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SERUM LIPID STATUS IN EUROPEAN ADOLESCENTS – ASSOCIATIONS WITH AGE, GENDER, MATURITY, BODY MASS INDEX, PERCENTAGE BODY FAT, AND FAT FREE MASS

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In der Schule haben wir gelernt, dass Lehrerwissen absolutes Wissen ist. Doch Wissenschaft kann niemals absolut sein. Sie ist die Kunst der Annäherung. Sie definiert nicht, sondern kreist ein, zieht keine Trennlinien, sondern schafft Übergänge, kennt keine Dogmen, sondern Entwicklungen. Sie kann nichts verifizieren, sondern nur durch Wegstreichen von Variablen ein möglichst klares Bild entwerfen. Selbst die Naturgesetze sind streng genommen Hypothesen. Wenn der Apfel jedes Mal zu Boden fällt, sobald man ihn loslässt, drängen sich absolute Aussagen regelrecht auf. Im Grunde resultieren die entsprechenden Gesetze aber nur aus identischen Versuchsreihen, die bis heute ausnahmslos das gleiche Ergebnis lieferten.

~ Frank Schätzing ~

für meine lehrer

Serum lipid status in European adolescents - associations with age, gender, maturity, body mass index, percentage body fat, and fat free mass

The development of cardiovascular diseases and atherosclerosis and its relation to lipids (e.g. cholesterol, lipoproteins, and fatty acids [FA]) is well established and begins in childhood and adolescence. Beside that, an adequate lipid profile is also important for normal growth and development in youth. However, less is known about how factors associated with adolescence like age, gender, maturity, body mass index (BMI), body fat (BF), and fat free mass (FFM) are influencing lipid profiles in healthy European adolescents.

Consequently, this thesis investigated the association of age, gender, maturity, BMI, BF, and FFM with the lipid profile of European adolescents within the *Healthy Lifestyle in Europe by Nutrition in Adolescence* (HELENA) Study. More precisely, the investigated associations of the factors mentioned above with clinical lipid parameters (i.e. total cholesterol (TC), high density lipoprotein cholesterol (HDL), low density lipoprotein cholesterol (LDL), triglycerides (TG), lipoprotein(a) [Lp(a)], and the apolipoproteins (apo) A-1 and B) as well as with several lipid and lipoprotein ratios are described in *chapter 4*. The relation of age, gender, maturity, BMI, BF, and FFM to the FA profile is presented in *chapter 5*. The results of the association of age, gender, maturity, BMI, BF, and FFM with several FA ratios are provided in *chapter 6*. Further, in *chapter 7* the relationships between selected FA and TC, HDL, LDL, TG, Lp(a), apo A-1 and B are summarized.

Generally, the results were as follows: the clinical lipid parameters were higher in girls than in boys. Furthermore, almost all absolute FA values were higher in girls than in boys whereas the relative FA profile showed less clear gender differences.

Triglyceride levels were positively associated with age in boys. Further, in both boys and girls some FA of the absolute FA profile and the relative FA profile were related to age.

Progression in maturity was associated with lower TC and HDL levels in boys and changes in TC, LDL, and apo B levels without clear direction in girls. Additionally, maturity was associated with fluctuations in the FA profile without clear pattern in boys and girls.

Body mass index was hardly associated with the lipid profile of boys. By contrast, TG levels and several lipid and lipoprotein ratios increased and HDL levels decreased with higher BMI status in girls.

Body fat was mainly associated with changes in the male subjects. In boys, TG, LDL, and several lipid and lipoprotein ratios increased, whereas HDL decreased with higher body fat. The FA profile and FA ratios showed minimal associations with body fat.

Fat free mass was positively associated with C20:5ω3c/C18:3ω3c ratios in boys and also associated with some FA in both sexes.

In *chapter 7*, individual serum FA (i.e. C16:0, C18:0, C18:1ω9c, C18:2ω6c, C18:3ω3c, C20:4ω6c, C20:5ω3c, and C22:6ω3c) were associated with clinical lipid parameters (i.e. TC, HDL, LDL, TG, Lp(a), apo A-1, and apo B) using multiple linear regression models. The effect of individual FA on several lipids and lipoproteins was minimal. The highest association was found between C16:0 and C18:0 with TG levels. In boys and girls, approximately 7% and 4% of the TG level variability could be related to C16:0 and C18:0. The other clinical lipid parameters showed minor associations (R^2 change=0.2%-3.5%) in both sexes.

Serum-Lipidstatus in europäischen Jugendlichen – Assoziationen mit Alter, Geschlecht, Reife, Body-Mass-Index, prozentualem Körperfettanteil und Magermasse

Das Auftreten von kardiovaskulären Erkrankungen und Atherosklerose steht in engem Zusammenhang mit Blutlipiden (z. Bsp. Cholesterol, Lipoproteinen, Fettsäuren [FS]), wobei erste Ausprägungen schon in früher Kindheit bzw. im Jugendalter diagnostiziert wurden. Andererseits sind adäquate Blutlipidwerte für ein normales Wachstum und eine entsprechende Entwicklung in diesem Alter von großer Bedeutung. Allerdings sind Faktoren, die die Adoleszenz beeinflussen können, wie Alter, Geschlecht, sexuelle Reife, Body-Mass-Index (BMI), prozentualer Körperfettanteil (BF) und die Magermasse (FFM), in gesunden europäischen Jugendlichen nicht hinreichend untersucht.
Daher war das Ziel dieser Arbeit, den Zusammenhang zwischen Alter, Geschlecht, Reife, BMI, BF und FFM und den Blutlipiden von europäischen Jugendlichen anhand der Daten der *Healthy Lifestyle in Europe by Nutrition in Adolescence* (HELENA) Studie zu untersuchen.
In *Kapitel 4* der Arbeit wurden die Assoziationen zwischen den oben genannten Faktoren und den klinischen Lipidparametern Gesamtcholesterin (TC), HDL, LDL, Triglyzeriden (TG), Lipoprotein(a) [Lp(a)], den Apolipoproteinen (apo) A-1 und B sowie verschiedenen Lipid- und Lipoproteinquotienten beschrieben. Weitere Schwerpunkte wurden auf die Zusammenhänge zwischen Alter, Geschlecht, Reife, BMI, BF sowie FFM und dem FS-Profil (*Kapitel 5*) und den FS-Quotienten (*Kapitel 6*) gelegt. In *Kapitel 7* wurde beschrieben, inwieweit ausgewählte FS einen Zusammenhang mit den klinischen Lipidparametern zeigten.
Aus der vorliegenden Arbeit lassen sich folgenden Ergebnisse zusammenfassen: Mädchen wiesen höhere Werte der klinischen Lipidparameter auf als Jungen. Dasselbe wurde für die meisten absoluten FS-Werte beobachtet. Darüber hinaus waren die FS der prozentualen Verteilung nicht eindeutig geschlechtsspezifisch erhöht.
Die TG-Werte der Jungen zeigten einen positiven Zusammenhang mit dem Alter. Weiterhin waren einige FS des relativen und absoluten FS-Musters bei Jungen und Mädchen mit dem Alter assoziiert.
Sinkende TC- und HDL-Werte waren bei männlichen Teilnehmern mit fortschreitender sexueller Reife zu beobachten. Im Gegensatz dazu waren bei den weiblichen Studienteilnehmern die TC-, LDL- und Apo B-Werte mit der sexuellen Reife assoziiert, wobei jedoch ein klarer Verlauf nicht zu erkennen war. Ein eindeutiges Muster konnte auch nicht in Bezug auf das FS-Profil festgestellt werden, obwohl einige FS mit dem Fortschreiten der sexuellen Reife in Verbindung gebracht werden konnten. Dies konnte sowohl für Jungen als auch für Mädchen gezeigt werden.
Die Blutlipidwerte der Jungen zeigten kaum Zusammenhänge mit dem BMI. Auf der anderen Seite waren die TG-Werte der Mädchen sowie die meisten Lipid- und Lipoproteinquotienten mit steigendem BMI erhöht und das HDL erniedrigt.
Der Körperfettanteil war hauptsächlich mit Blutlipiden der männlichen Teilnehmer assoziiert. Diese zeigten erhöhte TG- und LDL-Werte sowie erhöhte Lipid- und Lipoproteinquotienten mit erhöhtem BF. Zusätzlich war das HDL der Jungen mit höherem BF erniedrigt. Das FS-Profil und die FS-Quotienten zeigten nur geringe Zusammenhänge zum Körperfett.
Die Magermasse war positiv assoziiert mit dem C20:5ω3c/C18:3ω3c-Quotienten der Jungen. In beiden Geschlechtern zeigten einzelne FS Zusammenhänge mit der Magermasse.
In *Kapitel 7* wurde der Zusammenhang zwischen den FS C16:0, C18:0, C18:1ω9c, C18:2ω6c, C18:3ω3c, C20:4ω6c, C20:5ω3c sowie C22:6ω3c und TC, HDL, LDL, TG, Lp(a), apo A-1 und B mittels multipler, linearer Regressionsanalysen untersucht. Es zeigte sich jedoch, dass der Zusammenhang zwischen den Parametern minimal war. Lediglich die FS C16:0 und C18:0 zeigten einen wesentlichen Zusammenhang mit den TG-Werten. In Jungen und Mädchen war die Variabilität der TG-Werte zu 7% bzw. 4% durch diese FS zu erklären. Die restlichen Parameter waren mit den FS in Größenordnungen von 0.2%-3.5% assoziiert.

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List of Abbreviations

Fatty Acids		
Code	**Common name**	**Scientific name**
C12:0	Lauric acid	Dodecanoic acid
C14:0	Myristic acid	Tetradecanoic acid
C14:1ω5c	Myristoleic acid	*Cis*-9-Tetradecenoic acid
C15:0		Pentadecanoic acid
C15:1ω5c	Pentadecenoic acid	*Cis*-10-Pentadecenoic acid
C16:0	Palmitic acid	Hexadecanoic acid
C16:1ω7c	Palmitoleic acid	*Cis*-9-Hexadecenoic acid
C17:0		Heptadecanoic acid
C17:1ω7c	Heptadecenoic acid	*Cis*-10-Heptadecenoic acid
C18:0	Stearic acid	Octadecanoic acid
C18:1ω7c	Vaccenic acid	*Cis*-11-Octadecenoic acid
C18:1ω7t	Trans vaccenic acid	*Trans*-11-Octadecenoic acid
C18:1ω9c	Oleic acid	*Cis*-9-Octadecenoic acid
C18:2ω6c	Linoleic acid (LA)	*All-cis*-9,12-Octadecadienoic acid
C18:2ω6t	Linolelaidic acid	*All-trans*-9,12-Octadecadienoic acid
C18:3ω3c	α-Linolenic acid (ALA)	*All-cis*-9,12,15-Octadecatrienoic acid
C18:3ω6c	γ-Linolenic acid	*All-cis*-6,9,12-Octadecatrienoic acid
C18:4ω3c	Stearidonic acid	*All-cis*-6,9,12,15-Octadecatetraenoic acid
C20:0	Arachidic acid	Eicosanoic acid
C20:1ω9c	Eicosenoic acid	*Cis*-11-Eicosenoic acid
C20:2ω6c	Eicosadienoic acid	*All-cis*-11,14-Eicosadienoic acid
C20:3ω3c	Eicosatrienoic acid	*All-cis*-11,14,17-Eicosatrienoic acid
C20:3ω6c	Dihomo-γ-linolenic acid (DHGLA)	*All-cis*-8,11,14-Eicosatrienoic acid
C20:3ω9c	Mead acid	*All-cis*-5,8,11-Eicosatrienoic acid
C20:4ω6c	Arachidonic acid (AA)	*All-cis*-5,8,11,14-Eicosatetraenoic acid

The suffix “-ic acid” can be substituted for “-ate”
Fatty acids with the suffix “c” indicate *cis*, whereas the suffix “t” indicates *trans* configurations.

Fatty acids

Code	Common name	Scientific name
C20:5ω3c	Eicosapentaenoic acid (EPA)	*All-cis*-5,8,11,14,17-Eicosapentaenoic acid
C21:0		Heneicosanoic acid
C22:0	Behenic acid	Docosapentanoic acid
C22:1ω9c	Erucic acid	*Cis*-13-Docosenoic acid
C22:2ω6c	Docosadienoic acid	*All-cis*-13,16-Docosadienoic acid
C22:5ω3c	Docosapentaenoic acid	*All-cis*-7,10,13,16,19-Docosapentaenoic acid
C22:6ω3c	Docosahexaenoic acid (DHA)	*All-cis*-4,7,10,13,16,19-Docosahexaenoic acid
C23:0		Tricosanoic acid
C24:0	Lignoceric acid	Tetracosanoic acid
C24:1ω9c	Nervonic acid	*Cis*-15-Tetracosenoic acid

The suffix "-ic acid" can be substituted for "-ate"
Fatty acids with the suffix "c" indicate *cis*, whereas the suffix "t" indicates *trans* configurations.

Lipids, lipoproteins and apolipoproteins

Abbreviation	Name
Apo A-1	Apolipoprotein A-1
Apo B	Apolipoprotein B
HDL	High density lipoprotein cholesterol
LDL	Low density lipoprotein cholesterol
Lp(a)	Lipoprotein(a)
nonHDLC	Non high density lipoprotein cholesterol
TC	Total cholesterol
TG	Triglycerides

Other Abbreviations and Symbols	
Abbreviation/Symbol	**Name**
%	Percentage
∑	Sigma/summation sign
®	Registered Trademark
°C	Degrees Celsius
µL	Microlitre(s)
µmol	Micromole(s)
µV	Micro Volt
♀	Female symbol
♂	Male symbol
a.m.	ante meridiem (=in the morning)
AI	Atherogenic index
ANCOVA	Analysis of Covariance
ANOVA	Analysis of Variance
approx.	approximately
AVENA	Alimentación y Valoración del Estado Nutricional en Adolescentes (=Food and Assessment of the Nutritional Status of Adolescents)
BEFO	Behavioural and food choice study
BF	Body fat
BIA	Bioelectrical impedance analysis
BMI	Body mass index
bp	Boiling point
bzw.	beziehungsweise
CDS	Chromatography data system
CHD	Coronary heart disease
cm	Centimetre(s)
COMS	Crossover center study
CSS	Cross-sectional study
CV	Coefficient of variation
dL	Decilitre(s)

Other Abbreviations and Symbols	
Abbreviation/Symbol	**Name**
e.g.	exempli gratia (=for example)
et al.	*et alii* (=and others)
EU	European Union
FA	Fatty acid(s)
FAME/F.A.M.E.	Fatty acid methyl ester(s)
FFM	Fat free mass
FS	Fettsäure(n)
g	Gram(s)
GC	Gas chromatograph
h	Hour(s)
H_2	Hydrogen
HCl	Hydrochloric acid
HELENA	Healthy Lifestyle in Europe by Nutrition in Adolescence
i.e.	id est (=that is)
IEL	Institut für Ernährungs- und Lebensmittelwissenschaften (Institute of Nutrition and Food Sciences)
IS	Internal standard
kg	Kilogram(s)
L	Litre(s)
LCPUFA	Long-chain polyunsaturated fatty acids
LSEI	Lifestyle education intervention
mg	Milligram(s)
min	Minute(s)
mL	Millilitre(s)
mLU	Millilitre units
mm	Millimetre(s)
MM	Molecular mass
mmol	Millimole(s)

Other Abbreviations and Symbols

Abbreviation/Symbol	Name
MUFA	Monounsaturated fatty acids
n	Number of
N_2	Nitrogen
NCEP	National Cholesterol Education Program
ng	Nanogram(s)
NHANES	National Health and Nutrition Examination Survey
nmol	Nanomole(s)
p.m.	post meridiem (=in the afternoons)
PA	Peak area
PL	Phospholipid(s)
PUFA	Polyunsaturated fatty acids
r	Correlation coefficient
R^2	Coefficient of determination
SD	Standard deviation
SFA	Saturated fatty acids
SPSS	Statistical Package for Social Sciences
TEM	Technical errors of measurement
TLC	Thin-layer chromatography
UV	Ultra violet
vs	versus
WP	Work package
x *g*	x *gravity* (*g*=9.8 m/s²)
z. Bsp.	zum Beispiel
α	alpha
γ	gamma
ω	omega [Indicates the first double bond of carbon atoms beginning to count from the methyl end of a fatty acid]

Every human being is the author of his own health or disease.

~ Siddharta Gautama Shakyamuni ~

560-480 B.C.

1 General Introduction

The importance of reliable lipid data in adolescence

Adolescence is a life span characterized by rapid growth and development. Prerequisite for normal growth and development in this phase is an adequate provision of energy, macro- and micronutrients. A rapidly growing body is more sensitive to under- and/ or malnutrition than an adult. Moreover, an imbalanced nutrition in adolescence can already contribute to develop early signs and/ or risks for chronic diseases like atherosclerosis [1-7].

A balanced lipid profile is an important factor to maintain health in young age and to avoid early morbidity in later life. Therefore, blood lipid profiles in adolescents should be screened as early as possible to detect imbalances. A lipid profile screening should include the blood parameters mentioned below, which are suitable biomarkers and can be easily measured.

An objective evaluation of analytical data from cross-sectional studies in adolescents is, however, hampered by the fact that reliable reference data considering communicable and non-communicable determinants is not available. Consequently, the evaluation of blood lipid profiles in adolescence uses reference data of the American National Cholesterol Education Program (NCEP) for children and adolescents [8], which might lead to wrong conclusions for further reasons. First of all, the data is based on examinations of children and adolescents from families with hypercholesterolemia or premature cardiovascular disease, thus the subjects might not be healthy because of the familial predisposition. Secondly, the lipid values were defined in the 1980s and are addressed to the American adolescent population. Thus, reference data for Europe should include data based on examinations of apparently healthy European adolescents, considering communicable and non-communicable factors as well as being up-to-date.

With respect to fatty acid (FA) profiles, Decsi and Koletzko mentioned the importance of available and adequate reference data from a healthy population for the paediatric age group as prerequisite for the diagnosis of an altered FA status and the monitoring of a therapeutic intervention, but their proposed FA reference data was based on a Hungarian study group with a quite large age range (0-26 years) and a relatively small sample size (n=115). Even though, the authors categorized the study population into more specific age groups, the age range of the adolescent group (10 to 15 years) misrepresent adolescence and the study size became even smaller (n=25) [9]. Therefore, reference data should be based on a larger study group with a more appropriate age range.

Within adolescence, sexual hormones play an increasing role in controlling physiological processes and body composition, especially body fat distribution. Body mass index (BMI), percentage body fat (BF), and fat free mass (FFM) can be used to judge body composition. Moreover, sexual hormones affect lipid levels and other blood parameters. Thus, gender-specific differences in these parameters become more obvious in adolescence [10, 11]. Consequently, gender should be taken into account when investigating and interpreting data from cross-sectional studies in adolescence.

By contrast, age describes mainly the chronological development of individuals and is generally used as the main category to group adolescent subjects. As a consequence, significant findings are often related to age, even though the results might reflect changes due to sexual maturation [12]. Because of the particular role of sexual hormones in adolescence, as noted above, puberty has a main influence on lipid levels [12-14], thus maturity should be favoured over age for categorizations.

Blood lipid profiles in adolescents – descriptions and present knowledge

Blood lipids can be easily measured in plasma/ serum samples and are used as risk markers for chronic diseases like atherosclerosis [15-20]. Total cholesterol (TC), high density lipoprotein cholesterol (HDL), and low density lipoprotein cholesterol (LDL) indicate the cholesterol content of different blood lipoproteins. These lipoproteins act in specific ways as lipid transport system in the bloodstream. High levels of TC and LDL correlate with a higher risk for developing cardiovascular diseases, whereas high levels of HDL have a protective effect. Furthermore, the main apolipoprotein (apo) of the LDL particle, termed apo B, is associated with cardiovascular morbidity because of its essentiality for the binding of the LDL particle to the LDL receptor. Thus, an excessive number of apo B-containing particles promote the atherogenic process [21]. By contrast, apo A-1 is the major protein compound of HDL particles and supports the process of reverse cholesterol transport [21]. Triglyceride-rich lipoproteins are a major component of non-HDL particles and thus high triglyceride (TG) levels can be associated with atherogenic processes [22]. Lipoprotein(a) [Lp(a)] has no clear function in the human body, but is an independent risk marker for atherosclerosis [23]. The Lp(a) particle is strongly determined by genetics, and thus blood levels are quite constant during life [24]. Furthermore, lipid and lipoprotein ratios based on the already mentioned lipid and lipoprotein parameters can be used to assess atherogenic risks [25-27], because they reflect the particular relation of those lipid and lipoproteins.

A complete blood lipid profile should also include the measurement of FA. Generally, FA are important for optimal growth and development [28] including fetal and perinatal periods [29-31]. Fatty acids perform a variety of different tasks including the modification of cell membrane fluidity, and thus influencing pinocytosis, ion channel modulation, and membrane-associated receptor activities. Furthermore, FA can regulate gene expression due to the interaction with transcription factors as specific and non-specific ligands [32] and modulate inflammatory and immunological processes due to the provision of precursors for eicosanoids [33]. Fatty acids are proposed to have cholesterolemic effects on several lipoprotein classes, suggesting saturated FA (SFA) as hypercholesterolemic and polyunsaturated FA (PUFA) as hypocholesterolemic compared to SFA [34, 35].

Serum FA reflect the dietary FA intake as well as the endogenous FA metabolism and are mainly transported by lipoproteins as TG, cholesteryl esters, and phospholipids (PL). The metabolic situation of the last few days is reflected best by the FA pattern of serum PL [36, 37], because of the slower metabolic turnover of PL compared to other blood lipid classes (e.g. cholesteryl esters, TG or free FA) [38]. Thus, serum FA of the PL fraction are suitable in documenting the short-term FA status in humans [39]. To document the FA status according to the long-term situation, month to years, PL of the red blood cells, or FA of adipose and liver tissues are more suitable. However, these cells/ tissues were not available from the adolescent participants of the present project. Furthermore, FA can be used to calculate FA ratios. These FA ratios can indicate the relationship between FA families, for instance ω6/ω3 FA or C18:2ω6/C18:3ω3, because these FA families compete against the same enzymes for elongation and desaturation. Fatty acid ratios are also estimated, when the FA of the ratio are connected through enzymatic steps within the same FA family, for example C20:5ω3/C22:6ω3 or C18:2ω6/C20:4ω6. Thus, the obtained indices can help to identify the relation between several FA, which is important for the fulfilling of the physiologic functions within the human body.

Present studies highlight only the influence of single factors like BMI as communicable factor and/ or age and gender as non-communicable factors [40-45]. Studies covering a more complete spectrum including communicable determinants like BMI, BF, and FFM as well as the non-communicable factors age, sexual maturity, and gender are missing. Thus, the continued effect of these variables on blood lipids (e.g. lipoproteins, cholesterol, FA) is not known.

Furthermore, our knowledge of lipid profiles of children and adolescents is mostly based on studies in already diseased subjects, e.g. children with obesity and diabetes [43, 46-54]. Data available for healthy adolescents is mainly coming from surveys in America [41, 55, 56]. In Europe, only one known study examined healthy adolescents to present a reference lipid profile based on five representative Spanish cities [45]. The AVENA Study presented age- and sex-specific lipid and lipoprotein profiles of 581 adolescents aged 13 to 18.5 years. The 90^{th} percentile for TC was 191 mg/dL for males and 200 mg/dL for females. Females had higher HDL levels than boys and LDL levels ranged from 90 to 98 mg/dL in males and from 92 to 101 mg/dL in females. Triglyceride levels tended to increase gradually and to peak at 17 years of age for both sexes. Apolipoprotein A-1 and B-100 levels paralleled those of HDL and LDL values [45].

In general, comparisons of current studies are quite difficult because of the following reasons. First of all, comparing European countries is not possible, because there is only one known survey presenting reference data. The use of American data as European guideline may not be transferable due to discrepancies between European and American lifestyles and blood lipid profiles. Secondly, different methodological and analytical approaches have been used, which complicate comparisons on a global level. Thirdly, reliable reference data should consider gender and the impact of growth and development assessed as grade of puberty. Consequently, former trials evaluating an early atherogenic risk of children and adolescents could only be made by using cut-offs based on incorrect data, because of the already mentioned reasons. It cannot be excluded that this approach might under- or overestimate individual risks.

Presently, the FA profiles of healthy adolescents are not investigated sufficiently with respect to communicable and non-communicable factors and reliable reference data is missing. Similar to the before-mentioned difficulties in lipid research, FA research in adolescence is also focusing on subjects with diseases (e.g. diabetes, obesity, metabolic syndrome) [57-63]. Furthermore, methodological and analytical approaches differ and study comparisons are complicated due to the use of different blood lipid fractions (e.g. TG, cholesteryl esters, PL, red blood cell PL). As already pointed out, maturity and gender may also influence FA profiles of adolescents, but study results are still presented by age categories with and without consideration of gender. Summarized, the problems in lipid research mentioned before like transferability of study results, methodological differences and neglecting communicable and non-communicable factors apply to FA research as well.

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2 Purpose of the Thesis

As a consequence of the limited availability of European data, this thesis will investigate the lipid status of healthy European adolescents associated with communicable and non-communicable factors.
The data for this thesis is based on results of the cross-sectional part of the *Healthy Lifestyle in Europe by Nutrition in Adolescence* (HELENA) Study designed and performed in Europe. This project will be introduced in detail in the following chapter.

The outline of this work is presented in chapters to achieve a clear overview. The following questions are tried to be answered within these chapters:

- What is the current lipid and lipoprotein status of European adolescents?
- Are maturity, age, gender, body mass index and body fat associated with lipid and lipoprotein levels in European adolescents?

  (Chapter 4)
- What is the fatty acid status of European adolescents?
- Are maturity, age, gender, body mass index, body fat, and fat free mass associated with fatty acid levels in European adolescents?

  (Chapter 5)
- What is the current status of fatty acid ratios?
- Are maturity, age, gender, body mass index, body fat, and fat free mass associated with fatty acid ratios?

  (Chapter 6)

- Are the fatty acids C16:0, C18:0, C18:1ω9c, C18:2ω6c, C18:3ω3c, C20:4ω6c, C20:5ω3c, and C22:6ω3c associated with triglycerides, total cholesterol, cholesterol subclasses, lipoprotein(a), and apolipoproteins?

  (Chapter 7)

3 General Methodology

3.1 The HELENA Study - Study design and objectives

The ***He**althy **L**ifestyle in **E**urope by **N**utrition in **A**dolescence* (Acronym: HELENA; contract number: 007034) Study is a European multi-centre study supported within the frame of the 6th framework program (FP6-2003-Food-2-A, FOOD-CT-2005-007034) under the coordination of Prof. Luis A. Moreno Aznar (Universidad de Zaragoza). The study was performed in cooperation with ten cities all over Europe [1].

- Ghent University; Ghent (Belgium)
- Harokopio University; Athens (Greece)
- Istituto Nazionale di Ricerca per gli Alimenti e la Nutrizione; Rome (Italy)
- Karolinska Institutet; Stockholm (Sweden)
- Medical University of Vienna; Vienna (Austria)
- Research Institute of Child Nutrition Dortmund; Dortmund (Germany)
- Universidad de Zaragoza; Saragossa (Spain)
- Université de Lille 2; Lille (France)
- University of Crete School of Medicine; Heraklion (Greece)
- University of Pécs; Pécs (Hungary)

The general goals of the study were:

- To develop and harmonize innovative methods for the assessment of lifestyle habits of adolescents across Europe with special focus on diet, nutrition and physical activity.
- To assess dietary and physical activity patterns as well as nutritional status among European adolescents.
- To investigate knowledge and attitudes towards nutrition and physical activity among adolescents and to establish the main determinants of their food choice and preference.
- To describe biological parameters and give reference data of European adolescents for future studies.
- To describe social, genetic, pubertal, weight-dependent and gender differences and similarities across Europe.

- To identify adolescents at risk of eating disorders, dyslipidemia, obesity and/or type 2 diabetes.
- To develop a number of healthy foods and identify marketing strategies for consumers, in order to improve the diet of adolescents.
- To develop a Lifestyle Education Programme and test its efficacy for improving adolescents' health.

In order to organize the work efficiently, 14 Work Packages (WP) were introduced dealing with specific topics and research fields. Accordingly, the HELENA project was split in four experimental parts: (I) a crossover multi-centre study (COMS); (II) a lifestyle education intervention (LSEI); (III) a behavioural and food choice study (BEFO); (IV) a cross-sectional study (CSS). All work presented in this thesis was part of the CSS, specifically of WP 9 (*Haematological, biochemical, and immunologic data*) and WP 8 (*Body composition and obesity*).

The HELENA-CSS

Within the CSS, an adolescent group was assessed for food and nutrient intake, nutrition knowledge, physical activity and fitness, body composition, vitamin and mineral status, lipid and blood profile, and genetic variability. Therefore, methods were developed and harmonized in cooperation with all partners in order to obtain comparable data within the study centres. Subject recruitment was done by one research group or institute within the ten participating centres.

Study design and subject recruitment

Subjects with an age range of 12.5-17.49 years were recruited in ten cities across Europe. Selection of cities was based on two criteria: regional distribution and presence of an active research group assuring sufficient expertise and resources to successfully perform epidemiological studies. Within the study, Stockholm (Sweden) represented Northern Europe, Athens, Heraklion (Greece), Rome (Italy), Saragossa (Spain) Southern Europe, Pécs (Hungary) Eastern Europe, Ghent (Belgium), Lille (France) Western Europe, and Dortmund (Germany), Vienna (Austria) Central Europe. The sample size of 3,000 adolescents has been estimated using BMI (confidence level of 95%, and a ±0.3 error). Each partner was asked to include 150 male and female subjects per protocol, respectively.

Reliable and objective data concerning age and gender were obtained by analysing complete school classes. On the city level, diversity of the sample with respect to cultural and socio-economic aspects was achieved by performing a random proportional distribution of all schools taking into account the site of the school (district/ zone of the city) and the type of school (public or private). Blood sampling was performed in one third of the adolescents recruited (choice of whole classes representing the desired age of the subjects). The school and class random selection procedure, including the subset of classes for blood sampling, has been done centrally by one partner (i.e. Ghent) for all study centres. In case a selected school denied its participation, a school with comparable characteristics from a reserve list was taken.

Individual exclusion criteria were not being able to speak the local language and the simultaneous participation in another clinical trial. All protocols and informed consents for this study were reviewed and approved by an Ethics Review Committee in each country according to the Declaration of Helsinki and International Conferences on Harmonization for Good Clinical Practice. Quality control was assured throughout the whole project as described by Beghin *et al.* [2].

Prior to the start of the HELENA-CSS all methods have been tested in a pilot study to assure optimal sampling and to optimize transport logistics and analytics. These results have been described and published elsewhere more in detail [3].

Medical examination, blood sampling and anthropometric measurements

Prior to the study day of blood donation, body composition assessment and bioelectrical impedance analysis (BIA), participants were asked to abstain from eating and drinking later than 8 p.m. At the study day a medical doctor visited the school classes and asked all participants for medical history and acute diseases. A blood sampling questionnaire was used to assess fasting status, acute infections, allergies, vitamin and mineral supplements, and medication (see Appendix A). Maturity was assessed by means of Tanner stage [4]. Medical data and all information were recorded in a case report form for each participant (see Appendix B).

Blood sampling generally took place between 8-10 a.m. Approximately 30 mL of blood were collected from an antecubital vein in serum, heparin, and ethylene diamine tetraacetic acid monovettes® (Sarstedt AG & Co., Nümbrecht, Germany). These blood samples were used to assess the different biomarkers and blood parameters, including lipids and lipoproteins for the present thesis. After blood sampling a breakfast was offered for all participants.

Various anthropometric measures were performed using validated methods as published in detail previously [5]. Briefly, body weight was measured after blood sampling in underwear and without shoes with an electronic scale (Type SECA 861) to the nearest 0.1 kg, and height was measured barefoot in the Frankfort plane with a telescopic height measuring instrument (Type SECA 225) to the nearest 0.1 cm. A set of skinfold thicknesses (biceps, triceps, subscapular, suprailiac, thigh) were measured several times on the left side of the body, with a Holtain caliper (nearest 0.2 mm). For BIA measurements a classical tetrapolar technique was used by means of BIA 101 AKERN SRL. Standard instructions for BIA measurements were followed.

The intraobserver technical errors of measurement (TEMs) were between 0.12 and 2.9 mm for skinfold thicknesses. Intraobserver reliability for skinfold thicknesses was >69%. The intraobserver TEMs for skinfold thicknesses were <1; for BIA resistance TEMs were <0.1 Ω and <0.2 Ω for reactance. Intraobserver reliability values were >95%, 99% and 97% for skinfold thicknesses, BIA resistance and reactance. Interobserver TEMs for skinfold thicknesses ranged from 1 to 2 mm; for BIA they were 1.16 and 1.26 Ω for resistance and reactance. Interobserver reliability for skinfold thicknesses, BIA resistance and reactance were >90% [5].

Sample treatment and transport

Sample treatment and transport were tested in a pilot study to assure optimal sampling as described in detail previously [3]. After blood donation, serum samples were clotted at room temperature for at least 30 minutes followed by a centrifugation (2,500 x *g*, 10 minutes). Aliquots for FA analysis were stored locally at -20/ -80°C as soon as possible. After field work was done, all samples of one city were shipped on dry ice to the central laboratory (IEL) at the University of Bonn (Germany) and stored at -80°C until analysis. Aliquots for routine lipid parameters were sent to the IEL, stored in a refrigerator at 4-7°C overnight and analysed within 24 hours after blood collection in the University Hospital in Bonn. Aliquots for leptin analyses were also shipped to the IEL and stored locally at -80°C within 24 hours after blood donation. Leptin samples were shipped on dry ice to the laboratory of the Universidad Politécnica de Madrid (Spain) after all field work was done.

Heparin samples for insulin analyses were put directly on ice until centrifugation (2,500 x *g*, 10 minutes). Afterwards, samples were shipped under cooled conditions (4-7°C) to the IEL and stored at -80°C within 24 hours after blood drawing.

For all samples freeze-thaw cycles were avoided until analysis.

3.2 Materials

The following tables summarize all laboratory materials, equipments and FA standards used for FA analytics within the HELENA-CSS.

Table 3.1a: Used chemicals and gases

Name	Company	Order number
2',7'Dichlorofluorescein	Fluka, Deisenhofen, Germany	35848
Acetone	Carl Roth, Karlsruhe, Germany	7328.2
Air	Praxair GmbH, Düsseldorf, Germany	-
Ethanol	Carl Roth, Karlsruhe, Germany	P076.2
Hydrochloric acid fuming 37%	Carl Roth, Karlsruhe, Germany	X942.1
Helium 4.6	Praxair GmbH, Düsseldorf, Germany	-
Hydrogen 5.0	Praxair GmbH, Düsseldorf, Germany	-
Methanol	Carl Roth, Karlsruhe, Germany	HN41.1
n-Heptane	Carl Roth, Karlsruhe, Germany	CP78.1
Nitrogen 5.0	Praxair GmbH, Düsseldorf, Germany	-
Petroleum benzine 63-80°C	Carl Roth, Karlsruhe, Germany	5649.1
Petroleum ether 40-60°C	Carl Roth, Karlsruhe, Germany	T173.1
Trichloromethane/chloroform	Carl Roth, Karlsruhe, Germany	3313.2

Table 3.1b: Used authentic fatty acid standards*

Name	Company	Order number
1,2-Dipentadecanoyl-sn-glycero-3-phosphocholine	Sigma-Aldrich, Deisenhofen, Germany	P7285
Docosadienoic acid methyl ester	Sigma-Aldrich, Deisenhofen, Germany	D4034
Docosahexaenoic acid methyl ester	Sigma-Aldrich, Deisenhofen, Germany	D2659
F.A.M.E. Mix, C4-C24	Sigma-Aldrich, Deisenhofen, Germany	18919-1 AMP
Methyl 11,14,17-eicosatrienoate	Sigma-Aldrich, Deisenhofen, Germany	E6001
Methyl 5,8,11-eicosatrienoate	Sigma-Aldrich, Deisenhofen, Germany	E6013
Methyl 8,11,14-eicosatrienoate	Fluka, Deisenhofen, Germany	00813
Methyl arachidate	Fluka, Deisenhofen, Germany	10941
Methyl arachidonate	Sigma-Aldrich, Deisenhofen, Germany	A9298
Methyl behenate	Fluka, Deisenhofen, Germany	11940
Methyl butyrate	Fluka, Deisenhofen, Germany	19358
Methyl decanoate	Fluka, Deisenhofen, Germany	21479
Methyl docosapentaenoate	Fluka, Deisenhofen, Germany	17269
Methyl eicosadienoate	Fluka, Deisenhofen, Germany	17272
Methyl eicosapentaenoate	Fluka, Deisenhofen, Germany	17266
Methyl eicosenoate	Fluka, Deisenhofen, Germany	17263
Methyl erucate	Fluka, Deisenhofen, Germany	45659
Methyl heneicosanoate	Fluka, Deisenhofen, Germany	51535
Methyl heptadecanoate	Fluka, Deisenhofen, Germany	51633
Methyl heptadecenoate	Sigma-Aldrich, Deisenhofen, Germany	H9021
Methyl hexanoate	Fluka, Deisenhofen, Germany	21599
Methyl laurate	Fluka, Deisenhofen, Germany	234591
Methyl linoleate	Fluka, Deisenhofen, Germany	62280
Methyl linolelaidate	Fluka, Deisenhofen, Germany	62155
Methyl linolenate	Fluka, Deisenhofen, Germany	62200
Methyl myristate	Fluka, Deisenhofen, Germany	70129
Methyl myristoleate	Fluka, Deisenhofen, Germany	70121
Methyl octadecenoate	Fluka, Deisenhofen, Germany	46904

continued

Table 3.1b continued

Name	Company	Order number
Methyl octanoate	Fluka, Deisenhofen, Germany	21790
Methyl oleate	Fluka, Deisenhofen, Germany	75160
Methyl palmitate	Fluka, Deisenhofen, Germany	76159
Methyl palmitoleate	Fluka, Deisenhofen, Germany	76176
Methyl pentacosanoate	Fluka, Deisenhofen, Germany	76497
Methyl pentadecanoate	Fluka, Deisenhofen, Germany	76560
Methyl pentadecenoate	Sigma-Aldrich, Deisenhofen, Germany	P0315
Methyl stearate	Fluka, Deisenhofen, Germany	85769
Methyl stearidonate	Fluka, Deisenhofen, Germany	43959
Methyl tetracosanoate	Fluka, Deisenhofen, Germany	87115
Methyl tetracosenoate	Fluka, Deisenhofen, Germany	17265
Methyl trans-vaccenate	Sigma-Aldrich, Deisenhofen, Germany	V1381
Methyl tricosanoate	Fluka, Deisenhofen, Germany	91478
Methyl tridecanoate	Fluka, Deisenhofen, Germany	91558
Methyl undecanoate	Fluka, Deisenhofen, Germany	94118
Methyl γ-linolenate	Fluka, Deisenhofen, Germany	00238

* the precise nomenclature is shown in the list of abbreviations

Table 3.1c: Used laboratory equipment and materials

Name	Specification	Company
Analysis funnels	Lime-soda glass, 55 mm	Carl Roth, Karlsruhe, Germany
Centrifuge tubes	With thread and screw-cap, conical, 112x17 mm	Hecht-Assistant, Sondheim, Germany
Culture tubes	Schott DURAN, with screw-cap, 12x100 mm	Carl Roth, Karlsruhe, Germany
Eppendorf pipettes	Eppendorf Research®, 10-100µL, 100-1,000µL	Eppendorf, Hamburg, Germany
Filter papers	MN616 WA, 90 mm, hydrophobic phase separation papers	Macherey-Nagel, Düren, Germany
Medical gloves	Flexam, Latex, size M, REF 88471	Cardinalhealth, Illinois, USA
Micro-vials	conic	CS-Chromatographie Service GmbH, Langerwehe, Germany
Monovettes®	Serum, heparin, and ethylene diamine tetraacetic acid monovettes for different volumes	Sarstedt AG & Co., Nümbrecht, Germany
Pasteur pipettes	Lime-soda clear glass, uniform	Carl Roth, Karlsruhe, Germany
Pipette filler	-	Carl Roth, Karlsruhe, Germany
Pipette tips	For 10-100 µL, 100-1,000 µL pipettes	Eppendorf, Hamburg, Germany
Reaction tubes	Safe-lock tubes, 1.5 mL	Eppendorf, Hamburg, Germany
Reaction tubes	Tubes with screw-cap, 1.5 mL	Eppendorf, Hamburg, Germany
Syringes	Syringe Perfection, 10µL, 250 µL	SGE GmbH, Griesheim, Germany
Test tubes	With thread and plastic screw-cap, 98x16 mm	Schütt Labortechnik, Göttingen, Germany
TLC plates	SIL G-25, 20x20 cm	Macherey-Nagel, Düren, Germany
TLC-seperating chamber	glass	Carl Roth, Karlsruhe, Germany
Volumetric flasks	25, 50, 100, 200 mL	Carl Roth, Karlsruhe, Germany
Volumetric pipettes	1 mL, 2 mL, AR-glass	Hierschmann Laborgeräte, Eberstadt, Germany

Table 3.1d: Used machines and devices

Name	Specification	Company
Gas chromatograph	Varian CP-3900	Varian GmbH, Darmstadt, Germany
Autoinjector	Injector 1177	Varian GmbH, Darmstadt, Germany
Autosampler	Autosampler 8410	Varian GmbH, Darmstadt, Germany
Column	Zebron ZB-WAXplus, polyethylene glycol, 30 m x 0.25 mm x 0.25 µm	Phenomenex LTD, Aschaffenburg, Germany
Centrifuge	Heraeus Megafuge 1.0R	Thermo-Electron.Corporation, Waltham, USA
Thermostat	Reacti-Therm III, Heating/Stirring module	Pierce, Rockford, USA
Thermostat (additional equipment)	Reacti-Vap III	Pierce, Rockford, USA
Thin-layer fluorescent sprayer	Thin-layer fluorescent sprayer	E. Merck, Darmstadt, Germany
Ultrapure water-preparation system	Nanopure D 1797	Barstead, Bergisch-Galdbach, Germany
UV-System	Fluotest universal	Gerhardt, Bonn, Germany
Vacuum pump	Eppendorf 4151	Eppendorf, Hamburg, Germany
Vortex	Vortex-Genie®	Zimmermann Laborausrüster, Cologne, Germany

3.3 Analytical Methods

A Fatty acid determination of serum phospholipids

Figure 3.1 gives an overview of the complete analysis scheme. Serum samples were defrosted at room temperature. One mL of serum or if not available, the maximal sample volume, was transferred into culture tubes with Teflon caps. The precise sample volume was considered in the formula when calculating the FA concentration. Afterwards, 50 µL of internal standard (IS: 1,2-Dipentadecanoyl-sn-glycero-3-phosphocholine) were added. Throughout the procedure, samples were repeatedly flushed with nitrogen (N_2) and mixed with a Vortex®.

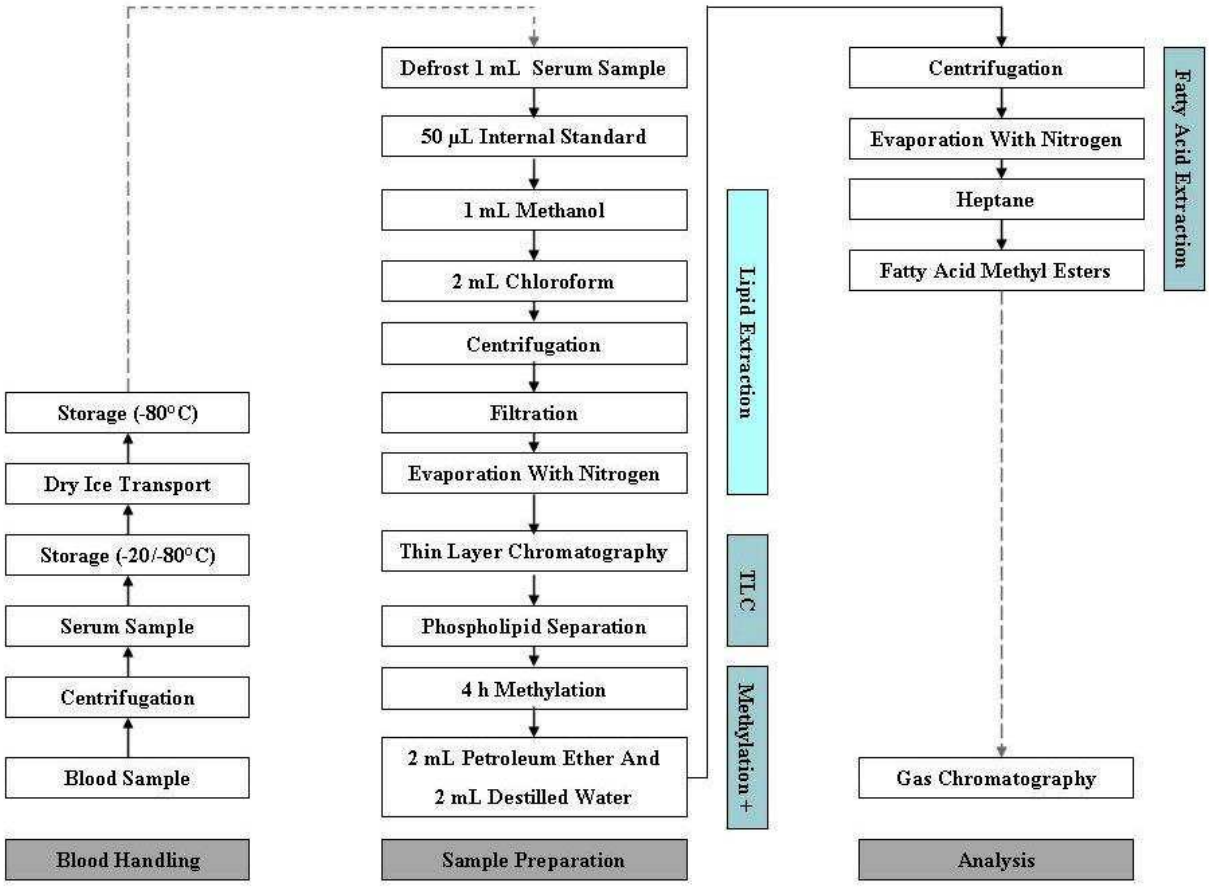

Figure 3.1: Flow chart of blood handling, sample preparation, and analysis of fatty acids.

TLC:Thin-layer chromatography

Lipid extraction, thin-layer chromatography and methylation

I. Lipid extraction

The FA extraction is based on a modified method by Folch [6] and Sperry and Brand [7]. For protein precipitation, one mL of methanol was added to the sample, and the samples were rested for 10 minutes. Next, two mL of chloroform were added. Ten minutes later, samples were centrifuged at 836 x *g* and 4°C for 10 minutes. After centrifugation, three phases were obtained and the upper methanol-containing phase was aspirated. The remaining protein band and the lipophil phase were mixed and filtrated through hydrophobic phase separation paper filters. The proteins and remaining aqueous solutions retained in the paper filters. The lipid phase, which contained the total amount of the extracted serum lipids, was collected in conical centrifugation tubes. Afterwards, the lipid phase was evaporated to dryness under constant N_2 flow. Dried samples were frozen overnight at -80°C.

II. Thin-layer chromatography

The TLC method including methylation and extraction was performed as described by Christophe and Matthijs [8]. The dried sample was dissolved in 150 mL of chloroform and spotted with a syringe on a TLC plate. Separation in PL, monoglyceride, non-esterified FA, cholesterol, diglycerid, TG, and cholesterol ester fractions took place in a TLC-separating chamber containing 100 mL of running solution (15% acetone + 85% petroleum benzine [bp 63-80°C]) (see figure 3.2). Thin-layer chromatography was completed when the solution front reached nearly 0.5 cm from the border (approximately after 45 minutes to 1 hour). Thin-layer chromatography plates were sprayed with a fluorescent solution, to visualize PL under UV light, containing 200 mg of 2',7'-dichlorofluorescein solved in 100 mL ethanol. The PL band was scraped off and transferred into culture tubes to be used for methylation.

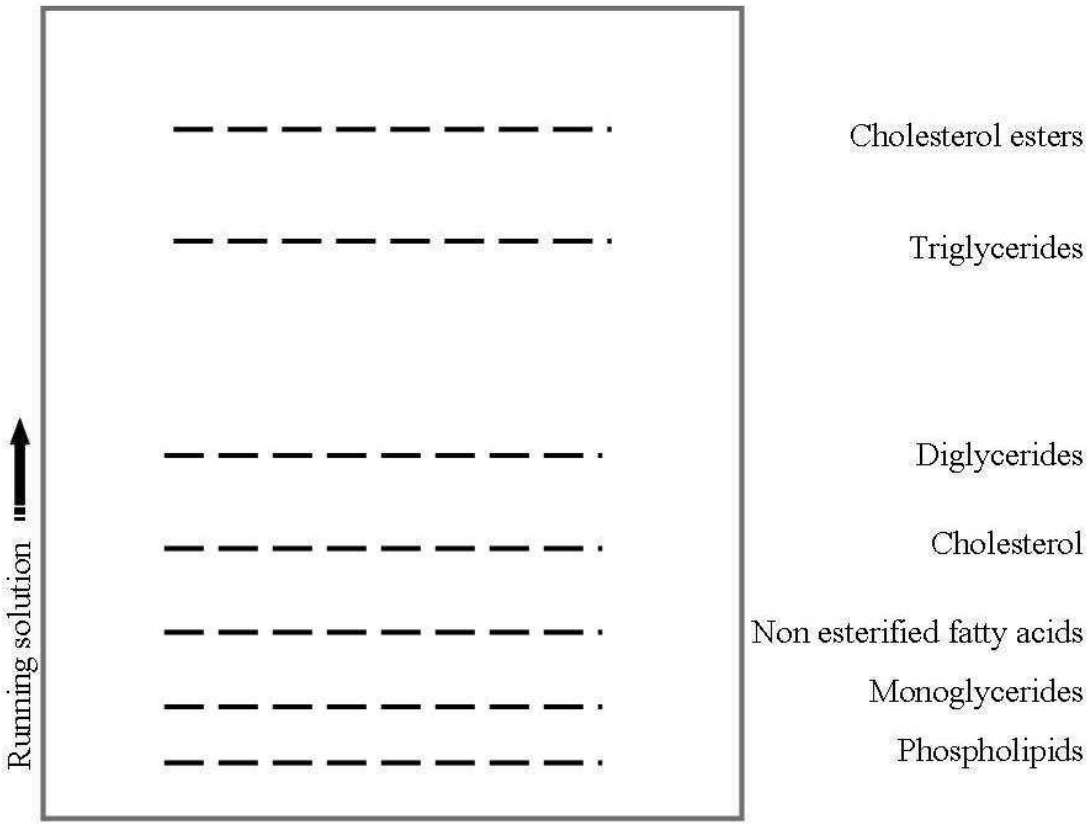

Figure 3.2: Illustration of the separation of serum lipid fractions by thin-layer chromatography (running solution: 15% acetone and 85% petroleum benzine [bp 63-80°C]; duration: approx. 1 hour).

III. Methylation and Extraction

Phospholipids were dissolved in two mL of methylating solution (mixture of four mL 37% hydrochloric acid and 100 mL methanol) and incubated (95°C, 4 h). The solution containing fatty acid methyl esters (FAME) was cooled at -20°C for 10 minutes. Two mL of petroleum ether (bp 40-60°C) and two mL of distilled water were then added for methyl ester extraction. Afterwards, the samples were centrifugated (836 x *g*, 4°C, 10 minutes). The top layer was transferred to conical centrifuge tubes and evaporated to dryness under constant N_2 flow. One hundred µL *n*-heptane were added to redissolve the dried FAME. Samples were transferred into micro-vials and one µL was injected in the gas chromatograph.

B Gas chromatography

Fatty acid methyl esters were analysed using gas chromatography. Equipment and analytical conditions are given in table 3.2.

Table 3.2: Gas chromatography – equipment and analytical conditions.

Gas chromatograph	CP-3900 (Varian GmbH)
Autosampler	8410 (Varian GmbH)
Injector	1177 (Varian GmbH)
Detector	Flame ionisation detector (FID)
Column	Zebron ZB-WAXplus, Polyethylene glycol, 30 m x 0.25 mm x 0.25 µm
Injection mode	Split/ splitless; split ratio 20
Injector temperature	200°C
Column pneumatics	Constant flow (4 mL/min)
Carrier gas	Helium
Detector temperature	250°C
Detector characteristics	Helium flow (makeup): 30 mL/min Hydrogen flow (reference): 30 mL/min Air flow: 300 mL/min
Injection volume	1 µL

Column oven program

Rate (°C/min)	Step (°C)	Time step (min)	Total time (min)
Initial	100	2.0	2.0
25.0	180	0.0	5.2
0.5	190	0.0	25.2
1.0	200	0.0	35.2
5.0	210	0.0	37.2
10.0	240	3.0	43.2

Typical chromatograms of a F.A.M.E. C4-C24 standard and a serum sample are presented in figures 3.3, 3.4 and 3.5, respectively.

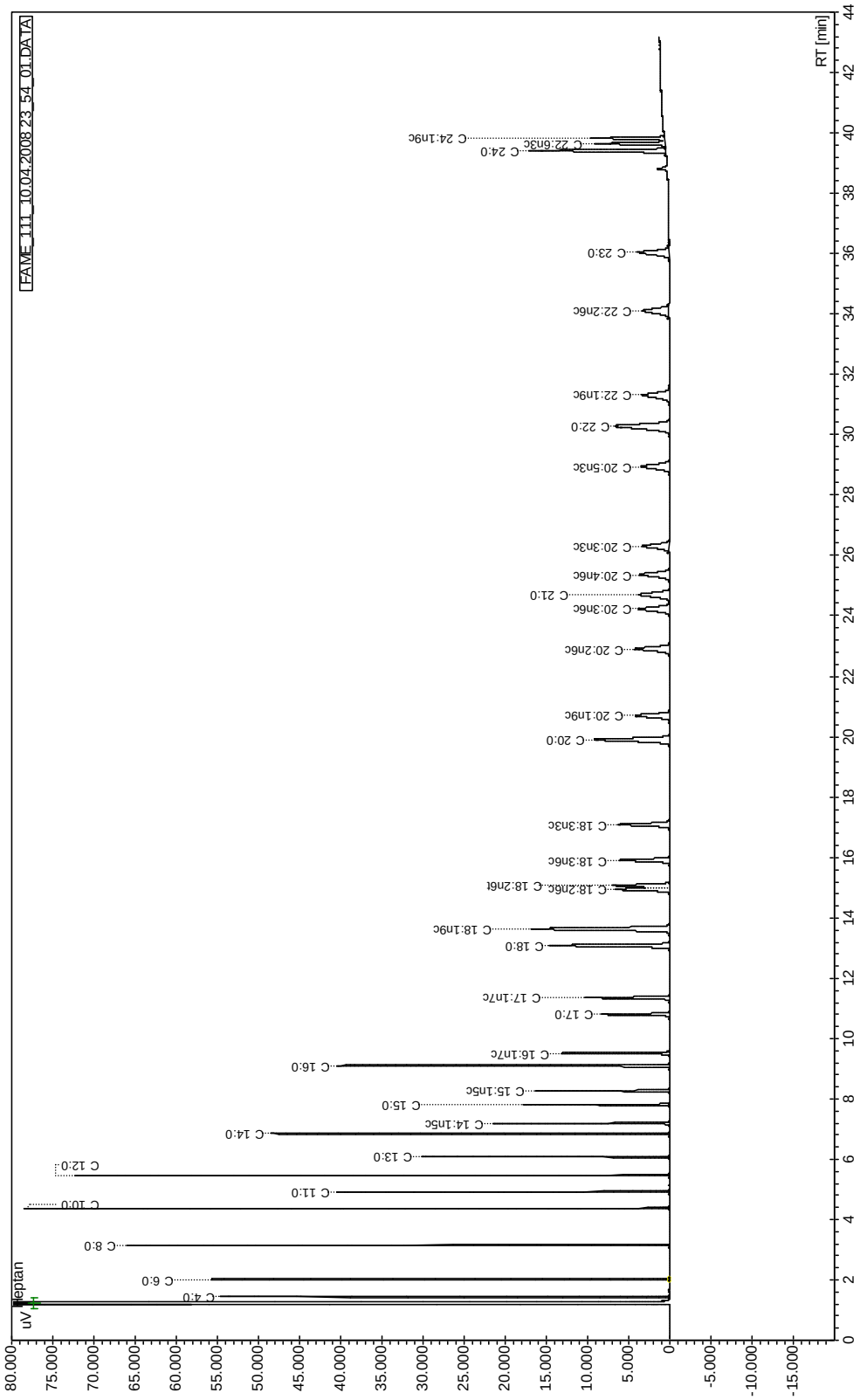

Figure 3.3: Typical GC chromatogram (FID, injection temperature 200°C, detector temperature 250°C) of the F.A.M.E. C4-C24 standard (100 mg in 10 mL *n*-heptane, injection volume 1 µL, split ratio 20) obtained with a run time of 44 min., helium as carrier gas (constant flow 4 mL/min) using a 30 m x 0.25 mm x 0.25 µm polyethylene glycol column.

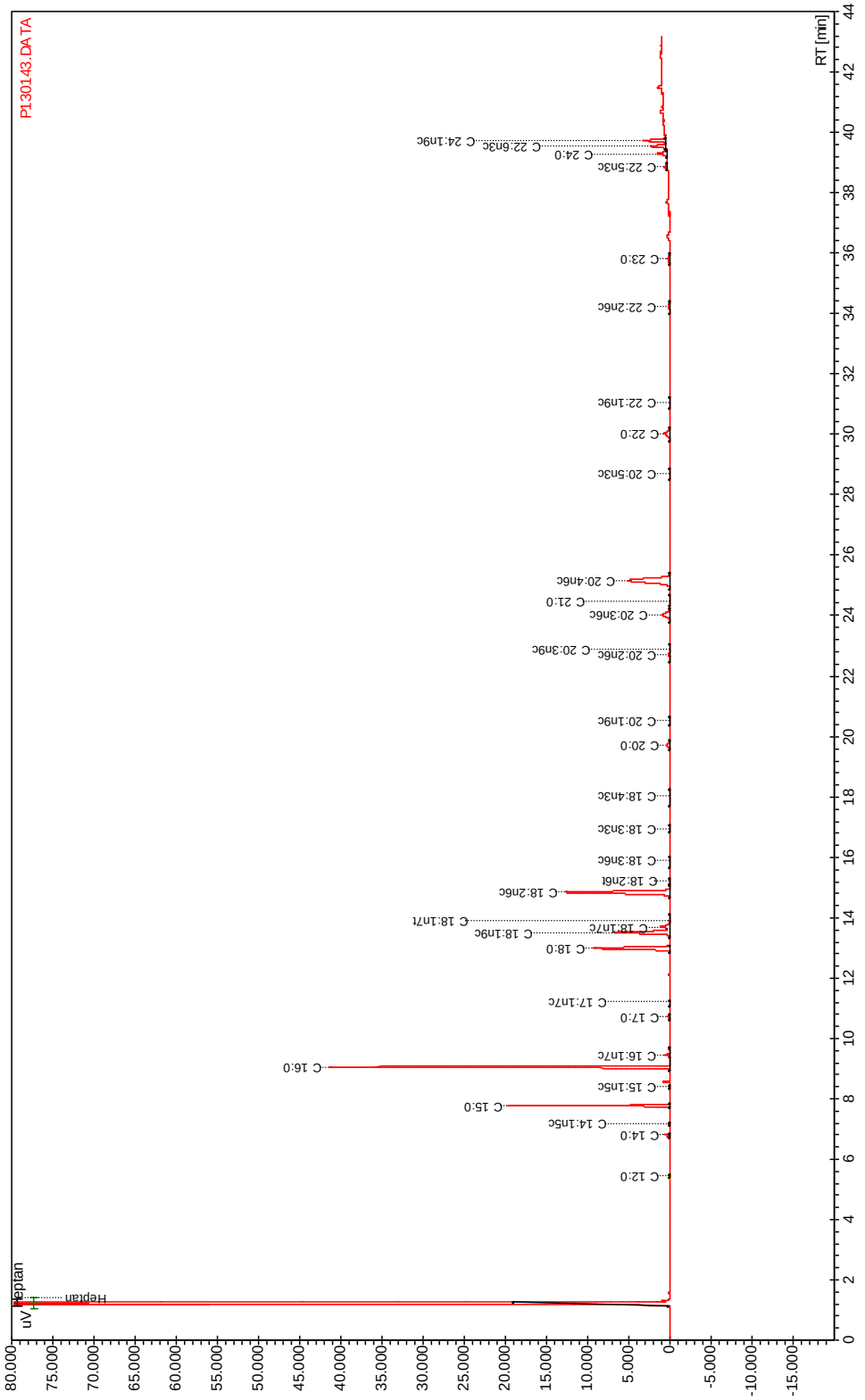

Figure 3.4: Typical GC chromatogram (FID, injection temperature 200°C, detector temperature 250°C) of a fatty acid profile of serum phospholipids based on 1 mL serum (injection volume 1 µL treated sample, split ratio 20) obtained with a run time of 44 min., helium as carrier gas (constant flow 4 mL/min) using a 30 m x 0.25 mm x 0.25 µm polyethylene glycol column.

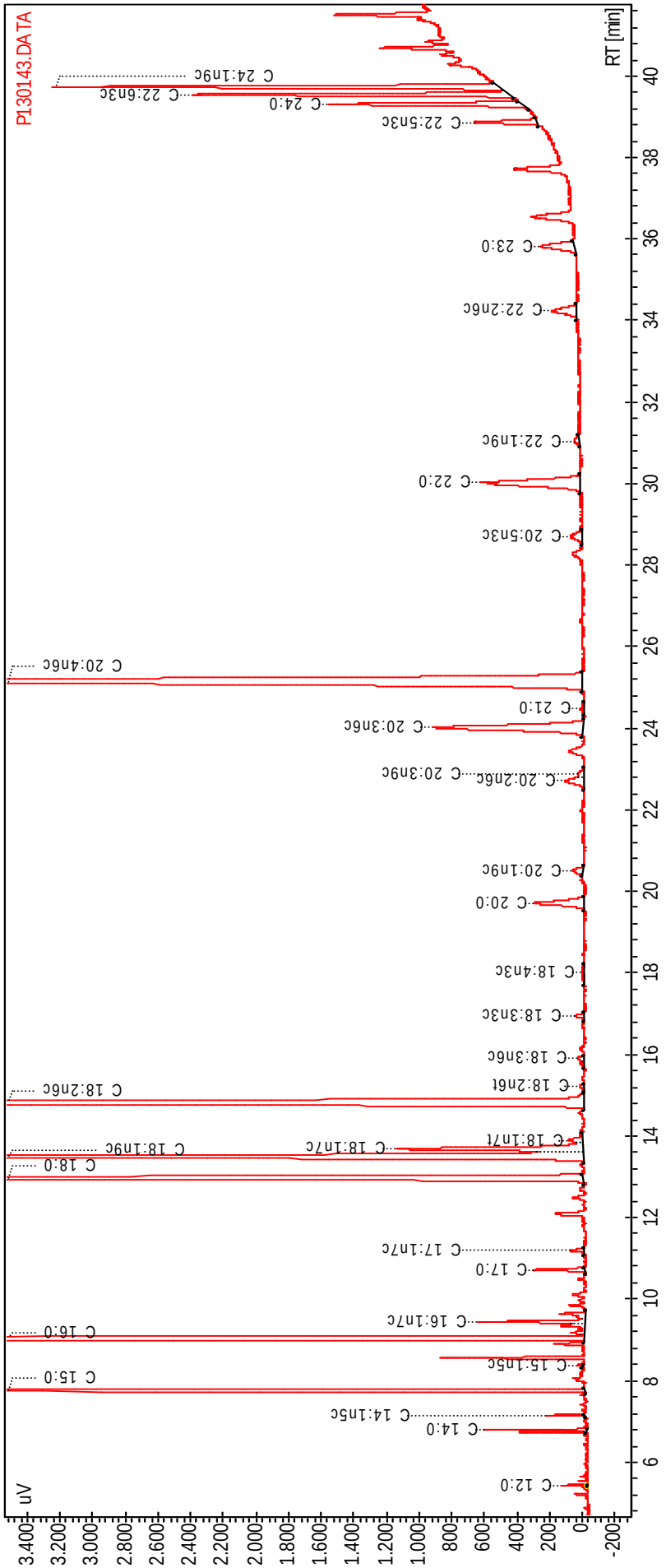

Figure 3.5 (Enlargement of figure 3.4): Typical GC chromatogram (FID, injection temperature 200°C, detector temperature 250°C) of a fatty acid profile of serum phospholipids based on 1 mL serum (injection volume 1 µL treated sample, split ratio 20) obtained with a run time of 44 min., helium as carrier gas (constant flow 4 mL/min) using a 30 m x 0.25 mm x 0.25 µm polyethylene glycol column.

Single authentic FAME reference standards were used to identify FA peaks of the sample. The retention times of the single FAME were measured and compared with the analysed peaks of the sample.

Valid identification during routine analysis was assured by analysing a F.A.M.E. C4-C24 standard every tenth sample. It was only feasible to calculate the absolute FA concentrations of those FA in the sample that were also included in the standard. The concentrations of those ten consecutive FA samples were calculated by using the mean concentrations of two F.A.M.E. C4-C24 standards, measured before and after those ten samples.

As mentioned earlier, 1,2-Dipentadecanoyl-sn-glycero-3-phosphocholine was chosen as IS. This was due to two reasons: (a) IS consists of two pentadecanoates (C15:0), which are also included in the FA composition of the F.A.M.E. C4-C24 standard; (b) pentadecanoate can be neglected in the evaluation of serum FA because it is only a minor component of blood FA profiles.

Preparation of the internal standard

One hundred mg IS (molecular mass [MM] 705.99) were solved in 40 mL of a methanol:chloroform solution. The methanol:chloroform solution consisted of 20 mL methanol and 40 mL chloroform (dilution factor 1:3). Thus, an IS concentration of 2.5 mg per one mL methanol:chloroform solution was obtained.

$$\text{Concentration of IS each 1,000 mL} = \frac{2{,}500\ \text{mg}}{705.99\ \text{MM}}$$

$$= 3.5411\ \text{mmol}$$

A concentration of 3.54 nmol IS per µL methanol:chloroform solution was obtained. Adding 50 µL of this solution to one mL serum sample resulted in an end concentration of 354.1 µmol pentadecanoates per L serum.

Calculation of the single fatty acids methyl esters of the F.A.M.E. C4-C24 standard

The following formula was used to calculate the concentration of the FAME of the standard. All important information to calculate the single FAME concentrations can be found in table 3.3 as well as the referring results (right column).

$$\text{FAME concentration (µmol/L)} = \frac{\text{Fatty acid methyl ester in mg/dL}}{\text{Molecular mass of the fatty acid methyl ester}} * 10 * 1{,}000$$

Table 3.3: Details of the single fatty acid methyl esters of the F.A.M.E. C4-C24.

Fatty acid methyl ester (FAME)	Weight% in the standard	Concentration in the diluted standard (mg/dL)	Molecular mass of the FAME	Concentration (µmol) per L standard
Butyric acid (C4:0)	4%	39.90	102.13	3906.79
Caproic acid (C6:0)	4%	39.80	130.18	3057.31
Caprylic acid (C8:0)	4%	39.90	158.24	2521.49
Capric acid (C10:0)	4%	39.90	186.3	2141.71
Undecanoic acid (C11:0)	2%	20.00	214.34	933.10
Dodecanoic acid (C12:0)	4%	39.90	214.34	1861.53
Tridecanoic acid (C13:0)	2%	20.00	228.37	875.77
Tetradecanoic acid (C14:0)	4%	39.90	242.4	1646.04
Cis-9-Tetradecenoic acid (C14:1)	2%	20.00	240.38	832.02
Pentadecanoic acid (C15:0)	2%	20.00	256.42	779.97
Cis-10-Pentadecenoic acid (C15:1)	2%	19.90	254.41	782.20
Hexadecanoic acid (C16:0)	6%	59.80	270.45	2211.13
Cis-9-Hexadecenoic acid (C16:1)	2%	20.00	268.43	745.07
Heptadecanoic acid (C17:0)	2%	20.30	284.48	713.58
Cis-10-Heptadecenoic acid (C17:1)	2%	20.00	282.46	708.06
Octadecanoic acid (C18:0)	4%	40.60	298.5	1360.13
Trans-9-Elaidic acid (C18:1)	2%	20.60	296.49	694.80
Cis-9-Octadecenoic acid (C18:1)	4%	39.90	296.49	1345.75
All-trans-9,12-Octadecadienoic acid (C18:2)	2%	20.00	294.47	679.19
All-cis-9,12-Octadecadienoic acid (C18:2)	2%	19.90	294.47	675.79
All-cis-6,9,12-Octadecatrienoic acid (C18:3)	2%	19.90	292.46	680.43
Eicosanoic acid (C20:0)	4%	39.90	326.56	1221.83
Cis-11-Eicosenoic acid (C20:1)	2%	20.00	324.54	616.26
All-cis-9,12,15-Octadecatrienoic acid (C18:3)	2%	20.00	292.46	683.85
Heneicosanoic acid (C21:0)	2%	20.00	340.58	587.23

continued

Table 3.3 continued

Fatty acid methyl ester (FAME)	Weight% in the standard	Concentration in the diluted standard (mg/dL)	Molecular mass of the FAME	Concentration (µmol) per L standard
All-cis-11,14-Eicosadienoic acid (C20:2)	2%	20.00	322.53	620.10
Docosapentanoic acid (C22:0)	4%	39.90	354.61	1125.18
All-cis-8,11,14-Eicosatrienoic acid (C20:3)	2%	19.90	320.51	620.89
Cis-13-Docosenoic acid (C22:1)	2%	19.90	352.59	564.39
All-cis-11,14,17-Eicosatrienoic acid (C20:3)	2%	20.40	320.51	636.49
All-cis-5,8,11,14-Eicosatetraenoic acid (C20:4)	2%	20.00	318.49	627.96
Tricosanoic acid (C23:0)	2%	19.90	368.64	570.48
All-cis-13,16-Docosadienoic acid (C22:2)	2%	20.00	350.58	570.48
Tetracosanoic acid (C24:0)	4%	39.90	382.66	1042.70
All-cis-5,8,11,14,17-Eicosapentaenoic acid (C20:5)	2%	20.20	316.48	638.27
Cis-15-Tetracosenoic acid (C24:1)	2%	20.00	380.65	525.42
All-cis-4,7,10,13,16,19-Docosahexaenoic acid (C22:6)	2%	19.90	342.51	581.00

Calculations - absolute fatty acid concentrations

This formula was used to obtain the fatty acid concentration in µmol per L serum:

$$\text{Concentration (µmol/L)} = \frac{\text{PA [Fatty acid in sample]}}{\text{PA [Fatty acid in standard]}} * \frac{\text{PA [C15 : 0 in standard]}}{\text{PA [C15 : 0 in sample]}} * \text{Factor}$$

$$\text{Factor} = \frac{\text{Concentration [C15 : 0 in sample]}}{\text{Concentration [C15 : 0 in standard]}} * \text{Concentration}_{\text{(FAME in standard)}} * \text{Dilution factor}$$

$$\text{Dilution factor} = \frac{1{,}000\,\text{µL}}{\text{Sample volume in µL}}$$

PA:Peak area (µV/min)

Calculations – absolute fatty acid concentrations as percentage distribution

The percentage distribution of the absolute concentrations of the FA profile was obtained using the calculated absolute concentrations of a single FA divided by the sum (∑) of all calculated FA, multiplied by the factor 100 (see example). The results are expressed as percentage of concentration (*conc%).*

Example:

$$\text{Oleic acid (}conc\%\text{)} = \frac{\text{Oleic acid (µmol/L)}}{\sum \text{of all fatty acids (µmol/L)}} * 100$$

Calculations - relative fatty acid profile

The relative FA profile, based on the peak area (PA), was obtained using two factors. Firstly, the PA of each FA of interest was calculated by integration, expressed in µV/min, with standard software (Galaxie CDS, version 1.8.4.1.) provided by Varian GmbH (Darmstadt, Germany). Secondly, the sum of all measured FA of interest was calculated. The calculation to obtain the individual relative FA in *area%* is shown in the example below. Within this calculations it was feasible to enlarge the FA profile with selected FA (C18:1ω7c, C18:1ω7t, C18:4ω3c, C20:3ω9c, and C22:5ω3c), which were not present in the F.A.M.E. C4-C24 standard.

Example:

$$\text{Oleic acid (}area\%\text{)} = \frac{\text{PA [Oleic Acid] (µV/min)}}{\text{PA [}\sum \text{of all calculated fatty acids] (µV/min)}} * 100$$

C Validation of fatty acid measurements

Precision

The precision of the used gas chromatography method was assured by measuring the same single FAME reference standard within three runs. Afterwards, means and standard deviations were used to calculate the coefficient of variation (CV) as shown below.

$$\text{Coefficient of variation (\%)} = \frac{\text{standard deviation}}{mean} * 100$$

Table 3.4a summarizes the CV of the intra-assay precision of each single FAME standard.
To obtain an overview about the variation within the different runs, the CV of the F.A.M.E. C4-C24 measurements were calculated for the complete sequence of each city (see table 3.4b). The CV includes day-by-day fluctuations and variations because of freshly prepared F.A.M.E. standards due to the time-consuming analysis of several days.

Table 3.4a: Intra-assay precision (n=3) of single fatty acid methyl ester standards measured by gas chromatography.

Single fatty acid methyl ester standard*	**Coefficient of variation (%)**
Docosadienoic acid methyl ester	2.8
Docosahexaenoic acid methyl ester	2.9
Methyl 11,14,17-eicosatrienoate	1.4
Methyl 5,8,11-eicosatrienoate	3.2
Methyl 8,11,14-eicosatrienoate	3.6
Methyl arachidate	1.6
Methyl arachidonate	1.5
Methyl behenate	4.4
Methyl butyrate	2.9
Methyl decanoate	3.0
Methyl docosapentaenoate	0.4
Methyl eicosadienoate	2.2
Methyl eicosapentaenoate	1.5
Methyl eicosenoate	3.2
Methyl erucate	2.9
Methyl heneicosanoate	2.7
Methyl heptadecanoate	3.4
Methyl heptadecenoate	1.3
Methyl hexanoate	3.2
Methyl laurate	2.4
Methyl linoleate	2.4
Methyl linolelaidate	0.7

continued

Table 3.4a continued

Single fatty acid methyl ester standard*	**Coefficient of variation (%)**
Methyl linolenate	2.4
Methyl myristate	2.3
Methyl myristoleate	1.7
Methyl octadecenoate	0.1
Methyl octanoate	1.4
Methyl oleate	1.5
Methyl palmitate	1.0
Methyl palmitoleate	1.8
Methyl pentacosanoate	2.9
Methyl pentadecanoate	3.0
Methyl pentadecenoate	1.2
Methyl stearate	3.5
Methyl stearidonate	1.7
Methyl tetracosanoate	0.9
Methyl tetracosenoate	1.2
Methyl trans-vaccenate	2.5
Methyl tricosanoate	1.8
Methyl tridecanoate	2.1
Methyl undecanoate	2.7
Methyl γ-linolenate	2.8

* the precise nomenclature is shown in the list of abbreviations

Table 3.4b: Coefficients of variation of the fatty acid methyl ester standard (F.A.M.E. C4-C24) for each study centre analysed by gas chromatography.

Fatty acid methyl ester*	**Coefficient of variation (%)**									
	A	**D**	**G**	**H**	**L**	**P**	**R**	**S**	**V**	**Z**
Docosadienoic acid methyl ester	10.7	6.5	6.6	3.5	14.5	8.1	11.0	9.8	11.5	8.0
Docosahexaenoic acid methyl ester	11.8	7.3	6.3	5.2	14.6	7.3	10.7	8.8	11.9	7.5
Methyl 8,11,14-eicosatrienoate	10.4	6.0	6.0	3.9	13.1	6.9	9.9	9.1	11.6	7.7
Methyl arachidate	11.3	5.3	5.4	3.7	11.4	4.9	10.9	9.0	7.9	10.2
Methyl arachidonate	11.1	6.3	5.7	3.9	12.2	6.9	10.7	8.0	11.9	7.6
Methyl behenate	11.1	5.9	6.6	4.2	11.2	6.4	10.2	9.7	9.0	15.7
Methyl eicosadienoate	11.2	6.0	5.9	4.0	13.4	7.1	10.8	8.6	12.0	8.4
Methyl eicosapentaenoate	11.0	5.6	5.8	4.0	12.6	6.6	10.5	8.8	11.8	8.0
Methyl eicosenoate	11.3	5.9	5.8	3.9	13.7	6.8	10.9	8.2	13.1	7.9
Methyl erucate	11.2	6.2	5.8	4.5	15.0	7.9	10.9	9.8	13.1	7.8
Methyl heneicosanoate	10.7	5.7	6.1	4.1	11.6	6.0	10.3	9.0	8.7	13.9
Methyl heptadecanoate	9.4	9.7	3.8	3.6	6.9	5.5	11.8	5.3	9.9	8.1
Methyl heptadecenoate	9.0	4.2	3.7	3.7	6.6	5.9	8.7	5.3	10.2	7.8
Methyl laurate	2.2	4.8	4.9	4.4	4.5	6.4	4.0	4.5	9.2	7.6
Methyl linoleate	10.7	5.7	5.2	3.2	8.4	8.4	9.3	8.7	8.7	6.5
Methyl linolelaidate	9.6	4.9	4.4	5.2	9.4	6.5	10.7	5.9	12.4	8.2
Methyl linolenate	9.8	4.7	4.3	3.7	9.1	6.0	9.9	6.3	10.9	10.2
Methyl myristate	4.2	4.3	4.3	4.1	3.7	6.0	4.6	4.1	9.6	7.8
Methyl myristoleate	3.9	4.3	4.2	4.1	3.7	6.0	4.6	4.1	9.5	7.6
Methyl oleate	10.2	4.8	4.3	3.6	9.1	6.2	9.9	6.4	10.9	7.5
Methyl palmitate	7.9	4.4	3.7	3.8	4.7	6.0	7.7	4.5	9.9	7.6
Methyl palmitoleate	7.4	4.0	3.6	3.7	4.6	5.9	7.8	4.4	10.1	7.5
Methyl pentadecanoate	6.0	4.3	3.9	4.0	3.6	6.1	6.6	4.2	9.9	7.7
Methyl pentadecenoate	5.7	4.0	3.8	3.9	3.6	5.9	6.0	4.0	9.7	7.7
Methyl stearate	10.5	5.0	4.7	3.7	9.1	5.4	10.0	6.7	8.7	7.1
Methyl tetracosanoate	10.4	7.2	9.1	4.2	11.8	6.4	10.8	8.4	9.6	19.1
Methyl tetracosenoate	10.1	7.0	6.3	4.5	14.7	7.8	10.7	8.9	12.7	7.4

continued

Table 3.4b continued

Fatty acid methyl ester*	Coefficient of variation (%)									
	A	**D**	**G**	**H**	**L**	**P**	**R**	**S**	**V**	**Z**
Methyl tricosanoate	10.8	7.0	8.1	5.5	12.3	6.9	9.8	8.9	10.2	17.3
Methyl γ-linolenate	9.8	4.5	4.2	3.8	8.5	6.1	9.5	6.1	10.8	7.8

* the precise nomenclature is shown in the list of abbreviations

A:Athens (analysed F.A.M.E.: n=13), D:Dortmund (n=14), G:Ghent (n=13), H:Heraklion (n=13), L:Lille (n=12), P:Pecs (n=15), R:Rome (n=12), S:Stockholm (n=13), V:Vienna (n=13), Z:Saragossa (n=13).

Stability of fatty acids

Prior to the study, the stability of the FA was tested. Blood samples were collected in ethylene diamine tetraacetic acid monovettes from six volunteers (mean age 29±3 years, 5 females), immediately centrifuged (2,500 x *g*, 10 minutes) and aliquoted. Aliquots were stored at room temperature or at approximately 7°C (refrigerator) over 24 hours. Samples were analyzed after eight and 24 hours in the IEL. Samples showed no stability over time at room temperature as well as at 7°C. Thus, for logistical and analytical reasons, serum blood was used instead of plasma and the samples had to be stored at -20/ -80°C in the local centre as soon as possible. No antioxidant or stabilizer was used to avoid FA oxidation because of the direct storage of the samples at -20/ -80°C.

D Routine lipid and lipoprotein determination

Serum TG, TC, HDL, and LDL were measured enzymatically on the Dimension RxL clinical chemistry system (Dade Behring, Schwalbach, Germany) using the manufacturer's reagents and instructions. Apolipoprotein A-1 and B concentrations were measured in an immunochemical reaction with a BN II analyzer (Dade Behring, Schwalbach, Germany) according to the manufacturer's instructions. The proteins contained in the serum sample form immune complexes with specific antibodies. These complexes scatter a light beam when passed through the sample. The intensity of the scattered light is proportional to the concentration of the relevant protein in the sample. The result is evaluated by comparison with a standard of known concentration. Serum Lp(a) was measured by means of particle-enhanced immunonephelometry using the BN II analyzer (Dade Behring).

E Validation of routine lipid measurements

Precision

The intraassay coefficient of variation of the TG, TC, HDL, LDL, apo A-1, apo B, and Lp(a) assay was 1.9%, 1.3%, 3.3%, 2.7%, 2.7%, 2.1%, and 3.9%, while the interassay coefficient was 2.2%, 1.8%, 3.9%, 3.6%, 3.4%, 3.2%, and 4.3%.

Stability of routine lipid parameters

To guarantee the stability of routine biochemistry analyses in fresh serum samples three samples with high – mean – low baseline values were tested over different time points during 24 hours after blood extraction. These stability tests were performed in the University Hospital in Bonn. The results are shown in table 3.5.

Table 3.5: Results from stability tests for lipids and lipoproteins.

Time after blood extraction (hours)		0	2	4	6	8	12	24	Median of deviation to time 0 (%)	Mean of deviation to time 0 (%)
	Sample									
Triglycerides	1	148	149	148	150	151	150	149	1.0	1.0
(mg/dL)	2	48	48	50	50	49	51	50	4.2	3.5
	3	105	103	107	107	108	108	106	1.9	2.1
TC	1	165	167	166	166	166	168	166	0.6	0.9
(mg/dL)	2	125	124	124	127	126	126	127	0.8	1.1
	3	63	62	62	62	63	63	63	0.8	0.8
HDL	1	31	32	31	32	31	31	33	1.6	2.2
(mg/dL)	2	33	33	32	34	33	34	34	3.0	2.0
	3	12	12	12	11	12	11	11	4.2	4.2
LDL	1	101	100	103	99	103	102	103	2.0	1.7
(mg/dL)	2	77	75	75	74	77	76	77	1.9	1.7
	3	29	29	30	29	30	30	30	3.4	2.3
Lp(a)	1	62	58	64	59	60	60	61	3.2	3.8
(mg/L)	2	62	58	60	59	63	60	62	3.2	3.2
	3	459	480	461	486	474	468	459	2.6	2.7
Apo A-1	1	1.37	1.4	1.39	1.39	1.38	1.39	1.4	1.5	1.6
(g/L)	2	0.82	0.86	0.85	0.8	0.79	0.85	0.84	3.7	3.5
	3	2	2.03	1.99	2.03	1.98	1.99	2.03	1.3	1.1
Apo B	1	0.35	0.34	0.35	0.37	0.35	0.35	0.36	1.4	1.9
(g/L)	2	0.95	0.98	0.97	0.97	0.97	0.96	0.95	2.1	1.8
	3	0.88	0.9	0.89	0.91	0.88	0.87	0.87	1.1	1.5

Apo:Apolipoprotein, HDL:High density lipoprotein cholesterol, LDL:Low density lipoprotein cholesterol, Lp(a):Lipoprotein(a), TC:Total cholesterol.

F Leptin and insulin determination

Leptin

Leptin concentrations were measured with an enzyme-linked immunosorbent assay kit (RayBio® Human Leptin ELISA Kit, RayBiotech Inc., Norcross, GA, USA) according to the manufacturer´s instructions and reagents using a sample volume of 100 µL serum. A Thermo Scientic Multiskan® EX (Hucoa Erlöss S.A., Madrid, Spain) was used as microplate photometer at a wave length of 450 nm. The sensitivity of the leptin assay was 6 pg/mL. The intraassay CV was <10% and the interassay CV <12%.

Insulin

Insulin concentrations were measured by a solid-phase two-site chemiluminescent immunometric assay with an Immulite 2000 analyzer (DPC Biermann GmbH, Bad Nauheim, Germany) using the manufacturer's reagents and instructions. A total amount of 100 µL heparin plasma was needed for analysis. The sensitivity of the insulin assay was 2 mLU/L. The interassay CV was 5.2%.

3.4 Statistics, Evaluation Procedures and Lipid Calculations

A Statistics

Generally, data analysis was performed using Statistical Package for Social Sciences (SPSS) version 17.0 for Windows (SPSS Inc., Chicago, Illinois, USA) and *p* values <0.05 were considered as statistically significant.
However, for the different topics of the chapters specific statistical methods were applied. Thus, in terms of simplification these specific methods are described within each chapter.

B Evaluation procedures

Within each chapter several non-communicable (i.e. age and maturity) and communicable (i.e. BMI, BF, and FFM) factors were determined and grouped. The specific classifications are listed below.

Age
To assess the influence of age, adolescents were stratified into four gender-specific age groups ranging from 12.5-13.99, 14-14.99, 15-15.99, and 16-17.49 years, respectively. A weighting factor was calculated in order to adjust the theoretical sampling to the observed sample in function of age and gender.

Maturity
Graduation into five stages of maturity ranging from no development (Tanner stage I) to complete development (Tanner stage V) was made using the method described by Tanner and Whitehouse [4]. If maturity revealed different results, when assessing gonads/ breasts and pubic hair, the higher grade was chosen.

Body mass index
Body mass index (weight [kg] divided by height [m] squared) categories were based on age- and sex-specific cut-off points created by Cole *et al*. [9, 10]. The BMI cut-off points correspond to adult cut-offs for underweight (≤18.5 kg/m²), normal weight (18.5-25 kg/m²), overweight (25-30 kg/m²), and obesity (>30 kg/m²).

Percentage body fat

Percentage BF was calculated using the formula of Slaughter *et al.* [11]. For girls, BF equals 0.61*(triceps+calf)+5.1, and for boys BF equals 0.735*(triceps+calf)+1. Normal BF correspond to <25% for boys and <35% for girls. High BF was defined as ≥25% for boys and ≥35% for girls [12].

Fat free mass

Fat free mass according to BIA measurements were expressed as quartiles for boys and girls. More precisely, the FFM quartiles (25^{th}, 50^{th}, and 75^{th}) were 38.5, 41.0, and 44.4 kg for girls, and 46.0, 52.0, and 57.8 kg for boys.

C Lipid Calculations

The calculations and estimations of lipid and lipoprotein ratios, FA families and FA ratios, which are used in the corresponding chapters, are summarized in the tables below.

Lipid and lipoprotein ratios

Table 3.6: Lipid and lipoprotein ratios based on the corresponding measurements.

Name/Abbreviation	Calculation
Apo B/Apo A-1 ratio	Apo B/Apo A-1
Apo B/LDL ratio	Apo B/LDL*100
Atherogenic Index	(TC-HDL)*Apo B/(HDL*Apo A-1)
LDL/HDL ratio	LDL/HDL
nonHDLC	TC-HDL
nonHDLC/HDL ratio	nonHDLC/HDL
TC/HDL ratio	TC/HDL
TG/HDL ratio	TG/HDL

Apo:Apolipoprotein, HDL:High density lipoprotein cholesterol, LDL:Low density lipoprotein cholesterol, nonHDLC:Non high density lipoprotein cholesterol, TC:total cholesterol, TG:Triglycerides.

Fatty acid ratios

Table 3.7: Fatty acid ratios based on the corresponding measurements.

Fatty acid ratio	**Calculation***
AA/LA	C20:4ω6c/C18:2ω6c
(DHGLA+AA)/LA	(C20:3ω6c+C20:4ω6c)/C18:2ω6c
EPA/ALA	C20:5ω3c/C18:3ω3c
AA/EPA	C20:4ω6c/C20:5ω3c
DHA/EPA	C22:6ω3c/C20:5ω3c
LA/ALA	C18:2ω6c/C18:3ω3c
AA/(EPA+DHA)	C20:4ω6c/(C20:5ω3c+C22:6ω3c)

ω6/ω3

$$\frac{\mathrm{C18:2}\omega 6c\ \&\ t + C18:3\omega 6c + C20:2\omega 6c + C20:3\omega 6c + C20:4\omega 6c + C22:2\omega 6c}{\mathrm{C18:3}\omega 3c + C18:4\omega 3c + C20:5\omega 3c + C22:5\omega 3c + C22:6\omega 3c}$$

Polyunsaturates/Saturates

$$\frac{\begin{array}{c}\mathrm{C18:2}\omega 6c\ \&\ t + C18:3\omega 6c + C20:2\omega 6c + C20:3\omega 6c + C20:4\omega 6c + C22:2\omega 6c + \\ \mathrm{C18:3}\omega 3c + C18:4\omega 3c + C20:5\omega 3c + C22:5\omega 3c + C22:6\omega 3c + \mathrm{C20:3}\omega 9c\end{array}}{\mathrm{C12:0 + C14:0 + C16:0 + C17:0 + C18:0 + C20:0 + C21:0 + C22:0 + C23:0 + C24:0}}$$

Monosaturates/Saturates

$$\frac{\mathrm{C14:1}\omega 5c + C15:1\omega 5c + C16:1\omega 7c + C17:1\omega 7c + C18:1\omega 7c\ \&\ t + C18:1\omega 9c + C20:1\omega 9c + C22:1\omega 9c + C24:1}{\mathrm{C12:0 + C14:0 + C16:0 + C17:0 + C18:0 + C20:0 + C21:0 + C22:0 + C23:0 + C24:0}}$$

*Calculations regarding the absolute fatty acid composition and fatty acids expressed as *conc%* did not include C18:1ω7c, C18:1ω7t, C18:4ω3c, C20:3ω9c, and C22:5ω3c.

AA:Arachidonic acid, ALA:α-linolenic acid, EPA:Eicosapentaenoic acid, DHA:Docosahexaneoic acid, DHGLA:Dihomo-γ-linolenic acid, LA:Linoleic acid.

Fatty acid families

Table 3.8: Fatty acid families based on single fatty acid calculation.

Name	Calculation*
Saturated fatty acids	∑ of C12:0, C14:0, C16:0, C17:0, C18:0, C20:0, C21:0, C22:0, C23:0 and C24:0
Monounsaturated fatty acids	∑ of C14:1ω5c, C15:1ω5c, C16:1ω7c, C17:1ω7c, C18:1ω7c, C18:1ω7t, C18:1ω9c, C20:1ω9c, C20:3ω9c, C22:1ω9c, and C24:1ω9c
Polyunsaturated fatty acids	∑ of C18:2ω6c, C18:2ω6t, C18:3ω6c, C18:3ω3c, C18:4ω3c, C20:2ω6c, C20:3ω9c, C20:3ω6c, C20:4ω6c, C20:5ω3c, C22:2ω6c, C22:5ω3c and C22:6ω3c
Long-chain polyunsaturated fatty acids	∑ of C20:3ω6c, C20:3ω9c, C20:4ω6c, C20:5ω3c, C22:5ω3c, and C22:6ω3c
trans fatty acids	∑ of C18:1ω7t and C18:2ω6t
ω3 fatty acids	∑ of C18:3ω3c, C18:4ω3c, C20:5ω3c, C22:5ω3c, and C22:6ω3c
ω6 fatty acids	∑ of C18:2ω6c, C18:2ω6t, C18:3ω6c, C20:2ω6c, C20:3ω6c, C20:4ω6c, and C22:2ω6c
ω9 fatty acids	∑ of C18:1ω9c, C20:1ω9c, C20:3ω9c, C22:1ω9c, and C24:1ω9c

*Calculations regarding the absolute fatty acid composition and fatty acids expressed in *conc%* did not include C18:1ω7c, C18:1ω7t, C18:4ω3c, C20:3ω9c, and C22:5ω3c.

Fatty acids with the suffix “c” indicate *cis*, whereas the suffix “t” indicates *trans* configurations.

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4 Serum lipid and lipoprotein status in European adolescents - associations with age, gender, maturity, body mass index, and percentage body fat

Short Summary

Background: Research has confirmed the crucial role of lipids and lipoproteins in the development of coronary heart diseases (CHD) and atherosclerosis, which is likely to start in childhood and adolescence. However, less is known about communicable (BMI and BF) and non-communicable factors (age, gender, and maturity) and their influence on lipids in healthy adolescents. Especially for Europe, lipid data is needed to clarify the association of age, gender, maturity, BMI, and BF content with growth and development in that particular period of life. This information will be conducive to the understanding of lipid patterns in youth. Further, the descriptive data may be used as physiological values of adolescents for lipid screenings in Europe.

Objective: To provide a complete lipid and lipoprotein profile of healthy European adolescents, analysing the association with age, gender, maturity, BMI, and BF.

Results: Lipid and lipoprotein parameters were higher in girls than in boys. Maturity was related to TC, HDL, apo A-1, and Apo B/LDL in boys and associated with TC, LDL, non high density lipoprotein cholesterol (nonHDLC), apo B, and most lipid and lipoprotein ratios (Apo B/Apo A-1, TC/HDL, LDL/HDL, nonHDLC/HDL, and the Atherogenic Index [AI]) in girls. By contrast, chronological age was only associated with male TG levels and the TG/HDL ratio. Body mass index was linked to TG, HDL, nonHDLC, and most lipid and lipoprotein ratios (Apo B/Apo A-1, TC/HDL, TG/HDL, LDL/HDL, nonHDLC/HDL, and the AI) in girls and not related to lipid and lipoprotein parameters in boys. Triglyceride, LDL, nonHDLC, and apo B levels as well as nearly all lipid and lipoprotein ratios (Apo B/Apo A-1, TC/HDL, TG/HDL, LDL/HDL, nonHDLC/HDL, and the AI) differed significantly between male BF groups. These observations were not present in girls. Further, reference data using percentiles (2.5^{th}-97.5^{th}) of healthy adolescents excluding smokers, underweight and obese subjects are provided.

Conclusion: The serum lipid profile of European adolescents depends more on gender, maturity, and body fat/ body weight status than on chronological age. The present normal values of apparently healthy European adolescents may be used for clinical use.

Introduction

Autopsy studies strongly indicated that the origin of artherosclerosis is located in early childhood and adolescence: fatty streaks are already present in early age and may progress to fibrous plaques after the second decade of life. The main determinants of plaque formation are smoking, hypertension, age, gender, race/ ethnicity and apo B-containing lipids [1-7]. Moreover, blood lipid concentrations (e.g. TG, TC, LDL, and HDL) and calculations of lipid and lipoprotein ratios such as TC/HDL, LDL/HDL, and TG/HDL can be used to assess the individual atherogenic risk [8-13].

The evaluation of blood lipid profiles in adolescence uses reference data of the American NCEP for children and adolescents [14]. For two reasons they might lead to wrong conclusions when applying them to healthy European adolescents. First of all, the data is based on examinations of children and adolescents from families with hypercholesterolemia or premature cardiovascular disease, thus the subjects might not be healthy because of the familial predisposition. Secondly, the reference data was defined in the 1980s and is addressed to the American adolescent population.

Furthermore, our knowledge of lipid profiles of children and adolescents is mostly based on European and non-European studies examining the lipid status in already diseased subjects, e.g. children with obesity or diabetes [13, 15-23]. Data available for healthy adolescents are mainly coming from surveys in America [24-26]. In Europe, only one Spanish study, based on five representative cities, examined healthy adolescents to present a reference lipid profile. This AVENA Study presented age- and sex-specific lipid and lipoprotein profiles of 581 adolescents aged 13 to 18.5 years [27]. However, reliable reference data considering the impact of growth, alterations in body composition and grade of puberty on lipid profiles in adolescence is lacking for Europe. Moreover, different methodological and analytical approaches have been used in these mentioned studies, which make comparisons on a global level difficult. Consequently, an objective evaluation of early atherogenic risks in children and adolescents living in Europe is not feasible. The alternative use of risk scores provided by NCEP may under- or overestimate individual risks in European adolescents.

Thus, reliable reference data for Europe should be assessed on the basis of examinations of apparently healthy European adolescents, considering communicable and non-communicable factors as well as being up-to-date.

The aim of the present study was, thus, to assess a detailed lipid profile of a healthy European adolescent population considering age, gender, sexual maturation, BMI, and percentage BF

applying harmonized methodology and analytics. The data representation will also include percentiles (2.5^{th}-97.5^{th}) as well as lipid and lipoprotein ratios, which may be used as a standard for European adolescents.

Subjects and Methods

A detailed description of study design, subject recruitment, medical examination, blood sampling, sample treatment, biochemical analysis, and evaluation procedures can be found in *chapter 3* (*General Methodology*).

Statistics

Numeric values are shown as mean±SD. Percentiles (2.5^{th}-97.5^{th}) were calculated to obtain reference data of healthy European adolescents excluding smokers, underweight and obese subjects. Blood analytes (dependent variables) were skewed and logarithmically transformated before statistical analysis. Differences in the main study characteristics of boys and girls were analysed using independent *t*-test (metric variables) and *chi square* test (categorical variables). Age, sexual maturation, BMI, and BF were grouped and defined as independent variables. For each of the dependent variables, a univariate analysis of variance (ANOVA) was used to analyse the associations with the independent variables. The Bonferroni *post-hoc tests* were used for sub-group analyses. All statistics were made separately for boys and girls. A *p*-value of <0.05 was considered as statistically significant and data analysis was performed by using Statistical Package for Social Sciences version 17.0 for Windows (SPSS Inc., Chicago, Illinois, USA).

To assure optimal validity of the results, a post hoc analysis for achieved power was conducted exemplarily with logarithmically transformated data for independent *t*-tests with a two-tailed significance of $p=0.05$ using GPower® version 3.0.10 [28]. The achieved power was >99% for all lipid parameters except for TG and Lp(a) with a power of 46% and 65%.

Results

The HELENA-CSS consisted of 1,097 adolescent subjects with blood measurements including complete data about basic characteristics (e.g. BMI and age). A total number of

1,084 blood samples was analysed completely for all lipid parameters. Apolipoprotein A-1, apo B and Lp(a) levels were determined in 1,056 samples because of sufficient blood volume for complete lipid analysis. Table 4.1 presents the basic characteristics of all study participants. Tables 4.2a-c show the percentiles (2.5^{th}-97.5^{th}) of the selected study group as well as for boys and girls. The present percentiles did not include smokers, underweight and obese participants to represent an apparently healthy population. Thus, data of 774 adolescents (369 boys and 405 girls) is presented in those tables.

Compared to boys, girls showed higher mean values for all lipid and lipoprotein parameters as highlighted in table 4.1.

With respect to age, there was an increase in TG levels in boys with higher age (p=0.013, table 4.3a). This effect was also present in the male TG/HDL ratio (table 4.3b). All other parameters remained unaffected by age in both boys and girls (tables 4.3a and 4.3b).

In boys, pubertal maturation was associated with TC, HDL and apo A-1 levels in a mostly inverse manner (table 4.4a). Additionally, Apo B/LDL increased slightly in the final stages of maturity (table 4.4b). By contrast, maturity was associated with TC, LDL, nonHDLC, and apo B levels without clear increasing/ decreasing tendency in girls (table 4.4a). Further, most female lipid and lipoprotein ratios (Apo B/Apo A-1, TC/HDL, LDL/HDL, nonHDLC/HDL, and the AI) increased between Tanner stage IV and V (table 4.4b).

Body mass index was associated with serum lipids and lipoproteins levels only in girls (table 4.5a). Girls tended to increase TG, nonHDLC and all of the risk indices except for Apo B/LDL, and tended to decrease HDL levels with higher BMI (tables 4.5a and 4.5b).

The results according to the assessment of percentage BF showed a different pattern. Significantly higher TG, LDL, and nonHDLC levels as well as lower HDL levels were found in boys with higher BF (table 4.5a). Additionally, all lipid and lipoprotein ratios showed higher values in the high BF group except for Apo B/LDL (table 4.5b). In girls, no relationship was observed between lipid parameters and BF content (tables 4.5a and 4.5b).

Discussion

With respect to gender, the most striking results were higher values in girls within each of the lipid variables analysed (table 4.1). This result is in accordance with findings in a Turkish study cohort of 2,896 schoolchildren aged 7-18 years [29]. In the Spanish AVENA Study (n=581, age 13-18.5 years), however, girls had only significantly higher HDL and TC levels

(p<0.05), whereas LDL, TG, nonHDLC, Lp(a), apo A-1, and apo B levels remained unaffected by gender [27]. In a Mexican study (1,076 girls and 770 boys, mean age 13.2±1 years), lipid parameters were increased in girls except for HDL [30]. These inconsistent results may be due to genetic variations and/ or different eating behaviours in Spain, Turkey, and Mexico. In the HELENA project because of the multinational design such effects are minimized. Moreover, different age categorizations may contribute to the different results, because with increasing age the influence of sex hormones becomes more obvious and is mirrored in altered lipid levels between sexes [31]. The absolute lipid concentrations of the present study showed some differences compared to the other study results. In HELENA, male participants had the lowest TG levels (65 mg/dL) compared to their Turkish and Mexican (88 mg/dL), and Spanish (73 mg/dL) counterparts. By contrast, the TG levels of the present European girls (73 mg/dL) lay in between the concentrations of Spanish (66 mg/dL), Turkish (82 mg/dL), and Mexican (97 mg/dL) girls. Further, Mexican adolescents had lower HDL values (♂=44 mg/dL; ♀=45 mg/dL) and lower TC levels (♂=150 mg/dL; ♀=156 mg/dL) compared to the HELENA participants (HDL: ♂=53 mg/dL, ♀=57 mg/dL; TC: ♂=154 mg/dL, ♀=167 mg/dL). No gender-specific differences in the lipid and lipoprotein ratios were found in other studies [27, 30]. In HELENA, the mean TG/HDL ratio was significantly higher in girls *versus* boys (p=0.01), whereas the other ratios (Apo B/Apo A-1, TC/HDL, LDL/HDL, Apo B/LDL, nonHDLC/HDL, and the AI) did not differ (data not shown). These results showed that, although girls had higher lipid concentrations, the proportions between those serum lipids and lipoproteins (expressed as ratio) are equal between European boys and girls (except for TG/HDL).

In the present study, age was only associated with TG levels in males (table 4.3a). The increase over age may reflect changes in eating habits during growth. For instance, an increased carbohydrate consumption induces an elevation of the serum TG levels. The TG increase over age was also found in most [29, 32, 33] but not all [27] studies. For example, serum TG levels of male Tehranian adolescents were higher in 17-19 year-olds compared to 11-13 year-olds including higher concentrations than in the HELENA Study (124 mg/dL *vs* 73 mg/dL) [32]. In contrast to the present results, most studies found also age-dependent changes within the other lipids for boys and girls [27, 29, 32, 33]. In HELENA, the association of TG levels with age in boys was mirrored in the TG/HDL ratio as well. All other ratios remained unaffected by age in both sexes (table 4.3b). These results are similar to the AVENA Study except for a decrease of LDL/HDL between 14 and 15 year-old Spanish girls

[27]. Thus, the proportions between serum lipids (expressed as ratios) are hardly associated with age in European adolescents.

However, it is likely that the age-associated findings in the studies mentioned before are related to pubertal progression, because the chronological development of individuals expressed by age mirrors partly the biological development. Indeed, age is an inappropriate marker for biological development and it is known that sexual maturation due to changes in sex hormones influences the lipid profile of adolescents [31, 34, 35]. These facts support the use of pubertal stages instead of age categories to investigate the influence of biological development in adolescence. In HELENA, boys showed decreased TC levels likely due to a decrease in HDL levels accompanied by apo A-1 alterations during puberty, whereas in girls TC, LDL, nonHDLC, and apo B levels were related to the grade of maturity (table 4.4a). Studies showed inconsistent findings probably due to sample size, classification of maturity, and geographic differences based on different genetic backgrounds, food patterns and lifestyles [15, 21, 36-38]. This should be kept in mind when comparing different study results. Lower HDL levels with advancing pubertal stages are in accordance with data from white boys of the Bogalusa Heart Study [38] and study results of Güven and Sanisoglu [15]. Güven and Sanisoglu measured the serum lipid profile with respect to pubertal progression in 182 obese girls and 158 obese boys aged 3.6-17.8 years. Sexual maturity was stratified into prepuberty, early puberty, mid-puberty, and late puberty. High density lipoprotein cholesterol was lower in late pubertal boys compared to pre- and early pubertal boys. By contrast, LDL gradually decreased parallel with pubertal progression in girls [15]. The study population based on obese subjects only, thus comparisons with the present study population should be made with caution and an influence of sex hormones on adipose tissue should be taken into account. In fact, a decrease of HDL in boys is seen as a consequence of higher testosterone levels compared to girls leading to a reduction of apolipoproteins associated with HDL [34]. Most studies did not evaluate lipid and lipoprotein ratios related to sexual maturity [21, 36-38]. In girls, there was an increament between Tanner stage IV and V for most lipid and lipoprotein ratios (Apo B/Apo A-1, TC/HDL, LDL/HDL, nonHDLC/HDL, and the AI), which was not apparent in males (table 4.4b). The gender-specific differences may be directly related to hormonal status, fat mass and/ or fat allocation. However, the measurement of sex hormones was not feasible. Thus, a direct relationship between hormones and lipids could not be investigated within the HELENA Study.

Beside the BMI status, percentage BF content was related to serum lipid status. The most striking finding was that female lipid parameters were associated with BMI alone, whereas

male serum lipids were related to BF content only (table 4.5a). More precisely, higher lipid and lipoprotein ratios (Apo B/Apo A-1, TC/HDL, TG/HDL, LDL/HDL, nonHDLC/HDL, and the AI) were observed in boys with high BF (≥25%), whereas in girls those ratios were increased with higher BMI status. Generally, these findings indicate that higher fat/ weight status is associated with a more unfavourable lipid profile and an increased atherogenic risk. It is not clear why male lipid levels are associated with BF content alone, whereas lipid parameters of girls are related solely to BMI status. Differences may be obtained due to the assessment methods. Fat allocation is different between boys and girls and this may be reflected in the different skinfold measurements. It may also be possible that the sub-group classification (two BF sub-groups *vs.* four BMI sub-groups) with different group sizes results in altered findings.

A French study by Klein-Platat *et al.* [39] found higher TG levels of 0.98 mmol/L (approx. 87 mg/dL) in 12 year-old overweight adolescents (n=60, BMI=23.3±0.3) compared to their normal weight counterparts with TG levels of 0.80 mmol/L (approx. 71 mg/dL; n=60, BMI 17.4±0.3), whereas HDL did not differ between obese (1.09 mmol/L; approx. 42 mg/dL) and normal weight (1.30 mmol/L; approx. 50 mg/dL) subjects. Body mass index and BF differed significantly between the groups, but only BMI was used for associations. In the present male subjects, HDL values were also not significantly altered with respect to BMI status. By contrast, lower HDL levels were observed in boys with higher BF; this effect was not investigated in the French study. The association between lipids and BMI status was observed in the French study group with both genders, thus gender-specific differences as observed in the present study might have disappeared [39]. A Swedish study (n=25) in obese (median age 12.0 years, median BMI 30.7 kg/m²) and non-obese (median age 12.6 years, median BMI 19.5 kg/m²) adolescents found no association with BMI except for lower HDL levels in obese girls. These results are not in accordance with the present findings; however, the results of the Swedish study may be limited because of the group size (5-9 subjects per group) [40]. In the Mexican study, the prevalence of different forms of dyslipidemia were significantly higher in obese and overweight than in normal weight boys and girls [30]. This supports the assumption that higher weight status leads to altered, unfavourable lipid profiles at younger ages. With respect to weight status, lipid and lipoprotein ratios showed the same pattern as observed for lipid parameters.

Beside gender, pubertal development and BF/ weight status are associated with changes in the adolescent lipid profile as seen in the HELENA Study as well as found by others [27, 29, 30,

32, 37-40]. These results suggest a strong association between those factors (i.e. gender, maturity, BMI, and BF) and serum lipid profiles during growth and development.

A few limitations of the present study deserve comment. Because of the CSS design, the temporal relationships are unknown; we cannot say anything about causality of the relationships.

The discussion about reference values for a healthy adolescent population and its application for lipid screening and prevention strategies is ongoing and the question, which percentiles/ cut-offs should be used, is not answered sufficiently yet. Moreover, it is still discussible how to develop reliable reference values for lipids in adolescence. However, within the HELENA study design it was not realizable to create reference values per se. Nonetheless, the data presented here is proposed to be used as physiological values of apparently healthy European adolescents for comparing the serum lipid profile in various disease conditions.

Additionally, a look at approaches of former reference data may be helpful to survey the present approach. The NCEP published blood cholesterol cut-offs for children and adolescents in 1992 [14]. In the age range of 2-19 years, TC was defined as high ≥200 mg/dL and normal <170 mg/dL. The lipoprotein levels of LDL and HDL were set as normal <110 mg/dL and >60 mg/dL, whereas high/ low levels were determined at ≥130 and ≤35 mg/dL. High TG levels were defined as ≥130 mg/dL and normal levels <90 mg/dL in the age range of 10-19 years. These cut-offs are based on the 75^{th} percentile for normal levels and the 95^{th} percentile for high levels, and for HDL on the 25^{th} and 5^{th} percentile, respectively. This approach has some limitations. These selected percentiles are fixed for an age range between 2 to 19 years, and the HELENA results showed fluctuations in the lipid and lipoprotein levels due to maturity, which make it reasonable to adapt the cut-offs to specific pubertal stages, or at least, but more inaccurate, to age categories. The selected percentiles are completely arbitrary. Further, the lipid screening of the American referent population was made in the early 1980s, but lifestyle and eating habits have changed. The reference data is based on data from children and adolescents from families with hypercholesterolemia or premature cardiovascular disease, and thus not applicable to a healthy population. Jolliffe and Janssen published age-specific lipid and lipoprotein thresholds for adolescents in 2008 [26]. They used data of an adolescent population (n=6,067) of the National Health and Nutrition Examination Surveys (NHANES) measured between 1988 and 2002. Their new approach was to create growth curves with the Lambda-Mu-Sigma method. They adapted the NCEP cut-offs for adults at age 20 and applied those particular percentiles to the age range of 12-20 year-old boys and girls. For example, 150 mg/dL is the borderline-high cut-off for TG in

adults defined by NCEP, which is equivalent to the 89th percentile at 20 years of age (entry into adulthood). This 89th percentile was then used to create the age-specific threshold for borderline-high TG at each age [41]. This approach is still discussible, because the risk factors of adults are not applicable to youth. Furthermore, the data measurement is based on a quite large period (1988-2002). How to overcome this problematic is not solved yet. The use of the 75th and 95th percentiles, 5th and 25th percentile in the case of HDL, by NCEP is very common and established.

However, a comparison of the American data with the present European data may identify possible differences in the absolute lipid values. Most American studies used NHANES data to present percentiles for concentrations of lipids and lipoproteins [25, 26, 41]. Ford *et al.* [25] used current NHANES data from 1999 to 2006. The 75th percentile of LDL was 104 mg/dL in 1,324 girls and 1,400 boys aged 12-17 years and the 95th percentile was 133 and 136 mg/dL in girls and boys. This is quite similar to the HELENA population; only the girls had approximately 10 mg/dL higher values in the 75th and 95th percentile (table 4.2c). The TC levels of 3,167 boys and 3,108 girls were 174/178 mg/dL in the 75th percentile and 212/214 mg/dL in the 95th percentile. The HELENA girls are in the same range (75th percentile 185 mg/dL and 95th percentile 214 mg/dL), whereas the boys have lower levels of 168 (75th percentile) and 198 (95th percentile) mg/dL. Kwiterovich took data from the Lipid Research Population Studies including 11,219 white subjects [24]. He used the mean, the 5th and the 95th percentile to categorize the population. The lipid profile of the 15-19 years-olds (1,980 males and 2,079 females), based on the selected percentiles (5th and 95th), differed somewhat compared to the current European population. Girls of the HELENA Study had 11 mg/dL higher TC levels in the 95th percentile. The male TG levels showed a decline of 11 mg/dL in the 5th percentile and a decline of 36 mg/dL in the 95th percentile compared with the American counterparts. The lipoprotein measurements of HDL and LDL were based on 300 male and 297 female Americans. The LDL and HDL levels were slightly different between the two study groups. The HDL levels of the male HELENA participants were 8-9 mg/dL higher in both percentiles, whereas female levels were 3-5 mg/dL higher compared to the American levels. The male LDL levels of the European group were 5 mg/dL lower in the 5th percentile, but nearly similar (3 mg/dL higher) in the 95th percentile. The present European girls showed higher LDL values in the 95th percentile of 143 mg/dL *vs.* 137 mg/dL in the American female group. Kwiterovich recommended the same lipid and lipoprotein cut-offs for adolescents as the NCEP, except for HDL. He defined normal levels of HDL by >45 mg/dL instead of >60 mg/dL as proposed by the NCEP.

There are some differences in the lipid concentrations with respect to the selected studies, which may reflect differences between American and European lifestyle habits and may be further dependent on the date of assessment. But the specific percentiles (75^{th}/ 95^{th} for TC, TG, LDL and 25^{th}/ 5^{th} for HDL) are established in the literature, although they are arbitrary and not connected to any risk factor. However, risk factors are difficult to determine in an apparently healthy adolescent population. Thus, those percentiles seem also to be appropriate to select borderline and high/ low lipid and lipoprotein concentrations within the reference profile of the present European adolescents.

Conclusion

The current cross-sectional data of an adolescent population of Europe brought some reliable insights into the relationship of lipids and gender, age, maturity, BMI, and BF. Lipid parameters are higher in female than in male European adolescents, thus gender-specific lipid research is more appropriate in adolescence. The use of chronological age in adolescence can lead to falsified assumptions with regard to growth and development. To avoid this bias, the use of biological age is recommended. Higher adipose tissue content, expressed as BMI or BF, leads to a more unfavourable lipid pattern. In general, the data suggests a stronger effect of sexual maturity, BMI, and BF on adolescents than age or gender alone. The results may be used as normal values of European adolescents.

Table 4.1: Basic characteristics of the study population as mean (standard deviation).

	Males	**Females**	***p* value***
n	513	584	
Age (years)	14.92 (1.25)	14.91 (1.20)	ns
Height (cm)	169.9 (9.9)	161.8 (7.0)	0.000
Weight (kg)	62.1 (13.9)	56.0 (10.2)	0.000
Body mass index (kg/m²)	21.4 (3.8)	21.3 (3.4)	ns
Tanner stage[1]	3.9 (1.0)	4.0 (0.8)	0.006[#]
Body fat (%)[2]	20.9 (10.5)	27.4 (7.6)	0.000
Triglycerides (mg/dL)[3]	65 (32)	73 (37)	0.000
Total cholesterol (mg/dL)[3]	154 (26)	167 (28)	0.000
nonHDLC (mg/dL)[3]	101 (25)	110 (26)	0.000
HDL (mg/dL)[3]	53 (10)	57 (11)	0.000
LDL (mg/dL)[3]	91 (24)	98 (25)	0.000
Lipoprotein(a) (mg/L)[4]	175 (215)	207 (256)	0.027
Apolipoprotein A-1 (g/L)[4]	1.45 (0.21)	1.55 (0.23)	0.000
Apolipoprotein B (g/L)[4]	0.63 (0.19)	0.68 (0.16)	0.000

**p* values (2-tailed) are based on independent *t*-test; [#] based on *Chi square* test.
[1]boys n=448, and girls n=524; [2]boys n=495, and girls n=560;
[3]boys n=512, and girls n=572; [4]boys n= 500, and girls n=556.
HDL:High density lipoprotein cholesterol, LDL:Low density lipoprotein cholesterol, nonHDLC: Non high density lipoprotein cholesterol, ns:non significant.

Table 4.2a: Percentiles (2.5^{th}-97.5^{th}) of lipids and lipoproteins of all European participants aged 12.5-17.49 years (n=774).

Percentile	Triglycerides (mg/dL)	Total cholesterol (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	Lipoprotein(a)* (mg/L)	Apolipoprotein A-1* (g/L)	Apolipoprotein B* (g/L)
2.5^{th}	28	110	36	50	24	1.10	0.40
5^{th}	30	118	39	58	24	1.20	0.40
10^{th}	35	128	43	65	24	1.20	0.50
20^{th}	42	138	47	75	26	1.30	0.50
25^{th}	45	142	49	78	35	1.40	0.60
30^{th}	47	145	50	81	50	1.40	0.60
40^{th}	54	155	52	87	69	1.40	0.60
50^{th}	59	161	55	93	102	1.50	0.60
60^{th}	65	167	58	98	144	1.50	0.70
70^{th}	74	174	61	105	213	1.60	0.70
75^{th}	79	178	62	110	255	1.60	0.70
80^{th}	86	183	64	114	311	1.70	0.80
90^{th}	105	197	70	127	474	1.80	0.90
95^{th}	124	209	74	139	698	1.90	0.90
97.5^{th}	159	223	79	149	876	2.00	1.00

HDL: High density lipoprotein cholesterol, LDL:Low density lipoprotein cholesterol, *n=752

Table 4.2b: Percentiles (2.5^{th}-97.5^{th}) of lipids and lipoproteins of male European adolescents aged 12.5-17.49 years (n=369).

Percentile	Triglycerides (mg/dL)	Total cholesterol (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	Lipoprotein(a)* (mg/L)	Apolipoprotein A-1* (g/L)	Apolipoprotein B* (g/L)
2.5^{th}	21	104	36	45	24	1.10	0.30
5^{th}	28	113	39	57	24	1.20	0.40
10^{th}	31	124	42	62	24	1.20	0.40
20^{th}	38	134	46	72	24	1.30	0.50
25^{th}	41	138	47	76	31	1.30	0.50
30^{th}	43	141	49	79	40	1.40	0.59
40^{th}	49	148	51	84	67	1.40	0.60
50^{th}	56	154	53	89	97	1.50	0.60
60^{th}	62	159	55	94	130	1.50	0.60
70^{th}	69	164	58	99	197	1.60	0.70
75^{th}	74	168	60	103	244	1.60	0.70
80^{th}	79	173	62	108	292	1.60	0.70
90^{th}	100	185	66	121	435	1.70	0.80
95^{th}	112	198	71	133	634	1.80	0.90
97.5^{th}	145	215	78	142	845	1.90	0.90

HDL: High density lipoprotein cholesterol, LDL:Low density lipoprotein cholesterol, *n=360

Table 4.2c: Percentiles (2.5^{th}-97.5^{th}) of lipids and lipoproteins of female European adolescents aged 12.5-17.49 years (n=405).

Percentile	Triglycerides (mg/dL)	Total cholesterol (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	Lipoprotein(a)* (mg/L)	Apolipoprotein A-1* (g/L)	Apolipoprotein B* (g/L)
2.5^{th}	31	117	36	53	24	1.20	0.40
5^{th}	34	123	40	59	24	1.20	0.40
10^{th}	40	131	45	69	24	1.30	0.50
20^{th}	46	142	49	78	32	1.40	0.50
25^{th}	48	148	51	81	44	1.40	0.60
30^{th}	51	155	52	84	53	1.40	0.60
40^{th}	56	161	55	91	71	1.50	0.60
50^{th}	61	167	57	97	109	1.50	0.70
60^{th}	68	175	60	103	156	1.60	0.70
70^{th}	79	181	62	111	228	1.70	0.70
75^{th}	84	185	64	115	275	1.70	0.80
80^{th}	91	191	66	121	350	1.70	0.80
90^{th}	109	202	72	131	528	1.80	0.90
95^{th}	139	214	77	143	702	1.90	1.00
97.5^{th}	167	231	81	152	918	2.00	1.00

HDL: High density lipoprotein cholesterol, LDL:Low density lipoprotein cholesterol, *n=392

Table 4.3a: Gender-specific lipids and lipoproteins as mean (standard deviation) in relation to age.

Age groups (years)	n	TG+ (mg/dL)	TC (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	NonHDLC (mg/dL)	Lp(a) (mg/L)	Apo A-1 (g/L)	Apo B (g/L)
Males									
12.5-13.99	130/129[a]	61 (34)[1]	159 (26)	56 (10)	93 (24)	103 (24)	193 (250)	1.48 (0.21)	0.64 (0.15)
14.0-14.99	131/126[a]	60 (28)[1]	154 (26)	53 (10)	91 (23)	100 (24)	192 (220)	1.46 (0.22)	0.62 (0.14)
15.0-15.99	131/126[a]	66 (31)	153 (27)	52 (10)	92 (27)	101 (27)	155 (167)	1.44 (0.21)	0.63 (0.16)
16.0-17.49	120/119[a]	73 (33)	149 (24)	50 (9)	87 (22)	98 (24)	159 (211)	1.44 (0.18)	0.62 (0.14)
Total	512/500[a]	65 (32)	154 (26)	53 (10)	91 (24)	101 (25)	175 (215)	1.45 (0.21)	0.63 (0.15)
p-value*		0.013	ns	ns	ns	ns	ns	ns	ns
Females									
12.5-13.99	146/138[a]	72 (41)	166 (27)	57 (11)	98 (24)	109 (25)	215 (262)	1.52 (0.22)	0.67 (0.15)
14.0-14.99	144/139[a]	72 (34)	167 (26)	57 (10)	97 (25)	109 (25)	209 (241)	1.55 (0.19)	0.67 (0.15)
15.0-15.99	146/144[a]	73 (28)	165 (29)	57 (10)	96 (26)	108 (26)	196 (252)	1.55 (0.24)	0.67 (0.16)
16.0-17.49	136/135[a]	77 (44)	170 (29)	57 (12)	100 (26)	113 (27)	211 (270)	1.57 (0.27)	0.71 (0.17)
Total	572/556[a]	73 (37)	167 (28)	57 (11)	98 (25)	110 (26)	207 (256)	1.55 (0.23)	0.68 (0.16)
p-value*		ns	ns	ns	ns	ns	ns	ns	ns

*Based on the ANOVA *F*-Test including centre, body mass index, percentage body fat, and maturity in the model; ns:not significant.
+Based on Bonferroni *post-hoc tests*: [1] $p<0.01$ compared to 16.0-17.49 years.
[a] n for Lp(a), apo A-1, and apo B.
Apo:Apolipoprotein; HDL:High density lipoprotein cholesterol; LDL: Low density lipoprotein cholesterol; Lp(a):Lipoprotein(a); NonHDLC:Non high density lipoprotein cholesterol; TC:Total cholesterol; TG:Triglycerides.

Table 4.3b: Gender-specific lipid and lipoprotein ratios as mean (standard deviation) in relation to age.

Age groups (years)	Apo B/Apo A-1	TC/HDL	TG/HDL[+]	LDL/HDL	Apo B/LDL	nonHDLC/HDL	Atherogenic Index
Males							
12.5-13.99	0.44 (0.12)	2.91 (0.61)	1.18 (0.90)[1]	1.73 (0.58)	0.70 (0.08)	1.91 (0.61)	0.90 (0.55)
14.0-14.99	0.44 (0.14)	2.95 (0.65)	1.20 (0.74)[1]	1.77 (0.63)	0.70 (0.05)	1.95 (0.65)	0.93 (0.80)
15.0-15.99	0.45 (0.15)	3.05 (0.78)	1.37 (0.82)	1.86 (0.76)	0.70 (0.08)	2.05 (0.76)	1.02 (0.86)
16.0-17.49	0.43 (0.11)	3.03 (0.70)	1.52 (0.86)	1.80 (0.63)	0.72 (0.08)	2.03 (0.70)	0.94 (0.58)
Total	0.44 (0.13)	2.99 (0.69)	1.31 (0.84)	1.79 (0.66)	0.70 (0.08)	1.99 (0.69)	0.95 (0.71)
p-value*	ns	ns	0.004	ns	ns	ns	ns
Females							
12.5-13.99	0.45 (0.13)	3.00 (0.63)	1.38 (1.08)	1.80 (0.59)	0.70 (0.08)	2.00 (0.63)	0.96 (0.62)
14.0-14.99	0.44 (0.11)	2.97 (0.62)	1.33 (0.83)	1.75 (0.58)	0.70 (0.08)	1.97 (0.62)	0.92 (0.53)
15.0-15.99	0.44 (0.12)	2.95 (0.55)	1.33 (0.59)	1.74 (0.54)	0.70 (0.08)	1.95 (0.55)	0.91 (0.51)
16.0-17.49	0.47 (0.13)	3.10 (0.71)	1.45 (1.04)	1.86 (0.66)	0.72 (0.08)	2.10 (0.71)	1.06 (0.70)
Total	0.45 (0.12)	3.00 (0.63)	1.37 (0.90)	1.79 (0.59)	0.70 (0.08)	2.00 (0.63)	0.96 (0.60)
p-value*	ns	ns	ns	ns	ns	ns	ns

*Based on the ANOVA *F*-Test including centre, body mass index, percentage body fat, and maturity in the model; ns:not significant.
[+]Based on Bonferroni *post-hoc tests*: [1] $p<0.001$ compared to 16.0-17.49 years.
Apo:Apolipoprotein; HDL:High density lipoprotein cholesterol; LDL: Low density lipoprotein cholesterol; NonHDLC:Non high density lipoprotein cholesterol; TC:Total cholesterol; TG:Triglycerides.

Table 4.4a: Gender-specific lipids and lipoproteins as mean (standard deviation) in relation to maturity.

Tanner stages	n	TG (mg/dL)	TC+ (mg/dL)	HDL+ (mg/dL)	LDL+ (mg/dL)	NonHDLC+ (mg/dL)	Lp(a) (mg/L)	Apo A-1+ (g/L)	Apo B+ (g/L)
Males									
I	7	70 (28)	163 (33)	51 (5)	104 (31)	112 (29)	199 (202)	1.44 (0.19)	0.70 (0.20)
II	34	68 (44)	168 (25)[1,2]	59 (12)[2,3]	97 (24)	109 (25)	142 (131)	1.56 (0.22)[1]	0.64 (0.16)
III	86	64 (30)	156 (25)	55 (11)	91 (22)	101 (22)	166 (197)	1.46 (0.23)	0.62 (0.14)
VI	186	68 (30)	153 (28)	52 (9)	90 (27)	101 (28)	191 (237)	1.43 (0.19)	0.63 (0.16)
V	133	63 (32)	149 (23)	52 (9)	88 (23)	97 (23)	181 (220)	1.46 (0.19)	0.61 (0.14)
Total	446	66 (32)	154 (26)	53 (10)	90 (25)	100 (25)	179 (217)	1.46 (0.20)	0.62 (0.15)
p-value*		ns	0.010	0.034	ns	ns	ns	0.013	ns
Females									
II	21	69 (36)	171 (23)	59 (10)	100 (20)	111 (22)	331 (364)	1.56 (0.21)	0.70 (0.16)
III	104	72 (41)	173 (30)	58 (11)	103 (28)[3]	114 (29)	187 (207)	1.56 (0.24)	0.69 (0.16)
IV	230	75 (39)	165 (28)	58 (11)	94 (24)	107 (25)	182 (232)	1.56 (0.22)	0.66 (0.16)[4]
V	159	75 (34)	167 (27)	55 (11)	100 (25)	112 (25)	230 (287)	1.54 (0.24)	0.70 (0.17)
Total	514	74 (38)	167 (28)	57 (11)	98 (25)	110 (26)	204 (254)	1.55 (0.23)	0.68 (0.16)
p-value*		ns	0.017	ns	0.005	0.005	ns	ns	0.023

*Based on the ANOVA *F*-Test including centre, body mass index, percentage body fat, and age in the model; ns:not significant.
+Based on Bonferroni *post-hoc tests*: [1] $p<0.05$ compared to Tanner stage IV; [2] $p<0.01$ compared to Tanner stage V; [3] $p<0.01$ compared to Tanner stage IV; [4] $p<0.05$ compared to Tanner stage V.
Apo:Apolipoprotein; HDL:High density lipoprotein cholesterol; LDL: Low density lipoprotein cholesterol; Lp(a):Lipoprotein(a); NonHDLC:Non high density lipoprotein cholesterol; TC:Total cholesterol; TG:Triglycerides.

Table 4.4b: Gender-specific lipid and lipoprotein ratios as mean (standard deviation) in relation to maturity.

Tanner stages	Apo B/Apo A-1[+]	TC/HDL[+]	TG/HDL	LDL/HDL[+]	Apo B/LDL[+]	nonHDLC/HDL[+]	Atherogenic Index[+]
Males							
I	0.48 (0.09)	3.16 (0.39)	1.36 (0.44)	2.02 (0.45)	0.67 (0.03)	2.16 (0.39)	1.07 (0.42)
II	0.42 (0.13)	2.94 (0.74)	1.30 (1.21)	1.72 (0.66)	0.67 (0.08)[2]	1.94 (0.74)	0.90 (0.63)
III	0.43 (0.12)	2.93 (0.57)	1.26 (0.81)	1.73 (0.53)	0.67 (0.08)	1.93 (0.57)	0.89 (0.50)
VI	0.45 (0.14)	3.00 (0.76)	1.38 (0.80)	1.79 (0.74)	0.70 (0.08)	2.00 (0.76)	1.00 (0.91)
V	0.42 (0.12)	2.95 (0.64)	1.31 (0.88)	1.77 (0.61)	0.70 (0.08)	1.95 (0.64)	0.89 (0.56)
Total	0.44 (0.13)	2.97 (0.68)	1.33 (0.86)	1.77 (0.65)	0.70 (0.08)	1.97 (0.68)	0.94 (0.72)
p-value*	ns	ns	ns	ns	0.013	ns	ns
Females							
II	0.46 (0.16)	2.95 (0.67)	1.25 (0.96)	1.76 (0.64)	0.70 (0.05)	1.95 (0.67)	0.99 (0.83)
III	0.45 (0.13)	3.02 (0.64)	1.31 (1.01)	1.83 (0.62)	0.67 (0.08)	2.02 (0.64)	0.99 (0.60)
IV	0.43 (0.12)[1]	2.92 (0.61)[1]	1.39 (0.94)	1.69 (0.57)[1]	0.70 (0.08)	1.92 (0.61)[1]	0.89 (0.54)[1]
V	0.47 (0.13)	3.10 (0.65)	1.44 (0.84)	1.88 (0.62)	0.70 (0.05)	2.10 (0.65)	1.05 (0.65)
Total	0.45 (0.13)	3.00 (0.63)	1.38 (0.92)	1.78 (0.60)	0.70 (0.08)	2.00 (0.63)	0.97 (0.61)
p-value*	0.018	0.004	ns	0.005	ns	0.005	0.007

*Based on the ANOVA *F*-Test including centre, body mass index, percentage body fat, and age in the model; ns:not significant.
[+]Based on Bonferroni *post-hoc tests*: [1] $p<0.05$ compared to Tanner stage V; [2] $p<0.01$ compared to Tanner stage IV.
Apo:Apolipoprotein; HDL:High density lipoprotein cholesterol; LDL: Low density lipoprotein cholesterol; NonHDLC:Non high density lipoprotein cholesterol; TC:Total cholesterol; TG:Triglycerides.

Table 4.5a: Gender-specific lipids and lipoproteins as mean (standard deviation) in relation to body mass index and body fat.

Body mass index and body fat	n	TG+ (mg/dL)	TC (mg/dL)	HDL+ (mg/dL)	LDL (mg/dL)	NonHDLC+ (mg/dL)	Lp(a) (mg/L)	Apo A-1 (g/L)	Apo B (g/L)
Males									
BMI									
Underweight	26	64 (33)	152 (23)	55 (8)	87 (21)	97 (23)	159 (196)	1.52 (0.17)	0.61 (0.14)
Normal weight	358/351 [a]	60 (27)	153 (25)	54 (10)	88 (23)	98 (23)	174 (222)	1.47 (0.21)	0.61 (0.14)
Overweight	90/87 [a]	70 (33)	155 (27)	50 (8)	96 (24)	106 (25)	183 (197)	1.40 (0.18)	0.66 (0.15)
Obesity	38/36 [a]	96 (46)	163 (32)	47 (9)	104 (30)	116 (32)	176 (199)	1.37 (0.19)	0.71 (0.18)
Total group	512/500[a]	65 (32)	154 (26)	53 (10)	91 (24)	101 (25)	176 (216)	1.45 (0.21)	0.63 (0.15)
p-value*		ns	ns	ns	ns	ns	ns	ns	ns
Body fat									
Normal	361/357 [a]	61 (27)	152 (24)	54 (10)	88 (22)	98 (22)	174 (226)	1.47 (0.21)	0.61 (0.14)
High	133/125 [a]	74 (40)	158 (29)	50 (9)	97 (25)	107 (27)	175 (181)	1.42 (0.20)	0.67 (0.16)
Total group	494/482 [a]	64 (31)	154 (25)	53 (10)	90 (23)	100 (24)	175 (215)	1.46 (0.21)	0.62 (0.15)
p-value*		0.002	ns	0.045	0.049	0.026	ns	ns	ns
Females									
BMI									
Underweight	33	66 (22)	159 (27)	57 (10)[2]	91 (26)	102 (25)[5]	275 (393)	1.56 (0.17)	0.64 (0.16)
Normal weight	425/414 [a]	70 (33)[1]	167 (28)	58 (11)[3,4]	97 (25)	109 (26)	203 (249)	1.57 (0.22)	0.67 (0.16)
Overweight	92/88 [a]	90 (54)	169 (27)	53 (11)	103 (23)	116 (24)	185 (211)	1.51 (0.25)	0.72 (0.15)
Obesity	22/21 [a]	74 (27)	164 (27)	48 (10)	105 (24)	116 (25)	280 (279)	1.36 (0.26)	0.75 (0.16)
Total group	572/556 [a]	74 (38)	167 (28)	57 (11)	98 (25)	110 (26)	203 (244)	1.55 (0.23)	0.68 (0.16)
p-value*		0.001	ns	0.000	ns	0.024	ns	ns	ns
Body fat									
Normal	460/454 [a]	73 (38)	167 (28)	58 (11)	97 (25)	109 (25)	209 (260)	1.56 (0.22)	0.67 (0.16)
High	88/79 [a]	73 (33)	166 (28)	53 (11)	102 (24)	113 (26)	213 (246)	1.47 (0.28)	0.70 (0.16)
Total group	548/533 [a]	73 (38)	166 (28)	57 (11)	98 (25)	109 (26)	210 (258)	1.55 (0.23)	0.68 (0.16)
p-value*		ns	ns	ns	ns	ns	ns	ns	ns

*Based on the ANOVA *F*-Test including centre, body mass index or percentage body fat, maturity and age in the model; ns:not significant.
+Based on Bonferroni *post-hoc tests*: [1] $p<0.01$ compared to overweight; [2] $p<0.05$ compared to obesity; [3] $p<0.001$ compared to overweight; [4] $p<0.001$ compared to obesity; [5] $p<0.05$ compared to overweight.
[a] n for Lp(a), apo A-1, and apo B.
Apo:Apolipoprotein; BMI:Body mass index; HDL:High density lipoprotein cholesterol; LDL: Low density lipoprotein cholesterol; Lp(a):Lipoprotein(a); NonHDLC:Non high density lipoprotein cholesterol; TC:Total cholesterol; TG:Triglycerides.

Table 4.5b: Gender-specific lipid and lipoprotein ratios as mean (standard deviation) in relation to body mass index and body fat.

Body mass index and body fat	Apo B/Apo A-1[+]	TC/HDL[+]	TG/HDL[+]	LDL/HDL[+]	Apo B/LDL	nonHDLC/HDL[+]	Atherogenic Index[+]
Males							
BMI							
Underweight	0.41 (0.11)	2.81 (0.52)	1.21 (0.65)	1.63 (0.49)	0.72 (0.08)	1.81 (0.52)	0.79 (0.43)
Normal weight	0.42 (0.12)	2.89 (0.65)	1.19 (0.70)	1.70 (0.63)	0.70 (0.08)	1.89 (0.65)	0.87 (0.64)
Overweight	0.48 (0.12)	3.17 (0.56)	1.47 (0.87)	1.96 (0.53)	0.70 (0.08)	2.17 (0.56)	1.10 (0.55)
Obesity	0.53 (0.18)	3.57 (0.97)	2.20 (1.38)	2.31 (0.89)	0.70 (0.08)	2.57 (0.97)	1.49 (1.34)
Total group	0.44 (0.13)	2.99 (0.70)	1.32 (0.85)	1.80 (0.66)	0.70 (0.08)	1.99 (0.70)	0.96 (0.72)
p-value*	ns	ns	ns	ns	ns	ns	ns
Body fat							
Normal	0.42 (0.12)	2.88 (0.61)	1.19 (0.66)	1.69 (0.60)	0.70 (0.08)	1.88 (0.61)	0.86 (0.61)
High	0.48 (0.12)	3.21 (0.73)	1.57 (1.09)	1.99 (0.64)	0.70 (0.08)	2.21 (0.73)	1.11 (0.65)
Total group	0.44 (0.12)	2.97 (0.66)	1.29 (0.82)	1.77 (0.62)	0.70 (0.08)	1.97 (0.66)	0.92 (0.63)
p-value*	0.022	0.003	0.001	0.008	ns	0.003	0.007
Females							
BMI							
Underweight	0.41 (0.11)[1,2]	2.83 (0.58)[2,3]	1.18 (0.44)[1]	1.64 (0.59)[2,3]	0.72 (0.08)	1.83 (0.58)[2,3]	0.81 (0.48)[2,3]
Normal weight	0.44 (0.12)[2,3,]	2.94 (0.59)[2,4]	1.28 (0.77)[4,5]	1.73 (0.56)[2,4]	0.70 (0.08)	1.94 (0.59)[2,4]	0.90 (0.52)[2,4]
Overweight	0.49 (0.12)	3.25 (0.62)	1.80 (1.36)	2.00 (0.58)	0.72 (0.08)	2.25 (0.62)	1.16 (0.66)
Obesity	0.57 (0.18)	3.53 (0.89)	1.64 (0.81)	2.30 (0.83)	0.72 (0.08)	2.53 (0.89)	1.59 (1.15)
Total group	0.45 (0.13)	3.01 (0.63)	1.38 (0.92)	1.80 (0.59)	0.70 (0.08)	2.01 (0.63)	0.97 (0.60)
p-value*	0.007	0.000	0.000	0.002	ns	0.000	0.001
Body fat							
Normal	0.44 (0.12)	2.95 (0.58)	1.35 (0.92)	1.74 (0.55)	0.70 (0.08)	1.95 (0.58)	0.92 (0.52)
High	0.49 (0.15)	3.23 (0.78)	1.48 (0.88)	2.01 (0.72)	0.70 (0.05)	2.23 (0.78)	1.18 (0.88)
Total group	0.45 (0.12)	3.00 (0.63)	1.37 (0.91)	1.78 (0.59)	0.70 (0.08)	2.00 (0.63)	0.96 (0.60)
p-value*	ns	ns	ns	ns	ns	ns	ns

*Based on the ANOVA *F*-Test including centre, body mass index or percentage body fat, maturity and age in the model; ns:not significant.
[+]Based on Bonferroni *post-hoc tests*: [1] $p<0.05$ compared to overweight; [2] $p<0.01$ compared to obesity, [3] $p<0.01$ compared to overweight; [4] $p<0.001$ compared to overweight; [5] $p<0.05$ compared to obesity.
Apo:Apolipoprotein; BMI:Body mass index; HDL:High density lipoprotein cholesterol; LDL: Low density lipoprotein cholesterol; NonHDLC:Non high density lipoprotein cholesterol; TC:Total cholesterol; TG:Triglycerides.

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5 Fatty acid status of the serum phospholipid fraction in European adolescents – associations with age, gender, maturity, body mass index, percentage body fat, and fat free mass

Short Summary

Background: Fatty acids are involved in many important tasks in physiology including modifications of cell membrane fluidity, gene expression, and eicosanoid synthesis, which affects inflammatory and immunological processes. Current research is focusing on FA patterns of diseased children/ adolescents, which likely show altered FA profiles. Less is known about the association of factors like age, gender, maturity, BMI, BF, and FFM with the FA composition of serum PL in healthy adolescents. These factors may influence the FA composition and consequently growth and development. Further, available and adequate reference data for the adolescent age group are an essential condition for the diagnosis of an altered FA status and the monitoring of a therapeutic intervention, but limited until now.

Objective: To investigate the association of age, gender, maturity, BMI, BF, and FFM with the serum FA composition of PL in a large European adolescent study population.

Results: The study population consisted of 1,026 adolescents aged 12.5-17.49 years. The FA profile (*conc%*) indicated differences for C16:1ω7c, C17:1ω7c, C18:1ω9c, C20:0, C20:3ω6c, C21:0, C23:0 C22:6ω3c, C24:1ω9c, ω3 FA, and ω9 FA between girls and boys. Changes in FA were associated with sexual maturation (C17:1ω7c, C18:3ω3c, C20:1ω9c, C20:2ω6c, C20:5ω3c, C22:1ω9c, and ω9 FA), age (C14:1ω5c, C18:0, C18:3ω6c, C20:2 ω6c, C23:0, and SFA), FFM (C16:0, C17:0, C18:2ω6c, C20:2ω6c, C20:4ω6c, C23:0, SFA, PUFA, and ω6 FA), and BF content (C24:1ω9c and *trans* FA) in boys. In girls, FA changes were related to sexual maturation (C17:1ω7c, C20:0, C21:0, C22:1ω9c, and C24:1ω9c), age (C16:0, C18:0, PUFA, *trans* FA, and ω3 FA), BMI (C18:2ω6c, C18:1ω9c, C21:0, and *trans* FA), and FFM (C18:0). The absolute FA composition (µmol/L) showed an altered pattern. Nearly all individual FA concentrations except for C15:1ω5c, C17:1ω7c, C18:1ω9c, C18:3ω6c, C20:3ω6c, and C20:5ω3c were higher in girls than in boys.

Conclusion: The presented FA profile may be used as a European standard. Further effort in the establishment and use of absolute FA values must be made. Gender differences should be considered and age, maturity, BMI, BF and FFM do affect serum FA profiles.

Introduction

Serum FA reflect the dietary FA intake as well as endogenous FA metabolism. Fatty acids are incorporated into PL and perform a variety of different tasks including the modification of cell membrane fluidity, and thus influencing pinocytosis, ion channel modulation, and membrane-associated receptor activities. Fatty acids can regulate gene expression as a result of the interaction with transcription factors as specific and non-specific ligands [1]. Moreover, FA modulate inflammatory and immunological processes due to the provision of precursors for eicosanoids (i.e. thromboxanes, leukotrienes, prostaglandins, and prostacyclins) in an ω3 and ω6 FA-dependent manner [2].

The metabolic situation of the last few days is reflected best by the FA pattern of serum PL [3, 4] because of the slower metabolic turnover of PL compared to other blood lipid classes (e.g. cholesteryl esters, TG or free FA) [5]. Thus, serum FA of the PL fraction are suitable in documenting the short-term FA status in humans [6].

Westernized eating habits do not meet the current fat recommendations and thus lead to an inadequate dietary fat supply. This fat supply is characterized by higher consumptions of SFA, *trans* FA, a decreased intake of α-linolenic acid (C18:3ω3; ALA), and an increased dietary intake of linoleic acid (C18:2ω6; LA) [7, 8]. This dietary fat supply reflects an unfavourable FA composition and will also be present in the serum FA profile assuming disadvantages for a normal growth and development in adolescence. This unfavourable FA composition may also contribute to the development of several diseases affecting today's civilizations. This implies atherosclerosis and CHD [2, 9-20] in adults as well as obesity [21-23], diabetes [24, 25], and the metabolic syndrome [26] in youth.

However, evaluating the risk or risk factors of a healthy adolescent population with respect to the FA profile is quite difficult. In a former step, the evaluation of factors that are physiologically more important in adolescence should be implied. This includes gender, age, sexual maturation, BMI, BF, and FFM. The assessment would clarify, which factors influence the FA profile in apparently healthy adolescents; however, this approach is still not sufficiently investigated. Further, Decsi and Koletzko [27] mentioned the importance of having available and adequate reference data from a healthy population for the adolescent age group as essential condition for the diagnosis of an altered FA status and the monitoring of a therapeutic intervention. But their data is based on a quite large age group (0-26 years) and a relative small sample size (n=115).

Generally, FA compositions are widely expressed as relative percentages but it may be less accurate than absolute concentrations. For instance, the relative percentage of total FA with high levels of non-essential FA may lead to a falsely decreased percentage of essential FA. Thus, the essential FA profile could be described inaccurately and important information may be unrecognized [28, 29].

Therefore, in the frame of this project the FA profiles were associated with age, gender, sexual maturity, BMI, BF, and FFM. Further, the HELENA Study assessed both the FA profile expressed as absolute concentrations and as percentages of the FA concentrations.

Subjects and Methods

A detailed description of study design, subject recruitment, medical examination, blood sampling, sample treatment, biochemical analysis, and evaluation procedures can be found in *chapter 3* (*General Methodology*).

Statistics

Numeric values are shown as mean±SD. Percentiles (5^{th}-95^{th}) were calculated to obtain reference standards of healthy European adolescents excluding smokers, underweight and obese subjects. Differences in the main study characteristics of boys and girls were analysed using independent *t*-tests (metric variables) and c*hi square* test (categorical variables). Comparison between individual FA in *area%* and *conc%* were made using paired *t*-tests. Blood analytes (dependent variables) were skewed and logarithmically transformated before statistical analysis. All statistics were performed separately for boys and girls. Age, sexual maturation, FFM, BMI and BF were determined as independent variables and grouped. Further, insulin and leptin were used as covariates in the following model. For each of the dependent variables (FA), a univariate analysis of covariance (ANCOVA) was used to analyse the association with the independent variables. The Bonferroni *post-hoc tests* were used for sub-group analyses. Data analysis was performed by using Statistical Package for Social Sciences version 17.0 for Windows (SPSS Inc., Chicago, Illinois, USA) and *p* values <0.05 were considered as statistically significant.

To test for the current power of the results, a post hoc analysis for achieved power was conducted exemplarily with logarithmically transformated data of selected FA concentrations (i.e. LA, arachidonic acid [AA], oleic acid, ALA, eicosapentaenoic acid [EPA],

docosahexaenoic acid [DHA]) for independent *t*-tests with a two-tailed significance of p=0.05 using GPower® version 3.0.10 [30]. The achieved power was >98% for LA, AA, and DHA, whereas ALA reached 84%, oleic acid 21%, and EPA 12%.

Results

The HELENA-CSS consisted of 1,097 adolescent subjects including blood measurements. Due to insufficient blood volume for complete analysis, FA data of 73 subjects were missing. Thus, 1,024 subjects contributed to the following results. Table 5.1 presents the basic characteristics of the study population, whereas tables 5.2a-c show the percentiles (5^{th}-95^{th}) of the total population as well as separately for boys and girls. To present percentiles of an apparently healthy population, tables 5.2a-c did not include smokers, underweight and obese participants. Thus, data of 733 adolescents (349 boys and 384 girls) were included in those tables.

The absolute FA values measured in PL are shown in table 5.3. Table 5.4 presents the FA profile in *area%* and *conc%* of male and female subjects. After comparing both percentaged FA profiles, significant differences were found (all p=0.000, paired *t*-test; data not shown). Thus, for the detailed analyses only the FA in *conc%* were used. This guaranteed more reliable results due to the more precise calculation of the FA profile as well as comparability with other studies.

Results of the fatty acid profile expressed as percentage of the concentration (conc%)

Based on the present FA profile including 33 measured FA, approximately 95% of the FA profile was determined by ten FA exceeding ≥1conc% of the total FA, i.e. C16:0, C18:0, C18:1ω9c, LA, C20:3ω6c, AA, C22:0, C24:0, DHA and C24:1ω9c. α-linolenic acid and EPA were below 1% in the present profile. The *trans* FA accounted for 0.1*conc%* for both boys and girls. Table 5.4 highlights the complete FA profile according to gender.

Girls had higher levels of C20:1ω9c, DHA, and ω3 FA, whereas lower levels of ω9 FA including C18:1ω9c were found in female profiles compared to male profiles (table 5.4). No differences were observed between male and female levels for LA, ALA, EPA, and ω6 FA.

Table 5.5 presents the FA composition of PL according to age. In boys, single FA accounting for <1.0conc% of total FA, i.e. C14:1ω5c, C18:3ω6c, C20:2ω6c, and C23:0 were associated

with age. Further, C18:0 and SFA differed within the four age categories. In girls, age was associated with C16:0, C18:0, PUFA, ω3 FA and *trans* FA (table 5.5).

Sexual maturation was associated with FA in both sexes as can be seen in table 5.6. Single FA accounting for <1.0*conc%* were more often associated with maturation than FA accounting for >1.0*conc%*. In boys, C17:1ω7c, ALA, C20:1ω9c, C20:2ω6c, EPA, and C22:1ω9c showed significant differences. Further, ω9 FA were significantly lower in Tanner stage I compared to Tanner stages III, IV, and V. In girls, pubertal progression was associated with altered levels of C17:1ω7c, C20:0, C21:0, C22:1ω9c, and C24:1ω9c (table 5.6).

The parameters of body composition (i.e. BMI, BF and FFM) were also associated with different FA levels in male and female European adolescents. In boys, C14:0 (p<0.019, *F*-test) and C18:0 (p<0.030, *F*-test) differed between the BMI groups. More precisely, underweight boys showed lower C14:0 values than their normal weight and overweight counterparts (p<0.05, *post hoc test*), and C18:0 decreased from underweight to normal weight (p=0.020, *post hoc test*). In girls, BMI was associated with C18:1ω9c, LA, AA, DHA, and long-chain PUFA (LCPUFA) (all p<0.05, *F*-tests). Oleic acid was lower in obese girls compared to their normal weight and overweight counterparts (p<0.011 and p<0.013, *post hoc tests*). Further, normal weight girls had higher values of LA compared to overweight and obese females (p<0.01 and p<0.05, *post hoc tests*). Moreover, AA increased significantly between normal weight and overweight girls (p=0.002, *post hoc test)*. The LCPUFA status (i.e. C20:3ω6c, AA, EPA, and DHA) increased with higher BMI, thus, overweight girls had higher levels than underweight and normal weight subjects (p<0.05 and p<0.01, *post hoc tests*).

Some changes in the male FA profile were observed with respect to percentage BF content. More detailed, C18:1ω9c, C22:0, and *trans* FA were higher in boys with a BF content of <25% (p=0.007, p=0.036, and p=0.011, *F*-tests), whereas DHA and C24:1ω9c were lower in male subjects of the same sub-group (p=0.028 and p=0.010, *F*-tests). In girls, no relationship between BF and individual FA was found.

In boys higher levels of EPA were associated with higher FFM quartiles (p=0.003, *F*-test). The 1st and 2nd quartile showed lower levels compared to the 3rd and 4th quartile (p<0.05, *post hoc tests*). By contrast, girls had higher levels of C22:1ω9c in the 4th quartile compared to the 1st quartile (p=0.044, *post hoc test*).

Results of the fatty acid profile expressed as concentrations (µmol/L)

Table 5.3 highlights the absolute FA composition according to gender. Compared to boys, all FA were higher in girls except for C15:1ω5c, C17:1ω7c, C18:1ω9c, C18:3ω6c, C20:3ω6c, and EPA.

In boys, changed FA levels due to age were seen in C14:1ω5c and C20:1ω9c. Both levels increased significantly from 14.0-14.99 years to 16.0-17.49 years ($p<0.02$, *post hoc tests*). Palmitic acid, C17:1ω7c, C20:1ω9c, C22:1ω9c, C22:2ω6c, C24:0, C24:1ω9c, MUFA, and ω9 FA increased from 12.5-13.99 years to 16.0-17.49 years significantly in female subjects ($p<0.05$, *post hoc tests*). Further, *trans* FA increased from 14.0-14.99 years to 16.0-17.49 years as well as C24:1ω9c and ω3 FA between the age groups 12.5-13.99 and 14.0-14.99.

Changes in the measured FA profile were also associated with sexual maturation. In boys, ALA and C20:1ω9c decreased between Tanner stage IV and V ($p=0.008$ and $p=0.025$, *post hoc tests*). Furthermore, EPA showed lower levels in Tanner stage III, IV, and V compared to Tanner stage II ($p=0.047$, $p=0.032$, and $p=0.010$, *post hoc tests*). By contrast, C20:0, C22:0, C22:1ω9c, C23:0, and C24:1ω9c decreased between Tanner stage III and IV in female participants ($p<0.05$, *post hoc tests*).

No association between the FA profile of boys and the BMI status could be found, whereas in girls some differences were observed. Normal weight girls showed a higher pattern of LA and *trans* FA compared to their overweight counterparts ($p<0.025$, *post hoc tests*). Additionally, LA and C18:1ω9c were lower in obese girls compared to girls with a BMI in the normal range ($p<0.023$, *post hoc tests*). Underweight girls had lower C21:0 levels than normal weight girls ($p=0.048$, *post hoc test*).

The FA composition of girls was not influenced by BF content, whereas *trans* FA were higher in boys with adequate BF ($p=0.022$, *F*-test), and C24:1ω9c was lower in the <25% BF group ($p=0.024$, *F*-test).

A relationship between FA and FFM was present in male and female FA profiles. In girls, C18:0 showed a significant decline between the 1st and the 4th quartile ($p=0.013$, *post hoc test*). In boys, C16:0, C17:0, LA, C20:2ω6c, AA, SFA, PUFA, and ω6 FA showed a decrease between the 2nd and 3rd FFM quartile ($p<0.022$, *post hoc tests*). Furthermore, C23:0 were lower in the 3rd quartile compared to the 1st quartile ($p=0.027$, *post hoc test*).

Discussion

The present study found some differences in the percentaged FA composition between boys and girls as shown in table 5.4. However, the essential FA LA and ALA as well as EPA and AA did not differ between sexes. Solely DHA was higher in female than in male adolescents. This finding may be due to a higher dietary intake of DHA in girls. Docosahexaenoic acid is found in fatty fish and seafood, and especially long-chain ω3 FA have been proposed as dietary intake markers of such food groups [31-33]. However, more reasonable are gender-specific differences in sex hormone concentrations, which may affect enzyme activities and have led to the present findings [34]. Other studies indicated no sex differences in their study populations [22, 23, 27, 35]. However, the present study population consists of a larger sample size and is more heterogeneous than the other study groups. Thus, it is likely that gender differences may only be detected in larger sample sizes or biased by the gender-specific food and FA intake of the participating countries [31]. Variations in the genetic background may also influence enzyme activities, which modify the present FA profile, and thus change some FA levels, especially of LCPUFA [36].

In most studies, differences in the FA profile due to age were not presented [21-24, 26, 35, 37, 38]. Nonetheless, in one Hungarian adolescent study group (n=115) age classifications were made, but there were no dissimilarities in the FA composition of PL between the age groups 5-10 years (n=18, 7.2±1.3 years), 10-15 years (n=25, 12.9±1.6 years), and 20-26 years (n=10, 23±2.8 years). Significant changes were only observed when comparing 20-26 year-olds with much younger age groups (i.e. 0-1 month, or 1-12 months), for example in C18:1ω9c, AA, DHA, and MUFA [27]. The present study found differences between the age groups for C16:0, C18:0, PUFA, ω3 FA, and *trans* FA in the female subjects, and in C14:1ω5c, C18:0, C18:3ω6c, C20:2ω6c, C23:0, and SFA in boys (table 5.5), probably as a result of altered dietary FA patterns.

Relationships between maturity and the present FA composition were observed. In girls, FA accounting for <1.0*conc%* like C17:1ω7c, C20:0, C22:0, C23:0 and C24:1ω9c differed during puberty. In boys, C17:1ω7c, ALA, C20:1ω9c, C20:2ω6c, EPA, C22:1ω9c, and ω9 FA were associated with sexual maturation (see table 5.6). These observations may be a consequence of altered dietary FA supply and/ or increasing sex hormone concentrations during puberty. Both possibilities could not be verified, because it was not feasible to measure sex hormones in the study population, and dietary FA evaluation did not contain such a broad spectrum of FA, as it is available in blood analytics to guarantee a reliable interpretation of the results.

However, none of the mentioned studies investigated the FA profile with respect to maturity; thus, no comparisons can be made.

Body composition is also linked to FA composition. In this study, changes in the FA profile with respect to BMI were more apparent in girls than in boys. On the other hand, BF was only related to FA profiles of male subjects. The majority of studies investigated the FA composition related to obesity and reported no sex differences within the individual FA, thus they analysed boys and girls together [23, 26, 35]. In HELENA, boys and girls were analysed separately making direct comparisons difficult. In the present study, underweight and/ or normal weight girls had higher levels of C18:1ω9c and LA as well as lower levels of AA and LCPUFA (i.e. C20:3ω6c, AA, EPA, and DHA) than their overweight and/ or obese counterparts. Another study found higher amounts of C18:1ω9c/ 7c and lower levels of AA and ω6 LCPUFA in obese subjects (n=22, body weight relative to normal weight for height=170±24%) compared to age-matched controls (n=25, mean age 13.7±1.4 years) of both sexes [23]. Others studies were not in accordance with the present findings [22, 26, 35]. However, in the HELENA Study higher levels of C18:1ω9c, *trans* FA and lower levels of DHA and C24:1ω9c were associated with boys having a BF content of <25%. This parameter was not investigated in other studies, thus comparisons cannot be made [22, 23, 26, 35].

As already mentioned in *chapter 4*, associations between body composition and lipid values are linked to gender. Changes in the FA profiles of girls are mainly associated with BMI status, while changes in the male FA profile are more present as a result of BF content categorization. The differences may be obtained due to the assessment methods. Fat allocation is different between sexes and this may be reflected in the different skinfold measurements. It may also be possible that the sub-group classification (two BF sub-groups *vs*. four BMI sub-groups) with different group sizes results in altered findings.

The current serum FA composition of European adolescents (12.5-17.49 years) is comparable with the FA profile of the Hungarian study group of Decsi and Koletzko [27]. Slight deviations can be explained by the fact that the HELENA FA profile consists of more measured FA (e.g. C22:1ω9c, C22:2ω6c, C23:0, C24:0, and C24:1ω9c), thus the percentaged proportion shifted. The HELENA Study results may be a more reliable approach for presenting physiological reference data of European adolescents because of the detailed FA spectrum, the larger study group, and the narrow age range. Thus, the current FA profile, presented as percentiles in the tables 5.2a-c, may be used as an appropriate reference standard for healthy European adolescents.

As already mentioned in the introduction of this chapter, the absolute FA profile could give additional, important information. However, the use of the absolute FA profile is very limited in current research. The relative FA composition is very well established because measurements of absolute amounts are more sophisticated and more expensive. Nevertheless, it may be important to analyse the absolute FA profile to avoid falsified results because of percentaged weighting. For instance, the relative percentage of total FA is dependent of the total number of measured FA as well as of their heights. Shifted proportions of non-essential FA may therefore easily alter the proportion of essential FA. As a consequence of the shifted proportion, the information value may be weaker or even incorrect [28, 29]. In the HELENA Study, nearly all absolute values of the FA, except for C15:1ω5c, C17:1ω7c, C18:1ω9c, C18:3ω6c, C20:3ω6c, and EPA, were higher in girls than in boys (table 5.3). This finding may be explained by the higher levels of transport lipoproteins in girls (table 5.1). The higher amounts of the essential FA ALA and LA in girls indicate different food choices (e.g. fish and seafood, walnuts, and green leafy vegetables) compared to boys. Further, the higher amount of DHA may be a result of higher elongase/ desaturase activities, probably influenced by the oestrogen status [39]. Moreover, the results between the absolute FA profile and the relative FA profile differed with respect to the association with age, maturity, BMI, BF, and FFM as pointed out in the results above. Therefore, it is not clear, which results are more reliable and/ or more usable as an approach in FA research. However, this cannot be decided within this thesis.

Lagerstedt *et al.* [29] presented a detailed FA profile of 37 subjects (boys and girls, aged 1-17 years). Their FA reference ranges are in accordance with the ranges of C16:0, C18:0, EPA, AA, C20:3ω6c, C20:0, DHA, C22:0, C24:1ω9c, C24:0, SFA, and ω3 FA found in the HELENA Study. The remaining FA (C12:0, C14:0, C16:1ω7c, C18:3ω6c, ALA, LA, C18:1ω9c, C22:1ω9c, MUFA, PUFA, and ω6 FA) were lower in the HELENA study population. These different findings may be explained by the smaller sample size and the larger age range of the other study as well as by differences in the methodological approach [29].

With respect to BMI, Larqué *et al*. [40] found higher *trans* FA in obese subjects (n=34, 9.41±0.36 years) compared to controls (n=20, 9.75±0.37 years). In the HELENA Study *trans* FA were higher in boys with normal BF content as well as lower in overweight girls than in their normal weight counterparts. The different study results indicate different food choices, because *trans* FA are solely found in industrially processed foods or milk and dairy products. This suggests a higher dietary intake of those food groups in the Spanish obese population

investigated by Larqué *et al.* compared to the European study group. The complete FA profile of the same Spanish study population were used by Gil-Campos *et al.* [41]. They found no significant differences in the FA profile of PL between obese and normal weight prepubertal children. We found an association between LA, *trans* FA, C18:1ω9c, and C21:0 and BMI only in girls. Oleic acid and LA were lower in obese and overweight subjects compared to girls with normal BMI. This effect was not apparent in the study population of Gil-Campos *et al.*, probably as a result of the mixed group design or the lower number of subjects (n=54).

A few limitations of the present study deserve comment. Because of the CSS design, the temporal relationships are unknown; one cannot say anything about causality of the relationships. Furthermore, dietary FA intake was not taken into account. Dietary FA data based on questionnaires or interviews are mostly biased by underreporting and become therefore unreliable [42-44]. Additionally, available food composition tables and food data bases lack of sufficient information especially for individual FA of several food groups, indicating that FA measured in serum are more reliable parameters for the assessment of the FA status.

Conclusion

The results indicate gender differences, which are trustable because of the large sample size, and should be taken into account in future studies. It has to be emphasized that the methodology of FA measurement and analysis differ between the research groups and need harmonization. Age, maturity, BMI, BF, and FFM are associated with FA and should be taken into account when interpreting the FA profile of adolescents. Further effort in the establishment and use of absolute FA values must be undertaken, because differences between absolute and percentaged FA profiles do exist.

Table 5.1: Basic characteristics of the study population as mean (standard deviation).

	Boys	**Girls**	***p* value***
n	475	551	
Age (years)	14.75 (1.19)	14.78 (1.15)	ns
Height (cm)	169.8 (9.8)	161.8 (7.0)	0.000
Weight (kg)	61.7 (13.7)	56.0 (10.2)	0.000
Body mass index (kg/m²)	21.3 (3.8)	21.3 (3.4)	ns
Tanner stage[1]	3.9 (1.0)	4.0 (0.8)	0.007[#]
Body fat (%)[2]	21.0 (10.5)	27.3 (7.6)	0.000
Fat free mass (kg)[3]	51.8 (9.6)	41.8 (7.0)	0.000
Insulin (mLU/L)[4]	9.9 (8.0)	10.7 (11.2)	ns
Leptin (ng/mL)[5]	9.30 (14.06)	28.29 (24.20)	0.000
Triglycerides (mg/dL)[6]	64 (31)	73 (37)	0.000
Total cholesterol (mg/dL)[6]	154 (26)	167 (28)	0.000
HDL (mg/dL)[6]	53 (10)	57 (11)	0.000
LDL (mg/dL)[6]	91 (25)	98 (25)	0.000
Apolipoprotein A-1 (g/L)[7]	1.45 (0.21)	1.55 (0.23)	0.000
Apolipoprotein B (g/L)[7]	0.62 (0.15)	0.68 (0.16)	0.000
Lipoprotein(a) (mg/L)[7]	176 (218)	209 (257)	0.021

**p* values (2-tailed) are based on an independent *t*-test; [#] based on *Chi square* test
[1]boys n=418, and girls n=495; [2]boys n=461, and girls n=529;
[3]boys n=469, and girls n=541; [4]boys n=472, and girls n=543;
[5]boys n= 436, and girls n=525; [6]boys n=475 and girls n=548;
[7]boys n= 464 and girls n=532.
HDL:High density lipoprotein cholesterol, LDL:Low density lipoprotein cholesterol.

Table 5.2a: Percentiles[#] of the total study population (n=733) excluding smokers, obese and underweight adolescents.

Percentile	**5th**	**25th**	**50th**	**75th**	**95th**
Fatty acid			*conc%*		
Saturates					
C12:0	0.01	0.03	0.04	0.05	0.11
C14:0	0.28	0.37	0.46	0.55	0.72
C16:0	29.42	31.00	32.01	32.89	34.56
C17:0	0.35	0.41	0.45	0.50	0.57
C18:0	12.19	13.12	13.78	14.46	15.46
C20:0	0.42	0.50	0.56	0.62	0.73
C21:0	0.00	0.00	0.03	0.04	0.06
C22:0	0.99	1.19	1.31	1.46	1.72
C23:0	0.36	0.46	0.53	0.61	0.73
C24:0	0.78	0.94	1.04	1.17	1.38
∑ SFA	47.93	49.32	50.27	51.08	52.49
Monounsaturates and ω9 Family					
C14:1ω5c	0.01	0.02	0.10	0.17	0.27
C15:1ω5c	0.02	0.04	0.05	0.06	0.08
C16:1ω7c	0.35	0.48	0.58	0.71	0.93
C17:1ω7c	0.05	0.08	0.10	0.11	0.16
C18:1ω9c	5.31	6.22	6.95	7.73	8.91
C20:1ω9c	0.10	0.12	0.14	0.16	0.20
C22:1ω9c	0.00	0.00	0.04	0.05	0.08
C24:1ω9c	1.38	1.68	1.93	2.17	2.65
∑ MUFA	7.82	9.01	9.88	10.83	12.20
∑ ω9 FA	7.09	8.24	9.06	9.90	11.13

continued

Table 5.2a continued

Percentile	**5th**	**25th**	**50th**	**75th**	**95th**
Fatty acid			*conc%*		
Polyunsaturates					
∑ PUFA	36.96	38.65	39.75	40.97	42.86
∑ LCPUFA	12.58	14.40	15.80	17.25	19.19
ω6 Family					
C18:2ω6c	18.99	21.44	22.98	24.78	27.13
C18:2ω6t	0.03	0.04	0.05	0.07	0.09
C18:3ω6c	0.06	0.07	0.08	0.10	0.17
C20:2ω6c	0.21	0.26	0.29	0.33	0.39
C20:3ω6c	1.97	2.50	2.91	3.38	4.13
C20:4ω6c	7.07	8.49	9.52	10.67	12.25
C22:2ω6c	0.24	0.28	0.31	0.35	0.42
∑ ω6 FA	32.66	34.93	36.29	37.76	40.05
ω3 Family					
C18:3ω3c	0.06	0.09	0.13	0.18	0.30
C20:5ω3c	0.15	0.30	0.42	0.60	1.08
C22:6ω3c	1.70	2.17	2.66	3.31	4.54
∑ ω3 FA	2.05	2.64	3.24	4.02	5.54
∑ *trans* FA	0.03	0.04	0.05	0.07	0.09

[#] rounded values

FA:Fatty acids; LCPUFA:Long-chain PUFA; MUFA:Monounsaturated FA; PUFA: Polyunsaturated FA; SFA:Saturated FA

Table 5.2b: Percentiles[#] of the male study population (n=349) excluding smokers, obese and underweight adolescents.

Percentile	**5th**	**25th**	**50th**	**75th**	**95th**
Fatty acid			*conc%*		
Saturates					
C12:0	0.02	0.03	0.04	0.05	0.11
C14:0	0.27	0.36	0.45	0.54	0.77
C16:0	29.37	31.04	32.14	33.00	34.47
C17:0	0.35	0.41	0.45	0.50	0.57
C18:0	12.27	13.09	13.65	14.33	15.48
C20:0	0.41	0.48	0.54	0.61	0.71
C21:0	0.00	0.00	0.02	0.04	0.06
C22:0	0.97	1.16	1.29	1.45	1.72
C23:0	0.35	0.45	0.52	0.60	0.72
C24:0	0.79	0.94	1.06	2.15	2.65
∑ SFA	47.83	49.22	50.29	51.30	52.64
Monounsaturates and ω9 Family					
C14:1ω5c	0.01	0.02	0.09	0.18	0.28
C15:1ω5c	0.02	0.04	0.05	0.06	0.09
C16:1ω7c	0.34	0.46	0.56	0.69	0.89
C17:1ω7c	0.05	0.08	0.10	0.12	0.17
C18:1ω9c	5.37	6.40	7.03	7.82	9.07
C20:1ω9c	0.10	0.12	0.14	0.16	0.20
C22:1ω9c	0.00	0.00	0.04	0.05	0.07
C24:1ω9c	1.35	1.67	1.90	2.15	2.65
∑ MUFA	7.93	9.12	9.98	10.98	12.30
∑ ω9 FA	7.15	8.39	9.16	9.98	11.25

continued

Table 5.2b continued

Percentile	**5th**	**25th**	**50th**	**75th**	**95th**
Fatty acid			*conc%*		
Polyunsaturates					
∑ PUFA	36.42	38.53	39.73	40.86	42.84
∑ LCPUFA	12.60	14.44	15.87	17.29	19.20
ω6 Family					
C18:2ω6c	18.75	21.43	22.85	24.56	26.86
C18:2ω6t	0.03	0.04	0.05	0.07	0.09
C18:3ω6c	0.06	0.07	0.08	0.10	0.18
C20:2ω6c	0.21	0.26	0.29	0.33	0.39
C20:3ω6c	2.03	2.55	3.01	3.43	4.07
C20:4ω6c	7.32	8.51	9.62	10.73	12.56
C22:2ω6c	0.23	0.28	0.31	0.35	0.41
∑ ω6 FA	32.28	34.91	36.35	37.84	40.03
ω3 Family					
C18:3ω3c	0.06	0.09	0.13	0.18	0.31
C20:5ω3c	0.15	0.30	0.45	0.62	1.05
C22:6ω3c	1.63	2.10	2.55	3.12	4.28
∑ ω3 FA	2.03	2.57	3.15	3.88	5.49
∑ *trans* FA	0.03	0.04	0.05	0.06	0.09

[#] rounded values

FA:Fatty acids; LCPUFA:Long-chain PUFA; MUFA:Monounsaturated FA; PUFA: Polyunsaturated FA; SFA:Saturated FA

Table 5.2c: Percentiles[#] of the female study population (n=384) excluding smokers, obese and underweight adolescents.

Percentile	**5th**	**25th**	**50th**	**75th**	**95th**
Fatty acid			*conc%*		
Saturates					
C12:0	0.02	0.03	0.04	0.05	0.10
C14:0	0.29	0.38	0.46	0.56	0.71
C16:0	29.44	30.92	31.92	32.77	34.67
C17:0	0.35	0.40	0.45	0.50	0.56
C18:0	11.93	13.14	13.87	14.54	15.45
C20:0	0.44	0.51	0.57	0.63	0.75
C21:0	0.00	0.00	0.03	0.04	0.06
C22:0	1.01	1.20	1.33	1.47	1.70
C23:0	0.37	0.47	0.54	0.61	0.74
C24:0	0.75	0.93	1.03	1.15	1.33
∑ SFA	48.05	49.39	50.27	50.98	52.23
Monounsaturates and ω9 Family					
C14:1ω5c	0.01	0.03	0.12	0.17	0.27
C15:1ω5c	0.02	0.04	0.05	0.06	0.08
C16:1ω7c	0.35	0.50	0.60	0.73	0.96
C17:1ω7c	0.05	0.07	0.09	0.11	0.15
C18:1ω9c	5.19	6.08	6.80	7.65	8.76
C20:1ω9c	0.10	0.12	0.14	0.16	0.20
C22:1ω9c	0.00	0.00	0.04	0.05	0.08
C24:1ω9c	1.40	1.68	1.96	2.17	2.65
∑ MUFA	7.77	8.90	8.92	10.73	12.09
∑ ω9 FA	7.02	8.13	8.99	9.83	11.07

continued

Table 5.2c continued

Percentile	**5th**	**25th**	**50th**	**75th**	**95th**
Fatty acid			*conc%*		
Polyunsaturates					
∑ PUFA	37.13	38.76	39.79	41.03	42.92
∑ LCPUFA	12.52	14.34	15.79	17.21	19.27
ω6 Family					
C18:2ω6c	19.07	21.46	23.08	24.92	27.28
C18:2ω6t	0.03	0.05	0.05	0.07	0.09
C18:3ω6c	0.06	0.07	0.08	0.10	0.17
C20:2ω6c	0.21	0.26	0.29	0.33	0.40
C20:3ω6c	1.95	2.45	2.87	3.33	4.20
C20:4ω6c	6.95	8.42	9.41	10.56	12.23
C22:2ω6c	0.24	0.28	0.31	0.35	0.44
∑ ω6 FA	32.76	34.94	36.24	37.62	40.10
ω3 Family					
C18:3ω3c	0.06	0.09	0.13	0.17	0.30
C20:5ω3c	0.14	0.30	0.41	0.57	1.14
C22:6ω3c	1.74	2.28	2.80	3.50	4.66
∑ ω3 FA	2.09	2.72	3.34	4.23	5.61
∑ *trans* FA	0.03	0.05	0.05	0.07	0.09

[#] rounded values

FA:Fatty acids; LCPUFA:Long-chain PUFA; MUFA:Monounsaturated FA; PUFA: Polyunsaturated FA; SFA:Saturated FA

Table 5.3: Gender-specific fatty acid composition of serum phospholipids as mean (SD)[#].

Fatty acid	Total (n=1,024)	Boys (n=475)	Girls (n=549)	*p* value*
		(µmol/L)		
Saturates				
C12:0	2 (2)	2 (2)	2 (2)	0.031
C14:0	18 (8)	17 (7)	19 (8)	0.000
C16:0	1,182 (212)	1,136 (191)	1,222 (221)	0.000
C17:0	17 (4)	16 (4)	17 (4)	0.000
C18:0	508 (99)	491 (99)	523 (97)	0.000
C20:0	21 (5)	19 (5)	22 (5)	0.000
C21:0	1 (1)	1 (1)	1 (1)	0.000
C22:0	49 (12)	47 (12)	51 (11)	0.000
C23:0	20 (5)	18 (5)	21 (5)	0.000
C24:0	39 (10)	38 (10)	39 (10)	0.044
∑ SFA	1,855 (324)	1,785 (309)	1,915 (326)	0.000
Monounsaturates and ω9 Family				
C14:1ω5c	4 (3)	4 (3)	4 (3)	0.006
C15:1ω5c	2 (1)	2 (1)	2 (1)	ns
C16:1ω7c	23 (11)	22 (9)	25 (11)	0.000
C17:1ω7c	4 (2)	4 (2)	4 (2)	ns
C18:1ω9c	259 (76)	255 (73)	263 (78)	ns
C20:1ω9c	5 (2)	5 (2)	6 (2)	0.000
C22:1ω9c	1 (1)	1 (1)	1 (1)	0.008
C24:1ω9c	71 (20)	68 (19)	75 (20)	0.000
∑ MUFA	370 (95)	359 (91)	379 (98)	0.000
∑ ω9 FA	337 (86)	329 (83)	344 (88)	0.001

continued

Table 5.3 continued

Fatty acid	Total (n=1,024)	Boys (n=475)	Girls (n=549)	*p* value*
		(µmol/L)		
Polyunsaturates				
∑ PUFA	1,470 (278)	1,412 (270)	1,520 (275)	0.000
∑ LCPUFA	586 (142)	565 (136)	604 (145)	0.000
ω6 Family				
C18:2ω6c	850 (178)	814 (170)	880 (179)	0.000
C18:2ω6t	2 (1)	2 (1)	2 (1)	0.000
C18:3ω6c	4 (2)	4 (2)	4 (2)	ns
C20:2ω6c	11 (3)	11 (3)	12 (4)	0.001
C20:3ω6c	111 (37)	109 (34)	114 (39)	ns
C20:4ω6c	354 (94)	345 (89)	363 (97)	0.000
C22:2ω6c	12 (4)	11 (3)	12 (4)	0.000
∑ ω6 FA	1,344 (258)	1,295 (249)	1,386 (257)	0.000
ω3 Family				
C18:3ω3c	5 (3)	5 (3)	6 (4)	0.003
C20:5ω3c	18 (13)	18 (13)	19 (14)	ns
C22:6ω3c	102 (40)	94 (38)	110 (40)	0.000
∑ ω3 FA	126 (50)	117 (48)	134 (51)	0.000
∑ *trans* FA	2 (1)	2 (1)	2 (1)	0.000

**P* values are based on independent *t*-test comparing boys and girls, ns:non significant.
FA:Fatty acids; LCPUFA:Long-chain PUFA; MUFA:Monounsaturated FA; PUFA: Polyunsaturated FA; SD:Standard deviation, SFA:Saturated FA
[#] rounded values

Table 5.4: Gender-specific fatty acid composition of serum phospholipids as mean (standard deviation)[#].

Fatty acid	Total (n=1,024)	Boys (n=475)	Girls (n=549)	*p* value*	Total (n=1,024)	Boys (n=475)	Girls (n=549)	*p* value*
	---------- *area%* ----------				---------- *conc%* ----------			
Saturates								
C12:0	>0.1	>0.1	>0.1	ns	>0.1	>0.1	>0.1	ns
C14:0	0.4 (0.1)	0.4 (0.1)	0.4 (0.1)	ns	0.5 (0.2)	0.5 (0.2)	0.5 (0.2)	ns
C16:0	27.5 (1.5)	27.4 (1.4)	27.5 (1.6)	ns	32.0 (1.6)	32.0 (1.5)	32.0 (1.7)	ns
C17:0	0.3 (0.1)	0.3 (0.1)	0.3 (0.1)	ns	0.4 (0.1)	0.5 (0.1)	0.4 (0.1)	ns
C18:0	13.1 (1.1)	13.1 (1.0)	13.1 (1.1)	ns	13.8 (1.1)	13.8 (1.0)	13.7 (1.1)	ns
C20:0	0.6 (0.1)	0.6 (0.1)	0.6 (0.1)	0.000	0.6 (0.1)	0.5 (0.1)	0.6 (0.1)	0.000
C21:0	>0.1	>0.1	>0.1	0.000	>0.1	>0.1	>0.1	0.000
C22:0	1.5 (0.2)	1.5 (0.2)	1.5 (0.2)	0.031	1.3 (0.2)	1.3 (0.2)	1.3 (0.2)	ns
C23:0	0.6 (0.1)	0.6 (0.1)	0.6 (0.1)	0.000	0.5 (0.1)	0.5 (0.1)	0.5 (0.1)	0.000
C24:0	1.3 (0.3)	1.4 (0.3)	1.3 (0.2)	0.000	1.1 (0.2)	1.1 (0.2)	1.0 (0.2)	0.000
∑ SFA	45.4 (1.5)	45.3 (1.6)	45.4 (1.4)	ns	50.3 (1.5)	50.2 (1.6)	50.3 (1.5)	ns
Monounsaturates and ω9 Family								
C14:1ω5c	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)	ns	0.1 (0.1=	0.1 (0.1)	0.1 (0.1)	ns
C15:1ω5c	>0.1	>0.1	>0.1	ns	0.1 (>0.1)	0.1 (>0.1)	>0.1	ns

continued

Table 5.4 continued

Fatty acid	Total (n=1,024)	Boys (n=475)	Girls (n=549)	*p* value*	Total (n=1,024)	Boys (n=475)	Girls (n=549)	*p* value*
	---------- *area%* ----------				---------- *conc%* ----------			
C16:1ω7c	0.5 (0.2)	0.5 (0.2)	0.5 (0.2)	0.005	0.6 (0.2)	0.6 (0.2)	0.6 (0.2)	0.006
C17:1ω7c	0.1 (>0.1)	0.1 (>0.1)	0.1 (>0.1)	0.008	0.1 (>0.1)	0.1 (>0.1)	0.1 (>0.1)	0.007
C18:1ω7c	1.5 (0.2)	1.4 (0.2)	1.5 (0.3)	0.031	nq	nq	nq	-
C18:1ω7t	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)	ns	nq	nq	nq	-
C18:1ω9c	10.0 (1.8)	10.2 (1.9)	9.8 (1.8)	0.001	7.0 (1.3)	7.1 (1.4)	6.9 (1.3)	0.001
C20:1ω9c	0.2 (0.1)	0.2 (0.1)	0.2 (0.1)	ns	0.1 (0.1)	0.1 (0.1)	0.1 (>0.1)	ns
C22:1ω9c	>0.1	>0.1	>0.1	ns	>0.1	>0.1	>0.1	ns
C24:1ω9c	2.4 (0.5)	2.3 (0.5)	2.4 (0.6)	0.024	1.9 (0.5)	1.9 (0.4)	2.0 (0.5)	0.018
C20:3ω9c	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)	0.000	nq	nq	nq	-
∑ MUFA	14.8 (2.1)	14.9 (2.2)	14.7 (2.0)	ns	10.0 (1.5)	10.1 (1.6)	9.9 (1.5)	ns
∑ ω9 FA	12.6 (1.9)	12.8 (2.0)	12.4 (1.8)	0.010	9.1 (1.4)	9.2 (1.4)	9.0 (1.4)	0.022
Polyunsaturates								
∑ PUFA	39.8 (2.0)	39.8 (2.0)	39.9 (1.9)	ns	39.8 (1.9)	39.7 (2.0)	39.8 (1.9)	ns
∑ LCPUFA	16.8 (2.4)	16.8 (2.3)	16.8 (2.4)	ns	15.8 (2.3)	15.8 (2.2)	15.8 (2.4)	ns

continued

Table 5.4 continued

Fatty acid	Total (n=1,024)	Boys (n=475)	Girls (n=549)	*p* value*	Total (n=1,024)	Boys (n=475)	Girls (n=549)	*p* value*
	---------- *area%* ----------				---------- *conc%* ----------			
ω6 Family								
C18:2ω6c	22.1 (2.6)	22.0 (2.6)	22.2 (2.6)	ns	23.0 (2.6)	22.9 (2.6)	23.1 (2.7)	ns
C18:2ω6t	0.1 (>0.1)	0.1 (>0.1)	0.1 (>0.1)	ns	0.1 (>0.1)	0.1 (>0.1)	0.1 (>0.1)	ns
C18:3ω6c	0.1 (>0.1)	0.1 (0.1)	0.1 (>0.1)	ns	0.1 (>0.1)	0.1 (0.1)	0.1 (>0.1)	ns
C20:2ω6c	0.3 (0.1)	0.3 (0.1)	0.3 (0.1)	ns	0.3 (0.1)	0.3 (0.1)	0.3 (0.1)	ns
C20:3ω6c	3.0 (0.7)	3.0 (0.7)	2.9 (0.7)	0.006	3.0 (0.7)	3.0 (0.7)	2.9 (0.7)	0.006
C20:4ω6c	9.6 (1.9)	9.7 (1.8)	9.5 (2.0)	ns	9.6 (1.9)	9.7 (1.8)	9.5 (2.0)	ns
C22:2ω6c	0.4 (0.1)	0.3 (0.1)	0.4 (0.1)	ns	0.3 (0.1)	0.3 (0.1)	0.3 (0.1)	ns
∑ ω6 FA	35.5 (2.4)	35.5 (2.4)	35.4 (2.4)	ns	35.4 (2.3)	36.4 (2.4)	36.3 (2.3)	ns
ω3 Family								
C18:3ω3c	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)	ns	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)	ns
C18:4ω3c	0.1 (0.3)	0.1 (0.1)	0.1 (0.4)	0.008	nq	nq	nq	-
C20:5ω3c	0.5 (0.3)	0.5 (0.3)	0.5 (0.3)	ns	0.5 (0.3)	0.5 (0.3)	0.5 (0.3)	ns
C22:5ω3c	0.7 (0.2)	0.7 (0.2)	0.7 (0.2)	0.011	nq	nq	nq	-
C22:6ω3c	2.9 (0.9)	2.8 (0.9)	3.0 (1.0)	0.000	2.8 (0.9)	2.6 (0.9)	2.9 (0.9)	0.000

continued

Table 5.4 continued

Fatty acid	Total (n=1,024)	Boys (n=475)	Girls (n=549)	*p* value*	Total (n=1,024)	Boys (n=475)	Girls (n=549)	*p* value*
	---------- *area%* ----------				---------- *conc%* ----------			
∑ ω3 FA	4.3 (1.3)	4.2 (1.2)	4.4 (1.4)	0.003	3.4 (1.1)	3.2 (1.1)	3.5 (1.2)	0.000
trans FA Family								
C18:1ω7t	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)	ns	nq	nq	nq	-
C18:2ω6t	0.1 (>0.1)	0.1 (>0.1)	0.1 (>0.1)	ns	0.1 (>0.1)	0.1 (>0.1)	0.1 (>0.1)	ns
∑ *trans* FA	0.2 (0.1)	0.2 (0.1)	0.2 (0.1)	ns	0.1 (>0.1)	0.1 (>0.1)	0.1 (>0.1)	ns

**P* values are based on independent *t*-test comparing boys and girls; ns:non significant, nq:not quantified.

FA:Fatty acids; LCPUFA: Long-chain PUFA, MUFA:Monounsaturated FA; PUFA: Polyunsaturated FA; SFA:Saturated FA.

[#] rounded values

Table 5.5: Gender-specific fatty acid composition of serum phospholipids (*conc%*) as mean (standard deviation)[#] in relation to age.

Fatty acid (*conc%*)	Boys					Girls				
	Age categorys					Age categorys				
	12.5-13.99 (n=126)	**14.0-14.99** (n=125)	**15.0-15.99** (n=125)	**16.0-17.49** (n=99)	***p*-value***	**12.5-13.99** (n=140)	**14.0-14.99** (n=142)	**15.0-15.99** (n=145)	**16.0-17.49** (n=122)	***p*-value***
Saturates										
C14:0	0.5 (0.1)	0.5 (0.1)	0.5 (0.1)	0.5 (0.2)	ns	0.5 (0.1)	0.5 (0.1)	0.5 (0.2)	0.5 (0.2)	ns
C16:0	32.0 (1.7)	31.8 (1.5)	32.3 (1.4)	31.9 (1.4)	ns	31.8 (1.3)	31.6 (1.4)[3]	32.3 (1.9)	32.7 (2.0)	0.005
C18:0	14.1 (1.0)	13.8 (0.9)	13.7 (1.1)[1]	13.5 (0.9)	0.024	14.1 (1.0)[4]	13.9 (0.9)[4]	13.6 (1.2)	13.2 (1.3)	0.000
C20:0	0.6 (0.1)	0.6 (0.1)	0.5 (0.1)	0.5 (0.1)	ns	0.6 (0.1)	0.6 (0.1)	0.6 (0.1)	0.6 (0.1)	ns
C22:0	1.5 (0.2)	1.3 (0.2)	1.3 (0.2)	1.3 (0.3)	ns	1.3 (0.2)	1.4 (0.2)	1.3 (0.2)	1.3 (0.3)	ns
C23:0	0.6 (0.1)[1]	0.5 (0.1)[1]	0.5 (0.1)	0.5 (0.1)	0.012	0.5 (0.1)	0.6 (0.1)	0.5 (0.1)	0.5 (0.1)	ns
C24:0	1.1 (0.2)	1.1 (0.3)	1.1 (0.2)	1.0 (0.2)	ns	1.0 (0.2)	1.0 (0.2)	1.0 (0.3)	1.0 (0.2)	ns
∑ SFA	50.6 (1.7)[1]	50.2 (1.5)	50.4 (1.4)	49.7 (1.6)	0.037	50.4 (1.1)	50.1 (1.4)	50.2 (1.4)	50.4 (1.9)	ns
Monounsaturates and ω9 Family										
C14:1ω5c	0.1 (0.1)	0.1 (0.1)[1]	0.1 (0.1)	0.1 (0.1)	0.014	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)	ns
C16:1ω7c	0.6 (0.2)	0.6 (0.2)	0.6 (0.2)	0.7 (0.3)	ns	0.6 (0.2)	0.6 (0.2)	0.6 (0.2)	0.7 (0.3)	ns
C18:1ω9c	7.0 (1.3)	7.3 (1.1)	7.1 (1.4)	7.3 (1.8)	ns	7.0 (1.1)	6.8 (1.1)	6.7 (1.2)	6.9 (1.7)	ns
C20:1ω9c	0.1 (>0.1)	0.1 (>0.1)	0.1 (0.1)	0.2 (0.1)	ns	0.1 (>0.1)	0.1 (>0.1)	0.1 (0.1)	0.2 (0.1)	ns

continued

Table 5.5 continued

Fatty acid (*conc%*)	Boys					Girls				
	Age categorys					Age categorys				
	12.5-13.99 (n=126)	**14.0-14.99** (n=125)	**15.0-15.99** (n=125)	**16.0-17.49** (n=99)	***p*-value***	**12.5-13.99** (n=140)	**14.0-14.99** (n=142)	**15.0-15.99** (n=145)	**16.0-17.49** (n=122)	***p*-value***
C24:1ω9c	1.9 (0.6)	1.9 (0.4)	1.9 (0.4)	1.9 (0.4)	ns	1.9 (0.5)	2.0 (0.4)	2.0 (0.4)	2.1 (0.5)	ns
∑ MUFA	9.9 (1.4)	10.2 (1.3)	10.0 (1.6)	10.3 (2.1)	ns	9.9 (1.4)	9.8 (1.3)	9.7 (1.5)	10.1 (1.8)	ns
∑ ω9 FA	9.0 (1.3)	9.4 (1.1)	9.1 (1.4)	9.4 (1.9)	ns	9.1 (1.2)	9.0 (1.2)	8.8 (1.3)	9.2 (1.7)	ns
ω6 Family										
C18:2ω6c	23.1 (2.7)	22.9 (2.4)	22.7 (2.6)	23.1 (2.6)	ns	23.2 (2.7)	23.1 (2.7)	22.4 (2.3)	22.8 (2.9)	ns
C18:3ω6c	0.1 (>0.1)[1]	0.1 (>0.1)	0.1 (0.1)	0.1 (0.1)	0.043	0.1 (>0.1)	0.1 (>0.1)	0.1 (>0.1)	0.1 (>0.1)	ns
C20:2ω6c	0.3 (0.1)[2]	0.3 (0.1)	0.3 (0.1)	0.3 (0.1)	0.034	0.3 (0.1)	0.3 (0.1)	0.3 (0.1)	0.3 (0.1)	ns
C20:3ω6c	3.1 (0.7)	3.1 (0.6)	3.0 (0.7)	3.0 (0.7)	ns	3.0 (0.6)	2.9 (0.8)	2.9 (0.7)	3.0 (0.9)	ns
C20:4ω6c	9.4 (2.0)	9.4 (1.7)	9.9 (1.9)	10.0 (1.6)	ns	9.3 (1.5)	9.5 (1.8)	9.6 (1.9)	9.6 (2.5)	ns
C22:2ω6c	0.3 (0.1)	0.3 (0.1)	0.3 (0.1)	0.3 (0.1)	ns	0.3 (0.1)	0.3 (0.1)	0.3 (0.1)	0.3 (0.1)	ns
∑ ω6 FA	36.3 (2.6)	36.2 (2.0)	36.4 (2.1)	36.9 (2.6)	ns	36.2 (2.2)	36.2 (2.4)	36.6 (2.3)	36.2 (2.5)	ns
Polyunsaturates										
∑ PUFA	39.5 (2.0)	39.6 (1.8)	39.7 (1.8)	39.9 (2.3)	ns	39.7 (1.8)[1]	40.0 (1.8)[1]	40.0 (1.9)	39.5 (2.1)	0.017
∑ LCPUFA	15.6 (2.4)	15.8 (2.1)	16.0 (2.3)	15.9 (2.0)	ns	15.6 (2.0)	16.0 (2.4)	15.8 (2.2)	15.8 (2.8)	ns

continued

Table 5.5 continued

Fatty acid (*conc%*)	Boys					Girls				
	Age categorys					Age categorys				
	12.5-13.99 (n=126)	**14.0-14.99** (n=125)	**15.0-15.99** (n=125)	**16.0-17.49** (n=99)	***p*-value***	**12.5-13.99** (n=140)	**14.0-14.99** (n=142)	**15.0-15.99** (n=145)	**16.0-17.49** (n=122)	***p*-value***
ω3 Family										
C18:3ω3c	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)	ns	0.2 (0.1)	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)	ns
C20:5ω3c	0.5 (0.3)	0.5 (0.4)	0.5 (0.3)	0.5 (0.3)	ns	0.5 (0.3)	0.6 (0.5)	0.4 (0.3)	0.5 (0.3)	ns
C22:6ω3c	2.7 (1.2)	2.7 (0.8)	2.6 (0.8)	2.4 (0.6)	ns	2.8 (1.0)	3.1 (1.0)	2.8 (0.8)	2.8 (0.7)	ns
∑ ω3 FA	3.3 (1.4)	3.3 (1.0)	3.3 (1.0)	3.0 (0.8)	ns	3.5 (1.2)	3.8 (1.4)[2]	3.4 (1.0)	3.4 (0.9)	0.043
∑ *trans* FA	0.1 (>0.1)	0.1 (>0.1)	0.1 (>0.1)	0.1 (>0.1)	ns	0.1 (>0.1)	0.1 (>0.1)	0.1 (>0.1)	0.1 (>0.1)	0.017

**P* values are based on the ANCOVA *F*-test including Tanner stages, body mass index groups, body fat groups, and fat free mass quartiles in the model, using leptin and insulin as covariates. ns:non significant.
Bonferroni *post hoc test*:[1] $p<0.05$ compared to age category 16.0-17.49 years;[2] $p<0.05$ compared to age category 14.0-14.99 years;[3] $p<0.01$ compared to age category 16.0-17.49 years;
[4] $p<0.001$ compared to age category 16.0-17.49 years.

[#] rounded values

FA:Fatty acids; LCPUFA:Long-chain PUFA; MUFA:Monounsaturated FA; PUFA: Polyunsaturated FA; SFA:Saturated FA.

Table 5.6: Fatty acid composition of serum phospholipids as mean (standard deviation)[#] according to sexual maturation.

Fatty acid (*conc%)*	Boys						Girls				
	Tanner stage						Tanner stage				
	I (n=7)	**II** (n=33)	**III** (n=82)	**IV** (n=171)	**V** (n=125)	***p*-value***	**II** (n=21)	**III** (n=100)	**IV** (n=221)	**V** (n=153)	***p*-value***
Saturates											
C16:0	32.3 (2.0)	31.7 (1.1)	31.9 (1.6)	32.1 (1.6)	32.1 (1.5)	ns	31.7 (1.1)	31.9 (1.5)	32.1 (1.6)	32.2 (2.2)	ns
C18:0	14.7 (0.5)	14.3 (1.0)	14.1 (1.1)	13.7 (1.0)	13.7 (0.9)	ns	14.2 (0.7)	13.9 (1.1)	13.7 (1.1)	13.7 (1.3)	ns
C20:0	0.6 (0.1)	0.6 (0.1)	0.5 (0.1)	0.5 (0.1)	0.5 (0.1)	ns	0.6 (0.1)	0.6 (0.1)[1]	0.6 (0.1)	0.6 (0.1)	0.007
C21:0	>0.1	>0.1	>0.1	>0.1	>0.1	ns	>0.1	>0.1	>0.1	>0.1	0.030
C22:0	1.5 (0.2)	1.4 (0.2)	1.3 (0.2)	1.3 (0.2)	1.3 (0.2)	ns	1.3 (0.2)	1.4 (0.2)	1.3 (0.2)	1.4 (0.2)	ns
C23:0	0.7 (0.1)	0.6 (0.1)	0.5 (0.1)	0.5 (0.1)	0.5 (0.1)	ns	0.6 (0.1)	0.6 (0.1)	0.5 (0.1)	0.5 (0.1)	ns
∑ SFA	52.2 (2.0)	50.6 (1.2)	50.6 (1.7)	50.1 (1.5)	50.2 (1.7)	ns	50.5 (0.9)	50.5 (1.4)	50.2 (1.5)	50.4 (1.6)	ns
Monounsaturates and ω9 Family											
C17:1ω7c	0.1 (>0.1)[1]	0.1 (>0.01)	0.1 (>0.1)	0.1 (>0.1)	0.1 (>0.1)	0.042	0.1 (>0.1)	0.1 (>0.1)	0.1 (0.1)[2]	0.1 (>0.1)	0.014
C18:1ω9c	5.9 (2.9)	6.9 (0.8)	7.1 (1.2)	7.4 (1.5)	6.9 (1.3)	ns	7.0 (0.9)	7.1 (1.0)	6.8 (1.5)	6.5 (1.0)	ns
C20:1ω9c	0.1 (0>0.1)	0.1 (>0.1)	0.1 (>0.1)	0.2 (0.1)	0.1 (>0.1)	0.006	0.1 (>0.1)	0.1 (>0.1)	0.2 (0.1)	0.1 (>0.1)	ns
C22:1ω9c	>0.1	>0.1	>0.1	>0.1	>0.1	0.030	>0.1	>0.1[1,2]	>0.1	>0.1	0.006
C24:1ω9c	1.7 (0.8)	1.9 (0.4)	1.9 (0.5)	1.9 (0.4)	1.9 (0.4)	ns	1.9 (0.5)	2.0 (0.6)[5]	2.0 (0.5)	2.0 (0.3)	0.005

continued

Table 5.6 continued

Fatty acid (*conc%)*	Boys						Girls				
	Tanner stage						Tanner stage				
	I (n=7)	**II** (n=33)	**III** (n=82)	**IV** (n=171)	**V** (n=125)	***p*-value***	**II** (n=21)	**III** (n=100)	**IV** (n=221)	**V** (n=153)	***p*-value***
∑ MUFA	8.6 (2.8)	9.9 (1.0)	10.0 (1.3)	10.4 (1.8)	9.8 (1.5)	ns	10.0 (1.3)	10.2 (1.3)	9.9 (1.7)	9.5 (1.2)	ns
∑ ω9 FA	7.8 (2.7)[2,4,5]	9.0 (0.9)	9.2 (1.2)	9.5 (1.6)	8.9 (1.4)	0.021	9.1 (1.2)	9.3 (1.2)	9.0 (1.5)	8.6 (1.1)	ns
Polyunsaturates											
∑ PUFA	39.2 (2.2)	39.5 (1.6)	39.4 (2.0)	39.5 (1.9)	40.0 (2.2)	ns	39.6 (1.2)	39.3 (1.8)	39.9 (1.9)	40.1 (2.1)	ns
∑ LCPUFA	17.3 (3.0)	16.2 (1.7)	15.3 (2.3)	15.8 (2.3)	15.8 (2.1)	ns	15.8 (1.6)	15.3 (2.5)	15.9 (2.5)	16.1 (2.2)	ns
ω6 Family											
C18:2ω6c	20.9 (3.6)	22.3 (2.4)	23.3 (2.6)	22.8 (2.5)	23.4 (2.8)	ns	22.8 (2.1)	23.1 (2.8)	23.1 (2.8)	23.2 (2.4)	ns
C20:2ω6c	0.3 (0.1)[2-4]	0.3 (0.1)	0.3 (0.1)	0.3 (0.1)	0.3 (0.1)	0.035	0.3 (>0.1)	0.3 (0.1)	0.3 (0.1)	0.3 (0.1)	ns
C20:4ω6c	10.2 (1.6)	9.8 (1.2)	9.3 (2.0)	9.6 (1.8)	9.7 (1.9)	ns	8.9.(1.2)	8.8 (1.9)	9.6 (1.9)	9.8 (2.1)	ns
C22:2ω6c	0.3 (>0.1)	0.3 (>0.1)	0.3 (0.1)	0.3 (0.1)	0.3 (0.1)	ns	0.3 (0.1)	0.3 (0.1)	0.3 (0.1)	0.3 (0.1)	ns
∑ ω6 FA	35.5 (2.8)	36.1 (2.0)	36.2 (2.4)	36.1 (2.3)	36.8 (2.6)	ns	35.4 (2.0)	35.6 (2.4)	36.5 (2.3)	36.6 (2.4)	ns
ω3 Family											
C18:3ω3c	0.1 (>0.1)	0.2 (0.1)	0.2 (0.1)	0.2 (0.1)[2]	0.1 (0.1)	0.019	0.2 (0.1)	0.2 (0.1)	0.2 (0.1)	0.1 (0.1)	ns

continued

Table 5.6 continued

Fatty acid (*conc%)*	Boys						Girls				
	Tanner stage						Tanner stage				
	I (n=7)	**II** (n=33)	**III** (n=82)	**IV** (n=171)	**V** (n=125)	***p*-value***	**II** (n=21)	**III** (n=100)	**IV** (n=221)	**V** (n=153)	***p*-value***
C20:5ω3c	0.6 (0.5)	0.6 (0.3)[1,2]	0.5 (0.3)	0.5 (0.3)	0.5 (0.4)	0.033	0.6 (0.3)	0.6 (0.4)	0.5 (0.4)	0.4 (0.3)	ns
C22:6ω3c	2.9 (1.4)	2.6 (0.8)	2.6 (0.9)	2.7 (0.9)	2.6 (0.8)	ns	3.4 (1.2)	3.0 (1.0)	2.8 (0.9)	2.9 (0.9)	ns
∑ ω3 FA	3.7 (1.9)	3.4 (1.0)	3.2 (1.1)	3.4 (1.1)	3.2 (1.0)	ns	4.1 (1.4)	3.7 (1.2)	3.4 (1.2)	3.5 (1.1)	ns
∑ *trans* FA	>0.1	>0.1	0.1 (>0.1)	0.1 (>0.1)	0.1 (>0.1)	ns	0.1 (>0.1)	0.1 (>0.1)	0.1 (>0.1)	0.1 (>0.1)	ns

**P* values are based on the ANCOVA *F*-test including age categories, body mass index groups, body fat groups, and fat free mass quartiles in the model, using leptin and insulin as covariates.
Bonferroni *post hoc test*: [1] $p<0.05$ compared to Tanner stage IV; [2] $p<0.05$ compared to Tanner stage V; [3] $p<0.05$ compared to Tanner stage II; [4] $p<0.05$ compared to Tanner stage III;
[5] $p<0.01$ compared to Tanner stage IV.
[#] rounded values
FA:Fatty acids; LCPUFA:Long-chain PUFA, MUFA:Monounsaturated FA; PUFA: Polyunsaturated FA; SFA:Saturated FA

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6 Fatty acid ratios in European adolescents – associations with age, gender, maturity, body mass index, percentage body fat, and fat free mass

Background: Essential FA LA and ALA have to be provided by the normal diet. These dietary FA are precursors for LCPUFA of the ω6 and ω3 family. Omega 6 and ω3 FA compete against the same enzymes for elongation and desaturation, thus a particular relation is necessary to obtain adequate amounts of both LCPUFA of the ω6 and ω3 series. This relation will maintain optimal health and development, especially important in the period of adolescence. Estimated ratios like ω6/ω3, LA/ALA, AA/EPA, AA/(EPA+DHA) are used to evaluate the relationship between ω6 and ω3 FA and can be calculated by dietary intake data or blood levels. If these ratios are influenced by FA intake alone or associated with age, gender, maturity, BMI, BF, and FFM is not investigated sufficiently, especially in adolescent subjects.

Objective: To investigate the association of FA ratios with age, gender, maturation, BMI, BF, and FFM based on serum FA of PL estimated by different calculation methods.

Results: Gender differences were seen between boys and girls and differed due to the calculation method. Male FA ratios showed no association with age. In girls, EPA/ALA ratios decreased with age significantly in both *conc%* and *area%* calculations. Maturation was associated with LA/ALA and EPA/ALA ratios in boys (*conc%* and *area%* calculations). In girls, ω6/ω3 (based on *area%*) was related to maturation. The ω6/ω3 ratio of boys (*conc%* calculation) showed some kind of U-shaped curve, whereas female ratios of AA/LA increased with BMI status based on FA measured in *conc%* and absolute values.

Conclusion: The associations of the different FA ratios with age, maturation, BF, FFM, and BMI are minimal and follow no clear pattern. Thus, it can be speculated that these FA ratios are mainly modified by dietary factors such as amount of fat and dietary FA pattern.

Introduction

Linoleic acid and ALA are essential FA and have to be provided by the normal diet. These two FA are termed parent essential FA because they are the starting point for the elongation-desaturation cascade leading to LCPUFA of the ω6 and ω3 family. Due to the fact that ω6 and ω3 FA compete against the same enzymes for elongation and desaturation, a particular relation, provided by the diet, is necessary to obtain a satisfactory amount of LCPUFA of both ω6 and ω3. This relation is prerequisite to ensure the fulfillment of the physiological functions LCPUFA are involved in. For instance, a balanced relation of ω6 and ω3 LCPUFA in different lipid fractions is particularly important for the regulation of the immune system and inflammation processes. Thus, for optimal health and development an adequate relationship/ ratio between ω6 and ω3 FA is needed in all lipid classes.

Fatty acid ratios are commonly calculated by using total ω6/ω3 FA or LA/ALA. Furthermore, other ratios are used to indicate the relationship between ω6 and ω3 FA (e.g. AA/EPA, AA/[EPA+DHA]) or, to show the relationships within the ω6 or ω3 family (e.g. DHA/EPA, AA/LA). Fatty acid ratios are based on dietary intake data or blood levels.

The current dietary ω6/ω3 ratio in countries with Westernized eating patterns is approx. 15:1-16:1 [1]. The recommended ratio of 5:1, suggested by some nutrition societies [2], indicates discrepancies between current ω6/ω3 FA intakes and recommendations. This is due to the high dietary intake of LA and the lower dietary intake of ALA, EPA, and DHA as well as due to the insufficient conversion of EPA and DHA from ALA in humans. Moreover, with respect to evolutionary aspects and the genetic background, which is adapted to a hunter-gatherer diet, a ω6/ω3 ratio of 2.4:1-1:1 is estimated as best possible. Thus, an even lower ratio would support optimal health [3]. As a consequence, increased ω6/ω3 ratios are related to several diseases of today's civilisations [4-8]. These increased ratios may also be present in adolescents with Westernized eating habits [9], suggesting a risk factor for later diseases and insufficient conditions for adequate growth and development. According to that, it is important to assess the FA ratios of adolescents, which are not assessed satisfactorily so far. If FA ratios are only determined by the dietary intake of ω6 or ω3 FA or if they are influenced by other parameters like age, gender, maturity, BMI, BF, and FFM is not clear yet. However, that could be of importance for improving FA ratios, and thus health, and assessing potential risk factors especially in adolescence.

Therefore, the HELENA Study assessed several FA ratios based on individual FA measured in serum PL in a healthy adolescent group. The ratios [AA/LA, (DHGLA+AA)/LA,

EPA/ALA, DHA/EPA, AA/EPA, LA/ALA, AA/(EPA+DHA), total ω6/ω3, Polyunsaturates/Saturates, and Monounsaturates/Saturates] were associated with age, gender, maturity, BMI, BF, and FFM. Furthermore, due to the different approaches to measure FA and to calculate their ratios afterwards, this chapter will also compare the results of FA ratios based on FA measured as *area%*, *conc%* and as absolute concentrations (*µmol/L*).

Subjects and Methods

A detailed description of study design, subject recruitment, medical examination, blood sampling, sample treatment, analytical methods, evaluation procedures, and calculation of the FA ratios can be found in *chapter 3* (*General Methodology*).

Statistics

Numeric values are shown as mean±SD. Differences in the main study characteristics of boys and girls were analysed using independent *t*-tests (metric variables) and *chi square* test (categorical variables). Fatty acids were skewed and logarithmically transformated before FA ratios were calculated. Fatty acid ratios were used as dependent variables in a gender-specific statistical analysis. Age, sexual maturation, FFM, BMI, and BF were determined as independent variables and grouped. Further, insulin and leptin were used as covariates in the present model. For each of the dependent variables, a univariate ANCOVA were used to analyse the association with the independent variables. The Bonferroni *post-hoc tests* were used for sub-group analyses. Data analysis was performed by using Statistical Package for Social Sciences version 17.0 for Windows (SPSS Inc., Chicago, Illinois, USA) and a *p* value of <0.05 was considered as statistically significant

Results

The HELENA-CSS consisted of 1,097 adolescent subjects including blood measurements. A number of 73 adolescents gave insufficient blood for FA analysis, thus 1,024 subjects contributed to the following results. Table 6.1 highlights the basic characteristics of the study population. Individual FA and their sums as basis for the different FA ratios are presented in the tables 6.2a-c followed by the gender-specific FA ratios in tables 6.3a-c. Table 6.3d shows

the differences between FA ratios based on *area%* and *conc%* within the male and female groups.

In detail, within the male groups as well as within the female groups, the comparison between FA ratios based on FA measured as *area%* and *conc%* showed significant differences (table 6.3d). All ratios except for DHA/EPA, AA/EPA, and AA/(EPA+DHA) were significantly different due to the calculation method. In male and female groups, ratios estimated by FA measured as *area%* were higher compared to ratios based on *conc%* calculations, except for ω6/ω3.

Fatty acid ratios based on calculations of fatty acids expressed as conc%

Gender-specific differences were observed for the (DHGLA+AA)/LA ratio (table 6.3a) with higher ratios in boys compared to girls.

With respect to age, female EPA/ALA ratios decreased significantly from 4.33±3.96 to 3.63±2.63 between 14.0-14.99 years and 16.0-17.49 years (p=0.041, *post hoc test*). All other FA ratios showed no association with age in girls. By contrast, no association was found between male FA ratios and age.

In boys, maturation was associated with LA/ALA (p=0.030, F-test) and EPA/ALA (p=0.036, F-test). The LA/ALA ratio increased from 184±98 to 221±111 between Tanner stage IV and V (p=0.023, *post hoc test*), whereas the EPA/ALA ratio decreased from 3.99±1.71 to 3.81±2.54 between Tanner stage II to IV (p=0.019, *post hoc test*). Girls showed no associations between maturity and FA ratios.

Gender-specific FA ratios categorized as BMI groups are presented in the tables 6.4a and 6.4b. A higher ratio of ω6/ω3 was present in underweight compared to normal weight boys. Moreover, the ω6/ω3 ratio showed some kind of U-shaped curve in boys, whereas girls showed no differences in the ratios according to the BMI categories. Additionally, AA/(EPA+DHA) were lower in obese compared to overweight boys (see table 6.4a). In girls, (DHGLH+AA)/LA and AA/LA ratios increased from normal weight to overweight (table 6.4b).

Differences between the FA ratios were neither observed in girls nor in boys when splitting the group according to percentage BF.

Fat free mass was associated with EPA/ALA in boys. The EPA/ALA ratio increased from the 1st quartile (3.56±2.27) to the 3rd (3.98±3.42) and 4th quartile (4.14±2.26, both p<0.04, *post hoc tests*). Fatty acid ratios of girls were not related to FFM quartiles.

Fatty acid ratios based on calculations of absolute fatty acid concentrations (µmol/L)

The ratios AA/(EPA+DHA) and ω6/ω3 showed gender-specific differences with higher ratios in male subjects. All other FA ratios remained unaffected by gender (table 6.3b).

The ω6/ω3 ratio was higher in 14.0-14.99 year-old compared to 12.5-13.99 year-old girls (p=0.015, *post hoc test*). Additionally, the M/S ratio decreased between 12.5-13.99 year-old girls and 16.0-17.49 year-old girls (p=0.041). The FA ratios of boys were not associated with age groups.

Fatty acid ratios showed an increase of the M/S ratio between Tanner stage I and IV (p=0.034, *post hoc test*) in boys. On the other hand, female ω6/ω3 ratios were associated with puberty (p=0.016, *F*-test).

The M/S ratio differed due to BMI status (p=0.038, *F*-test) in girls. Furthermore, the AA/LA ratio increased between normal weight and overweight girls (p=0.001, *post hoc test*). The FA ratios of boys remained unaffected by BMI status.

Body fat content was associated with male EPA/ALA ratios. The ratio increased with higher BF content (p=0.010, *F*-test). Female FA ratios did not differ significantly as a consequence of percentage BF categorization.

No association between FFM quartiles and FA ratios was found for boys and girls.

Fatty acid ratios based on calculations of fatty acids expressed as area%

The ratios AA/(EPA+DHA), (DHGLA+AA)/LA, and ω6/ω3 were significantly different between sexes. Higher ratios were observed in male subjects compared to females (table 6.3c).

The EPA/ALA ratio decreased with age in girls (p=0.034, *F*-test). Furthermore, the ω6/ω3 ratio of girls increased between the ages 12.5-13.99 and 14.0-14.99 (p=0.033, *post hoc test*). Male FA ratios were not associated with age as already observed in FA ratios based on *conc%* calculations.

The (DHGLA+AA)/LA, EPA/ALA, DHA/EPA, AA/EPA, and LA/ALA ratios of male subjects were associated with sexual maturation as specified in table 6.4c, whereas female FA ratios were not related to maturity.

Table 6.4d highlights the FA ratio results found in girls according to BMI status. In boys, ω6/ω3 differed between BMI groups (p=0.049, *F*-test).

Fatty acid ratios showed no association with BF content in boys, whereas in girls higher DHA/EPA and AA/EPA ratios were associated with lower BF content (p=0.047 and p=0.037, *F*-tests).

With respect to FFM, EPA/ALA increased between the 1st to 3rd, 1st to 4th, and 2nd to 4th quartile in boys (p=0.024, p=0.015 and p=0.042, *post hoc tests*), whereas female FA ratios remained unaffected by FFM.

Discussion

Generally, most studies did not include such a wide spectrum of FA ratios in their results as it is presented within this chapter [15-20]. Thus, it is difficult to compare the observed results with the literature especially for adolescents. Therefore, if possible further FA ratios were self-calculated on the basis of the available FA data of suitable publications and are included in this discussion. This allows a view on the absolute values but did not give any statistically significant information of the newly calculated FA ratios. Another difficulty was that some authors use dietary intake data mostly without calculating FA ratios [9, 21, 22]. However, dietary intake data is easier to assess but is biased by the kind of assessment (questionnaires, dietary recalls) and type of food composition table [9, 23]. Moreover, dietary intake cannot give a complete overview of the FA status of the human body, because the endogenous FA metabolism is not considered. Nonetheless, even within the blood FA measurements results differ, based on the chosen blood lipid fractions (e.g. serum/ plasma PL, cholesteryl esters, TG, red blood cell PL) or due to the methodological approach of FA analytics. The objectives and aims of a planned study, laboratory facilities and the budget of a study may account for those different choices.

However, we found gender-specific differences depending on the calculation basis of the FA. Fatty acid ratios based on *conc%* were significantly different for (DHGLA+AA)/LA whereas FA ratios based on absolute values showed significant differences for AA/(EPA+DHA) and ω6/ω3. Further, FA ratios estimated by FA expressed as *area%* included both of the results mentioned before. The different results, which have been found between the FA ratios, estimated by FA expressed as *conc%* and measured in absolute values, may be due to the logarithmically transformated values. The logarithmical transformation of the FA of both calculation methods resulted in different transformated values due to the different units (*µmol/L* and *conc%*). Those values were the basis for the estimation of the FA ratios, which then showed different results in the statistical analysis. This cannot be found in the tables 6.3a and 6.3b, because the tables show the raw data of the FA ratios. These FA ratios based on FA expressed as conc% and µmol/L are quite similar. Thus, it can be assumed that the

transformated data is responsible for the significant findings. This has to be kept in mind, when interpreting the present results. The comparison between FA ratios based on *area%* and *conc%* may therefore be a more reasonable choice to evaluate the results of the same study, because both approaches use percentaged values. The present gender differences may be explained by different eating habits between boys and girls especially of ω6 and ω3 FA. However, most studies in adolescents/ children investigating FA ratios did not separate between boys and girls [17, 20] or did not find gender-specific differences in their study groups [18, 19]. Thus, the present findings are not in accordance with other studies. In the HELENA Study, the LA/ALA ratio was 199 (table 6.3a) which was not found within the other studies. In two Hungarian studies, apparently healthy children, used as control groups, had LA/ALA ratios of 135 (n=25, mean age 12.9±1.6years) [19] and 254 (n=16, mean age 16.2±1.0) [20]. By contrast, American normal weight adolescents (n=190, mean age 15±1.2 years) had a ratio of 124 [18] (these ratios based on self-calculated PL FA provided by the publications). These differences may be a result of country-specific and age-dependent eating habits.

Age was associated with variations in the FA ratios of girls, whereas boys may not change their dietary FA intake habits over age or more precisely, the proportion of several FA do not alter. However, in girls, the decline of the EPA/ALA ratio might indicate lower intakes of EPA and/ or higher intakes of ALA and thus shifted dietary habits within the different age groups.

In boys, during maturity, the LA/ALA ratio based on *conc%* and *area%* calculations increased. Further, the AA/EPA ratio estimated by FA in *area%* increased as well. Maturity may be a better determinant than age to detect alterations in serum FA during growth in boys. Changed dietary eating patterns may be reflected in the shifted FA ratios. Hormonal changes may also account for fluctuations of FA levels (e.g. AA, EPA) and thus alter those FA ratios, because hormones might interact with elongation/ desaturation processes or influence food choices. Due to the fact that it was not feasible to measure sex hormones within the HELENA Study this question cannot be answered sufficiently.

Fatty acid ratios in adolescent subjects are commonly used to investigate differences in the FA profile between obese and normal weight subjects [17, 19, 20]. However, inconsistencies in those findings are present. Self-calculated LA/ALA ratios based on FA data of Decsi and coworkers [20] were 218, 213 and 254 for obese children with and without metabolic cardiovascular syndrome, and controls. The results show that the highest ratio in healthy controls reflects the most undesirable ratio within the three sub-groups. In another study of

Decsi *et al*. [19] LA/ALA ratios in 25 control subjects were 135. The LA/ALA ratio for the obese participants was not available, because ALA was not detectable in the obese sub-group (self-calculated values of both studies based on FA data in PL provided by the referring publication) [19]. In contrast to those Hungarian adolescents, the present European participants of the HELENA Study had LA/ALA ratios of 194 and 197 in normal weight boys and girls, whereas LA/ALA in obese boys and girls was 215 and 244 (see tables 6.4a and 6.4b). Those contrasting results of the studies may be a consequence of differences in the diet, FA analytics and probably group characteristics (e.g. nationality, age, group size).

Concerning the results of the different calculation methods, one can say that it is not clear, which method is the most reliable. However, some points can be highlighted: fatty acids and their ratios based on *conc%* are more reliable than FA expressed as *area%,* because *conc%* calculations take the molecular mass of each FA detected into account and thus do not simply refer to a peak area. Nevertheless, the percentaged FA profiles of both methods are influenced by the number of FA detected and the height of each FA peak, this approach may obscure some important findings. This point would be eliminated by using FA expressed as absolute concentrations, which are independent of other FA concentrations. Thus, changes in one specific FA concentration would not affect another FA concentration of the same profile. However, the most common method to express FA in the literature is the use of percentaged FA profiles.

The assessment of dietary FA was not included in this analysis, even though it may help to identify the contribution of dietary FA to the FA composition of PL. As already mentioned, most food composition data bases lack of reliability concerning FA data and the available spectrum of FA in different foods is insufficient in those data bases; thus, from this point of view, dietary FA data would weaken the results rather than strengthen them.

Conclusion

Although some FA ratios are associated with age, maturation, BMI, BF, and FFM in boys and girls, the results follow no clear pattern. Thus, the differences might best be explained by and are related to different eating habits in the different sub-groups. Thus, it can be speculated that FA ratios are mainly modified by dietary factors such as amount of fat and dietary FA pattern.

Table 6.1: Gender-specific characteristics of the study group as mean (standard deviation).

	Boys	**Girls**	***p* value***
n	475	551	
Age (years)	14.75 (1.19)	14.78 (1.15)	ns
Height (cm)	169.8 (9.8)	161.8 (7.0)	0.000
Weight (kg)	61.7 (13.7)	56.0 (10.2)	0.000
Body mass index (kg/m²)	21.3 (3.8)	21.3 (3.4)	ns
Tanner stage[1]	3.9 (1.0)	4.0 (0.8)	0.007[#]
Body fat (%)[2]	21.0 (10.5)	27.3 (7.6)	0.000
Fat free mass (kg)[3]	51.8 (9.6)	41.8 (7.0)	0.000
Insulin (mLU/L)[4]	9.9 (8.0)	10.7 (11.2)	ns
Leptin (ng/mL)[5]	9.30 (14.06)	28.29 (24.20)	0.000
Triglycerides (mg/dL)[6]	64 (31)	73 (37)	0.000
Total cholesterol (mg/dL)[6]	154 (26)	167 (28)	0.000
HDL (mg/dL)[6]	53 (10)	57 (11)	0.000
LDL (mg/dL)[6]	91 (25)	98 (25)	0.000
Apolipoprotein A-1 (g/L)[7]	1.45 (0.21)	1.55 (0.23)	0.000
Apolipoprotein B (g/L)[7]	0.62 (0.15)	0.68 (0.16)	0.000
Lipoprotein(a) (mg/L)[7]	176 (218)	209 (257)	0.021

**P* values (2-tailed) are based on an independent *t*-test; [#] based on *Chi square* test. ns:non significant
[1] boys n=418, and girls n=495, [2]boys n=461, and girls n=529;
[3] boys n=469, and girls n=541, [4]boys n=472, and girls n=543;
[5] boys n= 436, and girls n=525, [6]boys n=475 and girls n=548;
[7] boys n= 464 and girls n=532.
HDL:High density lipoprotein cholesterol, LDL:Low density lipoprotein cholesterol.

Table 6.2a: Selection of fatty acids (*conc%*) of serum phospholipids as mean (SD)[#].

Fatty acid	Total (n=1,024)	Boys (n=475)	Girls (n=549)	*p* value*
	---------- *conc%* ----------			
Saturates				
C16:0	32.0 (1.6)	32.0 (1.5)	32.0 (1.7)	ns
C18:0	13.8 (1.1)	13.8 (1.0)	13.7 (1.1)	ns
∑ SFA	50.3 (1.5)	50.2 (1.6)	50.3 (1.5)	ns
Monounsaturates				
C16:1ω7c	0.6 (0.2)	0.6 (0.2)	0.6 (0.2)	0.006
C18:1ω9c	7.0 (1.3)	7.1 (1.4)	6.9 (1.3)	0.001
∑ MUFA	10.0 (1.5)	10.1 (1.6)	9.9 (1.5)	ns
Polyunsaturates				
∑ PUFA	39.8 (1.9)	39.7 (2.0)	39.8 (1.9)	ns
∑ LCPUFA	15.8 (2.3)	15.8 (2.2)	15.8 (2.4)	ns
ω6 Family				
C18:2ω6c	23.0 (2.6)	22.9 (2.6)	23.1 (2.7)	ns
C18:3ω6c	0.1 (>0.1)	0.1 (0.1)	0.1 (>0.1)	ns
C20:3ω6c	3.0 (0.7)	3.0 (0.7)	2.9 (0.7)	0.006
C20:4ω6c	9.6 (1.9)	9.7 (1.8)	9.5 (2.0)	ns
∑ ω6	35.4 (2.3)	36.4 (2.4)	36.3 (2.3)	ns
ω3 Family				
C18:3ω3c	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)	ns
C20:5ω3c	0.5 (0.3)	0.5 (0.3)	0.5 (0.3)	ns
C22:6ω3c	2.8 (0.9)	2.6 (0.9)	2.9 (0.9)	0.000
∑ ω3	3.4 (1.1)	3.2 (1.1)	3.5 (1.2)	0.000

**P* values are based on independent *t*-test comparing boys and girls, ns:non significant, [#] rounded values
FA:Fatty acids; LCPUFA:Long-chain polyunsaturated FA, MUFA:Monounsaturated FA; PUFA: Polyunsaturated FA; SD:Standard deviation; SFA:Saturated FA.

Table 6.2b: Selection of fatty acids (*µmol/L*) of serum phospholipids as mean (SD)[#].

Fatty acid	Total (n=1,024)	Boys (n=475)	Girls (n=549)	*p* value*
	---------- *µmol/L* ----------			
Saturates				
C16:0	1,182 (212)	1,136 (191)	1,222 (221)	0.000
C18:0	508 (99)	491 (99)	523 (97)	0.000
∑ SFA	1,855 (324)	1,785 (309)	1,915 (326)	0.000
Monounsaturates				
C16:1ω7c	23 (11)	22 (9)	25 (11)	0.000
C18:1ω9c	259 (76)	255 (73)	263 (78)	ns
∑ MUFA	370 (95)	359 (91)	379 (98)	0.000
Polyunsaturates				
∑ PUFA	1,470 (278)	1,412 (270)	1,520 (275)	0.000
∑ LCPUFA	586 (142)	565 (136)	604 (145)	0.000
ω6 Family				
C18:2ω6c	850 (178)	814 (170)	880 (179)	0.000
C18:3ω6c	4 (2)	4 (2)	4 (2)	ns
C20:3ω6c	111 (37)	109 (34)	114 (39)	ns
C20:4ω6c	354 (94)	345 (89)	363 (97)	0.000
∑ ω6	1,344 (258)	1,295 (249)	1,386 (257)	0.000
ω3 Family				
C18:3ω3c	5 (3)	5 (3)	6 (4)	0.003
C20:5ω3c	18 (13)	18 (13)	19 (14)	ns
C22:6ω3c	12 (4)	11 (3)	12 (4)	0.000
∑ ω3	126 (50)	117 (48)	134 (51)	0.000

**P* values are based on independent *t*-test comparing boys and girls, ns:non significant, [#] rounded values
FA:Fatty acids; LCPUFA:Long-chain polyunsaturated FA, MUFA:Monounsaturated FA; PUFA: Polyunsaturated FA; SD:Standard deviation; SFA:Saturated FA.

Table 6.2c: Selection of fatty acids (*area%*) of serum phospholipids as mean (SD)[#].

Fatty acid	Total (n=1,024)	Boys (n=475)	Girls (n=549)	*p* value*
		---------- *area%* ----------		
Saturates				
C16:0	27.5 (1.5)	27.4 (1.4)	27.5 (1.6)	ns
C18:0	13.1 (1.1)	13.1 (1.0)	13.1 (1.1)	ns
∑ SFA	45.4 (1.5)	45.3 (1.6)	45.4 (1.4)	ns
Monounsaturates				
C18:1ω7c	1.5 (0.2)	1.4 (0.2)	1.5 (0.3)	0.031
C18:1ω9c	10.0 (1.8)	10.2 (1.9)	9.8 (1.8)	0.001
∑ MUFA	14.8 (2.1)	14.9 (2.2)	14.7 (2.0)	ns
Polyunsaturates				
∑ PUFA	39.8 (2.0)	39.8 (2.0)	39.9 (1.9)	ns
∑ LCPUFA	16.8 (2.4)	16.8 (2.3)	16.8 (2.4)	ns
ω6 Family				
C18:2ω6c	22.1 (2.6)	22.0 (2.6)	22.2 (2.6)	ns
C18:3ω6c	0.1 (>0.1)	0.1 (0.1)	0.1 (>0.1)	ns
C20:3ω6c	3.0 (0.7)	3.0 (0.7)	2.9 (0.7)	0.006
C20:4ω6c	9.6 (1.9)	9.7 (1.8)	9.5 (2.0)	ns
∑ ω6	35.5 (2.4)	35.5 (2.4)	35.4 (2.4)	ns
ω3 Family				
C18:3ω3c	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)	ns
C20:5ω3c	0.5 (0.3)	0.5 (0.3)	0.5 (0.3)	ns
C22:6ω3c	2.9 (0.9)	2.8 (0.9)	3.0 (1.0)	0.000
∑ ω3	4.3 (1.3)	4.2 (1.2)	4.4 (1.4)	0.003

**P* values are based on independent *t*-test comparing boys and girls, ns:non significant, [#] rounded values
FA:Fatty acids; LCPUFA:Long-chain polyunsaturated FA, MUFA:Monounsaturated FA; PUFA: Polyunsaturated FA; SD:Standard deviation; SFA:Saturated FA.

Table 6.3a: Gender-specific fatty acid ratios based on individual fatty acids (*conc%*) of serum phospholipids as mean (standard deviation) [#].

Fatty acid ratio	Total (n=1,024)	Boys (n=475)	Girls (n=549)	*p* value*
AA/LA	0.43 (0.11)	0.43 (0.11)	0.42 (0.11)	ns
(DHGLA+AA)/LA	0.56 (0.14)	0.57 (0.14)	0.55 (0.14)	0.006
EPA/ALA	3.76 (2.80)	3.86 (2.70)	3.68 (2.89)	ns
DHA/EPA	7.11 (3.64)	6.67 (3.35)	7.49 (3.68)	ns
AA/EPA	27.5 (18.9)	27.1 (18.5)	27.8 (19.3)	ns
LA/ALA	199 (103)	202 (102)	197 (103)	ns
AA/(EPA+DHA)	3.31 (1.65)	3.49 (1.87)	3.15 (1.41)	ns
ω6/ω3	12.0 (4.9)	12.5 (5.4)	11.6 (4.5)	ns
P/S	0.79 (0.06)	0.79 (0.06)	0.79 (0.06)	ns
M/S	0.20 (0.03)	0.20 (0.03)	0.20 (0.03)	ns

**P* values are based on independent *t*-test comparing boys and girls, *ns:non significant*.

Table 6.3b: Gender-specific fatty acid ratios based on individual fatty acids (*μmol/L*) of serum phospholipids as mean (standard deviation) [#].

Fatty acid ratio	Total (n=1,024)	Boys (n=475)	Girls (n=549)	*p* value*
AA/LA	0.43 (0.11)	0.43 (0.11)	0.42 (0.12)	ns
(DHGLA+AA)/LA	0.56 (0.14)	0.57 (0.14)	0.55 (0.14)	ns
EPA/ALA	3.76 (2.80)	3.86 (2.70)	3.68 (2.89)	ns
DHA/EPA	7.11 (3.64)	6.67 (3.55)	7.49 (3.68)	ns
AA/EPA	27.5 (18.9)	27.1 (18.5)	27.8 (19.3)	ns
LA/ALA	199 (103)	202 (102)	197 (103)	ns
AA/(EPA+DHA)	3.31 (1.65)	3.49 (1.87)	3.15 (1.41)	0.022
ω6/ω3	12.0 (4.9)	12.5 (5.4)	11.6 (4.5)	0.000
P/S	0.79 (0.06)	0.79 (0.06)	0.79 (0.06)	ns
M/S	0.20 (0.03)	0.20 (0.03)	0.20 (0.03)	ns

**P* values are based on independent *t*-test comparing boys and girls, *ns:non significant*. [#] rounded values
AA:Arachidonic acid, ALA:α-linolenic acid, DHA:Docosahexaenoic acid, DHGLA:Dihomo-γ-linolenic acid, EPA:Eicosapentaenoic acid, LA:Linoleic acid, M:Monounsaturates, P:Polyunsaturates, S:Saturates,

Table 6.3c: Gender-specific fatty acid ratios based on individual fatty acids (*area%*) of serum phospholipids as mean (standard deviation) [#].

Fatty acid ratio	Total (n=1,024)	Boys (n=475)	Girls (n=549)	*p* value*
AA/LA	0.45 (0.12)	0.45 (0.12)	0.44 (0.12)	ns
(DHGLA+AA)/LA	0.58 (0.14)	0.59 (0.14)	0.58 (0.14)	0.003
EPA/ALA	4.01 (2.98)	4.12 (2.86)	3.92 (3.08)	ns
DHA/EPA	7.60 (3.84)	7.15 (3.74)	7.99 (3.89)	ns
AA/EPA	27.9 (19.2)	27.6 (18.9)	28.1 (19.4)	ns
LA/ALA	206 (105)	208 (106)	204 (105)	ns
AA/(EPA+DHA)	3.16 (1.63)	3.34 (1.87)	3.01 (1.38)	0.000
ω6/ω3	9.1 (3.0)	9.3 (3.2)	8.9 (2.9)	0.008
P/S	0.88 (0.06)	0.88 (0.06)	0.88 (0.06)	ns
M/S	0.33 (0.05)	0.33 (0.06)	0.32 (0.05)	ns

**P* values are based on independent *t*-test comparing boys and girls, *ns:non significant.*

Table 6.3d: Gender-specific comparisons of fatty acid ratios based on *area%* and *conc%* of serum phospholipids as mean (standard deviation) [#].

Fatty acid ratio	Boys (n=475)		Girls (n=549)	
	area%	*conc%*	*area%*	*conc%*
AA/LA	0.45 (0.12)	0.43 (0.11) [1]	0.44 (0.12)	0.42 (0.11) [1]
(DHGLA+AA)/LA	0.58 (0.14)	0.57 (0.14) [1]	0.58 (0.14)	0.55 (0.14) [1]
EPA/ALA	4.01 (2.98)	3.86 (2.70) [1]	3.92 (3.08)	3.68 (2.89) [1]
DHA/EPA	7.60 (3.84)	6.67 (3.35)	7.99 (3.89)	7.49 (3.68)
AA/EPA	27.9 (19.2)	27.1 (18.5)	28.1 (19.4)	27.8 (19.3)
LA/ALA	206 (105)	202 (102) [1]	204 (105)	197 (103) [1]
AA/(EPA+DHA)	3.16 (1.63)	3.49 (1.87)	3.01 (1.38)	3.15 (1.41)
ω6/ω3	9.1 (3.0)	12.5 (5.4) [1]	8.9 (2.9)	11.6 (4.5) [1]
P/S	0.88 (0.06)	0.79 (0.06) [1]	0.88 (0.06)	0.79 (0.06) [1]
M/S	0.33 (0.05)	0.20 (0.03) [1]	0.32 (0.05)	0.20 (0.03) [1]

[1]*P* values are based on paired *t*-test with p=0.000 between *conc%* and *area%* of boys or girls. [#] rounded values
AA:Arachidonic acid, ALA:α-linolenic acid, DHA:Docosahexaenoic acid, DHGLA:Dihomo-γ-linolenic acid, EPA:Eicosapentaenoic acid, LA:Linoleic acid, M:Monounsaturates, P:Polyunsaturates, S:Saturates,

Table 6.4a: Fatty acid ratios (based on *conc%*) associated to body mass index in boys as mean (standard deviation)[#].

Fatty acid ratio	Boys				
	Body mass index				
	Underweight (n=25)	**Normal weight (n=332)**	**Overweight (n=84)**	**Obesity (n=34)**	***p*-value***
AA/LA	0.39 (0.08)	0.42 (0.11)	0.45 (0.12)	0.48 (0.15)	ns
(DHGLA+AA)/LA	0.52 (0.10)	0.55 (0.13)	0.60 (0.15)	0.64 (0.18)	ns
EPA/ALA	3.12 (1.80)	3.75 (2.75)	4.48 (2.89)	3.97 (2.03)	ns
DHA/EPA	7.86 (3.96)	6.56 (3.49)	6.86 (3.81)	6.43 (3.11)	ns
AA/EPA	34.6 (26.4)	26.6 (18.5)	26.4 (16.4)	28.4 (15.2)	ns
LA/ALA	209 (93)	194 (101)	225 (108)	215 (103)	ns
AA/(EPA+DHA)	3.83 (2.12)	3.43 (1.61)	3.31 (1.08)[3]	2.94 (1.18)	0.029
ω6/ω3	14.1 (6.1)[2]	12.4 (5.1)	11.8 (3.4)	14.3 (9.7)	0.017
P/S	0.80 (0.07)	0.79 (0.06)	0.79 (0.06)	0.79 (0.05)	ns
M/S	0.19 (0.03)	0.20 (0.04)	0.20 (0.03)	0.20 (0.03)	ns

**P* values are based on the ANCOVA *F*-test including maturity, age, centre, body mass index, body fat, and fat free mass as independent variables, with leptin and insulin as covariates. *ns:non significant.*
Bonferroni *post hoc test*: [1] $p<0.05$ compared to normal weight; [2] $p<0.01$ compared to normal weight; [3] $p<0.05$ compared to obesity.

[#] rounded values

AA:Arachidonic acid, ALA:α-linolenic acid, DHA:Docosahexaenoic acid, DHGLA:Dihomo-γ-linolenic acid, EPA:Eicosapentaenoic acid, LA:Linoleic acid, M:Monounsaturates, P:Polyunsaturates, S:Saturates,

Table 6.4b: Fatty acid ratios (based on *conc%*) associated to body mass index in girls as mean (standard deviation)[#].

Fatty acid ratio	Girls				
	Body mass index				
	Underweight (n=33)	**Normal weight (n=409)**	**Overweight (n=87)**	**Obesity (n=22)**	***p*-value***
AA/LA	0.37 (0.11)	0.41 (0.11)[3]	0.48 (0.11)	0.51 (0.12)	0.001
(DHGLA+AA)/LA	0.50 (0.12)	0.54 (0.13)[2]	0.62 (0.13)	0.65 (0.16)	0.031
EPA/ALA	3.46 (2.38)	3.49 (2.34)	3.86 (3.59)	6.76 (6.28)	ns
DHA/EPA	7.13 (3.24)	7.63 (3.85)	7.34 (3.20)	6.04 (2.03)	ns
AA/EPA	25.1 (16.4)	28.2 (20.4)	28.1 (15.9)	24.0 (11.3)	ns
LA/ALA	186 (109)	197 (102)	193 (91)	244 (138)	ns
AA/(EPA+DHA)	2.94 (1.18)	3.09 (1.23)	3.45 (2.16)	3.31 (0.98)	ns
ω6/ω3	11.6 (4.3)	11.6 (4.2)	11.5 (5.8)	11.1 (4.0)	ns
P/S	0.77 (0.07)	0.80 (0.06)	0.80 (0.05)	0.77 (0.05)	ns
M/S	0.20 (0.04)	0.20 (0.03)	0.20 (0.03)	0.19 (0.04)	ns

**P* values are based on the ANCOVA *F*-test including maturity, age, centre, body mass index, body fat, and fat free mass as independent variables, with leptin and insulin as covariates. *ns:non significant.*
Bonferroni *post hoc test*: [1] $p<0.05$ compared to obesity;[2] $p<0.05$ compared to overweight;
[3] $p<0.001$ compared to overweight.

[#] rounded values

AA:Arachidonic acid, ALA:α-linolenic acid, DHA:Docosahexaenoic acid, DHGLA:Dihomo-γ-linolenic acid, EPA:Eicosapentaenoic acid, LA:Linoleic acid, M:Monounsaturates, P:Polyunsaturates, S:Saturates,

Table 6.4c: Fatty acid ratios (based on *area%*) associated to sexual maturation in boys as mean (standard deviation)[#].

Fatty acid ratio	Boys					
	Sexual maturation					
	Tanner stage I (n=7)	Tanner stage II (n=33)	Tanner stage III (n=82)	Tanner stage IV (n=172)	Tanner stage V (n=122)	*p*-value*
AA/LA	0.54 (0.15)	0.47 (0.09)	0.43 (0.11)	0.45 (0.12)	0.45 (0.12)	ns
(DHGLA+AA)/LA	0.72 (0.21)	0.62 (0.12)	0.56 (0.13)	0.59 (0.14)	0.58 (0.14)	0.026
EPA/ALA	5.03 (3.99)	4.26 (1.81)[1]	3.86 (2.80)	4.07 (2.74)	4.16 (3.13)	0.027
DHA/EPA	6.59 (2.76)	5.17 (1.72)[2-4]	7.21 (3.86)	6.75 (3.42)	7.78 (4.17)	0.005
AA/EPA	23.83 (10.37)	19.92 (8.64)[2,3,5]	27.1 (19.7)	25.2 (16.9)	31.1 (22.5)	0.005
LA/ALA	176 (93)	172 (85)	205 (115)	190 (102)[5]	228 (98)	0.010
AA/(EPA+DHA)	3.14 (1.11)	3.17 (0.89)	3.25 (1.53)	3.12 (1.01)	3.34 (1.19)	ns
ω6/ω3	8.5 (3.0)	8.7 (2.5)	9.5 (3.3)	8.9 (2.6)	9.6 (2.8)	ns
P/S	0.84 (0.05)	0.88 (0.05)	0.87 (0.06)	0.88 (0.06)	0.88 (0.07)	ns
M/S	0.28 (0.09)	0.32 (0.03)	0.33 (0.04)	0.34 (0.06)	0.32 (0.05)	ns

[#] rounded values **P* values are based on the ANCOVA *F*-test including maturity, age, centre, body mass index, body fat, and fat free mass as independent variables, with leptin and insulin as covariates. *ns:non significant*.
Bonferroni *post hoc test*: [1] $p<0.05$ compared to Tanner stage IV;[2] $p<0.01$ compared to Tanner stage III; [3] $p<0.01$ compared to Tanner stage IV;
[4] $p<0.01$ compared to Tanner stage V; [5] $p<0.05$ compared to Tanner stage V.
AA:Arachidonic acid, ALA:α-linolenic acid, DHA:Docosahexaenoic acid, DHGLA:Dihomo-γ-linolenic acid, EPA:Eicosapentaenoic acid, LA:Linoleic acid, M:Monounsaturates, P:Polyunsaturates, S:Saturates,

Table 6.4d: Fatty acid ratios (based on *area%*) associated to body mass index in girls as mean (standard deviation)[#].

Fatty acid ratio	Girls				
	Body mass index				
	Underweight (n=33)	**Normal weight (n=409)**	**Overweight (n=87)**	**Obesity (n=22)**	***p*-value***
AA/LA	0.40 (0.12)	0.43 (0.12)[1]	0.50 (0.11)	0.53 (0.13)	0.000
(DHGLA+AA)/LA	0.52 (0.13)	0.56 (0.13)[2]	0.65 (0.13)	0.68 (0.16)	0.025
EPA/ALA	3.67 (2.50)	3.72 (2.48)	4.10 (3.87)	7.21 (6.69)	ns
DHA/EPA	7.65 (3.46)	8.14 (4.07)[3]	7.83 (3.43)[3]	6.46 (2.15)	0.021
AA/EPA	25.3 (16.4)	28.5 (20.6)[4]	28.3 (16.0)[4]	24.2 (11.2)	0.004
LA/ALA	191 (113)	203 (104)	198 (94)	253 (143)	ns
AA/(EPA+DHA)	2.81 (1.12)	2.95 (1.18)	3.30 (2.15)	3.16 (0.93)	ns
ω6/ω3	8.7 (3.0)	8.9 (2.9)	8.8 (2.6)	8.7 (2.9)	ns
P/S	0.85 (0.07)	0.88 (0.06)	0.88 (0.05)	0.85 (0.06)	ns
M/S	0.33 (0.06)	0.32 (0.05)	0.32 (0.05)	0.31 (0.07)	0.040

[#] rounded values

**P* values are based on the ANCOVA *F*-test including maturity, age, centre, body mass index, body fat, and fat free mass as independent variables, with leptin and insulin as covariates. *ns:non significant*.
Bonferroni *post hoc test*: [1] $p<0.001$ compared to overweight; [2] $p<0.05$ compared to overweight;
[3] $p<0.05$ compared to obesity; [4] $p<0.01$ compared to obesity.

AA:Arachidonic acid, ALA:α-linolenic acid, DHA:Docosahexaenoic acid, DHGLA:Dihomo-γ-linolenic acid, EPA:Eicosapentaenoic acid, LA:Linoleic acid, M:Monounsaturates, P:Polyunsaturates, S:Saturates,

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7 Individual serum phospholipid fatty acids and their association with lipid and lipoprotein concentrations in European adolescents

Short Summary

Background: The crucial role of lipids and lipoproteins in the development of CHD and atherosclerosis is well established and the origin of atherosclerosis is likely placed in childhood and adolescence. Fatty acids are proposed to have cholesterolemic effects on several blood lipids, suggesting SFA as hypercholesterolemic and PUFA as hypocholesterolemic compared to SFA. However, those studies are based on dietary FA intake data of adults and less is known about the association between serum FA concentrations measured in PL and blood lipid concentrations in healthy adolescents.

Objective: To investigate the influence of individual serum FA (C16:0, C18:0, C18:1ω9c, LA, ALA, AA, EPA, and DHA) on blood lipid concentrations (i.e. TC, HDL, LDL, TG, Lp(a), apo A-1, and apo B) in healthy European adolescents.

Results: The effect of individual FA (C16:0, C18:0, C18:1ω9c, LA, ALA, AA, EPA, and DHA) on several lipid and lipoprotein concentrations was investigated in 1,024 subjects (475 boys and 549 girls). Multiple linear regression analysis has been used. As main cause of variance the independent variables age, maturity, BMI, and the lipids (TG, TC, HDL, LDL, Lp(a), apo A-1, and apo B) except the lipid parameter, which has been defined as dependent variable (e.g. TG), have been included. Additionally, one FA (i.e. C16:0, C18:0, C18:1ω9c, LA, ALA, AA, EPA, or DHA) has been included as predictor in the current model. When including FA in the regression model, TG were mainly influenced by C16:0 and C18:0 (R^2 *change* 0.07 in boys and 0.04 in girls). The influence of the selected FA on cholesterol-containing lipoproteins can be neglected (R^2 *change* <0.004).

Conclusion: The selected serum FA were associated to a minor degree with the lipid and lipoprotein concentrations of healthy European adolescents. Thus, it might be interesting to focus on other nutrients that accompany dietary fat intake (e.g. fat soluble vitamins, plant sterols, polyphenols) as well as other nutrients in the diet (e.g. dietary fibres, carbohydrates, proteins) to explain the effect of dietary FA observed in other studies.

Introduction

Blood lipid (e.g. cholesterol) and lipoprotein (e.g. HDL, LDL) patterns play a qualitative and quantitative role in the development of atherosclerosis and CHD [1-5]. It is suggested that the beginning of atherosclerosis is placed in childhood and adolescence [6-8], where crucial metabolic changes (e.g. within lipid patterns) happen because of rapid growth and development.

Current research suggests cholesterolemic effects of FA, more precisely, a cholesterol increase caused by higher dietary intakes of SFA, whereas dietary PUFA decrease the cholesterol content of several lipoproteins compared to SFA [9, 10].

However, the cholesterolemic properties of dietary FA were investigated only in adults who were fed special diets focused on lipid-lowering effects [9, 10]. Beside that, study results, which found cholesterolemic effects of FA, are based only on dietary intake data. This data is critical for two reasons: (a) dietary intake data does not reflect the exact FA status of the body and (b) the data may be biased by the used assessment methods including questionnaires and food composition tables. These methods are still insufficient in assessing the exact dietary FA intake because of misreporting of the participants and lacking information about availability and content of individual FA in foods provided by the used food composition tables [11-13]. Furthermore, the intake of other dietary components may also lead to hypocholesterolemic effects and thus bias those mentioned findings, for instance the intake of dietary fibres [14, 15] or plant sterols [16, 17].

Consequently, it is important to investigate the association between serum FA and blood lipid concentrations to exclude those uncertainties.

In the frame of the HELENA Study, the serum FA profile of PL in an adolescent group was assessed. Serum PL reflect the FA content of the diet for at least a few days [18, 19]. Further, absolute concentrations instead of relative proportions were used. This approach enables the use of FA, which are independent of other FA measurements of the same FA profile, like it is present in percentaged profiles. The aim of the chapter was to assess the variability caused by selected individual FA concentration of C16:0, C18:0, C18:1ω9c, LA, ALA, AA, EPA, and DHA on TG, TC, HDL, LDL, apo A-1, apo B, and Lp(a) concentrations.

Subjects and Methods

A detailed description of study design, subject recruitment, medical examination, blood sampling, sample treatment, biochemical analysis, and evaluation procedures can be found in *chapter 3* (*General Methodology*).

Statistics

All statistical analysis procedures were carried out gender-specific using Statistical Package for Social Sciences version 17.0 for Windows (SPSS Inc., Chicago, Illinois, USA). Blood analytes were skewed and logarithmically transformated before statistical analysis. Basic characteristics were compared using independent *t*-tests (metric variables) and *chi square* test (categorical variables). Correlations were made using Pearson product-moment correlations (*r*) with 2-tailed significance between lipids, lipoproteins, and the selected FA. The association of C16:0, C18:0, C18:1ω9c, LA, ALA, AA, EPA, and DHA with lipid parameters were analysed comparing two linear regression models. As main cause of variance, model 1 included the independent variables age, maturity, BMI, and the lipid parameters of TG, TC, HDL, LDL, Lp(a), apo A-1, and apo B, except for the lipid parameter that was chosen as dependent variable in the current model. This model was compared with model 2 including the variables of model 1 additionally to one of the selected FA (predictors) of interest. The referring tables highlight the following indices to describe the results of the linear regression model. The adjusted coefficient of determination (R^2) explains the proportion of the variance in a dependent variable at a significant level (through a significance test of R^2) considering the complexity of the model. The standardized coefficient β is the average amount that the dependent variable increases when the independent variable increases one SD and other independent variables are held constant. The partial correlation measures the degree of association between the lipid variable and the selected FA when the other independent variables are removed. R^2 *change* was used to indicate the variability between the two models caused by the inclusion of the selected individual FA. Generally, *p* values <0.05 were considered as statistically significant.

Results

Serum FA data was available for 1,024 adolescent subjects (475 boys and 549 girls) within the HELENA-CSS. Basic characteristics of the study population are presented in table 7.1. Fasting blood lipid concentrations were significantly higher in girls than in boys as well as most FA. Only oleic acid and EPA failed to reach statistical significance between genders.

In table 7.2a, Pearsons correlations coefficients between FA and lipids/ lipoproteins in boys can be found. All FA correlated significantly with the listed lipids and lipoproteins except for Lp(a). Girls showed the same pattern as indicated in table 7.2b.

Results of the multiple linear regression analyses are shown in table 7.3a for TG, in table 7.3b for TC, in table 7.3c for HDL, in table 7.3d for LDL, in table 7.3e for Lp(a), in table 7.3f for apo A-1, and in table 7.3g for apo B.

For boys, the variables of model 1 (i.e. age, maturity, BMI, HDL, LDL, TC, Lp(a), apo A-1, and apo B) explained 44.7% of the variance of the TG concentrations. The strongest association, when including single FA in the model, was found between TG concentrations and C16:0 and C18:0. Palmitic acid, C18:0 as well as C18:1ω9c accounted for an additional change of 7.1%, 7.3%, and 3.5% of the adjusted R^2. The other FA (LA, ALA, EPA, DHA, and AA) led to a R^2 *change* of <2.3% (table 7.3a). With respect to TC, LDL, and HDL the adjusted R^2 of the models were 0.940, 0.878, and 0.937. The inclusion of FA in any of those models changed the results minimally. The selected FA were associated with changes of R^2 of approx. ≤0.3% of the TC, LDL, and HDL regression models (tables 7.3b-d). The inclusion of FA in the Lp(a) model failed to reach any statistical significance in boys. Further, age, maturity, BMI, HDL, LDL, TC, TG, apo A-1, and apo B accounted for 11.8% of the variance of Lp(a) concentrations (table 7.3e). The R^2 *change* of the apo regression models indicated an association of <0.5% by adding one FA (C16:0, C18:0, C18:1ω9c, LA, ALA, AA, EPA, or DHA) to the models. Generally, the adjusted R^2 of the models including age, maturity, BMI, HDL, LDL, TC, TG, Lp(a), and apo A-1 or apo B accounted for 77% and 88% of the variance of the apo A-1 and B concentrations (tables 7.3f and 7.3g).

For girls, the adjusted R^2 of model 1 (i.e. age, maturity, BMI, HDL, LDL, TC, Lp(a), apo A-1, and apo B) accounted for 47.6% of the variance of the TG concentrations. The strongest association, when including single FA in the model, was found between TG concentrations and C16:0 and C18:0. Palmitic acid and C18:0 achieved a R^2 *change* of 4.7% and 4.1% in the second model. The other FA (LA, ALA, and C18:1ω9c) accounted for an additional increase of <2.5% of the adjusted R^2 (table 7.3a). The models with TC, LDL, and HDL as dependent

variables showed adjusted R^2 values of 0.945, 0.936, and 0.890. The inclusion of selected FA in any of these models had minor effects and showed a R^2 *change* of ≤0.4% (tables 7.3b-d). In contrast to boys, the Lp(a) regression model of girls showed significant results for C16:0, C18:1ω9c, and AA. The highest R^2 *change* of 1.7% was observed when adding AA to the model. The other independent variables age, maturity, BMI, HDL, LDL, TC, TG, apo A-1, and apo B accounted for 4.4% of the variance of Lp(a) concentrations (table 7.3e). Differences in the adjusted R^2 values of the regression models using apo A-1 and B as dependent variable showed a R^2 *change* of ≤1.0% for the selected FA (C16:0, C18:0, C18:1ω9c, LA, ALA, AA, EPA, or DHA). Generally, the model including age, maturity, BMI, HDL, LDL, TC, TG, Lp(a), and apo A-1 or apo B accounted for 78% and 89% of the variance of the apo A-1 and B concentrations (tables 7.3f and 7.3g).

Discussion

Studies, which investigated cholesterolemic effects of FA, focused on dietary data in adulthood [9, 10, 20-25]. Fatty acids assessed by dietary recalls may be inaccurate when investigating cholesterolemic properties on blood lipid concentrations, because the data may be biased by misreporting and insufficient assessment of the true content of FA in foods. Biomarkers like serum FA of the PL fraction may be better parameters for dietary intake [26] and endogenous modifications and thus a more reliable approach.

A recently published study by Motoyama *et al.* [27] investigated the association of total serum ω6 and ω3 PUFA with lipids in three populations of men aged 40-49 years (whites n=261, Japanese n=285, and Japanese Americans n=212). Briefly, they made tertile groups of ω6 and ω3 PUFA for each population and compared age- and multivariable-adjusted tertile-specific concentrations of lipids (TG, LDL and HDL). Serum ω6 and ω3 PUFA were significantly inversely associated with TG levels across all three populations. Further, ω6 PUFA were significantly positively associated with HDL in whites and Japanese whereas EPA was significantly positively associated with HDL in whites. A significantly positive association of ω3 PUFA with HDL was found in Japanese. Both ω6 and ω3 PUFA were positively associated with LDL in Japanese but not in whites or Japanese Americans. However, whether these results are comparable with the current findings in European adolescents is questionable. Tertile groups were not applied in the HELENA Study, and whether the metabolism of adolescents follow similar metabolic patterns as observed in adults

is not clear. The few studies that found associations of serum FA and cholesterol levels in youth are solely based on the comparison of different TC cut-offs with FA compositions [28] or their correlations [29]. These studies do not allow a final evaluation of the cholesterolemic effects of serum FA and are not comparable with the present approach of a multiple linear regression analysis.

Within the HELENA Study, the inclusion of C16:0 or C18:0 into the linear regression model of TG led to the highest *R^2 change* values in both genders (table 7.3a). It might be reasonable to conclude that these two FA as a main part of dietary SFA in TG are also reflected in the serum TG levels and thus account for the variability of the TG levels. The deviating associations between boys and girls of the analysed FA with the TG concentrations may reflect differences in the dietary intake and/ or gender-specific discrepancies in FA metabolism [30]. Beside that, approx. 50% of the TG levels were explained by the parameters used in the present regression model (i.e. age, maturation, BMI, HDL, LDL, TC, Lp(a), apo B, and apo A-1) in both sexes (table 7.3a). Therefore, it can be suggested that other parameters, which were not part of the regression model, have a more important impact on TG levels (e.g. dietary fat or other nutrients).

The cholesterolemic effects of the selected FA (C16:0, C18:0, C18:1ω9c, LA, ALA, AA, EPA, and DHA) on TC, HDL, and LDL levels were minimal; the *R^2 change* value was <0.5% when adding one of those FA to the model. Due to the fact that apparently healthy adolescents were investigated, it may be possible that no cholesterolemic effects of those FA are observable. The HELENA participants showed acceptable lipid ranges and cholesterolemic effects of FA may possibly only be recognizable in adolescents with high lipid concentrations and/ or with higher FA concentrations. On the other hand, the serum FA proportion may be more important in regulating the cholesterol pool than individual FA concentrations.

However, the literature suggests effects of individual dietary FA or FA classes [9, 10]. Cellular mechanisms of these cholesterolemic effects are not fully understood. Studies using cultured cell lines proposed modifications within the LDL receptor activity and altered acyl-CoA:cholesterol acyltransferase enzyme activities caused by individual FA supplementation, although these studies show inconsistent results [31, 32].

Lipoprotein(a) was hardly associated with FA in European boys and girls. The reason for the general low impact of FA on Lp(a) levels may be the strong link to apolipoprotein(a) genotypes and Kringle repeats, which mainly define Lp(a) variability [33]. Thus, Lp(a) concentrations are determined early in life, stay relatively constant over time, and are generally independent of other factors. This is supported by the fact that the variables of the

used regression models accounted solely for 12% and 5% of the variability of Lp(a) concentrations in boys and girls (table 7.3e).

In general, the present basic regression models (model 1) explained quite sufficiently the variance of the different blood parameters without adding individual FA, except for Lp(a) and TG. These findings indicate an essential impact of age, maturation, and BMI as well as interaction effects of the lipids and lipoproteins on each other. However, one has to keep in mind that other factors may also be important in the regulation of lipid concentrations. For instance, hormones, genetic variations or lifestyle parameters (e.g. physical activity) could be of interest, but were not included in the model. Further, dietary fat intake itself was not available at the time of analysis. Moreover, dietary fat intake data may weaken the current results, based on reliable biomarkers, due to misreporting of the participants.

Another important point is that prior study results are mainly obtained through substitutions of FA classes in the diet [10]. These sudden dietary changes in the FA profile lead to modifications of the serum FA composition, which may shift the serum cholesterol pattern of lipoproteins. Additionally, these serum FA changes may result in prompt inhibitions or activations of metabolic pathways as response of these sudden dietary changes, and thus influencing more factors than the FA content alone (e.g. altered gene expression due to FA interactions). This may also alter factors which are important for the regulation of the cholesterol metabolism. In the HELENA-CSS, no special diet was introduced and no dietary patterns were modified for the study purpose, thus shifted interactions between FA and metabolic pathways as a consequence of intended dietary changes were not expected. Accordingly, the expected influence of FA on the cholesterol pattern of lipoproteins may not be present as it may be observed in lipid profile of subjects with recently changed eating habits.

Another reasonable explanation may be the intake of other dietary components affecting the serum lipid concentration [14-17, 34]. These dietary components may accompany dietary fat intake (e.g. fat soluble vitamins, plant sterols, polyphenols) and thus are ingested simultaneously. They have also lipid-lowering properties [16, 17, 34], but these effects could be covered or incorrectly associated to FA, because of the contemporary dietary intake. For instance, the simultaneously intake of plant sterols or tocopherols with dietary PUFA may enhance the distribution of cholesterol towards the enterohepatic cholesterol efflux. Moreover, dietary fibres, carbohydrates, proteins, or unknown components may affect lipid concentrations as well [14, 35].

Conclusion

In the present adolescent population, the associations between selected serum FA measured in PL and serum TG, TC, HDL, LDL, apo A-1, apo B, and Lp(a) parameters are minimal. Therefore, studies in adults, which found an association between dietary FA intake and blood lipids, may have another explanation. Moreover, the metabolism of adolescents may not follow in similar metabolic patterns as observed in adults. Furthermore, the lipid metabolism may be too strightly regulated to expect detectable changes due to one single FA. Additionally, investigations may focus on other nutrients that accompany dietary fat intake (e.g. fat soluble vitamins, plant sterols, polyphenols) as well as on other nutrients in the diet (e.g. dietary fibres, carbohydrates, proteins) to clarify their influence on lipid concentrations.

Table 7.1: Basic characteristics of the study population as mean (standard deviation).

	Boys	**Girls**	***p* value***
n	475	549	
Age (years)	14.84 (1.21)	14.90 (1.16)	ns
Height (cm)	169.8 (9.8)	161.8 (7.0)	0.000
Weight (kg)	61.7 (13.7)	56.0 (10.2)	0.000
Body mass index (kg/m²)	21.3 (3.8)	21.3 (3.4)	ns
Tanner stage[1]	3.9 (1.0)	4.0 (0.8)	0.007[#]
Body fat (%)[2]	21.0 (10.5)	27.3 (7.6)	0.000
Fat free mass (kg)[3]	51.8 (9.6)	41.8 (7.0)	0.000
Triglycerides (mg/dL)[4]	64 (31)	73 (37)	0.000
Total cholesterol (mg/dL)[4]	154 (26)	167 (28)	0.000
HDL (mg/dL)[4]	53 (10)	57 (11)	0.000
LDL (mg/dL)[4]	91 (25)	98 (25)	0.000
Apolipoprotein A-1 (g/L)[5]	1.45 (0.21)	1.55 (0.23)	0.000
Apolipoprotein B (g/L)[5]	0.62 (0.15)	0.68 (0.16)	0.000
Lipoprotein(a) (mg/L)[5]	175 (219)	209 (258)	0.017
C16:0 (µmol/L)	1,136 (191)	1,222 (221)	0.000
C18:0 (µmol/L)	491 (99)	523 (97)	0.000
C18:1ω9c (µmol/L)	255 (73)	263 (78)	ns
C18:3ω3c (µmol/L)	5 (3)	6 (4)	0.003
C18:2ω6c (µmol/L)	814 (170)	880 (179)	0.000
C20:4ω6c (µmol/L)	345 (89)	363 (97)	0.000
C20:5ω3c (µmol/L)	18 (13)	19 (14)	ns
C22:6ω3c (µmol/L)	94 (38)	110 (40)	0.000

**p* values (2-tailed) are based on an independent *t*-test; [#] based on *Chi square* test; ns:non significant
[1]boys n=417, and girls n=494; [2]boys n=461, and girls n=529;
[3]boys n=469, and girls n=541; [4]boys n=475, and girls n=547;
[5]boys n= 462, and girls n=531.

HDL:High density lipoprotein cholesterol, LDL:Low density lipoprotein cholesterol.

Table 7.2a: Pearson correlation coefficients (*r*) of lipids, lipoproteins and fatty acids for boys.

	C16:0	C18:0	C18:1ω9c	C18:2ω6c	C18:3ω3c	C20:4ω6c	C20:5ω3c	C22:6ω3c
Triglycerides	r=0.377**	r=0.404**	r=0.267**	r=0.149**	r=0.169**	r=0.229**	r=0.176**	r=0.202**
Total cholesterol	r=0.636**	r=0.589**	r=0.294**	r=0.414**	r=0.276**	r=0.464**	r=0.310**	r=0.445**
HDL	r=0.338**	r=0.310**	r=0.173**	r=0.378**	r=0.218**	r=0.222**	r=0.229**	r=0.223**
LDL	r=0.478**	r=0.432**	r=0.203**	r=0.256**	r=0.171**	r=0.312**	r=0.213**	r=0.339**
Apo A-1	r=0.430**	r=0.438**	r=0.202**	r=0.440**	r=0.203**	r=0.318**	r=0.254**	r=0.249**
Apo B	r=0.470**	r=0.430*	r=0.215**	r=0.258**	r=0.139**	r=0.354**	r=0.178**	r=0.312**
Lipoprotein(a)	r=0.054	r=0.086	r=-0.021	r=0.069	r=-0.017	r=0.038	r=-0.030	r=0.054

Significance (2-tailed): *$p<0.05$ and **$p<0.01$
Apo:Apolipoprotein, HDL:High density lipoprotein cholesterol, LDL:Low density lipoprotein cholesterol

Table 7.2b: Pearson correlation coefficients (*r*) of lipids, lipoproteins and fatty acids for girls.

	C16:0	C18:0	C18:1ω9c	C18:2ω6c	C18:3ω3c	C20:4ω6c	C20:5ω3c	C22:6ω3c
Triglycerides	r=0.404**	r=0.351**	r=0.230**	r=0.210**	r=0.231**	r=0.277**	r=0.050	r=0.219**
Total cholesterol	r=0.634**	r=0.528**	r=0.371**	r=0.420**	r=0.293**	r=0.429**	r=0.285**	r=0.423**
HDL	r=0.402**	r=0.357**	r=0.292**	r=0.436**	r=0.227**	r=0.271**	r=0.183**	r=0.182**
LDL	r=0.424**	r=0.344**	r=0.199**	r=0.228**	r=0.154**	r=0.241**	r=0.175**	r=0.286**
Apo A-1	r=0.545**	r=0.447**	r=0.358**	r=0.494**	r=0.228**	r=0.397**	r=0.179**	r=0.260**
Apo B	r=0.426**	r=0.313**	r=0.179**	r=0.219**	r=0.095*	r=0.286**	r=0.097*	r=0.257**
Lipoprotein(a)	r=0.017	r=0.000	r=-0.060	r=-0.016	r=0.024	r=-0.045	r=0.041	r=0.056

Significance (2-tailed): *p<0.05 and **p<0.01
Apo:Apolipoprotein, HDL:High density lipoprotein cholesterol, LDL:Low density lipoprotein cholesterol

Table 7.3a: Gender-specific results of the multiple linear regression model with **triglyceride** concentrations as dependent variable in association with selected individual fatty acids as predictor of triglyceride concentrations.

	Boys				Girls			
	Adjusted R^2 for Model 1 0.447 (p=0.000)				Adjusted R^2 for Model 1 0.476 (p=0.000)			
Predictor	**Standardized coefficient β**	***p* value**	**Partial Correlation**	**R^2 Change***	**Standardized coefficient β**	***p* value**	**Partial Correlation**	**R^2 Change***
C16:0	0.364	0.000	0.363	0.071	0.319	0.000	0.304	0.047
C18:0	0.366	0.000	0.369	0.073	0.256	0.000	0.284	0.041
C18:1ω9c	0.199	0.000	0.254	0.035	0.103	0.006	0.126	0.008
C18:2ω6c	0.155	0.000	0.180	0.017	0.193	0.000	0.223	0.025
C18:3ω3c	0.163	0.000	0.206	0.023	0.180	0.000	0.222	0.025
C20:4ω6c	0.017	ns	0.019	0.000	0.051	ns	0.057	0.002
C20:5ω3c	0.133	0.001	0.167	0.015	-0.041	ns	-0.051	0.001
C22:6ω3c	0.115	0.007	0.134	0.010	0.008	ns	0.009	0.000

Based on multiple linear regression analysis: *Difference between Model 1 (including age, maturity, centre, body mass index, HDL, LDL, TC, Lp(a), apo A-1, and apo B) and Model 2 (including Model 1 plus one individual fatty acid as predictor); *ns:non significant.*
Apo:Apolipoprotein, HDL:High density lipoprotein cholesterol, LDL:Low density lipoprotein cholesterol, Lp(a):Lipoprotein(a), TC:Total cholesterol.

Table 7.3b: Gender-specific results of the multiple linear regression model with **total cholesterol** concentrations as dependent variable in association with selected individual fatty acids as predictor of total cholesterol concentrations.

	Boys				Girls			
	Adjusted R^2 for Model 1 0.940 (p=0.000)				Adjusted R^2 for Model 1 0.945 (p=0.000)			
Predictor	**Standardized coefficient β**	***p* value**	**Partial Correlation**	**R^2 Change***	**Standardized coefficient β**	***p* value**	**Partial Correlation**	**R^2 Change***
C16:0	0.021	ns	0.060	0.000	0.048	0.003	0.137	0.001
C18:0	0.018	ns	0.052	0.000	0.029	0.038	0.094	0.000
C18:1ω9c	-0.004	ns	-0.014	0.000	0.041	0.001	0.154	0.001
C18:2ω6c	-0.006	ns	-0.021	0.000	-0.012	ns	-0.042	0.000
C18:3ω3c	0.009	ns	0.034	0.000	0.040	0.001	0.150	0.001
C20:4ω6c	0.063	0.000	0.221	0.003	0.061	0.000	0.214	0.002
C20:5ω3c	-0.004	ns	-0.015	0.000	0.057	0.000	0.228	0.003
C22:6ω3c	0.024	ns	0.085	0.000	0.072	0.000	0.273	0.004

Based on multiple linear regression analysis: *Difference between Model 1 (including age, maturity, centre, body mass index, HDL, LDL, TG, Lp(a), apo A-1, and apo B) and Model 2 (including Model 1 plus one individual fatty acid as predictor); *ns:non significant.*
Apo:Apolipoprotein, HDL:High density lipoprotein cholesterol, LDL:Low density lipoprotein cholesterol, Lp(a):Lipoprotein(a), TG:Triglycerides.

Table 7.3c: Gender-specific results of the multiple linear regression model with **high density lipoprotein cholesterol** concentrations as dependent variable in association with selected individual fatty acids as predictor of high density lipoprotein cholesterol concentrations.

	Boys				**Girls**			
	Adjusted R^2 for Model 1 0.878 (p=0.000)				**Adjusted R^2 for Model 1 0.890 (p=0.000)**			
Predictor	**Standardized coefficient β**	***p* value**	**Partial Correlation**	**R^2 Change***	**Standardized coefficient β**	***p* value**	**Partial Correlation**	**R^2 Change***
C16:0	0.071	0.004	0.141	0.002	0.018	ns	0.036	0.000
C18:0	0.023	ns	0.046	0.000	0.028	ns	0.065	0.000
C18:1ω9c	0.045	0.015	0.120	0.002	-0.016	ns	-0.041	0.000
C18:2ω6c	0.049	0.015	0.121	0.002	0.067	0.000	0.167	0.003
C18:3ω3c	0.046	0.014	0.122	0.002	0.006	ns	0.015	0.000
C20:4ω6c	-0.046	0.028	-0.110	0.001	-0.036	ns	-0.089	0.001
C20:5ω3c	0.040	0.035	0.105	0.001	-0.047	0.004	-0.130	0.002
C22:6ω3c	0.026	ns	0.064	0.000	-0.065	0.000	-0.169	0.003

Based on multiple linear regression analysis: *Difference between Model 1 (including age, maturity, centre, body mass index, TG, LDL, TC, Lp(a), apo A-1, and apo B) and Model 2 (including Model 1 plus one individual fatty acid as predictor); *ns:non significant.*
Apo:Apolipoprotein, LDL:Low density lipoprotein cholesterol, Lp(a):Lipoprotein(a), TC:Total cholesterol, TG:Triglycerides.

Table 7.3d: Gender-specific results of the multiple linear regression model with **low density lipoprotein cholesterol** concentrations as dependent variable in association with selected individual fatty acids as predictor of low density lipoprotein cholesterol concentrations.

	Boys				**Girls**			
	Adjusted R^2 for Model 1 0.937 (p=0.000)				**Adjusted R^2 for Model 1 0.936 (p=0.000)**			
Predictor	**Standardized coefficient β**	***p* value**	**Partial Correlation**	**R^2 Change***	**Standardized coefficient β**	***p* value**	**Partial Correlation**	**R^2 Change***
C16:0	0.025	ns	0.069	0.000	0.002	ns	0.006	0.000
C18:0	0.016	ns	0.043	0.000	0.020	ns	0.060	0.000
C18:1ω9c	0.004	ns	0.015	0.000	-0.014	ns	-0.049	0.000
C18:2ω6c	0.018	ns	0.060	0.000	0.022	ns	0.070	0.000
C18:3ω3c	0.014	ns	0.051	0.000	0.005	ns	0.016	0.000
C20:4ω6c	-0.059	0.000	-0.199	0.002	-0.057	0.000	-0.185	0.002
C20:5ω3c	0.027	0.044	0.100	0.001	-0.003	ns	-0.011	0.000
C22:6ω3c	0.015	ns	0.052	0.000	-0.025	ns	-0.085	0.000

Based on multiple linear regression analysis: *Difference between Model 1 (including age, maturity, centre, body mass index, HDL, TG, TC, Lp(a), apo A-1, and apo B) and Model 2 (including Model 1 plus one individual fatty acid as predictor); *ns:non significant.*
Apo:Apolipoprotein, HDL:High density lipoprotein cholesterol, Lp(a):Lipoprotein(a), TC:Total cholesterol, TG:Triglycerides.

Table 7.3e: Gender-specific results of the multiple linear regression model with **lipoprotein(a)** concentrations as dependent variable in association with selected individual fatty acids as predictor of lipoprotein(a) concentrations.

	Boys				Girls			
	Adjusted R^2 for Model 1 0.118 (p=0.000)				Adjusted R^2 for Model 1 0.044 (p=0.000)			
Predictor	**Standardized coefficient β**	***p* value**	**Partial Correlation**	**R^2 Change***	**Standardized coefficient β**	***p* value**	**Partial Correlation**	**R^2 Change***
C16:0	-0.119	ns	-0.088	0.007	-0.150	0.025	-0.102	0.010
C18:0	-0.082	ns	-0.061	0.003	-0.097	ns	-0.077	0.006
C18:1ω9c	-0.074	ns	-0.073	0.005	-0.118	0.019	-0.106	0.011
C18:2ω6c	-0.028	ns	-0.026	0.001	-0.101	ns	-0.084	0.007
C18:3ω3c	-0.017	ns	-0.017	0.000	0.042	ns	0.037	0.001
C20:4ω6c	-0.098	ns	-0.088	0.007	-0.164	0.003	-0.136	0.017
C20:5ω3c	-0.075	ns	-0.074	0.005	0.049	ns	0.046	0.002
C22:6ω3c	-0.026	ns	-0.024	0.000	0.027	ns	0.024	0.001

Based on multiple linear regression analysis: *Difference between Model 1 (including age, maturity, centre, body mass index, HDL, LDL, TC, TG, apo A-1, and apo B) and Model 2 (including Model 1 plus one individual fatty acid as predictor); *ns:non significant.*
Apo:Apolipoprotein, HDL:High density lipoprotein cholesterol, LDL:Low density lipoprotein cholesterol, TC:Total cholesterol, TG:Triglycerides.

Table 7.3f: Gender-specific results of the multiple linear regression model with **apolipoprotein A-1** concentrations as dependent variable in association with selected individual fatty acids as predictor of apolipoprotein A-1 concentrations.

	Boys				Girls			
	Adjusted R^2 for Model 1 0.774 (p=0.000)				**Adjusted R^2 for Model 1 0.778 (p=0.000)**			
Predictor	**Standardized coefficient β**	***p* value**	**Partial Correlation**	**R^2 Change***	**Standardized coefficient β**	***p* value**	**Partial Correlation**	**R^2 Change***
C16:0	0.082	0.015	0.121	0.003	0.153	0.000	0.213	0.010
C18:0	0.153	0.000	0.230	0.012	0.067	0.018	0.108	0.003
C18:1ω9c	-0.002	ns	-0.004	0.000	0.057	0.022	0.104	0.002
C18:2ω6c	0.074	0.007	0.133	0.004	0.061	0.022	0.104	0.002
C18:3ω3c	-0.028	ns	-0.054	0.001	-0.026	ns	-0.047	0.000
C20:4ω6c	0.088	0.002	0.157	0.005	0.103	0.000	0.176	0.007
C20:5ω3c	0.020	ns	0.039	0.000	0.031	ns	0.058	0.001
C22:6ω3c	0.004	ns	0.007	0.000	0.062	0.015	0.111	0.003

Based on multiple linear regression analysis: *Difference between Model 1 (including age, maturity, centre, body mass index, HDL, LDL, TC, Lp(a), TG, and apo B) and Model 2 (including Model 1 plus one individual fatty acid as predictor); *ns:non significant.*
Apo:Apolipoprotein, HDL:High density lipoprotein cholesterol, LDL:Low density lipoprotein cholesterol, Lp(a):Lipoprotein(a), TC:Total cholesterol, TG:Triglycerides.

Table 7.3g: Gender-specific results of the multiple linear regression model with **apolipoprotein B** concentrations as dependent variable in association with selected individual fatty acids as predictor of apolipoprotein B concentrations.

	Boys				Girls			
	Adjusted R^2 for Model 1 0.875 (p=0.000)				Adjusted R^2 for Model 1 0.886 (p=0.000)			
Predictor	**Standardized coefficient *β***	***p* value**	**Partial Correlation**	**R^2 Change***	**Standardized coefficient *β***	***p* value**	**Partial Correlation**	**R^2 Change***
C16:0	0.014	ns	0.028	0.000	0.002	ns	0.003	0.000
C18:0	0.014	ns	0.028	0.000	-0.035	ns	-0.079	0.001
C18:1ω9c	0.020	ns	0.052	0.000	-0.026	ns	-0.067	0.000
C18:2ω6c	0.016	ns	0.037	0.000	0.017	ns	0.041	0.000
C18:3ω3c	-0.028	ns	-0.072	0.001	-0.071	0.000	-0.187	0.004
C20:4ω6c	0.054	0.010	0.128	0.002	0.031	ns	0.075	0.001
C20:5ω3c	-0.023	ns	-0.061	0.000	-0.082	0.000	-0.227	0.006
C22:6ω3c	-0.222	ns	-0.053	0.000	-0.054	0.002	-0.138	0.002

Based on multiple linear regression analysis: *Difference between Model 1 (including age, maturity, centre, body mass index, HDL, LDL, TC, TG, Lp(a), and apo A-1) and Model 2 (including Model 1 plus one individual fatty acid as predictor); *ns:non significant.*
Apo:Apolipoprotein, HDL:High density lipoprotein cholesterol, LDL:Low density lipoprotein cholesterol, Lp(a):Lipoprotein(a), TC:Total cholesterol, TG:Triglycerides.

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8 General Discussion

The focus of this thesis is set on the lipid status of adolescents across Europe and their associations with several non-communicable (i.e. gender, age, and sexual maturity) and communicable (i.e. BMI, BF, and FFM) parameters. In the previous chapters lipids results (e.g. for cholesterol, TG, or FA) were described separately according to the association with those non-communicable and communicable factors. Within this chapter, the discussion will sum up the relationships between the non-communicable or communicable factors and the complete measured lipid profile of European adolescents. Further, the limitations and strengths of the HELENA Study, the different FA calculation methods, and the application of reference data will be discussed.

The first non-communicable factor that will be discussed here is *gender*. In HELENA, gender differences within the lipid variables were the most striking results. Compared to boys, girls showed higher lipid levels measured in routine diagnostics (e.g. TC, HDL, LDL, or TG), as highlighted in table 4.1. Moreover, nearly all absolute FA concentrations, except for C15:1ω5c, C17:1ω7c, C18:1ω9c, C18:3ω6c, C20:3ω6c, and EPA, were higher in girls than in boys (table 5.3). This finding may be related to the higher levels of routine lipid parameters observed in girls, and may point to higher amounts of and/ or larger transport lipoproteins in females. However, differences in the percentaged FA profile (*conc%*) between boys and girls are less obvious (table 5.4). Girls showed higher proportions of C20:1ω9c, DHA, and total ω3 FA, whereas total ω9 FA and in particular C18:1ω9c were decreased. The essential FA LA and ALA as well as EPA and AA did not differ between sexes. With respect to FA ratios, some gender-specific differences were found (i.e. [DHGLA+AA]/LA, AA/[EPA+DHA], and ω6/ω3) with higher ratios in boys, but these results were linked to the calculation method (for details see *chapter 6*). With regard to the lipid and lipoprotein ratios, only the mean TG/HDL ratio was significantly higher in girls compared to boys, whereas the other ratios (Apo B/Apo A-1, TC/HDL, LDL/HDL, Apo B/LDL, nonHDLC/HDL, and the AI) did not differ between genders. Generally, these findings show that gender differences exist, especially in the absolute values of blood lipids. Even though, these gender differences become less obvious in comparison with lipid and lipoprotein ratios, percentaged FA profiles, or FA ratios.

The results of the routine diagnostic lipid parameters are in accordance with one study [1], but not with others [2, 3]. Discrepancies may be a consequence of different sub-group classifications. The separation of boys and girls into different age ranges leads to different

group sizes, which may affect the study results. Further explanations may be the influence of country-specific eating patterns or genetic variability. Gender differences in FA profiles were not observed in other studies [4-7]. However, the present study population consists of a larger sample size and is more heterogeneous than the other study groups. Thus, it is likely that gender differences may be detected only in larger sample sizes or biased by the gender-specific food and FA intake of the participating countries [8]. Furthermore, most studies, which investigated FA ratios in adolescents/ children, did not separate between boys and girls [9, 10] or did not find gender-specific differences in their study groups [4, 5].

Gender differences between the clinical lipid parameters may be explained by changes in the sex hormone levels, especially during adolescence. This assumption is supported by findings in other study populations [11-13]. Gender-specific sex hormone levels may also contribute to the different results found in the FA profiles, because sex hormone concentrations may affect enzyme activities of the FA metabolism [14]. However, the impact of sex hormones on the FA composition is less investigated in adolescents. In HELENA, the measurement of sex hormones was not feasible, thus this point cannot be judged sufficiently with the available data. Nonetheless, if was feasible to judge the state of puberty of the HELENA participants according to their physical signs, which will be discussed later on. Moreover, gender differences in serum FA may be related to gender-specific eating patterns including different food choices of the adolescents. Especially the essential FA of the ω6 and ω3 family have to be provided by the normal diet. For instance, the intake of long-chain ω3 FA, found in fatty fish and seafood, may be higher in female adolescents compared to male adolescents. Those FA have been proposed as dietary intake markers of such food groups [8, 15, 16].

As a consequence of the gender-specific differences in the blood lipid concentrations, all other communicable and non-communicable determinants were discussed for boys and girls separately, which makes comparisons to other study results in some cases difficult.

Age is a non-communicable factor, which is commonly used in the literature, to categorize children/ adolescents into several age groups for later comparisons. In HELENA, age was associated with shifted TG levels in boys (table 4.3a), and, as a consequence, with changes in the male TG/HDL ratio. Further, the present study found associations of age with several FA (*conc%*) namely C16:0, C18:0, PUFA, ω3 FA, and *trans* FA in female subjects. In boys, a relation between age and C14:1ω5c, C18:0, C18:3ω6c, C20:2ω6c, C23:0, and SFA was present (table 5.5). Moreover, the absolute FA composition of boys differed in C14:1ω5c and C20:1ω9c due to age, whereas in girls differences in C16:0, C17:1ω7c, C20:1ω9c, C22:1ω9c, C22:2ω6c, C24:0, C24:1ω9c, MUFA, ω9 and ω3 FA were observed. By contrast, FA ratios

with regard to age categories differed only in girls. Changes in the EPA/ALA, ω6/ω3, and/ or M/S ratio have been shown, depending on the calculation method.

Higher TG levels with increasing age were also found in most [1, 17, 18] but not all [2] studies. Moreover, all mentioned studies found also age-dependent changes within the other lipid levels of boys and girls [1, 2, 17, 18]. By contrast, FA profiles were basically not analysed with a special focus on age [4, 5, 7, 9, 10, 19, 20], thus information concerning age and FA status is scarce. Only one known study used age groups to categorize their study population, but the study found no differences between the age groups 5-10 years, 10-15 years, and 20-26 years. Significant changes were only observed when comparing 20-26 year-olds with much younger age groups (i.e. 0-1 month or 1-12 months), for example in C18:1ω9c, AA, DHA and MUFA [6]. The association between FA ratios and age is even less investigated.

It can be suggested that the main reason for alterations in the lipid profile of adolescents, observed due to age, is associated with changed eating pattern. Moreover, it is more likely that the age-associated findings in the studies mentioned before are related to *maturity*. Age describes mainly the chronological development of individuals and is not a reliable indicator of physiological/ biological development per se. Adolescents can be in different pubertal stages, but having the same age. Thus, in studies using age as a criteria instead of pubertal stages to group adolescents, significant physiological changes in the lipid profile are incorrectly referred to age, even though the age-associated observations are addressed to puberty [21]. Other researchers confirmed the link between puberty/ sexual hormones and changes in lipid profiles [11, 22-25]. Therefore, the HELENA Study assessed beside chronological age also the biological age expressed as Tanner stages.

In the female HELENA population, there was an increase between Tanner stage IV and V for most lipid and lipoprotein ratios (Apo B/Apo A-1, TC/HDL, LDL/HDL, nonHDLC/HDL, and the AI), which was not apparent in males (table 4.4b). Further, boys showed associations between TC, HDL, and apo A-1 levels and maturity in a mostly inverse manner. Contrariwise, in girls, associations between TC, LDL, nonHDLC, and apo B levels and maturity were present, but without clear increase or decrease during maturity (table 4.4a). Relationships between maturity and the present FA composition were observed. In girls, FA values accounting for <1.0*conc%* of the FA profile, like C17:1ω7c, C20:0, C22:0, C23:0, and C24:1ω9c, differed during puberty. In boys, C17:1ω7c, ALA, C20:1ω9c, C20:2ω6c, EPA, C22:1ω9c, and ω9 FA were associated with sexual maturation (see table 5.6). Absolute FA values (ALA, EPA, and C20:1ω9c) decreased with progressing puberty in boys, whereas in

girls C20:0, C22:0, C22:1ω9c, C23:0, and C24:1ω9c decreased with higher Tanner stages. In boys, during maturity, the LA/ALA ratio based on *conc%* and *area%* calculations increased. Furthermore, the AA/EPA ratio estimated by FA in *area%* increased as well. The female FA profiles (*conc%* and *area%*) were not associated with maturity.

Previous studies showed inconsistent findings concerning routine lipid parameters and their ratios, probably due to sample size, classification of maturity, and geographic differences based on different genetic backgrounds, eating patterns and lifestyle habits [13, 26-29]. In fact, a decrease of HDL is seen as a consequence of higher testosterone levels in boys, compared to girls, leading to a reduction of apolipoproteins associated with HDL [24]. Moreover, maturity proceeds gender-specifically and thus hormone concentrations are different between sexes and lead to different lipid levels, body fat content and/ or fat allocation. However, the measurement of sex hormones was not feasible, making precise conclusions impossible.

None of the mentioned studies investigated the FA profile with respect to maturity; thus, no comparisons can be made [4, 5, 7, 9, 10, 19, 20]. In HELENA, the observations may be a consequence of increasing sex hormone concentrations during puberty and/ or altered dietary FA supply. Both possibilities could not be verified, because neither data on sex hormone status nor dietary FA intake data was available.

Body mass index, *percentage BF*, and *FFM* were chosen as communicable factors within this thesis. The most striking finding was that female clinical routine lipid parameters were associated with BMI alone, whereas male serum routine lipid parameters were only related to BF content (table 4.5a). Additionally, higher lipid and lipoprotein ratios (Apo B/Apo A-1, TC/HDL, TG/HDL, LDL/HDL, nonHDLC/HDL, and the AI) were observed in boys with high percentage BF (≥25%), whereas in girls those ratios were increased with higher BMI status. In the male participants HDL values were not significantly altered with respect to BMI status. By contrast, lower HDL levels were observed in boys with higher percentage BF. In girls, HDL decreased with increasing BMI, but this decrease was not observed according to percentage BF in girls. The absolute FA composition of girls showed associations between LA, *trans* FA, C18:1ω9c, and C21:0 and BMI. Oleic acid and LA were lower in obese and overweight subjects compared to girls with normal BMI. In this study, changes in the absolute FA profile with respect to BMI were only apparent in girls, not in boys. In the percentaged FA profile (*conc%*) underweight and/ or normal weight girls had higher levels of C18:1ω9c, LA and lower levels of AA and LCPUFA (i.e. C20:3ω6c, AA, EPA, and DHA) than their overweight and/ or obese counterparts. Contrariwise, BF as grouping factor was only related

to changes in the male FA profile (absolute and *conc%*). In the percentaged FA profile (*conc%*), higher levels of C18:1ω9c, *trans* FA and lower levels of DHA and C24:1ω9c were associated with boys having a BF content of <25%.

Generally, these findings indicate that higher fat/ weight status is associated with a more unfavourable lipid profile and an increased atherogenic risk. This was also observed in a Mexican study where the prevalence of different forms of dyslipidemia were significantly higher in obese and overweight than in normal weight boys and girls [3]. However, it is not clear why, with respect to body composition parameters, male lipid levels are associated with BF content only, whereas changes in female lipid parameters are related solely to BMI status. Differences may be obtained due to the assessment methods. Fat allocation shows gender-specific differences, which may be reflected in the different skinfold measurements [30, 31]. It may also be possible that the sub-group classification (two BF sub-groups *vs*. four BMI sub-groups) with different group sizes results in altered findings as a consequence of specific eating patterns or genetic differences in those sub-groups. The majority of studies investigated the FA composition related to obesity and reported no sex differences within the individual FA, thus they analysed both sexes together [4, 5, 9]. In HELENA, boys and girls were analysed separately making direct comparisons difficult. The present findings of the absolute FA composition were not apparent in the study population of Gil-Campos *et al.*, which may be a result of the mixed group design or the lower number of subjects in that study [32]. Fatty acid ratios are often implemented to detect differences in the FA content due to weight status [5, 9, 10]. Further, as estimates of desaturase activities, they may point to functional impairments of those enzymes in obesity [4, 33]. However, current research including the present findings are inconsistent [5, 9]. Those contrasting results may be a consequence of differences in the diet, genetics, FA analytics, and probably group sizes.

The association between selected individual FA (i.e. C16:0, C18:0, C18:1ω9c, LA, ALA, AA, EPA, and DHA) and several blood lipid and lipoprotein concentrations was investigated using multiple linear regression analyses. Triglyceride levels were mainly associated with C16:0 and C18:0 (R^2 *change* of 0.07 in boys and 0.04 in girls). The association between selected FA and the cholesterol-containing lipoproteins can be neglected (R^2 *change* <0.004).

Previous study results are based on dietary data in adults and found cholesterolemic effects of FA [34-41], these studies cannot be compared with the HELENA results. The general problematic of dietary FA data consists of two points: (a) FA assessed by dietary recalls may be biased by misreporting leading to inaccurate evaluations and (b) food composition tables

cannot specify the true value of all possible FA in foods. Moreover, the investigation of the influence between dietary FA and blood lipids may not lead to correct conclusions, because that approach excludes the influence of physiologically important steps in FA metabolism (e.g. digestion, absorption, elongation, and desaturation of FA). These steps influence the amount and kind of FA in the body and thus any possible function of FA including the assumed cholesterolemic effects of FA on blood lipids. Therefore, biomarkers like serum FA of the PL fraction may be better parameters. These biomarkers consider dietary intake and endogenous modifications of the last days. A recently published study by Motoyama *et al.* [42] investigated the association of total serum ω6 and ω3 PUFA with lipids in three populations of men aged 40-49 years. Serum ω6 and ω3 PUFA were significantly inversely associated with TG levels across all three populations (whites, Japanese, Japanese Americans). Further, ω6 PUFA were significantly positively associated with HDL in whites and Japanese whereas EPA was significantly positively associated with HDL in whites. A significantly positive association of ω3 PUFA with HDL was found in Japanese. Both ω6 and ω3 PUFA were positively associated with LDL in Japanese but not in whites or Japanese Americans [42]. However, whether these results are transferable to European adolescents is questionable. Tertile groups were not applied in the HELENA Study, and whether the metabolism of adolescents follow similar metabolic patterns as observed in adults is not clear. The few studies that found associations between serum FA and cholesterol levels in youth are solely based on the comparison of different TC cut-offs with FA compositions [43] or their correlations [44]. These studies do not allow a final evaluation of the cholesterolemic effects of serum FA and are not comparable with the present study results.

Nonetheless, the literature suggests cholesterolemic effects of individual dietary FA or FA classes [40, 41]. Possible cellular mechanisms of these cholesterolemic effects are not fully understood. Studies using cultured cell lines proposed modifications within the LDL receptor activity and altered acyl-CoA:cholesterol acyltransferase enzyme activities caused by individual FA supplementation, however, these studies show inconsistent results [45, 46]. Another reasonable explanation may be the intake of other dietary components affecting the serum lipid concentration [47-51]. These dietary components may accompany dietary fat intake (e.g. fat soluble vitamins, plant sterols, and polyphenols) and thus are ingested simultaneously. These components have also lipid-lowering properties [49-51], but because of the contemporary dietary intake, these effects could be covered or incorrectly associated to FA intake. For instance, the simultaneous intake of plant sterols or tocopherols with dietary PUFA may enhance the distribution of cholesterol towards the enterohepatic cholesterol

efflux. Moreover, dietary fibres, carbohydrates, proteins, or yet unknown components may affect lipid concentrations as well [47, 52].

A few *limitations* of the present study deserve comment. Because of the cross-sectional design, the temporal relationships are unknown; one cannot say anything about causality of the relationships. Furthermore, dietary FA intake was not taken into account. Dietary FA data based on questionnaires or interviews is mostly biased by misreporting and therefore become imprecise [53-56]. Additionally, available food composition tables and food data bases lack of sufficient information concerning amount and spectrum of individual FA to calculate the FA amount of certain foods separately. An alternative approach could be the correlation of serum FA patterns with foods and food groups, instead of calculating the content of single FA in particular foods. The FA pattern, like the percentage of SFA, MUFA, and PUFA, of foods and food groups, for instance, of butter, meat products, or plant oils are well known and would give some additional information concerning the association of dietary FA patterns and the serum FA composition. However, at the time of the analyses for the present thesis, this data was not available. A further critical fact of food composition tables is that newly developed food products are not included completely. Those food products are enriched with, for instance, ω3 FA, polyphenols, or dietary fibres to increase the nutritional value of the food products. These products are hardly assessed in the data bases, but may influence the lipid profiles in one or another way due to the fact that they are consumed by adolescents. These points indicate that FA measured in serum are more reliable parameters for the assessment of the FA status than dietary FA alone. Furthermore, it has to be mentioned that until the completion of this thesis is was not feasible to measure the sexual hormones of the participants, although, those measurements would help to specify relationships between different biomarkers and the state of maturity. However, it is intended to do hormone measurements in further analyses of remaining blood samples.

Some *strengths* of the HELENA Study should also be stated here. The HELENA Study is the first European multicentre, cross-sectional study, realizing a standardized methodology, to cover and connect a wide range of different research fields, which deal with the health and fitness status of adolescents. This includes as main aspects: (a) dietary, nutrition and eating attitudes, (b) physical activity and fitness, (c) body composition, (d) haematology, nutrition physiology and immunonutrition, and (e) genetics. With this background, is is quite understandable that this thesis can only cover a small part of the HELENA Study. Further, the reliability of the data as well as the quality of the project are a consequence of harmonized

and standardized methods and materials, centralized sample recruitment, biochemical analyses, and database management, including a pilot study to assure an optimal study performance.

Within this thesis different results were found with respect to the calculation method of the FA and their ratios. Due to the study design of the HELENA project, it was not possible to judge, which calculation method is the most reliable to interpret the results of FA profiles. However, the following points may highlight the main differences: first of all, FA and their ratios based on *conc%* may be more reliable than FA expressed as *area%*, because *conc%* calculations take into account the molecular mass of each FA detected and thus do not refer simply to a peak area. Nevertheless, the percentaged FA profiles of both methods are influenced by the number of FA included in the profile and the height of each FA peak. The dependency of FA within the percentaged FA profiles may obscure some important findings. The just mentioned problematic with percentaged FA profiles would be eliminated by using FA expressed as absolute concentrations, which are independent of other FA concentrations. Thus, changes in the concentration of one specific FA would not affect another FA of the same profile. Nonetheless, the most common method in the literature to express FA is the use of percentaged FA profiles, even though the absolute FA profile could give additional, important information. The reason could be the more sophisticated and more expensive measurements of absolute FA amounts. Nevertheless, it may be important to analyze the absolute FA profile to avoid falsified results because of percentaged weighting. For instance, shifted proportions of non-essential FA may easily alter the proportion of essential FA. As a consequence of the shifted proportion, the information value may be weaker or even incorrect [57, 58].

The discussion concerning reference values for a healthy adolescent population and its application for lipid screening and prevention strategies is ongoing and the question, which percentiles/ cut-offs should be used is not answered sufficiently yet. Moreover, it is still discussible how to develop reliable lipid reference values for adolescents. However, within the HELENA study design it was not realizable to create reference values per se. But the presented lipid concentrations and FA profiles (relative and absolute) are proposed to be used as physiological values of apparently healthy European adolescents. They may be applied as some kind of reference data for the comparison with other adolescent lipid concentrations, for

instance for the diagnosis of altered lipid and FA profiles of diseased subjects or the monitoring of therapeutic interventions.

The NCEP published blood cholesterol cut-offs for children and adolescents (2-19 years) in 1992, which will be the standard in clinical use [59]. These cut-offs for TC, LDL and TG levels are set at the 75^{th} percentile for normal levels and at the 95^{th} percentile for high levels, in the case of HDL at the 25^{th} and 5^{th} percentile. This approach has some limitations one has to keep in mind. First of all, the cut-offs are valid for the complete mentioned age range, neglecting possible physiological changes during adolescence. Further, the selected percentiles are completely arbitrary. Moreover, the lipid screening based on an American referent population, thus the cut-offs are addressed to the American population. The assessment was made in the early 1980s, but lifestyle and eating habits likely have changed during the last 30 years. However, more important is the fact that the reference values are based on data from children and adolescents from families with hypercholesterolemia or premature cardiovascular disease, and thus are not applicable to a healthy population. A more recent approach was made by Jolliffe and Janssen in 2008. They created age-specific lipid and lipoprotein thresholds for adolescents [60]. Data of 6,067 adolescents of NHANES (1988-2002) was used to obtain percentile curves with the Lambda-Mu-Sigma method. They adapted the NCEP cut-offs for adults at age 20 and applied those particular percentiles to the age range of 12-20 year-old boys and girls. With this approach the percentile curves are more adapted to age-specific differences, which will be present in 12-20 year-olds. However, this approach is still discussible, because the risk factors of adults are not applicable to youth. Furthermore, age is not the main determinant associated with changes during that particular period of life. Maturity categorized into Tanner stages would be the best choice. Although, the selection of the already mentioned percentiles for lipid cut-offs by NCEP is arbitrary and not connected to any risk factor, those cut-offs are established and frequently used. Still, risk factors are difficult to determine in an apparently healthy adolescent population and the adoption of American data for the evaluation of European lipid profiles may not be appropriate.

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9 Conclusions

The HELENA results show that lipid research in the period of adolescence should be made separated for gender, because differences are present as reported for routine lipid parameters, lipoproteins, and most absolute FA concentrations.

The use of chronological age in adolescence can lead to falsified assumptions with regard to growth and development. To avoid this bias, the use of biological age is recommended especially for the analysis of lipid parameters and lipoproteins (i.e. TC, HDL, LDL, TG, Lp(a), apo A-1, apo B).

Higher adipose tissue content, expressed as BMI or BF, leads to a more unfavourable lipid pattern as seen in routine lipid and lipoprotein parameters and their ratios. However, this association was less clear between individual FA and parameters of body composition. The observations, that changes of the female lipid levels are more present due to BMI status, whereas changes in the male lipid levels are more often seen by BF categorization, need clarification.

Although some FA ratios are associated with age, maturity, BMI, BF, and FFM in boys and girls, the results follow no clear pattern. The differences may be explained by and could be more related to different eating habits in the different sub-groups. Thus, it can be speculated that FA ratios are more likely modified by dietary factors such as amount of fat and dietary FA pattern.

The association between the selected serum FA measured in PL and serum TG, TC, HDL, LDL, apo A-1, apo B, and Lp(a) parameters are minimal. Therefore, studies in adults, which found an association between dietary FA intake and blood lipids may not be correct. On the other hand, the metabolism of adolescents may not follow similar metabolic patterns as observed in adults. Moreover, investigations may focus on other nutrients that accompany dietary fat intake (e.g. fat soluble vitamins, plant sterols, polyphenols) as well as on other nutrients in the diet (e.g. dietary fibres, carbohydrates, proteins) to clarify their influence on lipid concentrations

It has to be emphasized that the methodology of FA measurement and analysis differs between research groups and needs harmonization. Further effort in the establishment and use of absolute FA values must be undertaken, because differences between absolute and relative FA profiles do exist.

Finally, the selected communicable and non-communicable factors should be taken into account when interpreting data of adolescent lipid profiles.

Appendix

A Blood sampling questionaire

Healthy Lifestyle in Europe by Nutrition in Adolescence in 13-16 years adolescents across Europe.

Cross-sectional study (HELENA-CSS) and Pilot Study.

BLOOD SAMPLING QUESTIONNAIRE

From UL2/Bonn November 2005

Subject number: H 2 ....

Number of centre | Number of school | Number of class | Number of subject

Subjects initials: .....

first name/second name /family name

Evaluation date: ....

Day | Month | Year

Please ask the adolescent **before** taking the blood samples and tick the appropriate box to answer each question.

1. Has the adolescent fasted for at least 10 hours?

Yes ☐ (1) No ☐ (0)

Please specify what time he had his last meal, snack or drink (other than water):

hh mm

2. Did the adolescent come to school by motorised transport (car, bus, …)?

Yes ☐ (1) No ☐ (0)

Or walking?

Yes ☐ (1) No ☐ (0)

3. Did the adolescent have a common cold or other infectious disease during the last week?

Yes ☐ (1) No ☐ (0)

If yes, please specify type of disease

(one letter per case)

Answer will be coded (Coding instructions will be provided) by investigator before electronic data capture.

If yes, please indicate for how long: days

4. Did the adolescent have any symptoms of allergy during the last week?

Examples of symptoms: Red itchy, watery eyes or sneezing/congestion/runny nose or urticaria or abdominal pain or asthma or other.

Yes ☐ (1) No ☐ (0)

If yes, please indicate for how long: ..... days

If yes, please indicate which kind of allergy?

- ❍ Pollen
- ❍ Dust
- ❍ Food allergens
- ❍ Animals
- ❍ Others:

for others, please specify :

.....

one letter per case

Answer will be coded (Coding instructions will be provided) by investigator before electronic data capture

.....

5. Did the adolescent take any medication during the last month?

Yes ☐ (1) No ☐ (0)

If yes, please specify when, what was taken and doses (table):

	Duration (days)	Name of the medication	Quantity per day
1			
2			
3			
4			
5			

6. Did the adolescent take any supplement (vitamin/minerals) during the last month?

Yes ☐(1) No ☐ (0)

If yes, please specify when, what was taken and doses (table):

	Duration (days)	Name of the supplement	Dose per day
1			
2			
3			
4			
5			

7. Has the adolescent been vaccinated during the last two weeks?

Yes ☐ (1) No ☐ (0)

If yes, please specify when and what vaccine:

Date of vaccination: ....
dd mm year

Type:

.....

one letter per case

Answer will be coded (Coding instructions will be provided) by investigator before electronic data capture

.....

. (Only for girls) Is the girl menstruating at the moment?

Yes ☐ (1) No ☐ (0)

Please specify first day of last menstruation:

Date: ....
dd mm year

9. Has there been any complications during the blood extraction?

Yes ☐(1) No ☐ (0)

If yes, specify the problem:

.....

one letter per case

Answer will be coded (Coding instructions will be provided) by investigator before electronic data capture

.....

And fill an adverse event form in the Case Report Form !!!!

END OF THE BSQ

B Case report form

Healthy Lifestyle in Europe by Nutrition in Adolescence in 13-16 years adolescents across Europe.

Cross-sectional study (HELENA-CSS) and Pilot Study.

CASE REPORT FORM

Subject number : H2 [ ]

Subjects initials : .....

first name/second name /family name

GENERAL INSTRUCTIONS FOR THE INVESTIGATOR

General Instructions for the Investigator (see SOP of the CRF)

When completing this CRF, please

- Use a ballpoint, fine-tip pen (permanent ink), black or blue.
- Make sure that all entries are clear and legible, preferably in block letters.
- Use English and specific medical terminology.
- Make corrections by drawing a single line through the incorrect item, which must remain legible, and write the correct data next to it.
- Do not use correction fluids or any other method of erasure!
- Date and initial for all corrections and changes.
- Tick closed boxes and circles ☒

Do not use

- abbreviations and acronyms

Subject Initials : first name-middle-family/last name (ex)

Jean Marcel DUPONT J M D

- If, there is no second name, put a "-"
- Subject initials must remain consistent throughout the CRF.
-

Enrolment Code

- Subjects will be assigned an enrolment code number at inclusion : study code Helena CSS – H1 - city number (see Appendix) -number of study inclusion

Subject number: H2 ....

Number of centre | Number of school | Number of the class | Number of subject

Exemple **H2** 0 4 0 1 0 5 1 0

Missing Data

- If some information has been impossible to obtain, please strike out the field/box and explain briefly next to the field/box why the information is missing.

Historical dates

- If a historical date, or part of a historical date, has been impossible to obtain, please fill in as much as is known and strike out the remaining field(s)

Sic

- Indicate data which are unusual or unexpected but still correct by noting 'Sic' next to that data. Sic can be understood as 'so is correct.'

SELECTION OF SUBJECTS

Inclusion Criteria

	Yes	**No**
Male and female subjects aged 13 – 16 ± 2 years	☐	☐
Schooling in one of the participating cities	☐	☐
Informed consent form signed by the parents and/or the legal representative.	☐	☐

Exclusion Criteria

Subject participating simultaneously in another research trial	☐	☐

Subject number : ☐

Date of consent : __/__/2 0 0__
day month year

Visit Date : __/__/2 0 0__
day month year

<u>Name and signature of the investigator :</u>

☐

PHYSICAL EXAMINATION

1) Date of Birth : ⌊__⌋ ⌊__⌋ **1 9** ⌊__⌋⌊__⌋

day month year

2) Sex : Male ☐(1) Female ☐(2)

3) Medical personal history (including traumatic accidents):

None ☐(0)

Or Medical diagnosis (see coding annex): (1)

	Year of diagnosis /or intervention	Past medical or surgical history (coding)
1	⌊__⌋⌊__⌋⌊__⌋	/
2	⌊__⌋⌊__⌋⌊__⌋	/
3	⌊__⌋⌊__⌋⌊__⌋	/
4	⌊__⌋⌊__⌋⌊__⌋	/
5	⌊__⌋⌊__⌋⌊__⌋	/
6	⌊__⌋⌊__⌋⌊__⌋	/
7	⌊__⌋⌊__⌋⌊__⌋	/

4) Current treatment (medication or supplements)

Has the subject taken any treatment for more than 7 days during the last 30 days ?

Yes ☐(1) No ☐ (0)

If yes, please specify when and what was taken):

	During of medication			What Medication (INNs)	CODE (see appendix IV)
	Start date (dd/mm/year)	End date (dd/mm/year)	On going		
1	/ /	/ /	☐		/
2	/ /	/ /	☐		/
3	/ /	/ /	☐		/
4	/ /	/ /	☐		/
5	/ /	/ /	☐		/
6	/ /	/ /	☐		/
7	/ /	/ /	☐		/
8	/ /	/ /	☐		/

Has the subject taken any treatment in the last 24 hours? (except when on going is ticked)

Yes ☐(1) No ☐ (0)

Fever (>38°C) during the last 24 hours Yes ☐ (1) No ☐ (0)

5) Allergy

Has patient any allergy? Yes ☐ (1) No ☐ (0)

If yes, please indicate which type of allergen?

- ❍ Pollen
- ❍ Dust
- ❍ Food
- ❍ Animal
- ❍ Other:

If Other, please specify

.....

(one letter per case)

Which is/are the clinical signs?

- ❍ Atopic dermatitis
- ❍ Urticaria
- ❍ Allergic rhinitis
- ❍ Asthma
- ❍ Other

If Other, please specify

.....

(one capital letter per case)

6) Clinical examination of following items and tick the appropriate box :

	Normal	*Abnormal*	*If abnormal findings, specify*
a) General condition	☐ (1)	☐ (0)	……
b) Abdominal palpation	☐(1)	☐(0)	……
c) Pulmonary auscultation	☐(1)	☐(0)	……...
d) Cardiac auscultation	☐(1)	☐(0)	……...

e) Other abnormalities if detected

……

(one capital letter per case)

7) Pubertal status (see appendix I)

Girls

a) breast					**b) pilosity**				
1	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**
☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

Boys

a) gonade					**b) pilosity**				
1	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**
☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

Only for girls, if adaptable

Have you already your menses?

Yes ☐ (1) No ☐ (0)

Age of menarchy: ……. ……

Months Year

Date of last menstruation: ⌊__⌋ ⌊__⌋ ⌊__⌋⌊__⌋⌊__⌋

dd mm Year

Daily oral contraception? Yes ☐(1) No ☐ (0)

Or

Patch contraception? Yes ☐(1) No ☐ (0)

CLINICAL PARAMETERS

8) Anthropometry

a) Height : ⌊__⌋⌊__⌋⌊__⌋ **cm**

b) Weight : ⌊__⌋⌊__⌋⌊__⌋ **kg**

9) <u>Bio-impedancemetry</u> :

measurement

Resistance **Reactance**

⌊__⌋⌊__⌋⌊__⌋ ⌊__⌋⌊__⌋

- **<u>% of Body Density by Bop Pod (optional):</u>**

measurement

⌊__⌋⌊__⌋’ ⌊__⌋

10) SKIN FOLD THICKNESS

	1st measurement	2nd measurement	3nd measurement
a) Bicipital (mm)	⌊__.__⌋, ⌊__⌋	⌊__.__⌋, ⌊__⌋	⌊__.__⌋, ⌊__⌋
b) Tricipital (mm)	⌊__.__⌋, ⌊__⌋	⌊__.__⌋, ⌊__⌋	⌊__.__⌋, ⌊__⌋
c) Sub-scapular (mm)	⌊__.__⌋, ⌊__⌋	⌊__.__⌋, ⌊__⌋	⌊__.__⌋, ⌊__⌋
d) Suprailiac (mm)	⌊__.__⌋, ⌊__⌋	⌊__.__⌋, ⌊__⌋	⌊__.__⌋, ⌊__⌋
e) Thigh (mm)	⌊__.__⌋, ⌊__⌋	⌊__.__⌋, ⌊__⌋	⌊__.__⌋, ⌊__⌋
f) Calf (mm)	⌊__.__⌋, ⌊__⌋	⌊__.__⌋, ⌊__⌋	⌊__.__⌋, ⌊__⌋

11) CIRCUMFERENCES

	1st measurement	2nd measurement	3nd measurement
a) Arm (mm)	⌊__.__⌋, ⌊__⌋	⌊__.__⌋, ⌊__⌋	⌊__.__⌋, ⌊__⌋
b) Biceps (mm)	⌊__.__⌋, ⌊__⌋	⌊__.__⌋, ⌊__⌋	⌊__.__⌋, ⌊__⌋
c) Waist (mm)	⌊__.__.__⌋, ⌊__⌋	⌊__.__.__⌋, ⌊__⌋	⌊__.__.__⌋, ⌊__⌋
d) Hip (mm)	⌊__.__.__⌋, ⌊__⌋	⌊__.__.__⌋, ⌊__⌋	⌊__.__.__⌋, ⌊__⌋
e) Proximal thigh (mm)	⌊__.__⌋, ⌊__⌋	⌊__.__⌋, ⌊__⌋	⌊__.__⌋, ⌊__⌋

12) VITAL SIGNS

a) Blood pressure (mmHg) :

Systolic 1	Diastolic 1	Systolic 2	Diastolic 2
⌊__⌋⌊__⌋⌊__⌋	⌊__⌋⌊__⌋⌊__⌋	⌊__⌋⌊__⌋⌊__⌋	⌊__⌋⌊__⌋⌊__⌋

b) Heart Rate (beats/min) : ⌊__⌋⌊__⌋⌊__⌋

13) Do the subject participate to accelerometry assessment ?Yes ☐(1) No☐

BLOOD SAMPLES

Laboratory Assessments, blood

	Yes	No
Subject allocated to a blood samples class ?	☐	☐

	Yes	No
Blood samples have been collected (including specific questionnary ?)	☐	☐

Sample Date ⌊__⌋⌊__⌋ / ⌊__⌋⌊__⌋ / ⌊__⌋⌊__⌋⌊__⌋⌊__⌋

dd mm Year

	Yes	No
Did the adolescent have fever/cold the day of blood samples?	☐	☐

Please fill the appropriate Laboratory request form HELENA-CSS

SPECIFIC QUESTIONNAIRES FOR HELENA-CSS

Tick when done	Date	Yes	No
General Questionnary for Adolescents Self European Socio-economic Status			
	/ /	☐	☐
Nutrition Knowledge (NKT-C)	/ /	☐	☐
Eating Behaviour (EWI-C)	/ /	☐	☐
YANA-C	/ /	☐ first	☐
	/ /	☐ second	☐
Food choice and preference	/ /	☐	☐
Determinants of Healthy Eating (HE) Determinants of physical activity (PA)			
	/ /	☐	☐
Physical Activity (PAQ)	/ /	☐	☐
Questionnaire for Parents (QP)	/ /	☐	☐
Blood sample questionnary	/ /	☐	☐
Accelerometer	/ /	☐	☐
Physical fitness tests	/ /	☐	☐
Sedentary questionnary	/ /	☐	☐

ADVERSE EVENTS (see Appendix 4 for definitions)

DESCRIBE THE ADVERSE EVENTS BELOW:

1st event :

It is a serious adverse event? ☐ **YES** ☐ **NO**

Description of the event including date and hour of occurrence, duration, and hour of resolution, and consequences for the subject.

_____
_____
_____
_____
_____
_____

2nd event :

It is a serious undesirable event? ☐ **YES** ☐ **NO**

Description of the event including the date and the hour of beginning, the duration, and hour to end, and the consequences for the subject.

_____
_____
_____
_____
_____
_____

3rd event :

It is a serious undesirable event? ☐ **YES** ☐ **NO**

Description of the event including the date and the hour of beginning, the duration, and hour to end, and the consequences for the subject.

_____
_____
_____
_____
_____

CAUTION: IF A SERIOUS ADVERSE EVENT OCURRED, YOU MUST MAKE A STATEMENT ON SPECIFIC FORM (SEE NEXT PAGE!) FOR YOUR LOCAL REGULATORY AGENCY.

SERIOUS ADVERSE EVENT (SAE) REPORT

SUBJECT IDENTIFICATION OF SAE

SAE date ⌊__⌋ / ⌊__⌋ / ⌊____⌋
dd mm Year

Awareness of SAE by the Investigator ⌊__⌋/⌊__⌋/⌊____⌋
dd mm Year

Death ☐

Hospitalisation ☐

Life threatening ☐ persistent or significant disability/Incapacity

If death

Probable cause of death……

Date of death ⌊__⌋/⌊__⌋/⌊____⌋ Autopsy performed : Yes ☐ No ☐
dd mm Year

If hospitalized

Date of hospitalization ⌊__⌋/⌊__⌋/⌊____⌋
dd mm Year

Date of discharge ⌊__⌋/⌊__⌋/⌊____⌋
dd mm Year

Appendix B

Symptoms, course……

…….

Diagnostic investigations results

……...

Treatment of AE……

Other comments……...

Investigator's initials ……..

STUDY TERMINATION

Has the subject completed all exams and questionnaires of the study?

Yes ☐ No ☐

If no, please specify:

Essential for each subject:

	Yes	No
1. Inclusion criteria	☐	☐
2. Weight and height	☐	☐
3. Informed consent signed	☐	☐

Optional (each subject must complete at least 75% of the following, i.e. 12 measurements)

1. Clinical examination (validity assessed by the Medical Doctor)
   Yes ☐ No ☐
2. Anthropometric measurements (all the measurements)
   Yes ☐ No ☐
3. BIA
   Yes ☐ No ☐
4. General Questionnary for adolescents Self European Socio-economic Status (not fully blank)
   Yes ☐ No ☐
5. Nutrition Knowledge (NKT-C)(75% of the questions responded)
   Yes ☐ No ☐
6. Eating Behaviour (EWI-C) (75% of the questions responded)
   Yes ☐ No ☐
7. YANA-C (2 days)
   Yes ☐ No ☐

8. Food choice and preference (75% of the questions responded)

Yes ☐ No ☐

9. Determinants of Healthy Eating (HE) + Determinants of physical activity (PA)

Yes ☐ No ☐

10. Physical Activity (PAQ)

Yes ☐ No ☐

11. Questionnary for parents (QP) (75% of the questions responded)

Yes ☐ No ☐

12. Blood Sampling (including questionnaire)

Yes ☐ No ☐

13. Accelerometry assessment (minimum criteria defined into the protocol)

Yes ☐ No ☐

14. Physical fitness tests

Yes ☐ No ☐

Main reason (only one) for premature discontinuation

Withdrawal of the subject ☐

Adverse event ☐
Please make sure that AE form is completely filled

Subject lost of follow-up ☐

Other ☐
Please specify.....

Name and signature of the person checking the CRF

Date and signature

└─┴─┘ / └─┴─┘ / └─┴─┴─┴─┘

Name of the investigator: ……
Date and signature

└─┴─┘ / └─┴─┘ / └─┴─┴─┴─┘

List of Publications and Presentations

Original Articles and Reviews

A. Spinneker, R. Sola, V. Lemmen, M.J. Castillo, K. Pietrzik and M. González-Gross
Vitamin B_6 status, deficiency and its consequences – an overview.
Nutr Hosp, 2007;22:7-24

González-Gross M, Breidenassel C, Gómez S, Ferrari M, Béghin L, **Spinneker A**, Díaz LE, Maiani G, Demailly A, Al-Tahan J, Albers U, Wärnberg J, Stoffel-Wagner B, Jiménez-Pavón D, Libersa C, Pietrzik K, Marcos A, Stehle P, on behalf of the HELENA study group.
Sampling and processing of fresh blood samples within a European Multicenter Nutritional study – Evaluation of biomarker stability during transport and storage.
Int J Obes (2008) 32, S66-S75

Communications presented to congresses

Spinneker A, González-Gross M, Sola R, Castillo MJ, Pietrzik K
Einfluss von 5-MTHF- und PGA-Supplementen auf Parameter des Vitamin B_{12}-Status von spanischen Heimbewohnern
43rd Kongress der DGE (Deutsche Gesellschaft für Ernährung)
Stuttgart, Germany; March 9-10, 2006.

Spinneker A
Healthy Lifestyle in Europe by Nutrition in Adolescence
News in agricultural and food research
Brussels, Belgium; April 27th, 2006

Spinneker A
Logistics and procedures for collection, handling, shipment and storage of biological samples in HELENA
Oral presentation at the IDEFICS-Symposium "Technologies for data collection"
Bremen, Germany; September 2nd, 2006

Spinneker A, Albers U, González-Gross M, Sola R, Castillo MJ, Pietrzik K
Difficulty in proving the masking of cobalamin by folic acid supplementation – PGA vs 5-MTHF
10th European Nutrition Conference (FENS)
Paris, France; July 10-13, 2007

Breidenassel C, Al-Tahan J, **Spinneker A**, Segoviano M, Gómez S, Ferrari M, Beghin L, Pietrzik K, Stehle P, González-Gross M on behalf of the HELENA Study group
Assessment of nutritional biomarkers in fresh blood samples in an international multi-centre study: a challenge for a handling and transport system
10th European Nutrition Conference (FENS)
Paris, France; July 10-13, 2007

Segoviano M, Breidenassel C, **Spinneker A**, Al-Tahan J, Díaz LE, Ferrari M, Béghin L, Stoffel-Wagner B, González-Gross M, Stehle P
Gewinnung und Verarbeitung von Frischblutproben im Rahmen einer Europäischen Multicenter-Studie – Untersuchungen zur Stabilität ausgewählter Biomarker während Transport und Lagerung.
45th Kongress der DGE (Deutsche Gesellschaft für Ernährung)
Bonn, Germany; March 13-14, 2008

Spinneker A, González-Gross M, Breidenassel C, Al-Tahan J, Stoffel-Wagner B, Gómez, Martínez S, Venneria E, Castillo M, Béghin L, Stehle P, on behalf of the HELENA Study group
Serum lipid and lipoprotein status is more influenced by biological than chronological age in European Adolescents
ECO 2009 Pre-Congress Meeting on: "Health behaviour and status in European adolescents"
Amsterdam, The Netherlands; May 6th, 2009

Al-Tahan J, Albers U, Valtuena J, **Spinneker A**, Breidenassel C, Azzini E, Diaz LE, Marcos A, Gottrand F, Kunert J, Manios Y, Pietrzik K, Stehle P, González-Gross M on behalf of the HELENA study group.
Folate and vitamin B12 status in European adolescents: associations with gender, age, and maturity
ECO 2009 Pre-Congress Meeting on: "Health behaviour and status in European adolescents"
Amsterdam, The Netherlands; May 6th, 2009

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Anyone who has never made a mistake has never tried anything new.

~ Albert Einstein ~

1879-1955