1.02 ± 5.70	79	6.31 ± 5.76

* Difference between groups at baseline ($P < 0.05$).

HOMA-IR compared to CG boys ($P = 0.004$), whereas no difference between groups was found for girls (Fig. 1). There were no other significant group differences in change scores from baseline to postintervention. Long-term effects of the intervention were measured comparing IG and CG change scores from baseline to follow-up. There was a significant sex × group interaction for SBP (Table 2). The change scores of SBP were significantly lower for IG boys compared to CG boys ($P = 0.010$), but this was not found for girls (Fig. 2). There were no other significant differences in change scores from baseline to follow-up. After Bonferroni correction, no differences were found between groups.

Dropout and missing data. At baseline, 696 children participated, 613 participated postintervention (≈88%) and 441 participated at follow-up (≈63%). Most dropouts were caused by families moving away from the school districts. There were no significant differences at baseline between children who participated postintervention and children who dropped out. Children who participated at follow-up had significantly lower baseline BMI *z*-scores (−0.2) and waist circumference (−2.9 cm) and a higher baseline $\dot{V}O_{2peak}$ ($1.7\ mL \cdot kg^{-1}$) compared to children who dropped out ($P > 0.006$) in both groups.

Blood was obtained from ≈69% of the children participating at baseline, 74.4% of the children participating postintervention and 87.8% of the children participating at follow-up. At baseline, children without blood samples had higher waist circumference (~1 cm) compared to children

TABLE 2. Change scores (mean ± SD) and adjusted differences between IG and CG.

		Change Baseline to Postintervention				Change Baseline to Follow-up			
Variables	Group	n	Change Scores ± SD	Adjusted Difference (95% CI)	*P*	n	Change Scores ± SD	Adjusted Difference (95% CI)	*P*
BMI ($kg \cdot m^{-2}$)	IG	355	1.31 ± 1.23			257	3.40 ± 1.94		
	CG	247	1.15 ± 1.20	0.12 (−0.38 to 0.13)	0.322	176	3.07 ± 1.78	***0.34 (−0.70 to 0.01)***	***0.057***
z_{BMI} for age	IG	355	0.044 ± 0.50			257	−0.21 ± 0.53		
	CG	247	−0.006 ± 0.54	0.032 (−0.14 to 0.08)	0.551	176	−0.24 ± 0.56	0.027 (−0.16 to 0.11)	0.678
Sum of four skinfolds (mm)	IG	354	7.52 ± 9.71			255	10.20 ± 13.34		
	CG	243	6.00 ± 9.30	1.48 (−3.64 to 0.68)	0.167	173	8.38 ± 13.13	1.90 (−6.92 – 3.12)	0.436
Waist (cm)	IG	356	6.65 ± 4.63			257	12.19 ± 5.40		
	CG	245	6.64 ± 5.00	0.10 (−1.27 to 1.47)	0.881	177	11.81 ± 4.81	0.21 (−1.39 – 0.98)	0.719
Mean PA (counts per minute)	IG	245	−61 ± 230			157	−257 ± 209		
	CG	152	−77 ± 229	19 (−105 to 67)	0.508	108	−257 ± 242	1.2 (−82 – 78)	0.975
MVPA ($min \cdot d^{-1}$ >1500 counts per minute)	IG	245	−0.4 ± 38.7			157	−35.2 ± 34.8		
	CG	152	−11.2 ± 36.7	11.8 (−30.1 to 6.5)	0.192	108	−37.6 ± 41.9	1.6 (−16.3 – 13.1)	0.816
Cardiorespiratory fitness ($\dot{V}O_{2peak}$) ($mL \cdot kg^{-1} \cdot min^{-1}$)	IG	328	2.64 ± 5.93			231	1.95 ± 8.74		
	CG	224	2.04 ± 6.88	0.46 (−3.29 to 2.37)	0.738	149	1.02 ± 8.24	0.56 (−3.56 to 2.44)	0.697
SBP (mm Hg)	IG	355	5.34 ± 8.20			257	11.97 ± 9.20		
	CG	247	6.75 ± 8.14	***−1.60 (−0.29 to 3.48)***	***0.092***	177	13.53 ± 8.86	−1.8 (−3.5− −0.1)	*0.038
Insulin resistance (HOMA-IR)	IG	226	0.47 ± 0.78			193	2.12 ± 1.64		
	CG	138	0.60 ± 0.85	−0.142 (−0.31 to 0.03)	*0.010	105	1.91 ± 1.45	0.24 (−0.74 to 0.26)	0.329
TC/HDL ratio	IG	233	−0.54 ± 0.70			196	−0.14 ± 0.76		
	CG	137	−0.58 ± 0.58	0.026 (−0.25 to 0.20)	0.809	107	−0.21 ± 0.65	0.060 (−0.31 to 0.20)	0.633
TG ($mmol \cdot L^{-1}$)	IG	229	−0.062 ± 0.33			193	0.20 ± 0.40		
	CG	137	−0.025 ± 0.30	−0.043 (−0.057 to 0.14)	0.377	107	0.14 ± 0.41	0.06 (−0.20 to 0.07)	0.348
Sum of *z*-scores	IG	203	0.67 ± 3.91			165	6.24 ± 5.45		
	CG	121	0.80 ± 3.70	−0.29 (−1.11 to 1.69)	0.667	88	5.98 ± 5.89	0.48 (−3.15 to 2.19)	0.708

Change scores from baseline to postintervention and from baseline to follow-up presented by group (mean and SD). The adjusted difference between groups in change scores is the net effect of the intervention: IG change scores minus CG change scores adjusted for sex and maturation (mean and 95% confidence intervals (CI)). Borderline significant differences between IG and CG change scores are marked with bold and italic.

* Significant group × sex interaction *P* value presented. Analyses done with split by sex are presented in Figures 1 and 2.

who gave blood ($P = 0.025$). postintervention and at follow-up, children without blood samples had lower $\dot{V}O_{2peak}$ compared to children who gave blood (−2.6 and −2.7 $mL \cdot kg^{-1}$, respectively; both $P > 0.04$). There were no other significant differences between children who gave versus those who did not.

DISCUSSION

CoSCIS was proposed as a way to enhance and improve PA and thereby health in Danish children in public schools. The plan was to test the largest possible change in PA in schools, which we considered politically realistic to implement nationwide. Also, the magnitude and costs of the intervention elements in CoSCIS were realistic and did not exceed what could be implemented on large-scale populations. Positive results could therefore provide politicians with an idea of a relatively simple way to improve health in school-age children. Thus, results from the CoSCIS are potentially important in planning and implementing public health strategies.

Surprisingly, doubling the amount of PE had no significant effect on overall PA levels in IG. One possible explanation is that IG children compensated for the increased school-time PA during the remainder of the day. It is also notable that the amount of MVPA at baseline was quite high in this population (both in the IG and CG). The increase in PA in an already-active population may not induce sufficiently large, measurable metabolic changes. Conjecture suggests that, if the children were less active, there may have been a greater effect of the intervention.

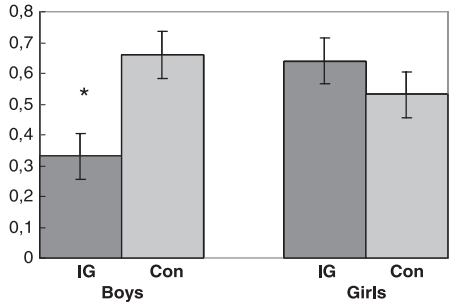

FIGURE 1—HOMA-IR change scores (Δ) from baseline to postintervention for IG and CG presented by sex. *P = 0.004 IG boys versus CG boys. No significant difference was found for girls. Only change scores from baseline to postintervention is presented because an interaction between group and sex was found in this analysis (Table 2).

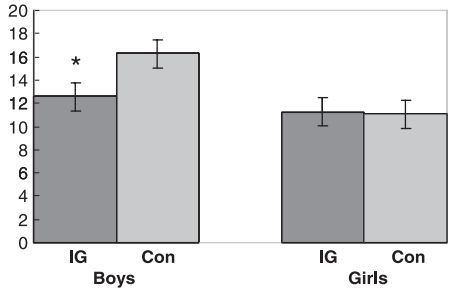

FIGURE 2—Systolic blood pressure change scores (Δ) from baseline to follow-up for IG and CG presented by sex. *P = 0.010 IG boys versus CG boys. No significant difference was found for girls. Only change scores from baseline to follow-up is presented because an interaction between group and sex was found in this analysis (Table 2).

However, although not statistically significant, the IG did-decrease their MVPA less compared to CG from baseline to postintervention the difference amounted to ~12 min·d^{-1}. Because MVPA is related to CVD risk factors (1,10), this modest, 12-min difference could explain some of the small positive metabolic changes we found in this study. The lack of statistically significant difference could have at least two contributory causes. First, although the use of accelerometers and questionnaires to assess PA is the best available method at this time, these kinds of data have a large variation because of measurement error, noncompliance with wearing, and the device's inability to measure some activities, such as swimming, which was part of the PE intervention. Second, dropout rate on this variable was also considerably larger than many of the other variables because of the strict inclusion criteria.

We did not find any positive intervention effect on $\dot{V}O_{2peak}$. Researchers have suggested that, to improve the $\dot{V}O_{2peak}$ of children, the focus has to be on high-intensity exercise with three to five training sessions per week (31,34). Most population-based long-term intervention studies with PE intervention comparable to CoSCIS fail to achieve improvements in $\dot{V}O_{2peak}$ (7,20), which emphasizes the difficulty in implementing sufficiently intense large-scale interventions to meet these criteria. Others do, however, find an effect on fitness (19,28,37). The intervention in the study by Kriemler et al. (19) consisted of two extra PE lessons of 45 min, giving a total number of five weekly PE lessons (225 min·wk^{-1}), several short PA breaks during the day, plus 10 min of exercise homework for 9 months. They found a significantly greater improvement in fitness in the IG compared to the CG. In addition, Resaland et al. (28) found a significant intervention effect on $\dot{V}O_{2peak}$ after a 2-yr school-based PA intervention. Their intervention consisted of 60-min daily PA lessons (300 min·wk^{-1}), with focus on keeping the intensity moderate to vigorous. This was compared to a CG receiving normal curriculum of 45 min of PE twice a week (90 min·wk^{-1}). These studies suggest that it is possible to improve fitness in normal pediatric populations but that intensity, frequency, and duration must be considered. The intervention in the present study was focused on increasing the overall PA levels in PE classes and on enhancing the motivation for and enjoyment of PA. Obvious differences between the interventions in CoSCIS and the studies of Kriemler et al. and Resaland et al. are the number of minutes of PE/PA lessons (180 vs 225 and 300 min) and the distribution throughout the week (two times per week vs five times per week). In addition, if the 90 min of extra PA we provided is divided by seven weekdays, the number of extra minutes expected is ~12 min·d^{-1}, whereas the other studies mentioned above would have proven >25 min·d^{-1} of activity. These results suggest that, in an already-fit group, 12 min·d^{-1} may not be sufficient to cause a change.

There were no positive intervention effects on any measure of body composition. CoSCIS was not specifically focused on overweight and obesity prevention. Some school-based interventions have had an effect on overweight and obesity outcomes (12–14), whereas most large-scale studies do not find any effects (5,18,20,32). An extensive review on studies addressing obesity prevention programs in children concluded that most studies show limited success in preventing childhood obesity (11). This emphasizes the difficulty in preventing overweight and obesity even in interventions with this specific aim.

Although we did not find any significant group differences in measurements of fatness, PA, or $\dot{V}O_{2peak}$, we did find indicators for positive intervention effects on some CVD risk factors, especially for boys. We found a borderline smaller increase in SBP from baseline to postintervention compared to controls. This difference was significant at the 4-yr follow-up but only for boys. Strong et al. (34) reviewed more than 850 studies on exercise in youth and found four studies showing an effect on blood pressure in children with elevated blood pressure but no effect on children with normal blood pressure. However, some studies have found short-term improvements in blood pressure after exercise interventions in children (24). These results, together with our results, suggest that it is possible to generate positive alterations on blood pressure in both normotensive and hypertensive children.

A smaller increase in HOMA-IR from baseline to postintervention was observed for the boys in the IG compared to CG, but this difference did not persist to follow-up. Previous studies have reported positive intervention effects on glucose and/or insulin levels (12,30,37), whereas others have not found an effect on these variables (7). The newer large-scale intervention study, The HEALTHY Study, found that the improvement in insulin levels in intervention schools compared to control schools was accompanied by improvements in measures of fatness (12). However, other studies on overweight and obese children have found improvements in indices of insulin sensitivity, independent of changes in body composition (3,26). These results, together with our results, suggest that it is possible to make favorable changes in insulin sensitivity by targeting PA, even without changes in fatness or fitness. It is possible that the better maintenance of mean PA and MVPA in IG compared to CG, although not statistically significant, could have mediated these changes. We can only speculate about why we are finding better results forthe boys compared to the girls. One possible explanation for the sex differences in the intervention effect could be the slightly greater increase in all body fatness measures in girls in IG compared to CG girls, which was not found for the boys. A larger fat mass is associated with insulin resistance and maybe any effect of the PA intervention on this variable is superseded by the gain in fat mass seen in the girls.

We did not find any intervention effects on blood lipids, similar to the results from most (20,39) but not all other intervention studies in children (21). The review by Strong et al. (34) found evidence for a weak relationship between PA and blood lipids, especially for HDL-C and TG. They concluded that there seems to be a minimum threshold of 40 min·d^{-1} of activity 5 d·wk^{-1} (total 200 min·wk^{-1}) to

achieve an improvement in blood lipid profile. The PE intervention of CoSCIS (180 min·wk^{-1} given in two sessions) did not meet this threshold, and this may be one reason why we did not find any effect on blood lipids.

To the best of our knowledge, only one other school-based intervention study has analyzed the effect on a clustered risk score consisting of *z*-scores of waist circumference, mean blood pressure, glucose, TG, and inverted HDL cholesterol (19). They found a significantly larger decrease in the cardiovascular risk score in the IG compared to the CG, which is in contrast to our findings. Results from the European Youth Heart Study shows that both low cardiorespiratory fitness and high levels of body fat are strong predictors for clustering of CVD risk factors in children (2). The fact that we did not find an effect of the intervention on the sum of *z*-scores may be caused by the lack of intervention results on these variables. In support of this, the study by Kriemler et al. (19) also found positive intervention results in measures of fatness and $\dot{V}O_{2peak}$.

The main strengths of our study were the length of the intervention, the inclusion of a 4-yr follow-up post-intervention and the use of accurate methods of measuring PA, $\dot{V}O_{2peak}$, and CVD risk factors. One of the limitations of the study was the quasi-experimental study design. A randomized controlled design would have been stronger but was not practically a possibility at the time the study was started. However, we did attempt to match IG and CG on sociodemographic characteristics, which could be considered a strength. In addition, we did not have a stringent control with the content of the extra PE sessions. For example, we did not examine the amount of time actually spent on PA in the sessions or the individual student participation rate. We do, however, know that the students received the extra PE sessions because PE is mandatory in Denmark. Furthermore, in the youngest classes, the children are not allowed to leave the school during the day, and all children present at a given day are attending PE. The observed (but nonsignificant) difference in MVPA ($\sim$12 min·d^{-1}) between IG and CG could also indirectly shows that the intervention was delivered as intended. Although the lack of stringent control over intervention components is a limitation of our study, it could be stated that our results reflect the real-life school system. The results are therefore more generalizable and easy to apply on a population basis.

Another factor influencing our results was the dropout rate. We found that the children who dropped out were heavier and had poorer $\dot{V}O_{2peak}$ than those who remained. This observation was found in both IG and CG. Because the fattest and most unfit children are the ones most expected to gain from an intervention like CoSCIS, this could theoretically cause an underestimation of the true intervention effect. Also, a dropout rate similar to this study is very common and difficult to avoid in large-scale school-based interventions. The amount of missing data was larger for some variables, namely, blood variables, cardiorespiratory fitness, and PA owing to the unpleasant and comprehensive nature of these measurements. This could have limited our power to detect differences between groups in these variables. Finally, a general problem with school-based interventions is that they are not sustained during vacation. A study on overweight American children showed that all improvements in $\dot{V}O_{2peak}$, percentage body fat, and insulin concentration gained in a 9-month intervention were lost during summer vacation (6).

This is the first Danish large-scale school-based PA intervention using the best-available methods in measuring PA, $\dot{V}O_{2peak}$, and CVD risk factors. Other school-based intervention studies providing more substantial effects on CVD risk factors and $\dot{V}O_{2peak}$ have conducted an intervention more focused on high intensity and had a greater overall amount of PA (e.g., Foster et al. [12] and Katz et al. [28]). However, these interventions could be difficult to implement on a population basis because of the large time and resource expenditure, and further research is needed to investigate how interventions should be conducted to obtain positive intervention results in rather healthy youth populations. Future PA intervention studies should specifically focus on the influence of duration, frequency, and intensity of the programs on the development in health in children. For this purpose, studies with a stronger design and more comprehensive interventions compared to CoSCIS have now been started in Scandinavia.

CONCLUSIONS

This study found that a 3-yr school-based exercise intervention resulted in positive changes in SBP and HOMA score in the IG compared with the CG, especially for the boys. We did not find any effects on PA, $\dot{V}O_{2peak}$, and fatness, and no differences were statistically significant after Bonferroni corrections. Therefore, our results indicate that a doubling of PE exposure and providing training and equipment may not be sufficient to induce major changes in CVD risk factors in healthy populations, at least not when administered as two double lessons per week. Furthermore, if positive changes should be sustained, the increased amount of PE exposure should probably continue throughout the children's school-life.

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The authors declare that they have no competing interests.

The results of the present study do not constitute endorsement by the American College of Sports Medicine.

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Paper II

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Clustered cardiovascular disease risk factors track from childhood to adolescence

Running title: Tracking of clustered CVD risk in youth

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Abstract

Clustering of cardiovascular disease (CVD) risk factors has been found in children as young as 9 years of age. However, the stability of this clustering over the course of childhood has yet to be determined. The purpose of this study was to determine tracking of clustered CVD risk from young school-age through adolescence and examine differences in tracking between levels of overweight/obesity and cardiorespiratory fitness. Six year-old children (n = 434) were measured three times in 7 years. Anthropometrics, blood pressure and VO_{2peak} were measured. Fasting blood samples were analyzed for traditional CVD risk factors. A clustered risk-score (z-score) was constructed by adding sex-specific z-scores for systolic blood pressure, homeostatic model assessment (HOMA-IR), triglyceride, skinfolds and inverse values of HDL-cholesterol and VO_{2peak}. Significant tracking coefficients were found between clustered z-score at all time intervals (r = 0.514, 0.559 and 0.381 between ages 6 to 9, 9 to 13 and 6 to 13 yrs, respectively, all P< 0.0001). Tracking was higher for low-fit children, whereas no clear pattern was found for different levels of body-fat. In conclusion, we found that clustered z-score is a fairly stable characteristic through childhood and implementation of preventive strategies could therefore start at early school-age.

Abbreviations

Body mass index (BMI)

Cardiorespiratory fitness (CRF and VO_{2peak})

Cardiovascular disease (CVD)

Clustered risk-score (z-score)

Confidence intervals (CI)

Homeostatic model assessment (HOMA-IR)

High-density lipoprotein cholesterol (HDL)

Standard deviation (SD)

Sum of the four skinfolds (S4SF)

Triglyceride (TG)

Introduction

"High risk" for future cardiovascular disease (CVD) is difficult to define in children, as no hard endpoints such as manifest disease or death has yet occurred. Clustering of individual risk factors in the same individual has been suggested as a good method to assess CVD risk level in apparently healthy children (1). Earlier studies from our group found no clustering of CVD risk factors in 6 year old children, whereas at age 9 yrs three or more CVD risk factors was found in 3.33 times as many participants than expected (2). However, clustering of CVD risk factors in children is only of interest if it is a stable characteristic. A recent review of the literature regarding tracking of CVD risk factors from childhood to adulthood conclude that despite the differences in methodology, studies have consistently found tracking of cardiometabolic risk factor clustering from childhood or adolescence to adulthood (3). However, the researchers concluded that the shorter-term stability of clustered risk factors over the course of childhood and adolescence has yet to be elucidated. Furthermore, clustering of CVD risk factors in children have been found associated with lifestyle factors such as obesity (1;4-7) and cardiorespiratory fitness (CRF) (1;6-8). To our knowledge only one study in youth has examined the effect of obesity on tracking of clustered risk (9) and no studies have looked at the effect of CRF. Therefore, the aim of this study was to evaluate tracking of clustered CVD risk in youth using three time-points from age 6 to age 13 yrs. Secondly, to examine the how different levels of overweight/obesity and CRF effects the precision of tracking.

Methods

Subject for this study were participants in The Copenhagen School Child Intervention Study, which was started in 2001 and included all children attending kindergarten class in two communities in the area of Copenhagen (46 kindergarten classes in 18 public schools). Written informed consent was obtained from the parents/guardian of 706 children (69% of the population) and 696 actually participated in the study at baseline. Following the intervention, the children were re-tested in 2004/2005 at age 9 yrs and followed-up again in 2008 at age 13 yrs. The study has been described in details elsewhere (10-12). In the present analysis we included all children with complete measures of CVD risk factors at least at two of the three time-points (n = 434). The study was approved by the ethical committee, University of Copenhagen.

Since the complete methodology has been previously published, the methodology presented here includes only those variables of interest. Height, body weight and waist circumference were measured. Body mass index (BMI) was calculated ($kg \bullet m^{-2}$) and BMI z-scores were computed based on WHO recommendations (13). Bicipital, tricipital, subscapular and suprailiac skinfolds were measured using standard procedures with a Harpenden skinfold caliper (Harpenden, West Sussex, UK). The sum of the four skinfolds (S4SF) was used as a measure of body fatness (14).

Cardiorespiratory fitness (VO_{2peak}) was assessed using a continuous running protocol on a treadmill until exhaustion. VO_{2peak} was measured directly on an AMIS 2001 Cardiopulmonary Function Test System (Innovision, DK 5260 Odense) at age 6 and 9 yrs and using the COSMED $K4b^2$ portable metabolic system (COSMED, Rome, IT) at age 13 yrs. Both systems provide valid measures of VO_2 when validated against the Douglas bag method (15;16). Detailed criteria for an accepted test has been reported earlier (11). Blood pressure was measured in the sitting position after 15 minutes of rest with a Dinamap XL vital signs blood pressure monitor (Critikron, Inc., Tampa, FL) using appropriate sized cuffs. The mean of the last three of 5 measurements taken over 10 minutes was used for analysis.

Fasting blood samples were taken between 08:00 – 09:30 h and glucose was analyzed immediately (Hemocue, Sweden). The remainders of the samples were centrifuged; plasma aliquoted within 30 min, kept at -20^{o}C, and later stored at -80^{o}C until analyzed. Insulin was analyzed spectrophotometrically using an enzyme linked immunosorbent assay (DAKO Insulin, Code no. K6219). Insulin resistance was estimated according to homeostasis model assessment (HOMA-IR) as glucose ($mmol \cdot 1^{-1}$) multiplied by insulin ($mU \cdot 1^{-1}$) divided by 22.5 (17). Blood lipids were analyzed on a COBAS FARA (Roche, Switzerland) using spectrophotometry (ABX diagnostics, Montpellier, France).

Statistical analysis

A composite CVD risk score (clustered z-score) was constructed by adding the sex-specific z-scores for systolic blood pressure, HOMA-IR, triglyceride (TG), S4SF and the negative z-scores for high-density lipoprotein cholesterol (HDL) and CRF. Rationale for choosing these variables for the clustered z-score has been described elsewhere (18). In analysis with stratification for S4SF, S4SF was removed from the clustered z-score and in analysis with stratification for CRF, this variable was removed from the clustered z-score.

No differences in the clustered z-score between intervention and control group of the CoSCIS was evident (10), so the groups were pooled for all analyses. For descriptive purposes means and standard

deviations for all variables at age 6, age 9 and age 13 yrs were computed. BMI, waist circumference, S4SF, HOMA-IR and TG were positively skewed and therefore transformed (natural log) for the analyses. Tracking coefficients were calculated between the clustered z-score at age 6 to 9 yrs, age 6 to 13 yrs and age 9 to 13 yrs using Pearson correlations (19). This was also done for each of the CVD risk factors, to determine which factors were the most important in the tracking of the clustered z-score. High risk cases were defined as having a clustered z-score above 1 standard deviation (SD) corresponding to ~ 16, 14 and 12 % of the population at ages 6, 9 and 13 yrs, respectively. This choice of assigning high-risk is conservative, and was based on an earlier finding indicating that CVD risk factors cluster in around 11% of a normal European pediatric population (18). Logistic regression analysis was performed and odds ratios between children in three different risk categories; low risk (< median z-score), moderate risk (median to 1 SD) and high risk (≥ 1 SD) at one time-point for being at high risk at the second time-point. Finally, to evaluate the influence of CRF and fatness (S4SF), children were grouped in tertiles based on these variables and clustered z-score (without CRF or S4SF, respectively) tracking coefficients were calculated within each tertile using Pearson correlations. All analyses were performed using the Statistical Package for the Social Sciences version 19 (SPSS, Chicago, IL).

Results

The general characteristics of the sample at the age of 6, 9 and 13 yrs are presented in Table 1. BMI, S4SF, systolic blood pressure and HOMA-IR increased with age. CRF increased from age 6 to age 9 yrs, triglycerides increased from age 9 to age 13 yrs and HDL cholesterol increased from age 6 to age 9 yrs and then decreased from age 9 to age 13 yrs. Tracking coefficients between clustered z-score at age 6 to 9, 9 to 13 and 6 to 13 yrs are presented in Table 2. Moderate correlations were found between clustered z-score at age 6 to 9 and 9 to 13 yrs, whereas the coefficient between age 6 and 13 yrs was somewhat weaker. These coefficients did not change substantially when analyses were done for each sex separately (data not shown). For the single risk factors highest tracking coefficients were seen for S4SF, followed by systolic blood pressure and HDL cholesterol. Tracking coefficients for HOMA-IR and TG were low to moderate. The tracking coefficients for CRF were high from 6 to 9 yrs and 9 to 13 yrs, but low from 6 to 13 yrs.

Logistic regression showed that children with moderate and high risk, respectively, in clustered z-score at first time-point had a 6.1 and 21.2 times greater risk, respectively, of having a clustered z-score above 1 SD at the second time-point between the ages 6 to 9 yrs, 4.51 and 30.8 times greater risk between the ages 9 to 13 yrs, and 2.3 and 4.9 times greater risk between the ages 6 to 13 yrs, compared to children with low risk in clustered z-score (Table 3).

When children were grouped based on their level of CRF, tracking coefficients were higher in the 1st tertile (least fit) at all three time intervals (*r*-values 0.501-0.670). The coefficients were strong in the 1st and 2nd tertiles and low in the 3rd tertiles (*r*-values 0.253-0.396). When children were grouped based on their S4SF, we found lowest tracking coefficients in the 1st tertile (least fat) at all three time intervals. However, the picture was not as clear as for CRF. Between age 6 and 9 yrs there was no significant coefficient in the 1st tertile (*r*-value 0.068), a strong coefficient in the 2nd tertile (*r*-value 0.499) and moderate coefficient in the 3rd (*r*-value 0.340). Between age 6 and 13 yrs the coefficients were weak in all tertiles (non-significant in the first) (*r*-values 0.201, 0.314 and 0.233 in 1st, 2nd and 3rd tertile, respectively). Between age 9 and 13 the coefficients were weak in the 1st and 2nd tertile (*r*-values 0.317 and 0.297) and stronger in the 3rd (*r*-value 0.492).

Discussion

Tracking is a term used to describe the development of a characteristic over time and involves both the longitudinal stability of this variable and the ability of one measurement to predict the value of following measurement(s) (19). This present study examined the short-term tracking of a clustering of CVD risk in a normal youth population from early school-age to start adolescence. We found moderate to high level of tracking between all the measured time points and the risk of having a high sum of z-score at a second time point was between 2.3 and 30.8 times increased for children having a high sum of z-score at the first time point. Similarly, the only other study examining the stability of clustered CVD risk factors from childhood (age 9 y) to adolescence (age 15 y) in a sample of Swedish and Estonian children found moderate overall tracking coefficient for boys and girls, respectively (20). Also studies looking at clustered CVD risk factor tracking from childhood or adolescence to adulthood displayed similar results (21-24). We have earlier shown that CVD risk factors did not cluster at age 6 yrs in this cohort (25), but at the age of 9 yrs clustering was evident in the same cohort (2). Therefore, it was unexpected to find a high degree of tracking between the ages 6 to 9 yrs and 6 to 13 yrs and the

results indicate that a relative high level of several CVD risk factors at this low age is predictive for high future metabolic risk. Juhola and colleagues (26) using data from the Cardiovascular Risk in Young Finns Study, investigated sensitivity and specificity rates for predicting abnormal CVD risk factors as an adult from values obtained at ages 3, 6, 9, 12, 15 and 18 years. They found that for obesity and blood pressure values at all childhood ages were predictive for the adult value. For HDL-cholesterol there was no difference between age groups for females, but for males HDL levels at ages 6, 9 and 12 years were most predictive for adult values, compared to ages 3, 15 and 18. All together these results suggest that clustering of CVD risk factors is a fairly stable characteristic from early school-age to adulthood. We did not find any sex differences in tracking of clustered CVD risk (data not shown), which is similar to results from some (20;21), but not all studies (27).

In the present study all of the single risk factors included in the summed z-score had a positive tracking coefficient (Table 2), which means that they all contributed to the tracking coefficient for the sum of z-score. However, tracking coefficients of single risk factors varied from low to high, whereas the tracking coefficients for the clustered z-score varied from moderate to high. In agreement with our findings, Bao and colleagues (24) found that tracking expressed as an inter-age correlation was higher for a clustered risk score (r = 0.64) compared to the single risk factors (r = 0.34-0.57); conversely, others did not find this difference between individual and clustered risk factors (21;22). Based on these results, there is no indication of a much higher tracking of a clustered risk score, compared to the single risk factors. However, the clustered score has been suggested as a good way to assess overall health in healthy pediatric populations, as it uses all available information (no cut-points, but a continuous score) and is not as influenced by measurement error and day-to-day variations as the single risk factors.

Clustering of CVD risk factors have been found related to CRF in cross-sectional studies in youth (1;6;8;23;28). We analyzed the stability of the summed CVD risk factors within each tertile of CRF and found that a lower baseline CRF level was associated with a higher tracking coefficient of clustered z-score and this was consistent for all time intervals. This implies that within the least fit group, the stability of metabolic health is greater compared to within the most fit group. Conjecture suggests that it takes more effort to positively change health affecting behavior (e.g. lose weight or increase physical activity and fitness) compared to change behavior in a way that negatively influence your health (e.g. by gaining fat and decreasing physical activity). To our knowledge, no other studies have examined the

effect of CRF level on the degree of tracking of clustered CVD risk factors in this age group and our results should therefore be verified in other cohorts.

In the present study, children were grouped based on their S4SF and no clear picture was found between groups in tracking of clustered CVD risk factors, which could suggest that the level of fatness may not be pivotal for the stability of CVD risk factor clustering over time in a normal youth population. This is not to say, that overweight does not play an important role in the development of clustering of risk factors, a fact that has been demonstrated in several studies. In cross-sectional studies overweight and obesity have consistently been found related to clustering of CVD risk factors in children and adolescents (1;4-6) and convincing evidence suggests that overweight and obesity tracks from childhood into adulthood (29). Furthermore, some studies have found that overweight or obesity in childhood is associated with increased risk of later development of CVD risk factor clustering (30;31). However, in one of these studies, the association disappeared when fatness was removed from the clustered risk score. Thus, the authors concluded that the effect of fatness in childhood on the cluster of CVD risk factors in adolescence is a result of adiposity tracking (30). In support of this, a recent review concludes that there is not much evidence for childhood obesity to be an independent risk factor for adult CVD risk, because the relationship is attenuated or no longer present when adjusting for adult obesity (32). Likewise, Chen and co-workers demonstrated that longitudinal changes in clustering of CVD risk factors assessed by incremental area (area under the curve) were decreased by approximately 50%, when adjusted for BMI (9). These results suggest that fatness is an important part of a causal chain leading to clustered CVD risk. It is however, not possible to conclude anything about causation; it could be that fatness is an intermediate and adjusting for an intermediate may remove the associations between the investigated exposure and the outcome.

The strength of this study is the relatively large cohort followed for a 7 years period with three measurements from early school-age to start adolescence. A limitation is the relatively lean and healthy population studied which could limit the generalizability of the results. However, as mentioned, we have previously found that in this cohort CVD risk factors cluster in some children already from the age of 9 yrs, and that this cluster is related to CRF and fatness (2). In addition, the size of the tracking coefficient is highly dependent upon the reproducibility of the measurement and its error variation. In that regard S4SF is easier to reproduce compared to some of the other variables measured e.g. VO_{2peak}.

In conclusion, moderate to strong tracking was found for clustered z-score in a normal population measured three times from age 6 to age 13 yrs. Furthermore, children with a higher clustered z-score at first measurement had an increased risk of having a clustered z-score above 1 SD at the second measurement. This means that a high level in several CVD risk factors in early school-age is already predictive for the development of the clustering of CVD risk factors seen in the older age-groups. Tracking of clustered z-score differed between tertiles of fitness with children in the lowest fitness group displaying the highest tracking coefficients. The picture was not as clear for tertiles of fatness. We believe that the results of the present study have important clinical implications, as the results show stability of clustering of CVD risk factors across school-age and points to implementation of preventive strategies starting in early childhood.

Acknowledgments

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Table 1. Physical characteristics of participants by age.

Variables	Age 6 years mean (SD)	Age 9 years mean (SD)	Age 13 years mean (SD)
Age (years)	6.76 (0.35)	9.56 (0.36)	13.37 (0.34)
BMI ($kg \cdot m^2$)	15.90 (1.58)	17.06 (2.20)	19.11 (2.74)
zBMI for age	0.21 (0.94)	0.22 (1.02)	-0.05 (1.05)
Sum of 4 skinfolds (mm)	25.70 (8.23)	31.66 (14.30)	34.73 (17.29)
VO_{2peak} ($ml \cdot kg^{-1} \cdot min^{-1}$)	46.97 (5.94)	49.80 (6.99)	49.17 (8.36)
Systolic Blood Pressure (mmHG)	97.31 (7.35)	103.45 (8.47)	110.35 (8.63)
Insulin resistance (HOMA-IR)	0.79 (0.67)	1.27 (0.68)	2.78 (1.55)
HDL cholesterol ($mmol \cdot L^{-1}$)	1.49 (0.26)	1.60 (0.33)	1.46 (0.32)
Triglyceride ($mmol \cdot L^{-1}$)	0.59 (0.24)	0.55 (0.24)	0.79 (0.35)
Clustered Z-score	-0.19 (2.92)	-0.25 (3.34)	-0.09 (3.48)

Table 2. Tracking coefficients or Pearson correlations (*r*) between clustered z-scores and the single risk factors at different ages.

	6 to 9 years	9 to 13 years	6 to 13 years
	r	*r*	*r*
Clustered Z-score	0.514	0.559	0.381
Sum 4 skinfolds	0.858	0.774	0.668
VO_{2peak}	0.524	0.561	0.294
Systolic BP	0.538	0.499	0.441
HOMA	0.317	0.213	0.248
HDL	0.347	0.686	0.414
TG	0.230	0.327	0.195*

All *P*-value < 0.0001, unless marked. *: *P*-value = 0.001

Table 3. Risk of high clustered z-score (above 1 SD) based on former risk level of clustered z-score.

	Odds ratio	95% CI	p-value
Age 6 to 9 yrs			
Low risk at age 6 yrs	1		
Moderate risk at age 6 yrs	6.10	2.37-7.93	<0.0001
High Risk at age 6 yrs	21.22	7.93-56.80	<0.0001
Age 9 to 13 yrs			
Low risk at age 9 yrs	1		
Moderate risk at age 9 yrs	4.51	1.71-11.89	0.002
High Risk at age 9 yrs	30.80	9.73-97.50	<0.0001
Age 6 to 13 yrs			
Low risk at age 6 yrs	1		
Moderate risk at age 6 yrs	2.32	1.04-5.19	0.040
High Risk at age 6 yrs	4.86	1.92-12.34	0.001

Low risk (≤ median), moderate risk (> median < 1SD) and high risk (≥ 1SD). Low is set as reference.

Paper III

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Inflammatory markers and clustered cardiovascular disease risk factors in Danish Adolescents

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Running head: CVD risk and low grade inflammation

Keywords: cytokines, CRP, adiponectin, cardiorespiratory fitness, fatness

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Abstract

Aims: To evaluate the associations between inflammatory markers and clustering of cardiovascular disease (CVD) risk factors, and to examine how CVD risk and inflammatory markers are related to fatness and cardiorespiratory fitness in adolescents.

Methods: Body mass and height, skinfolds and blood pressure of 413 adolescents (mean age: 13.4±0.3 years) were measured. Circulating fasting levels of glucose, insulin, lipids, adiponectin, C-reactive protein (CRP), tumor necrosis factor (TNF)α, soluble TNF receptor-1 (sTNFR1), interleukin (IL)-6 and interleukin-1 receptor antagonist (IL-1Ra) were measured.. Fitness was measured in progressive tests to exhaustion. CVD risk was expressed as a clustered z-score, summing standardized values of individual risk factors.

Results: The clustered z-score was negatively associated with adiponectin and positively associated with CRP, IL-6 and TNFα levels (*P*-values all < 0.05). The associations with adiponectin, CRP, IL-6 were stronger for the fattest adolescents. No associations were found for sTNFR1 or IL-1Ra. The high-fitness group had lower clustered z-score, adiponectin and IL-6 levels compared to low-fitness group, and the high-fatness group had higher clustered z-score and CRP, but lower fitness and adiponectin, compared to low-fatness group.

Conclusions: In adolescents, CVD risk was associated with adiponectin, TNFα, CRP and IL-6, and related to both fitness and fatness.

Background

Cardiovascular disease (CVD) is one of the main causes of global mortality and disease-related morbidity [1;2]. CVD is related to several well-known risk factors, including central obesity, insulin resistance, hypertension and dyslipidaemia; all components in the metabolic syndrome (MetS) [3;4]. The underlying mechanisms linking these risk factors to each other and to CVD have been subject for extensive investigation in the last decades [5]. One of the proposed hypotheses is that the excess visceral adipose tissue acts as a secretory gland releasing an excessive amount of adipokines, including cytokines and hormones. This creates a state of low-grade inflammation promoting oxidative stress, damage to the endothelium of blood vessels and to the islet cells in the pancreas [3;5-7].

Inflammation is an appropriate response to tissue injury or pathogen exposure. However, when becoming chronic, as in obesity-related chronic low-grade inflammation, it can have detrimental effects [6;7]. The inflammatory processes are controlled by cytokines produced by many cell types, including adipocytes and cells of the immune system. Some cytokines are considered to have mainly pro-inflammatory properties such as tumor necrosis factor (TNF) –α and interleukin (IL) -1β. Other cytokines, such as IL-10, and cytokine-binding proteins, including soluble TNFα receptors (sTNFR) and IL-1 receptor antagonist (IL-1Ra), are considered anti-inflammatory. In particular, sTNFR1 are secreted in response to cytokine-induced inflammation, and are thought to function as a natural inhibitor blocking the biological effects of TNFα [8]. IL-1Ra is a peptide that binds to the IL-1 receptors, acting as a competitive antagonist for the pro-inflammatory cytokines IL-1α and -β [9]. Moreover, some cytokines, such as IL-6, have both pro-inflammatory and anti-inflammatory actions. IL-6 has pronounced pro-inflammatory effects being one of the principal mediators of clinical manifestations of tissue injury and inducers of fever, leukocytosis, thrombocytosis and increased plasma levels of acute phase proteins. Furthermore, Il-6 stimulates plasma cytosis and hypergammaglobulinemia [10] The anti-inflammatory effects of IL-6 include inhibition of TNFα and stimulation of the production of IL-1Ra and IL-10 [11]. An optimal outcome of immune activation is thought to depend upon the balance between immune-activating cytokines and anti-inflammatory mediators.

Apart from the cytokines, other markers are associated with low-grade inflammation. Among them is CRP, a protein released from hepatocytes [12], and adiponectin, a protein exclusively secreted from adipocytes, but negatively related to the amount of body fat and with anti-inflammatory properties [13].

In youth, markers of low-grade inflammation have been linked to all of the individual CVD risk factors [14-19]; as well as to the MetS [20-22]. The presence of MetS in youth is typically determined using specific age and sex cut-points. This dichotomization of the risk factors used in MetS definitions, however, may not be the best way to assess cardiovascular health in children, as it reduces the available information, and no consensus exists regarding cut-off levels [23]. Clustering of CVD risk factors in the same individual, assessed by summing standardized values of the individual risk factors, is a continuous variable, and has been suggested as a good method to assess CVD risk level in apparently healthy children [23;24]. Four studies in youth have previously examined the relation between low-grade inflammation and a clustering of CVD risk factors and found associations for some, but not all, investigated markers of low grade inflammation [15;18;25;26]. No study has, to our knowledge, included the anti-inflammatory natural blockers of pro-inflammatory cytokines, sTNFR1 and IL-1Ra, in this type of analysis.

Therefore, the primary aim of the present investigation was to evaluate the relationships between clustered CVD risk and a wide range of inflammatory markers, including both immune activating cytokines and anti-inflammatory mediators, in a normal Danish adolescence population, and to examine whether these correlations were evident in only the overweight adolescents. Furthermore, since convincing evidence suggests a relationship between the level of inflammatory markers and lifestyle related factors such as level of body fatness [15-18] and cardiorespiratory fitness (VO_{2peak}) [27;28], our secondary aim was to investigate whether CVD risk and inflammatory marker levels differed between groups of fatness and cardiorespiratory fitness.

Methods

Participants

All children starting school (6-7 years of age) in 2001 from 2 suburbs of Copenhagen, Denmark, were invited to participate in a 3-year, controlled intervention study of physical activity and health; the Copenhagen School Child Intervention Study. The study has been described in details elsewhere [29] and only measures pertinent to this study are described here. Data for this cross-sectional study is from the follow-up measurement in 2008 (mean age 13.4 yrs ± 0.3). Complete data for the clustered z-score

and the inflammatory markers were available for 413 subjects. Since no difference in clustered z-score was found between intervention and control group [29], data from the two groups were pooled for all analyses.

Ethics

The Ethics Committee of Copenhagen approved the study. All subjects gave assent, and their parents or guardians signed an informed consent.

Measurements

Tests were performed between 8:00 AM and 02:00 PM at the adolescent's schools in the two communities. Fitness tests were performed in a mobile unit, and all other tests were performed in a gym or a classroom. Body mass was measured to the nearest 0.1 kg (Seca 882, Brooklyn, NY). Body height was measured to the nearest 1 mm without shoes (Harpenden stadiometer, West Sussex, UK). BMI was computed using body mass in kilograms divided by height in meters squared (BMI = kg/m^2). Skinfolds (biceps, triceps, subscapular and suprailiac) were measured in triplicate to the nearest mm with caliper (Harpenden, West Sussex, UK) and the mean of 3 measurements was used for subsequent analysis. The sum of four skinfolds (S4SF) was calculated and used as an estimate of body fatness [30]. Sexual maturation was assessed by self-report using a scale of pictures of breast and genital development for girls and boys, respectively [31]. Blood pressure was measured with a Dinamap XL vital signs BP monitor (Critikron, Tampa, FL) after the child had been at rest for 15 min. Five measurements were made over 10 minutes, with the mean of the last 3 measurements being recorded. Cardiorespiratory fitness (VO_{2peak}) was measured during a continuous, progressive, graded treadmill run using the COSMED $K4b^2$ portable metabolic system (COSMED, Rome, IT). The adolescents were instructed to run until exhaustion. Standard objective criteria were used to determine if the test was performed satisfactory. The same experienced researcher performed all tests. For further details of the VO_{2peak}-test see [29].

Blood Sampling and Analyses

Blood samples were obtained between 7 and 10 AM after an overnight fast. Fasting compliance was verbally verified by on-site researchers. Glucose was measured immediately after sampling (Hemocue, Ängelholm, Sweden). The rest of the samples were centrifuged; the plasma was aliquoted within 30

minutes, kept at −20°C, and later stored at −80°C. The stored samples were analyzed at the Institute for Inflammation Research, University Hospital of Copenhagen, Denmark. Insulin was analyzed spectrophotometrically using an enzyme-linked immunosorbent assay (ELISA) (DAKO Insulin, code no. K6219, Glostrup, Denmark). Blood lipids were analyzed on a Cobas Fara (Roche, Basel, Switzerland) using spectrophotometry (ABX Diagnostics, Montpellier, France).
Inflammatory markers were analyzed with high-sensitivity immunoassays or multi-plex kits. For IL-6, Quantikine High-Sensitivity ELISA with a detection limit of 0.5 pg·mL−1 (R&D, Minneapolis, MN) was used. For TNFα, Quantikine High-Sensitivity ELISA with a detection limit of 0.1 $pg \cdot mL^{-1}$ (R&D, Minneapolis, MN) was used. sTNFR1 (the total amount of free receptor plus the total amount of receptor bound to TNFα), IL-1Ra and adiponectin was measured on a Luminex 100 (Luminex Corporation, Austin, TX, USA) using Invitrogen kits (Invitrogen Corporation, California, USA), with detection limits of 15 $pg \cdot mL^{-1}$, 30 $pg \cdot mL^{-1}$ and 0.058 $ng \cdot mL^{-1}$ for sTNFR1, IL-1Ra and adiponectin, respectively. C-reactive protein was determined using particle-enhanced turbidimetric immunoassay: Tina-quant CRP (latex) high-sensitivity assay (Cobas, Roche Diagnostics, Mannheim, Germany) with a detection limit of 0.1 $mg \cdot L^{-1}$. All essays were used according to the manufacturer's instructions.

Data Management

Homeostasis model assessment (HOMA-IR) was computed as follows: HOMA = insulin (micro units per milliliter) × glucose (millimoles per liter)/22.5 and was used as an estimate of insulin resistance [32]. We constructed a continuous score representing a composite CVD risk factor profile (clustered z-score) by summing standardized residuals (z-score) "by sex" of following six (non-transformed) variables; the ratio of total cholesterol to high-density lipoprotein cholesterol (HDLc), triglyceride (TG), HOMA-IR, systolic blood pressure, S4SF, and inverse of VO_{2peak} (in milliliters per kilogram per minute). The entire sample was divided using a median split by sex on fatness (S4SF) and cardiorespiratory fitness (VO_{2peak}). The adolescents were subsequently divided into four groups, based on these splits; high fitness/low fatness, high fitness/ high fatness, low fitness/low fatness, low fitness/high fatness.

Statistical Analysis

For descriptive purposes, mean and standard deviations were computed for all variables by fitness/fatness groups. Since waist, sum of four skinfolds, the HOMA-IR, CRP, sTNFR1, IL-1Ra, TNFα and IL-6 had a skewed distribution, their values were transformed using the natural log. Differences between fitness/fatness groups were analyzed in a general linear model adjusted for sex and pubertal stage. Fitness-by-fatness interaction was tested, but where no interaction was found, the interaction term was removed from the final model. If there was an interaction the adolescents were stratified by either fatness or fitness groups. As the level of fatness and fitness differed between e.g. the two low fat groups, post hoc analysis with linear regression models were made to analyze the association between cardiorespiratory fitness (as a continuous variable) and key variables adjusting for fatness (S4SF), sex and pubertal stage. Similarly, linear regression models were made to analyze the association between S4SF (as a continuous variable) and key variables adjusted for cardiorespiratory fitness, sex and pubertal stage.

Associations between the inflammatory markers and the clustered z-score were assessed using partial correlations adjusting for sex and puberty for the entire cohort and stratified by median split for S4SF by sex. Significant correlations were further explored by dividing the adolescents into quartiles of the inflammatory marker (1^{st} quartile lowest level and 4^{th} quartile highest level) for logistic regression analyses. Odds ratios were calculated for having a clustered z-score above 1 SD based on level of the inflammatory marker. Finally, the inter-correlations between all inflammatory markers were assessed using simple correlations. Statistical significance was held at $P < 0.05$. Analyses were conducted using SPSS for Windows version 18.0.

Results

The general characteristics of the sample are presented by fitness/fatness groups in Table 1. After adjusting for sex and pubertal status, the high-fitness group had lower BMI (only in the high fat group) and S4SF, higher VO_{2peak} and systolic blood pressure, higher HDLc concentration and lower clustered z-score, adiponectin and IL-6 levels than the low fitness group. Adolescents in the high fat group were younger, had higher BMI, S4SF, systolic blood pressure, HOMA-IR, clustered z-score and CRP level, but lower fitness, HDLc concentration and adiponectin level compared to the low fat group.

Post hoc analyses using linear regression were made to evaluate associations between variables and VO_{2peak} as a continuous variable, adjusted for S4SF, sex and pubertal status. VO_{2peak} was negatively

correlated to clustered z-score (β = -0.387, $P < 0.0001$), adiponectin (β = -0.251, $P < 0.0001$) and IL-6 (β = -0.151, $P = 0.029$) and positively correlated to systolic blood pressure ($\beta = 0.155$, $P = 0.024$), whereas no correlations were found between VO_{2peak} and BMI or HDLc levels.

Linear regression analysis between variables and S4SF (as a continuous variable), adjusted for VO_{2peak}, sex and pubertal status, displayed significant negative correlations for age (β-coefficient = -0.129, P-value = 0.043), HDLc (β = -0.247, $P < 0.0001$) and adiponectin (β = -0.348, $P < 0.0001$) and positive correlations for BMI ($\beta = 0.786$, $P < 0.0001$), systolic blood pressure ($\beta = 0.139$, $P = 0.028$), HOMA-IR ($\beta = 0.343$, $P < 0.0001$), clustered z-score ($\beta = 0.430$, $P \geq 0.0001$) and CRP ($\beta = 0.400$, $P < 0.0001$).

Partial correlations, adjusted for sex and pubertal status, between clustered z-score and the inflammatory markers are presented in Table 2. Significant, but weak, correlations were found between clustered z-score and circulating levels of adiponectin, CRP, IL-6 and TNFα. When analyzed in groups based on median split of S4SF by sex, correlations between the clustered z-score and adiponectin and CRP were only significant for adolescents with S4SF values in the upper half. This group also exhibited a greater correlation coefficient for IL-6 and lower correlation coefficient for TNFα compared to adolescents with S4SF values in the lower half, but these differences were non-significant.

As shown in table 3, adolescents in the 1st quartile (the lowest levels) of adiponectin had greater risk of having a clustered z-score above 1 SD compared to adolescents in the 4th quartile (the highest levels of adiponectin). Odds ratios for high clustered z-score were significantly higher for the 3rd and 4th quartiles of CRP and IL-6 compared to the 1st quartile, whereas risk of having a clustered z-score above 1 SD was increased in all three upper quartiles of TNFα, when compared to the 1st quartile.

Table 4 shows the correlations between the inflammatory markers. The anti-inflammatory markers adiponectin, sTNFR1 and IL-1Ra were correlated, as were the pro-inflammatory markers CRP, IL-6 and TNFα. Furthermore, adiponectin was inversely correlated to CRP.

Discussion

Even though manifest CVD does not occur before adulthood, the process of atherosclerosis for some individuals starts in childhood and progresses throughout life [33]. In adults convincing evidence suggest that low-grade inflammation has a fundamental role in all stages of the atherosclerotic process [34]. How markers of low-grade inflammation relate to CVD risk in children and adolescents is, however, not yet fully elucidated.

We evaluated the associations between a clustered z-score and 6 different markers of low-grade inflammation. The score correlated with circulating CRP, TNFα and IL-6 levels and correlated inversely with adiponectin. No correlations were found for IL-1Ra and sTNFR1. The risk of having a clustered z-score above 1 SD was increased in adolescents within the highest quartiles of CRP, IL-6 and TNFα and within the lowest quartile of adiponectin.

In agreement with our finding, others have found a significant inverse correlation between clustered z-score and adiponectin in 9-year old children [25], and others have reported that adiponectin predicted the number of MetS features [20;22]. Adiponectin is also inversely correlated to the individual risk factors in youth such as insulin resistance [19;21;35], lipid profile and blood pressure [36]. In adults, adiponectin has been found negatively related to the development of both metabolic syndrome [37] and CVD [38]. The protective mechanisms of adiponectin are not completely revealed;, however, in vitro experiments have shown that adiponectin reduces the expression of adhesion molecules in endothelial cells and suppresses proliferation and migration of smooth muscle cells into the intima-media. Furthermore, animal models have shown that adiponectin ameliorates insulin resistance and increases fatty acids oxidation (for review see [13]). Our results and the results of others suggest that these insulin-sensitizing and anti-inflammatory properties of adiponectin might impact the overall risk profile already at young ages. We found that adiponectin was negatively related to S4SF, replicating the results of others [17;21;28]. Furthermore, we only found a significant correlation between adiponectin and clustered z-score in the group with S4SF values above the median, which emphasizes that fatness is important for the association. However, our results do not explain whether adiposity is the cause, a mediator or a confounder in the mechanistic chain. Paradoxically, we also found that fitness was negatively correlated to adiponectin after adjusting for fatness. The same was found in a study by Nemet and colleagues [28], whereas Rubin and colleagues found a positive correlation [19]. Theoretically, a positive relationship between adiponektin and fitness makes most sense if one of the ways exercise is being cardioprotective, is by improving the adipokine profile and adjusting for fatness might be misleading (see below).

We found a correlation between level of CRP and the clustered z-score, which is in agreement with previous studies in youth [15;18;26]. A new study found that carotid intima-media thickness was related to the level of CRP in 7-year old children [39]. These results indicate that CRP is related to the

early development of systemic complications and could be used as a marker for CVD risk detection in youth. In adults, the level of CRP has been found to predict development of CVD [12;40]. *In vitro* and *in vivo* studies have found that CRP induces endothelial cell activation and dysfunction [41;42], impairs endothelial vasoreactivity [42] and is directly involved in insulin resistance [43]. However, a recent study doing mendelian randomization analyses of four important CRP genes in almost 200.000 participants, found no indications of CRP per se as a causal factor in coronary heart disease [44]. Therefore, the precise role of CRP in the development of CVD risk factors in humans remains unknown. In our study, CRP was positively correlated to fatness, which resembles findings from several studies in youth [15-18;21;26;45]. Moreover, a significant correlation between CRP and the clustered z-score was only found in the group with S4SF values above the median, and fatness seems to be an important factor in the association. CRP was not significantly correlated to VO_{2peak} after adjusting for S4SF. Other studies have found negative correlations between VO_{2peak} and CRP in youth [26;27;45;46]. Two of these studies find, like we do, that the correlation was not significant after adjusting for fatness [27;46]. However, this does not necessarily eliminate fitness as an important factor, because fatness could be an intermediate between fitness and low-grade inflammation and adjusting for fatness could therefore be misleading.

We found a significant correlation between circulating IL-6 levels and the clustered z-score, as opposed to other studies [15;26]. However, in support of our results, one study did find an increasing level of IL-6 with increasing number of MetS components present [20]. The discrepancy between these studies illustrates the existing controversy regarding the effect of IL-6. On the one hand, IL-6 has consistently been observed in patients with type 2 diabetes mellitus, and as a consequence has been related to insulin resistance and/or impaired glucose disposal [11], but on the other hand administration of IL-6 to healthy humans did not impair muscle glucose uptake or whole-body glucose disposal [47]. Furthermore, reports have suggested both pro- and anti-inflammatory effects of IL-6 [11]. Therefore, the cross-sectional relationship between IL-6 and clustered z-score in the present study should be interpreted with caution and may not indicate causality.
In the present study IL-6 was negatively related to fitness, whereas previously studies in youth did not find any relation [26;28]. The circulating level of IL-6 can be affected both chronically by release from the adipose tissue in overweight, but also acutely by release from muscle cells during and after bouts of

exercise [11]. As blood samples were taken first thing in the morning in our study, they were probably not affected by any acute exercise bouts, but are more likely reflecting body fat, which is also strongly correlated to cardiorespiratory fitness expressed in milliliters VO_2 per kilogram [48]. We did not find any correlation between fatness and IL-6 in the present study.

We found a significant correlation between the clustered z-score and TNFα, which contradicts the results of previous studies on youth which did not find any correlation [15;26]. TNFα was not correlated to fatness. Furthermore, the correlation between the clustered z-score and TNFα was strongest in the group with S4SF values below the median, but did not reach statistical significance in either group. This indicates that the relationship between CVD risk and TNFα might be independent of adiposity in youth. In adults, high systemic levels of TNFα is consistently found related to obesity, insulin resistance and type 2 diabetes [49], but the specific role of TNFα in the development of CVD is not completely revealed [5].

The binding of TNF to membrane-bound TNFR1 triggers a series of intracellular events including inflammatory responses [50]. sTNFR1 is secreted in response to stimulation with TNFα as well as with IL-1β, IL-6, and IL-8, and sTNFR1 is therefore considered a marker of cytokine-induced inflammation. sTNFR1 binds TNFα, thereby preventing TNFα binding to cellular TNFR's, thus reducing the biological effects of TNF. TNFα is difficult to measure, partly because of the very low levels in normal youth and partly because of its unstable nature. TNFα exerts greatest impact locally and is metabolized quite fast. As the concentration of TNFα was measured in the blood, it might not reflect the actual tissue level and this could cause discrepancies in results. In contrast, sTNFR1 is considered a more robust marker of cytokine induced inflammation, as TNFRI has a much longer half-life and is present at higher concentrations in the blood compared to TNFα [8]. We did not find any correlation between clustered z-score and sTNFR1 in this cohort, however, which might imply that any inflammation in these adolescents is still insufficient to induce an accompanying release of anti-inflammatory mediators from leukocytes and other tissues. This is supported by the fact that we did not find any correlation between clustered z-score and the anti-inflammatory protein IL-1Ra. Furthermore, the anti-inflammatory mediators were inter-correlated, as were the pro-inflammatory mediators, whereas the only correlation between pro- and anti-inflammatory mediators was an inverse correlation between adiponectin and CRP. Our finding is of potential interest, since release of sTNFR1 and IL-1Ra is usually accompanying elevation in pro- inflammatory cytokines both in children and adults with

inflammatory diseases (e.g. [51;52]). It may therefore be speculated that cytokine release from fat-tissue, as opposed to cytokine release during leukocyte activation, is insufficiently counter-balanced by anti-inflammatory molecules with a risk of continuous low-grade inflammation, which could ultimately result in CVD.

The strength of the present study is the simultaneous measurement of a number of inflammatory markers, the traditional CVD risk factors, fatness and the direct measurement of VO_{2peak} in a normal adolescent population. The study has some weaknesses, however; first, the cross-sectional nature of the study does not allow any explanations of causality. Longitudinal studies on the development of clustered CVD risk and inflammatory markers in youth are warranted, in order to determine the timeline in development of fatness, low-grade inflammation and CVD risk factors. Secondly, the study population contained a low number of overweight and obese individuals and the general level of CVD risk factors is low (Table 1). It is possible that stronger associations between clustered z-scores and inflammatory markers would have been found in a population containing a larger number of overweight individuals and individuals with a higher level in CVD risk factors. However, we have earlier reported that in this cohort ~14% of the children had an impaired risk profile at age 9 years [24], which suggest that the processes leading to an impaired risk profile begin at an early age.

Conclusion

The complex mechanisms leading to impaired metabolism and the possible roles of the inflammatory markers are still under investigation. Our results showed a significant correlation between the clustered z-score and circulating levels of CRP, TNFα and IL-6, and an inverse correlation with adiponectin, whereas no correlations were found for IL-1Ra and sTNFR1. The results for CRP and adiponectin resemble results from previous studies, whereas the correlations between IL-6, TNFα and the clustered z-score contrast with the findings of others. For CRP, adiponectin and IL-6 the correlations to clustered z-score were strongest in the fattest half of the adolescents, whereas the opposite was found for TNFα. However, it should be emphasized, that this may not reflect causality, as fatness theoretically could be the cause, a mediator or a confounder in the association between CVD risk and low grade inflammation.

This is the first study to examine the relation between clustered z-score and the anti-inflammatory markers IL-1Ra and sTNFR1 in youth, and interestingly, in contrast to a number of inflammatory

diseases, up-regulation of pro-inflammatory signals were not accompanied by an increase in these anti-inflammatory molecules.

VO_{2peak} was negatively correlated to clustered z-score, adiponectin and IL-6 levels. S4SF was positively correlated to clustered z-score and CRP levels and negatively to adiponectin levels, which implies that life style modifications that alters fitness level and body composition can positively influence the level of markers of low grade inflammation and maybe, thereby, the metabolic risk profile in adolescents.

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Table 1. Mean and SD for key variables in groups based on median split for fatness and fitness. P values for differences between fitness group (adjusted for fatness) and fatness groups (adjusted for fitness).

Variables	HiFi/LoFa (n=150)	HiFi/HiFa (n=55)	LoFi/LoFa (n=56)	LoFi/HiFa (n=152)	*P* value fitness	*P* value S4SF
Age (years)	13.38 (0.34)	13.28 (0.32)	13.41 (0.30)	13.34 (0.35)	ns	0.022
BMI ($kg \cdot m\text{-}^{2}$)	17.65 (1.54)	19.58 (1.77)	17.78 (1.82)	21.16 (2.66)	0.001*	< 0.0001
Sum of 4 skinfolds (mm)	23.10 (4.63)	40.62 (11.05)	25.12 (5.34)	48.53 (17.80)	< 0.0001	< 0.0001
VO_{2peak} ($ml \cdot kg^{-1} \cdot min^{-1}$)	55.93 (6.43)	53.34 (5.72)	44.58 (4.98)	42.56 (5.40)	< 0.0001	< 0.0001
Sysolic blood pressure (mmHG)	110.70 (8.23)	111.76 (9.24)	106.54 (7.23)	111.00 (8.86)	0.005	0.006
Insulin resistance (HOMA-IR)	2.36 (1.16)	2.84 (1.53)	2.68 (1.69)	3.12 (1.47)	ns	< 0.0001
Total cholesterol ($mmol \cdot L^{-1}$)	4.13 (0.66)	4.14 (0.80	4.16 (0.73)	4.11 (0.65)	ns	ns
HDL cholesterol ($mmol \cdot L^{-1}$)	1.54 (0.32)	1.42 (0.32)	1.45 (0.29)	1.37 (0.30)	0.035	0.019
Triglycerides ($mmol \cdot L^{-1}$)	0.76 (0.42)	0.78 (0.38)	0.77 (0.27)	0.82 (0.39)	ns	ns
Clustered Z-scores	-2.17 (2.30)	0.032 (2.89)	-0.57 (2.18)	2.33 (3.51)	< 0.0001	< 0.0001
Adiponectin ($\mu g \cdot mL^{-1}$)	12.88 (7.69)	9.81 (7.16)	14.54 (8.16)	12.08 (7.70)	0.066	0.002
CRP ($mg \cdot L^{-1}$)	0.45 (0.67)	0.60 (0.67)	0.47 (0.76)	0.87 (1.39)	ns	< 0.0001
IL-6 ($pg \cdot mL^{-1}$)	0.52 (0.47)	0.60 (0.54)	0.92 (2.05)	0.68 (0.49)	0.005	ns
IL-1Ra ($ng \cdot mL^{-1}$)	2.92 (10.13)	2.62 (7.09)	2.05 (3.32)	2.65 (7.69)	ns	ns
TNFα ($pg \cdot mL^{-1}$)	0.59 (0.41)	0.55 (0.32)	0.62 (0.70)	0.66 (0.63)	ns	ns
sTNFR1 ($pg \cdot mL^{-1}$)	1.20 (2.60)	0.94 (0.37)	1.03 (0.64)	1.02 (0.45)	ns	ns

All analyses adjusted for sex and pubertal stage.
HiFI: High fitness group, LoFat: Low fatness group, HiFa: High fatness groups, LoFi: Low fitness group (all based on median split for cardiorespiratory fitness and sum of 4 skin folds, respectively)
BMI: body mass index, HDL: high density lipoprotein, CRP: C-reactive protein, IL-6: interleukin-6, IL-1Ra: interleukin-1 receptor antagonist, TNFα: tumor necrosis factor alpha, sTNFR1: soluble TNF receptor-1
ns: non significant
*Interaction between fit*fat groups (*P* = 0.003). Significant difference between low and high fit groups only in high fat group (*P* = 0.001).

Table 2. Pearson correlations between clustered z-score and the inflammatory markers.

	All		*Below median for S4SF*		*Above median for S4SF*	
	r	*p*	*r*	*p*	*r*	*p*
Adiponectin	-0.145	0.003	-0.052	0.464	**-0.139**	**0.048**
CRP	**0.247**	**<0.0001**	-0.030	0.671	**0.296**	**<0.0001**
IL-6	**0.151**	**0.002**	0.091	0.197	0.124	0.079
IL-1Ra	0.008	0.870	0.015	0.838	0.020	0.774
TNFα	**0.101**	**0.042**	0.116	0.101	0.053	0.457
sTNFR1	0.038	0.450	0.061	0.386	0.005	0.943

All analysis adjusted for sex and pubertal status. *r*- and *P*-values for the whole group and for groups based on median split for S4SF.

S4SF: sum of 4 skinfolds, CRP: C-reactive protein, IL-6: interleukin-6, IL-1Ra: interleukin-1 receptor antagonist, TNFα: tumor necrosis factor alpha, sTNFR1: soluble TNF receptor-1

Table 3. Risk of high clustered z-score (above 1SD) based on level of the inflammatory marker. Only markers showing a significant association to clustered z-score are included.

	Odds ratio	95% Confidence Interval	p-value
Adiponectin			
1st quartile	2.612	1.388-4.915	0.003
2nd quartile	1.178	0.603-2.303	0.632
3rd quartile	1.825	0.962-3.463	0.066
4th quartile	1		
CRP			
1st quartile	1		
2nd quartile	1.065	0.536-2.118	0.857
3rd quartile	2.199	1.148-4.215	0.018
4th quartile	3.233	1.755-5.958	<0.0001
IL-6			
1st quartile	1		
2nd quartile	1.938	0.987-3.805	0.055
3rd quartile	2.082	1.075-4032	0.030
4th quartile	2.809	1.462-5.399	0.002
TNFα			
1st quartile	1		
2nd quartile	1.987	1.027-3.841	0.041
3rd quartile	2.376	1.236-4.567	0.009
4th quartile	2.478	1.279-4.804	0.007

Odds ratios for having a clustered z-score above 1 standard deviation (SD) according level of the inflammatory marker (quartiles) adjusted for sex and pubertal status. The lowest quartile was set as reference, except for adiponectin where highest quartile was used as reference.

CRP: C-reactive protein, IL-6: interleukin-6, TNFα: tumor necrosis factor alpha,

Table 4. Pearson correlations between the inflammatory markers.

	r-values (*P*-values)					
	Adiponectin	CRP	IL-6	TNFα	IL-1Ra	sTNFR1
Adiponecti n	1					
CRP	**-0.139 (P = 0.005)**	1				
IL-6	0.069 (P = 0.160)	**0.409 (P ≤ 0.0001)**	1			
TNFα	-0.017 (P = 0.734)	**0.138 (P = 0.005)**	**0.132 (P =0.007)**	1		
IL-1Ra	**0.191 (P ≤ 0.0001)**	0.022 (P = 0.656)	-0.014 (P = 0.782)	0.076 (P = 0.124)	1	
sTNFR1	**0.167 (P = 0.001)**	0.052 (P = 0.295)	-0.020 (P = 0.683)	0.075 (P = 0.128)	**0.524 (P ≤ 0.0001)**	1

CRP: C-reactive protein, IL-6: interleukin-6, IL-1Ra: interleukin-1 receptor antagonist, TNFα: tumor necrosis factor alpha, sTNFR1: soluble TNF receptor-1
Significant findings are marked with bold

Paper IV

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Research Article

Sex Differences in the Association between Level of Childhood Interleukin-6 and Insulin Resistance in Adolescence

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The purpose of this study was to determine whether levels of interleukin-6 (IL-6) in childhood are related to insulin resistance in adolescence. Further, to explore how fatness and cardiorespiratory fitness (VO_{2peak}) moderate this relationship. *Methods*. 292 nine-year-old children ($n = 292$) were followed for 4 years. Anthropometrics and VO_{2peak} were measured. Fasting blood samples were analyzed for IL-6, insulin, and glucose. Homeostasis model assessment (HOMA-IR) was used as a measure of insulin resistance. *Results*. For girls but not boys, levels of IL-6 at age 9 yrs correlated with HOMA-IR at age 13 yrs: $r = 0.223$, $P = 0.008$. Girls with IL-6 levels within the highest quartile at age 9 yrs had an odds ratio of 3.68 (CI = 1.58–8.57) being in the highest quartile of HOMA-IR four years later. *Conclusion*. In this cohort, IL-6 levels in childhood were related to insulin resistance in adolescence, but only for girls.

1. Background

It is well established that increased levels of fatness are associated with low-grade inflammation in adults [1, 2], as well as in children [3]. Low-grade inflammation has been proposed as a mechanism linking obesity with systemic complications such as insulin resistance and type 2 diabetes [4, 5]. Among the inflammatory markers, IL-6 has emerged as one of the potential mediators between obesity and insulin resistance/diabetes [6–8]. However, reports have suggested both pro- and anti-inflammatory effects of IL-6 [9, 10]. IL-6 is a cytokine produced in various tissues including adipose tissue, muscle tissue, and immune cells. Resting, circulating levels of IL-6 have been found positively related to fatness [7, 11] and negatively related to the level of fitness and physical activity [12].

The cross-sectional relationship between IL-6 and insulin resistance in youth has been investigated, but no relationship has been found [13–16]. One longitudinal study, investigated the effect of a weight loss program on inflammatory markers in obese children [17]. They found a weak but significant correlation between change scores of IL-6 and HOMA-IR over one year. To our knowledge, no studies have investigated the longitudinal relation between IL-6 and HOMA over thecourse of childhood and early adolescence in a normal pediatric population. Therefore, the aim of the present study was to evaluate associations between IL-6 and HOMA-IR in a longitudinal design, following children through early adolescence. Furthermore, we aimed at exploring whether fatness or VO_{2peak} moderate these relationships. We hypothesized that in a normal pediatric population (1) a high level of IL-6 in childhood is, not was related to an elevated HOMA-IR in

adolescence and (2) a high level of IL-6 in childhood tracks into adolescence.

2. Methods

2.1. Participants. All children in kindergarten classes (6-7 years of age) from 2 suburbs of Copenhagen, Denmark, were invited to participate in a 3-year, controlled intervention study of physical activity and health: the Copenhagen School Child Intervention Study. Data were collected at baseline, following the intervention, and 4 years after the intervention. The study has been described in details elsewhere [18, 19]. Data used in this paper is from the postintervention measurement in 2003/2004 (age 9 yrs) and the follow-up measurement in 2008 (age 13 yrs). Complete data on IL-6 from both assessments were available for 292 subjects. Since no differences between IL-6 levels in the intervention and control group were found, data from the two groups were pooled for all analyses.

2.2. Ethics. The ethical committee of Copenhagen approved the study. Written informed consent was obtained from the parents/guardians.

2.3. Measurements. Tests were performed at all 18 schools in the two communities. Fitness test was performed in a mobile unit, and all other tests were performed in a gym or a classroom between 8:00 AM and 02:00 PM. Fasting blood samples were collected before 10 AM. Body mass was measured to the nearest 0.1 kg (Seca 882, Brooklyn, NY). Body height was measured to the nearest 1 mm in bare feet (Harpenden stadiometer, West Sussex, UK). Body mass index (BMI) was calculated as weight multiplied by height squared. Skinfolds (biceps, triceps, subscapular and suprailiac) were measured to the nearest mm with caliper (Harpenden, West Sussex, UK) according to criteria presented by De Lorenzo and coworkers [20]. The mean of 3 measurements was used for subsequent analysis. The sum of four skinfolds (S4SF) was calculated and used as an estimate of body fatness [21]. Waist circumference was measured at the end of a normal expiration, midway between the lower rib margin and the anterior iliac crest. Sexual maturation was assessed by self-report using a scale of pictures of breast and genital development for girls and boys, respectively [22]. 5 boys and one girl were unwilling to have their sexual maturation assessed.

Blood samples were collected in the morning after a minimum of 8 hours verified fasting. Children did not have their blood drawn if they suffered from any infectious disease or had fever. Samples were immediately analyzed for glucose (Hemocue, Sweden). The rest of the samples were centrifuged, and the plasma was initially frozen at -20°C and then stored at -80°C the same day. Insulin was analyzed using an enzyme-linked immunosorbent assay (DAKO Insulin, Code no. K6219; DAKO, Glostrup, Denmark). Homeostasis model assessment (HOMA-IR) was used as an estimate of insulin resistance [23]: ([glucose ($\text{mmol}\cdot\text{L}^{-1}$) $\times$ insulin ($\text{mU}\cdot\text{L}^{-1}$)]/22.5). IL-6 was analyzed with high-sensitivity immunoassays (Quantikine High-Sensitivity ELISA) with a detection limit of 0.5 $\text{pg}\cdot\text{mL}^{-1}$ (R&D, Minneapolis, MN). Cardiorespiratory fitness (VO_{2peak}) was measured during a progressive treadmill running test until exhaustion. VO_{2peak} was measured using an AMIS 2001 Cardiopulmonary Function Test System (Innovision, DK 5260 Odense) at age 9 yrs and using the COSMED K4b^2 portable metabolic system (COSMED, Rome, IT) at age 13 yrs. Both systems have been found to give reliable measures of VO_2 uptake, when validated against the Douglas bag method [24, 25]. For a test to be valid, at least one of three objective physiological criteria should be fulfilled; heart rate higher than 200 beats/min, respiratory exchange ratio equal to or higher than 1.00, or a plateau of VO_2 defined as an increase of less than 2.1 $\text{mL}\cdot\text{min}^{-1}\cdot\text{kg}^{-1}$ [26]. Moreover, the test leader should subjectively consider the child exhausted. If a child ran to exhaustion, but did not attain a valid measurement only because of equipment failure, we estimated VO_{2peak} from running time to exhaustion and sex in a regression equation using all the valid measurements, as earlier reported [19]. Approximately 10% of children had their VO_{2peak} calculated.

2.4. Data Analysis. Means and standard deviations for all variables were computed by sex for descriptive purposes. BMI, waist circumference, S4SF, HOMA-IR, and IL-6 were positively skewed and therefore transformed (natural log) for the analyses. Independent samples t-tests were used to test for differences between sexes.

Pearson correlations were used for longitudinal bivariate associations between IL-6 at age 9 yrs and HOMA-IR at age 13 yrs, adjusting for HOMA-IR at age 9 yrs. Then, in a stepwise fashion the correlation were adjusted for (1) S4SF (2) fitness, and (3) both S4SF and fitness at age 13 yrs. Subsequently, children were divided into groups based on their maturation (Tanner stages 1–3 and 4-5), and the correlations were performed stratified for this variable. To further explore the data, logistic regression was performed. The children were divided into sex-specific quartiles of HOMA-IR and IL-6 for the logistic regression. Odds ratios were calculated for being in the upper quartiles of IL-6 and HOMA-IR at age 13 yrs, based on being in the upper quartile of IL-6 at age 9 yrs, compared to the three lower quartiles. The analyses of HOMA were adjusted for HOMA at age 9 yrs to account for any differences already present at baseline. Finally, cross-sectional correlations between IL-6 and HOMA-IR both at age 9 yrs and at age 13 yrs were assessed and adjusted for (1) S4SF, (2) fitness, and (3) S4SF and fitness. All analyses were performed using the statistical package for the social sciences version 15 (SPSS, Chicago, IL).

3. Results

The general characteristics of the samples at the age of 9 and 13 yrs are presented by sex in Table 1. Generally, boys were slightly older and taller than girls and had a greater waist circumference ($P < 0.05$). Boys had a greater aerobic fitness

Table 1: Characteristics of participants by sex and age: mean and standard deviation.

Variables	Boys		Girls	
	9 years	13 years	9 years	13 years
Age (years)	9.6 (0.4)	13.4 (0.3)	9.5 (0.3)	13.3 (0.3)
Height (cm)*	140.6 (5.7)	164.9 (8.0)	138.8 (6.8)	162.2 (6.8)
Weight (kg)	33.7 (6.0)	51.7 (10.0)	32.7 (6.1)	50.5 (8.5)
BMI ($kg \cdot m^{-2}$)	17.0 (2.2)	18.9 (2.7)	16.9 (2.2)	19.2 (2.6)
Normal weight/overweight/obese (%)	88.1/9.9/2.0	88.7/9.3/2.0	87.2/12.1/0.7	90.7/9.3/0.0
Waist circumference (cm)*	62.1 (6.3)	68.3 (7.0)	60.6 (6.2)	65.7 (5.4)
Sum of four skinfolds (mm)*	27.5 (12.5)	31.2 (17.8)	33.8 (15.8)	37.4 (15.6)
Tanner stages (1–5)	1.1 (0.2)	3.4 (0.8)	1.4 (0.6)	3.8 (0.8)
Aerobic fitness ($mL \cdot kg^{-1} \cdot min^{-1}$)*	53.4 (6.8)	53.0 (7.9)	47.6 (5.9)	45.7 (7.2)
IL-6 ($pg \cdot mL^{-1}$)	0.8 (1.0)	0.6 (0.5)	1.1 (1.3)	0.8 (1.4)
HOMA score*	1.2 (0.6)	2.5 (1.4)	1.3 (0.7)	2.9 (1.5)

* $P < 0.05$, girls versus boys.

Table 2: Pearson correlations between IL-6 at age 9 yrs and IL-6 and HOMA-IR at age 13 yrs, (1) unadjusted, (2) adjusted for sum of 4 skinfolds (S4SF), (3) VO_{2peak} (mL/kg), and (4) both S4SF and VO_{2peak} at age 13 yrs.

Risk factors age 13 yrs	IL-6 age 9 yrs			
	Unadjusted	Adjusted for S4SF	Adjusted for VO_{2peak}	Adjusted for S4SF & VO_{2peak}
IL-6				
Boys	0.130	0.138	0.136	0.133
Girls	**0.360***	**0.349***	**0.402***	**0.388***
HOMA-IR[a]				
Boys	0.053	0.065	0.044	0.055
Girls	**0.223***	**0.199***	**0.223***	**0.211***

* P value < 0.04. Significant correlations in bold face.
[a] adjusted for HOMA age 9 yrs.

Table 3: Pearson correlations between IL-6 at age 9 yrs and IL-6 and HOMA-IR at age 13 yrs in groups based on sexual maturation and adjusted for HOMA age 9 yrs.

		n	r
Boys	Tanner stages 1–3	83	0.006
	Tanner stages 4-5	63	0.107
Girls	Tanner stages 1–3	41	0.286†
	Tanner stages 4-5	99	**0.218***

Significant correlations in bold face. *P value = 0.031. †P value = 0.077, borderline significant.

Table 4: Risk of high IL-6 and HOMA-IR level in adolescence based on IL-6 level in childhood.

		95% confidence	
	Odds ratio	Interval	P value
IL-6 at age 13			
Boys	0.90	0.38–2.12	0.81
Girls	**2.55**	**1.12–5.80**	**0.03**
HOMA-IR at age 13[a]			
Boys	0.93	0.39–2.22	0.87
Girls	**3.68**	**1.58–8.57**	**0.003**

Odds ratios for being in the highest quartile of CVD risk factors age 13 yrs according to being in the upper quartile of IL-6 at age 9 yrs. Significant odds ratios are presented in bold.
[a]: adjusted for HOMA at age 9 yrs.

and lower S4SF (both $P < 0.001$), as well as lower HOMA-IR ($P < 0.02$), compared to girls. At ages 9 and 13 yrs, the girls were on average ~0.4 Tanner stages ahead of the boys ($P < 0.001$).

Relationships between circulating IL-6 levels at age 9 yrs and IL-6 and HOMA-IR at age 13 yrs are presented in Table 2. A significant correlation was found between levels of IL-6 at age 9 yrs and at age 13 yrs for the girls, but not for the boys. Furthermore, the girls also displayed correlations between IL-6 levels at age 9 yrs and HOMA-IR four years later. These correlations were weakened after adjusting for fatness, and fitness and fatness combined at age 13 yrs. Table 3 shows these correlations stratified by Tanner stages. Only the most mature girls (Tanner stages 4-5) displayed a significant correlation between IL-6 levels at age 9 yrs and HOMA-IR at age 13 yrs.

Logistic regression was performed to assess the risk of ending up in the highest quartile of HOMA-IR at age 13 yrs after having had systemic IL-6 levels within the upper quartile at age 9 yrs. The results are presented in Table 4. Girls

with IL-6 levels within the upper quartile at age 9 yrs had an odds ratio of 2.55 for having IL-6 levels within the upper quartile at age 13 yrs and an odds ratio of 3.86 for being in the upper quartile of HOMA-IR, compared with the rest of the girls. There were no other significant increased risks ($P > 0.05$).

There were no significant cross-sectional correlations between IL-6 levels and HOMA-IR at age 9 yrs or at age 13 yrs, irrespective of adjustment for fatness and fitness. HOMA age 9 yrs was correlated with HOMA age 13 yrs for girls ($r = 0.242$, $P = 0.004$), but not for boys ($r = 0.143$, $P = 0.083$).

4. Discussion

To our knowledge this is the first study to examine the longitudinal relation between IL-6 and HOMA-IR in a normal pediatric population. A correlation was observed between circulating IL-6 levels at age 9 yrs and HOMA-IR at age 13 yrs for girls, but not for boys. In support of a longitudinal relationship between IL-6 and HOMA-IR, Roth and colleagues found a weak but significant correlation between change scores of IL-6 and HOMA-IR over one year in an intervention study on obese children [17]. This result, together with our finding in girls, could suggest a role of IL-6 in the longitudinal development of insulin resistance. However, we did not find any correlations between IL-6 and HOMA-IR in the cross-sectional analyses, in accordance with findings of other cross-sectional studies on IL-6 and insulin level or HOMA-IR in youth [13–15, 27].

The specific role of IL-6 in the pathogenesis of insulin resistance is still controversial. It has been shown that IL-6 suppresses the production of TNF-α, a cytokine involved in the pathogenesis of insulin resistance and CVD [10]. Concomitantly, TNF-α causes IL-6 production and release of IL-6 to the circulation [9]. Therefore, hypothetically it is possible that TNF-α actually induces impaired glucose metabolism, while high systemic levels of IL-6 reflect a high local production of TNF-α and are not directly involved in the pathogenesis of insulin resistance [9]. Unfortunately, we were not able to include other measures of low-grade inflammation, for example, TNF-α, TNF-modifying mediators, or CRP in this study, which makes it impossible for us to make any interpretations regarding the mechanisms by which low-grade inflammation influence the development of insulin resistance. Our findings should therefore just be interpreted as descriptive.

Body fat is related to HOMA-score [28], as well as to systemic IL-6 levels in some studies of pediatric populations [16, 29–31], but not all [14, 17, 27, 32]. Fatness could therefore potentially influence the relation between IL-6 and HOMA-IR. Adjusting for S4SF at age 13 yrs weakened the correlation found in girls between IL-6 at age 9 yrs and HOMA-IR at age 13 yrs in this study, but systemic IL-6 levels remained as an independent predictor of high HOMA-IR at age 13 yrs. A study by Tam and colleagues found no significant differences in IL-6 level between normal weight and overweight/obese groups at age 8 yrs, but when followed up at age 15 yrs, overweight/obese girls had significantly higher levels of IL-6, compared to normal-weight girls. No differences were seen for boys [33]. This study supports our finding of a sex difference in relation to IL-6, which has also been found in studies on adult populations [34, 35]. Furthermore, their results, together with our results, suggest that in girls elevated IL-6 production develops over a prolonged period of time and is, possibly, influenced by sexual maturation or estrogens. The girls in our study were on average 0.4 tanner stages ahead of the boys at the same age (Table 1), which may account for some of the sex differences we found in the relations between IL-6 and HOMA-IR. When the girls in our study were split into groups of maturation stages (Tanner stages 1–3 and Tanner stages 4-5), only the mature girls displayed a significant correlation between IL-6 and HOMA-IR (Table 3). However, this difference in significant findings between sexual maturation groups was probably caused by a lack of power due to the small sample size in the less mature group. Actually, the correlation coefficient in the less mature group was higher compared to the more mature.

In the present study, adjustment for VO_{2peak} did not alter the correlations between IL-6 and HOMA-IR. Likewise, Rubin et al. did not find any effect of adjusting for maximal aerobic power in the association between HOMA-IR and IL-6 [13]. Other studies have investigated the relationship between IL-6 and fitness in youth and did not find any correlation [15, 16].

The main strength of the present study is the longitudinal design with a four-year period between the two measurements. To our knowledge, no other researchers have followed children for this length of time. One limitation of this study is the low number of overweight and obese individuals in the study population and low mean values for both HOMA-IR and IL-6 levels (Table 1). However, we have earlier reported that in this cohort ~14% of the children had an adverse CVD risk profile at age 9 yrs [36]. Also, we did not include other inflammatory markers, for example, TNF-α and TNF-modifying mediators in this study, thus we cannot exclude that some confounders related to both IL-6 and insulin resistance are affecting the associations we investigated. Therefore, no conclusions regarding the mechanisms by which low-grade inflammation affect insulin resistance can be drawn. Future studies could therefore focus on other inflammatory markers more closely related to the pathogenic processes in youth, for example, CRP and TNF-α. Furthermore, a more diverse population including a higher number of overweight and obese children and more children with adverse health outcomes could strengthen the scientific impact of the results. Also the genetic aspects in the relation between low-grade inflammation and the pathogenesis of insulin resistance should be investigated in future studies. Finally, we did not control for exercise immediately prior to blood sampling. However, blood sampling was done early in the morning, and it was not likely that any of the children had performed prolonged exercise prior to sampling. Furthermore, although we excluded children with any signs of infections or fever, undetected subclinical infection could have caused an increase in IL-6 levels in some children. We did, however, try to run all analyses

omitting outliers and that did not change our results substantially.

In conclusion, the results of this study show that IL-6 levels in childhood track into adolescence, especially for girls. Further, IL-6 levels in childhood are related to HOMA-IR four years later for girls, but not for boys. These relationships remained significant after adjusting for fatness and fitness. We did not find any cross-sectional association between IL-6 and HOMA-IR at either age 9 or age 13 yrs, suggesting that in these age groups the level of IL-6 is not directly related to HOMA-IR.

Abbreviations

CVD:	Cardiovascular disease
IL-6:	Interleukin-6
VO_{2peak}:	Cardiorespiratory fitness
HOMA-IR:	Homeostatic model assessment
CRP:	C-reactive protein
TNF-α:	Tumor necrosis factor-α
BMI:	Body mass index
S4SF:	The sum of four skinfolds.

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Appendix: Intervention effects on subgroups

Table 1. Subgroup based on sum of four skinfolds (at or above 1 SD) at baseline. Change-scores (mean & SD) and adjusted differences between IG and CG.

		At or above 90th percentile for waist at baseline							
		Change baseline to post-intervention				Change baseline to follow-up			
Variables	Group	n	Change-scores (SD)	Adjusted difference (95% CI)	*P-value*	n	Change-scores (SD)	Adjusted difference (95% CI)	*P-value*
BMI (kg/m^2) *	IG	29	2.45 (0.29)			15	4.94 (0.63)		
	CG	29	1.70 (0.29)	0.75 (-0.08 – 1.57)	0.075	17	4.13 (0.60)	0.81 (-0.92 – 2.55)	0.347
zBMI for age	IG	29	0.18 (0.15)			15	-0.51 (0.18)		
	CG	29	-0.31 (0.15)	***0.50 (0.07 - 0.93)***	***0.025***	17	-0.22 (0.17)	-0.29 (-0.80 - 0.22)	0.247
Sum of 4 skinfolds (mm)	IG	29	6.18 (0.44)			15	18.63 (5.04)		
	CG	29	4.86 (0.53)	1.32 (-0.03 – 2.68)	0.055	17	15.14 (4.78)	3.50 (-10.49 – 17.48)	0.613
Waist (cm)	IG	29	9.9 (1.32)			15	12.84 (2.32)		
	CG	29	8.5 (1.33)	1.45 (-2.29 – 5.20)	0.441	17	13.22 (2.21)	-0.38 (-6.84 – 6-07)	0.904
Mean PA (counts/min)	IG	16	-19 (62)			7	-230 (75)		
	CG	19	-63 (57)	44 (-131 - 219)	0.611	12	-298 (59)	68 (-133 – 269)	0.482
MVPA (min/day above 1500 counts/min) *	IG	16	14.9 (10.1)			7	-36.4 (17.6)		
	CG	19	-13.3 (9.23)	***28.2 (-0.32 – 56.7)***	***0.052***	12	-48.7 (13.9)	12.3 (-34.7 – 59.2)	0.586
Cardiorespiratory fitness (VO_{2peak}) (ml kg^{-1} min^{-1})	IG	27	-0.29 (5.93)			13	0.51 (2.13)		
	CG	26	-1.06 (6.88)	0.0.77 (-2.35 – 3.89)	0.623	14	-0.16 (2.05)	0.67 (-5.37 -6.71)	0.821
Systolic Blood Pressure (mmHG)	IG	28	8.71 (1.45)			14	7.78 (2.63)		
	CG	29	8.02 (1.92)	0.70 (-4.80 - 6.20)	0.801	18	10.27 (2.38)	-2.49 (-9.66- 4.68)	0.483
Insulin resistance (HOMA-IR)	IG	15	0.61 (0.29)			10	3.84 (0.96)		
	CG	14	0.90 (0.30)	-0.29 (-1.16 - 0.57)	0.493	10	2.82 (0.97)	1.01 (-1.92 – 3.94)	0.474
Cholesterol/HDL-ratio	IG	15	-0.27 (0.18)			10	0.080 (0.24)		
	CG	14	-0.38 (0.18)	0.11 (-0.42 - 0.63)	0.678	10	0.32 (0.24)	-0.24 (-0.98- 0.49)	0.495
Triglyceride mmol/l	IG	15	-0.10 (0.33)			10	0.22 (0.07)		
	CG	14	0.029 (0.30)	-0.13 (-0.38- 0.11)	0.274	10	0.21 (0.07)	0.011 (-0.20 – 0.22)	0.913
Sum of Z-scores	IG	14	2.70 (1.47)			8	9.62 (2.19)		
	CG	13	3.32 (1.52)	-0.66 (-5.03 – 3.71)	0.758	8	7.25 (2.19)	2.37 (-4.50 – 9.23)	0.467

Change-scores from baseline to post-intervention and from baseline to follow-up presented by group (mean and standard deviations (SD)). The adjusted difference between groups in change-scores is the net effect of the intervention; intervention group change-scores minus control group change-scores adjusted for sex and maturation (mean and 95% confidence intervals (CI)). Significant and borderline significant differences between intervention and control group change-scores are marked with bold and italic.

*: Becomes significant when using M;cCarthy cutpoints

Table 2. Subgroup based on fitness (at or below -1 SD) at baseline. Change-scores (mean & SD) and adjusted differences between IG and CG.

		Change baseline to follow-up							
		Below 90th percentile for waist at baseline				**At or above 90th percentile for waist at baseline**			
Variables	**Group**	**n**	**Change-scores (SD)**	**Adjusted difference (95% CI)**	***P-value***	**n**	**Change-scores (SD)**	**Adjusted difference (95% CI)**	***P-value***
BMI (kg/m^2)	IG	47	1.80 (0.19)			32	4.47 (0.33)		
	CG	35	1.48 (0.22)	0.32 (-0.26 – 0.90)	0.278	19	2.91 (0.43)	***1.56 (0.50 -2.65)***	***0.006***
zBMI for age	IG	47	0.23 (0.09)			32	-0.21 (0.10)		
	CG	35	-0.20 (0.11)	***0.43 (0.14 – 0.71)***	***0.004***	19	-0.37 (0.14)	0.16 (-0.18 – 0.50)	0.352
Sum of 4 skinfolds (mm)	IG	47	11.79 (1.76)			32	14.49 (2.63)		
	CG	35	10.23 (2.03)	1.57 (-3.78 – 6.91)	0.561	19	6.16 (3.48)	8.33 (-0.41 – 17.08)	0.061
Waist (cm)	IG	48	8.71 (0.85)			32	13.95 (1.22)		
	CG	33	7.50 (1.02)	1.21 (-1.43)	0.364	19	10.62 (1.62)	3.33 (-0.74 – 7.39)	0.106
Mean PA (counts/min)	IG	27	-127 (44)			14	-213 (52)		
	CG	18	-44 (55)	-82 (-228 -63)	0.260	9	-153 (65)	-59 (-235 – 118)	0.494
MVPA (min/day above 1500 counts/min)Y	IG	27	-5.91 (6.12)			14	-25.49 (7.00)		
	CG	18	-3.94 (7.54)	-1.97 (-22.12 – 18.19)	0.845	9	-28.93 (8.89)	3.44 (-20.53 – 27.41)	0.767
Cardiorespiratory fitness (VO_{2peak}) (ml kg^{-1} min^{-1})	IG	47	5.64 (0.87)			29	7.40 (1.52)		
	CG	33	7.00 (1.04)	-1.37 (-4.05 – 1.32)	0.314	18	6.14 (2.00)	1.26 (-3.84 – 6.36)	0.620
Systolic Blood Pressure (mmHG)	IG	48	6.53 (1.39)			31	10.85 (1.57)		
	CG	35	7.44 (1.63)	-0.914 (-5.18 – 3.35)	0.671	19	13.07 (1.98)	-2.22 (-7.29 – 2.85)	0.383
Insulin resistance (HOMA-IR)	IG	33	0.49 (0.16)			25	2.35 (0.32)		
	CG	15	0.45 (0.24)	0.045 (-0.54 – 0.63)	0.878	10	0.62 (0.53)	***1.73 (0.53 – 2.94)***	***0.006***
Cholesterol/HDL-ratio	IG	35	-0.39 (0.11)			25	-0.045 (0.16)		
	CG	15	0.44 (0.17)	0.057 (-0.35 – 0.47)	0.783	10	-0.20 (0.26)	0.157 (-0.44 – 0.76)	0.597
Triglyceride mmol/l	IG	35	-0.032			25	0.167 (0.063)		
	CG	15	0.077	-0.032 (-0.22 – 0.15)	0.722	10	0.149 (0.104)	0.018 (-0.22 – 0.26)	0.877
Sum of Z-scores	IG	32	1.21 (0.81)			22	5.88 (1.25)		
	CG	15	0.71 (1.23)	0.50 (-2.48 – 3.48)	0.737	9	2.73 (2.05)	3.15 (-1.65 – 7.95)	0.189

Change-scores from baseline to post-intervention and from baseline to follow-up presented by group (mean and standard deviations (SD)). The adjusted difference between groups in change-scores is the net effect of the intervention; intervention group change-scores minus control group change-scores adjusted for sex and maturation (mean and 95% confidence intervals (CI)). Significant differences between intervention and control group change-scores are marked with bold and italic.