Diplomarbeit

Leberveränderungen bei übergewichtigen Kindern und Jugendlichen:

Der Fatty Liver Index als Möglichkeit zur Diagnose und Verlaufskontrolle

eingereicht von

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Zusammenfassung

Ziele: Die Prävalenz von Übergewicht und Adipositas und deren Folgeerkrankungen - unter anderem der nicht-alkoholischen Fettleber-Erkrankung (NAFLD) - nimmt in allen Altersklassen zu, so dass derzeit weltweit epidemische Ausmaße erreicht werden. Übergewicht und Adipositas treten bereits im Kindesalter auf und bleiben in der Regel bis ins Erwachsenenalter bestehen. Daher ist es von großer Wichtigkeit, bereits frühzeitig mit Prävention und Behandlung zu beginnen. Ziel dieser vorliegenden Studie war es, die Auswirkungen des Interventionsprogramms MODUL insbesondere auf die Leber und auf NAFLD zu untersuchen.

Methoden: Dies ist eine retrospektive Analyse von Daten, die in dem Interventions-programm MODUL erhoben wurden. Insgesamt nahmen 25 übergewichtige und adipöse (Body Mass Index (BMI) >95. Perzentile) Kinder und Jugendliche (11 Buben und 14 Mädchen im Alter von 12 bis 15 Jahren) an dem 19-wöchigen Programm teil. Funktionswerte der Leber sowie Parameter, die im Zusammenhang mit der Pathogenese von NAFLD stehen, wurden als solche identifiziert und im Verlauf des Programms wiederholt bestimmt. Diese Parameter umfassten: Nahrungsaufnahme [gesamte Kalorienaufnahme und die Kalorienaufnahme in Form von Fett, Proteinen und Kohlenhydraten], Leberenzyme [Alanin Transaminase (ALT), Aspartate Transaminase (AST), γ-Glutamyltransferase (GGT) und den Quotienten von AST zu ALT], anthropometrische Parameter [Körpergewicht, BMI, Taillenumfang, Gesamtkörperfett, viszerales Fett und subkutanes Fett], Stoffwechselparameter [Gesamt-cholesterin, High-density Lipoprotein (HDL)- und Low-density Lipoprotein (LDL)-Cholesterin, Triglyceride (TG) und Nüchternblutzucker] und Entzündungsparameter [C-reaktives Protein (CRP) und Interleukin 6 (IL-6)] sowie Parameter, die Aufschluss geben über das Verhältnis von Oxidantien zu Antioxidantien [Malondialdehyd, α-Tocopherol und den Quotienten von α-Tocopherol zu Cholesterin]. Der Fatty Liver Index (FLI), bestehend aus den Parametern BMI, Taillenumfang, GGT und TG, wurde in der Literatur als der beste Marker für die nicht-alkoholische Fettleber (NAFL), die mildeste Form der NAFLD, identifiziert und zum ersten Mal in einem Interventionsprogram für übergewichtige und adipöse Kinder und Jugendliche angewandt, um die Änderungen von NAFL zu beobachten und zu quantifizieren.

Ergebnisse: Wir zeigten positive Effekte des MODUL Programms anhand eines statistisch signifikanten Rückgangs ($P<0.05$) von Körpergewicht, BMI, Taillenumfang, Gesamt-

körperfett, subkutanem Fett, viszeralem Fett, TG, GGT, AST, ALT und dem FLI. Der FLI nahm von 45.3 ± 26.3 auf 20.6 ± 23.2 signifikant ab (P<0.001). Der größte Unterschied wurde in den ersten vier Wochen des Programms beobachtet. Der Anteil der Teilnehmer, für die NAFLD ausgeschlossen werden konnte (FLI <30), wurde nahezu verdoppelt (von 40 % auf 75 %) und der Anteil der Teilnehmer, die mit großer Wahrscheinlichkeit an NAFLD erkrankt waren (FLI >60), reduzierte sich von anfangs 24 % auf nur 10 % am Ende des Programms.

Konklusion: Die Teilnahme an dem Interventionsprogramm MODUL wirkt sich positiv auf eine durch Übergewicht hervorgerufene NAFLD aus. Programme dieser Art werden für übergewichtige und adipöse Kinder und Jugendliche empfohlen, da sowohl Übergewicht reduziert werden kann, also auch Folgekrankheiten, insbesondere auch die NAFLD, verhindert bzw. therapiert werden können. Es muss hervorgehoben werden, dass die positiven Effekte eines Gewichtsverlustes bereits nach nur vier Wochen auftreten.

Schlüsselwörter: Übergewicht, Adipositas, nicht-alkoholische Fettleber-Erkrankung, Fatty Liver Index, Gewichtsreduktion.

Abstract

Objective: The prevalence of overweight and obesity and associated conditons including non-alcoholic fatty liver disease (NAFLD) is increasing in all age groups and currently epidemic proportions are reached worldwide. Overweight and obesity may already occur in childhood and tend to persist to adulthood. Therefore it is very important to start prevention and treatment early in life. The aim of the present study was to investigate the impact of the obesity intervention program MODUL on the liver and on NAFLD.

Method: This is a retrospective analysis of data collected in the obesity intervention program MODUL. A total of 25 overweight and obese (Body Mass Index (BMI) >95th centile) children and adolescents (11 boys and 14 girls at the age of 12-15 years) were enrolled in the program which lasted 19 weeks. Markers of the liver function and markers related to the pathogenesis of NAFLD were identified and monitored. Variables studied included: food intake [total energy intake and energy intake from fat, proteins and carbohydrates], liver enzyme activities [alanine transaminase (ALT), aspartate transaminase (AST), γ-glutamyltransferase (GGT) and the ratio of AST to ALT], anthropometry [body weight, BMI, waist circumference (WC), total adipose tissue mass, visceral adipose tissue mass and subcutaneous adipose tissue mass], metabolic markers [total cholesterol, high-density lipoprotein (HDL) cholesterol and low-density lipoprotein (LDL) cholesterol, triglycerides (TG) and glucose] and markers of inflammation [C-reactive protein (CRP) and interleukin 6 (IL-6)] and oxidant-antioxidant status [malondialdehyde, α-tocopherol and the ratio of α tocopherol to cholesterol]. The Fatty Liver Index (FLI), combining the variables BMI, WC, GGT and TG, was indentified to be the best performing marker for the non-alcoholic fatty liver (NAFL), the mildest form of NAFLD. For the first time the FLI has been applied in an interventional program for overweight and obese children and adolescents to monitor and quantify changes related to NAFL.

Results: We demonstrated beneficial effects of the program as evidenced by a statistically significant decrease ($P<0.05$) of body weight, BMI, WC, total fat mass, subcutaeous adipose tissue mass, visceral adipose tissue mass, TG, GGT, AST, ALT and the FLI. The FLI decreased significantly ($P<0.001$) from 45.3 ± 26.3 to 20.6 ± 23.2. The changes were most pronounced during the first four weeks of intervention. The percentage of participants for whom NAFLD could be ruled out (FLI <30) had almost doubled (from 40 % to 75 %)

and the number of those with suspected NAFLD (FLI >60) had decreased from 24 % to only 10 % at the end of the program.

Conclusion: Participation in the obesity intervention program MODUL has beneficial effects on obesity-related NAFLD as evidenced by a significant reduction of the FLI. Similar programs are recommended for overweight and obese children and adolescents as overweight and related comorbidities including NAFLD are prevented or treated. It needs to be stressed that the beneficial effects of weight loss may occur within a period of only four weeks.

Key words: Overweight, obesity, non-alcoholic fatty liver disease, Fatty Liver Index, weight loss.

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Abbreviations

AAR	Ratio of AST to ALT
AASLD	American Association for the Study of Liver Diseases
ALT	Alanine transaminase
ATII	Angiotensin II
AST	Aspartate transaminase
AUC	Area under the receiver operating characteristic curve
B	Intervention break (during the MODUL Program)
BMI	Body Mass Index
CK-18	Cytokeratin 18
CRN	Clinical research network
CRP	C-reactive protein
CT	Computed tomographie
DGE	Deutsche Gesellschaft für Ernähung (engl.: German Association for Nutrition)
FFA	Free fatty acids
FLI	Fatty Liver Index
GGT	γ-Glutamyltransferase
HA	Hyaluronic acid
HCC	Hepatocellular carcinoma
HDL	High-density lipoprotein
HIV	Human immunodeficiency virus
HOMA-IR	Homeostasis model assessment insulin resistance
HPLC	High performance liquid chromatography
IL-6	Interleukin 6
IR	Insulin resistance
LDL	Low-density lipoprotein
M	Module (Intervention period during the MODUL Program)
MDA	Malondialdehyde
MODUL	Mitmachen Ohne Diät, Ultraleicht (engl.: join us without diet - ultra easy)
MRI	Magnet resonance imaging
NAFL	Non-alcoholic fatty liver

NAFLD	Non-alcoholic fatty liver disease
NAS	NAFLD activity score
NASH	Non-alcoholic steatohepatitis
ROC	Receiver operating characteristic curve
ROS	Reactive oxygen species
SAT	Subcutaneous adipose tissue
SD	Standard deviation
T	Time points of measurement (during the MODUL Program)
T2DM	Type 2 diabetes mellitus
TCR	Ratio of α-tocopherol to cholesterol
TG	Triglycerides
TZD	Thiazolidinediones
TNF-α	Tumor necrosis factor-alpha
VAT	Visceral adipose tissue
VLDL	Very low-density lipoprotein
WC	Waist circumference

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1 Background

The World Health Organization (WHO) designates obesity as one of the most important health threats (68, 130). It is considered to be the fastest growing health risk worldwide and currently about 7 % of the entire world population are either overweight or obese (56). The prevalence is highest in the United States but also in Europe epidemiological data are alarming and there is a clear upwards trend (68). Obesity is not restricted to industrialized countries but affects also the developing world (130).

The gain of excess body fat usually starts early in life and the prevalence is increasing in all age groups (60). In Austria, about 46 % of adults and 13-16 % of the 3-10-year-olds are currently overweight or obese (56).

Secondary disorders include cardiovascular disease, degenerative joint disease, sleep apnea, chronic renal dysfunction and adverse metabolic traits such as type 2 diabetes mellitus (T2DM) (65, 73).

Overweight and obesity also have adverse effects on the liver and may cause non-alcoholic fatty liver disease (NAFLD). NAFLD has only been discovered about 40 years ago (71). Its significance has been underestimated for a long time (96) and still has not received sufficient attention by many general practitioners (34). However, it is currently the leading cause of liver disease in the industrialized world (37, 86).

The main causes for overweight and obesity and related comorbidities, including NAFLD, are physical inactivity and inadequate nutrition, also referred to as "life style disorders", in the western world (27). Therefore, lifestyle changes are the most promising strategy for prevention and treatment. In this thesis the obesity intervention program called MODUL will be presented. The focus will be on the effects of the MODUL Program on NAFLD and on the Fatty Liver Index (FLI) in 12-15-year-old children and adolescents.

1.1 Overweight and obesity

1.1.1 Definition for adults and children

Overweight and obesity are defined as increased body fat mass and the degree is described by the Body Mass Index (BMI):

$$BMI = \frac{weight\,(kg)}{height^2\,(m^2)}$$

For adults, a BMI of 18.5-24.9 kg/m² is considered normal weight, 25.0-29.9 kg/m² overweight and 30.0 kg/m² and above obesity. There are three grades of obesity: grade I (BMI of 30.0-34.9 kg/m²), grade II (BMI of 35.0-39.9 kg/m²) and grade III (BMI of 40.0 kg/m² and above) (37).

Given that the distribution of body fat mass is age-related, the diagnostic power of the absolute value of BMI is only poor in children and adolescents. Therefore, the definition of childhood obesity is based on age- and sex-specific reference values that are visualized as centile curves (Figures 1 and 2). Usually, overweight is defined as BMI above the 90th centile and obesity as BMI above the 97th centile (46, 56, 78).

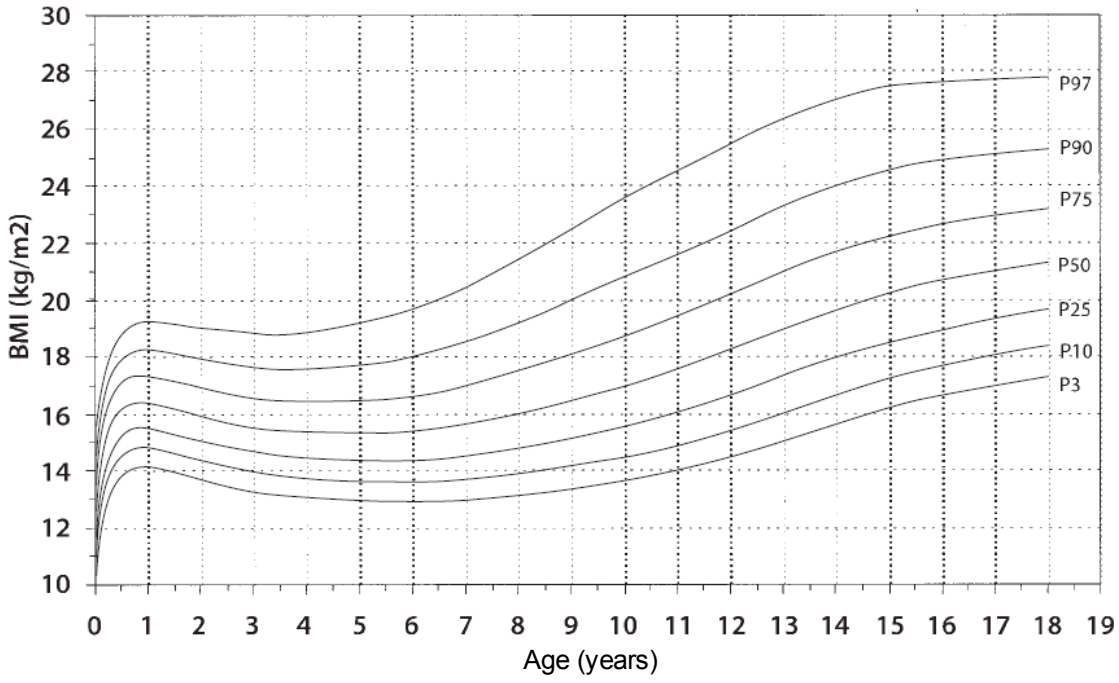


Figure 1: Centile curves for the BMI of girls at the age of 0-18 years from Kromeyer-Hauschild et al. (64)

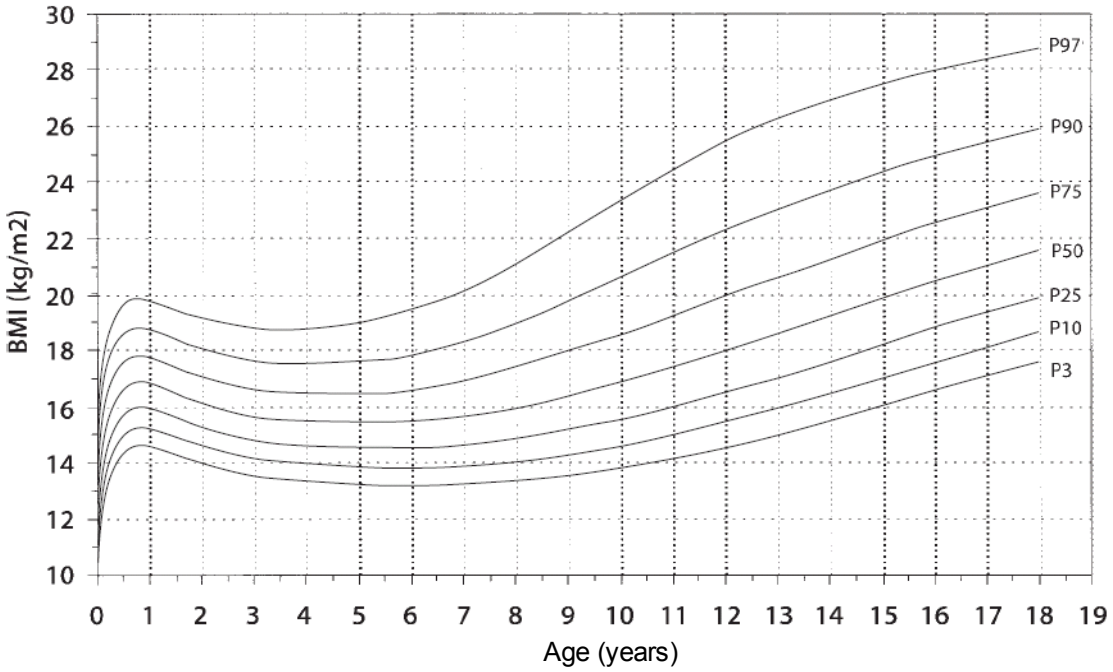


Figure 2: Centile curves for the BMI of boys at the age of 0-18 years from Kromeyer-Hauschild et al. (64)

Also the cut-off values of the 85th and 95th centile, respectively, are frequently used (37, 55, 60, 68). There is no generally accepted definition yet (68). In the present study the cut-off values of the 90th and the 97th centile are used.

1.1.2 Prevalence in adults and children

According to an epidemiological study conducted by the Institute for Social Medicine of Vienna in 1999, 37 % of Austrian adults are overweight and 9 % are obese. The same study reported that about 13-14 % of the 3-10-year-old girls and 16 % of the boys of the same age group are above the 90th centile for BMI (56). According to another cross-sectional study, 20 % of the 13-year-old Austrian boys and girls are overweight, based on the cut-off of the 85th centile for BMI (68). For Germany, Imhof et al. (47) reported a prevalence of overweight of 15 % for girls and 12 % for boys, based on the cut-off of the 90th centile for BMI.

These data are particularly alarming as the prevalence is increasing in all age groups (56, 60). Furthermore, childhood obesity is not a transient condition, but in most cases it persists to adulthood (56, 65, 68, 60).

Girls start to gain excess body fat as early as at the age of five, while boys do so at the age of eight (65). Adolescence is also a critical period for the onset of obesity. Therefore, it is very important to start prevention and treatment early in life (68).

1.1.3 Consequences

Obese people have an increased overall mortality rate (80). The prevalence of the following secondary disorders increases in accordance with body weight gain: degenerative joint disease, dyspnea, T2DM, dyslipoproteinemia, essential hypertension, cardiovascular disease, cholelithiasis, gastroesophageal reflux, sleep apnea (37) and exacerbation of asthma (60). The effects of overweight on the liver are summarized as non-alcoholic fatty liver disease (NAFLD) (73). Furthermore, obese people often have to deal with harassment (80) and in many cases they suffer from poor self-esteem (60). Obesity also predisposes to thrombosis and increases the risk to develop complications following surgery (55).

In children, the most common secondary disorders include hypercholesterinemia, essential hypertension, T2DM, degenerative joint disease, behavior disorder, sleep apnea and NAFLD (55).

1.2 Obesity, the metabolic syndrome and NAFLD

The metabolic syndrome consists of insulin resistance (IR), hyperinsulinemia, impaired glucose tolerance or T2DM, elevated blood pressure, dyslipoproteinemia and visceral obesity (37). NAFLD is strongly associated with the metabolic syndrome (63, 87, 86, 108) and its risk factors (43) and there is a great body of evidence suggesting that it may even be one of its manifestations (27, 57, 76, 101, 110). The Asian-Pacific Working Party for NAFLD confirmed that NAFLD is indeed part of the metabolic syndrome (15). Given that the metabolic syndrome predicts an increased risk for cardiovascular disease, there is also a strong association between NAFLD and cardiovascular disease (63) even in the pediatric population (31, 108). However, NAFLD may also occur in the absence of the metabolic syndrome (19, 123).

1.3 NAFLD

Hepatic steatosis, as a consequence of alcohol abuse, has been known for a long time. However, that it may also occur independently from alcohol intake, has first been described by Ludwig et al. (71) in 1980. The significance of NAFLD has only been recognized within the last ten years (96). Today it is the leading cause of liver disease in industrialized countries (37, 86).

1.3.1 Definition

The diagnosis of NAFLD requires the exclusion of several other causes of hepatic injury, including alcohol abuse and the intake of metals, cytotoxic antibiotics or other cytotoxic drugs, as well as inborn errors of metabolism such as Wilson's disease and infectious diseases like hepatitis B and C and human immunodeficiency virus (HIV) infection (86). Clinicians will be able to exclude most of these potential causes during history taking. In some cases however, NAFLD will be diagnosed despite of other underlying conditions (96). So far, there is no agreement on the level of alcohol consumption that may exclude the diagnosis *non*-alcoholic liver disease (27). However, this is of little relevance in children.

1.3.1.1 The spectrum of NAFLD: NAFL, NASH, fibrosis and cirrhosis

NAFLD represents a wide spectrum of conditions ranging from simple steatosis, the so called non-alcoholic fatty liver (NAFL), to non-alcoholic steatohepatitis (NASH), liver fibrosis and cirrhosis (79, 86) (Figure 3). Several authors suggest that a large rate of "cryptogenic cirrhosis", i.e. cirrhosis of unknown origin, may be caused by NAFL and NASH and therefore should be integrated in the spectrum of NAFLD as well (79, 110). Whether there is a causal relation between NAFLD and hepatocellular carcinoma (HCC) is discussed controversially (27, 22).

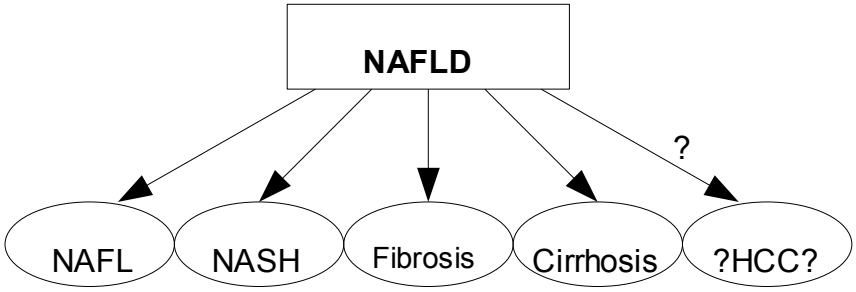


Figure 3: The spectrum of NAFLD

1.3.1.2 Histological features

Histologically, NAFLD is very similar to alcoholic liver disease (71, 79). It is defined as a significant fat accumulation in the liver exceeding about 5-10 % by weight (81).

Following are the histological features of NAFL and NASH, which in children is devided into NASH type 1 and NASH type 2. In NAFL, there is an accumulation of lipids in large and small droplets within the hepatocytes. For the diagnosis of NASH the NASH clinical research network (CRN) has proposed that three features need to be present: (i) macrovesicular steatosis in zone 3 which borders on the central vein, (ii) hepatocyte ballooning and (iii) mixed lobular inflammation (61, 86) (Figure 4).

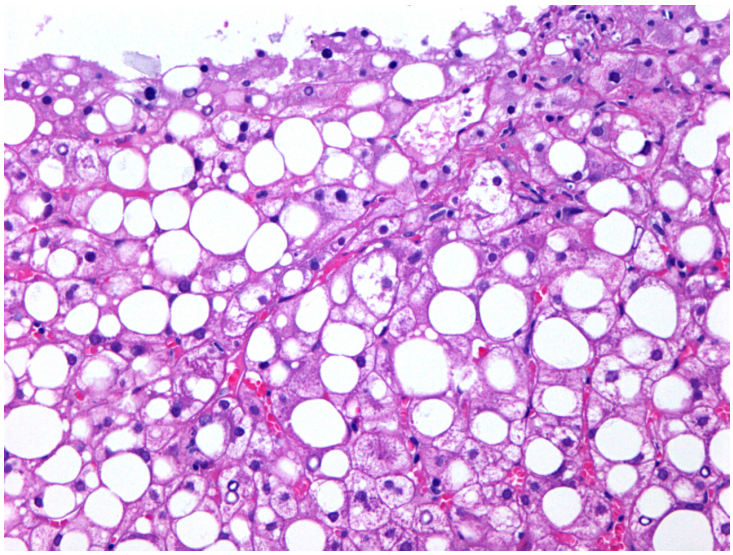


Figure 4: NASH, hematoxylin-eosin staining, magnification 200-fold, courtesy of Prof. Karoline Lackner, Institute for Pathology, Medical University of Graz, Austria

In children there are two different types of NASH. The findings described above are summarized as NASH type 1. NASH type 2 never affects adults, but it represents the more common form of NASH in the pediatric population. It is characterized by macrovesicular steatosis with portal inflammation and/or fibrosis without evidence of cellular injury or lobular inflammation. Children with NASH type 2 tend to be younger and more obese compared to children with NASH type 1. It is not known whether NASH type 2 evolves into NASH type 1 as the children grow up (8).

Besides the mandatory findings of macrovesicular steatosis, hepatocyte ballooning and mixed lobular inflammation, there are some additional features that are also common but not necessary for the diagnosis of NASH. These include mild to moderate portal inflammation, acidophilic bodies, lipogranulomas, glycogenated nuclei, Mallory's hyaline in ballooned hepatocytes, mild siderosis and megamitochondria. NASH can occur either isolated or together with fibrosis (86) (Figure 5).

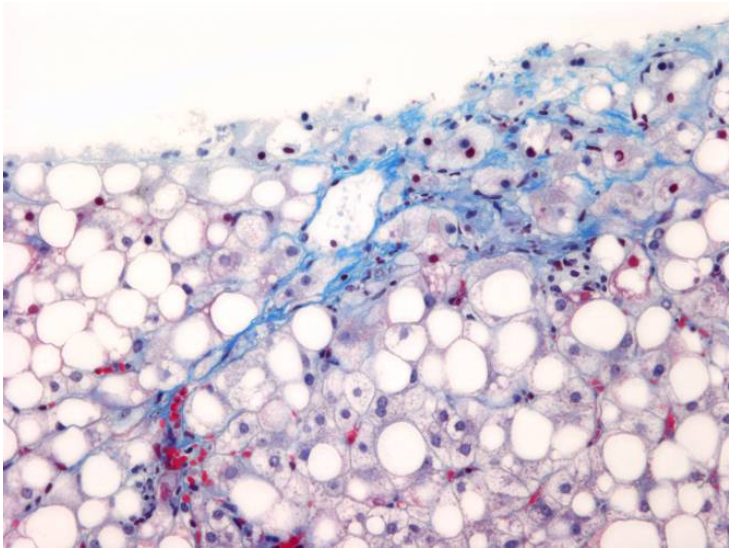


Figure 5: NASH with pericellular fibrosis, chromotrop anilin blue staining, magnification 200-fold, courtesy of Prof. Karoline Lackner, Institute for Pathology, Medical University of Graz, Austria

The CRN designed and validated a scoring system to evaluate the severity of histological findings: the NAFLD activity score (NAS). It comprises 14 histological features, four of which were evaluated semi-quantitatively: steatosis [0-3], lobular inflammation [0-2], hepatocellular ballooning [0-2] and fibrosis [0-4]. The values for NAS range from 0 to 8. NAS values between 0 and 3 are predictive for NAFL and NAS values ≥5 are predictive for NASH (61).

1.3.2 Prevalence

It is very difficult to obtain reliable data on the prevalence of NAFLD. Patients are usually asymptomatic and biopsy, the gold standard for diagnosis, can hardly be performed in epidemiological studies (22). Therefore, the available data are based on tests lacking sensitivity and specificity (27). Another problem is that many studies are based on clinical samples that may not be representative for the general population (60).

Despite these limitations, NAFLD is believed to be the most common liver disease in the Western world (79). Its prevalence is increasing in accordance with the general increase in the BMI in all age groups (96).

Although NAFLD can affect all ages, genders and ethnic groups (86) it is not distributed evenly among these groups. While earlier it was believed that NAFLD is more common in women, later this theory was proved to be false. Conversely, NAFLD is more prevalent in males, both in adults and children (27, 96, 73). Its prevalence is highest among Hispanics, followed by Caucasians. African Americans are affected least (27, 51) (Figure 6).

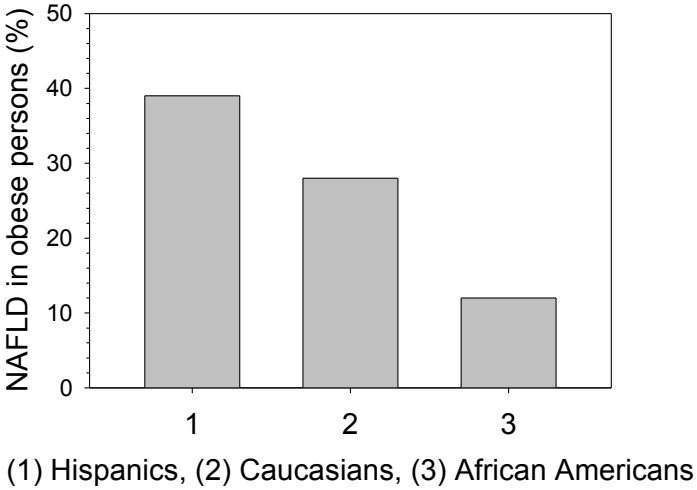


Figure 6: Prevalence of NAFLD in obese persons of different races according to Kallwitz et al. (51)

In developed countries, about 15-30 % of the adult population may have NAFLD (86, 96, 110) (Figure 7). The prevalence of NAFLD in obese individuals (BMI >30) is estimated to be 65-75 % (96) and in morbidly obese (BMI >35) even 85-90 % (96, 110). Interestingly, in lean patients NAFL may be present in 35 % and NASH in 2.7 % (81). NASH may make up about 10-33 % of NAFLD (27) and an upward tendency can be observed. The prevalence of NASH may range between 1.2 and 7 % in the general population (27, 79, 96) (Figure 7) and even between 15 and 20 % in obese subjects (27, 96). The morbidly obese may be affected in 25-36 % (86).

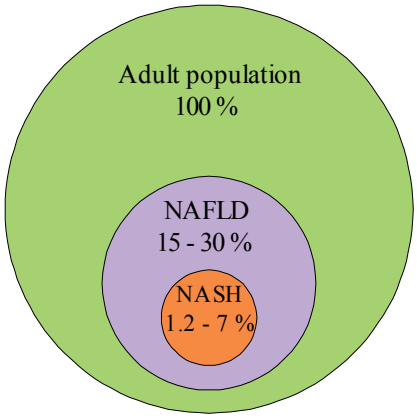


Figure 7: Prevalence of NAFLD and NASH in the general adult population

In unselected groups of children high-quality studies on the prevalence of NAFLD are lacking (96). In 2002, the American Association for the Study of Liver Diseases (AASLD) estimated a prevalence in the pediatric population of 1-2 % (81). According to several studies from the United States and Asia, the prevalence may be slightly higher and range between 2.5 and 9.5 % (96). Recent data from the United States National Health and Nutrition Examination Survey suggest a prevalence of 8 % in the population aged 1-19 years (89). In obese children the prevalence may range between 53 %, as shown for Italy and 77 %, as reported for China (105).

Obesity-related NAFLD is more frequent among boys than girls (47). It is often diagnosed at the age of 11-14 years, but serious cases of NAFLD can already occur in preschool children. The youngest child with diagnosed NAFLD has been no older than two years (73). Since the prevalence of obesity in children is rising (105), NAFL and even NASH will appear more frequently in younger individuals (27, 86).

Only little attention has been paid on NAFLD in normal-weight children. It seems to be of a lower grade and might be associated with other factors than the metabolic variables in obesity-related NAFLD (5).

1.3.3 Risk factors

NAFLD has been considered to be a disease of the "West". Altered socioeconomic circumstances and changes in food intake, food composition and physical activity play a role (27). According to the AASLD, the most reliable risk factors are age higher than 40-50 years, severe obesity, diabetes and hyperlipidemia, especially hypertriglyceridemia (81). Even though NAFLD may also occur in lean persons (63) and in absence of any risk factors (79), the majority of individuals is either overweight or obese (96). Particularly visceral fat in particular is a major determinant and it is even more predictive than overall obesity and the BMI. The risk for NAFL is increased by rapid weight loss, jejunoileal bypass surgery and total parenteral nutrition (79). Schwimmer et al. (107) showed that NAFLD is partly heritable. As already mentioned above, male gender and Hispanic origin also predispose for NAFLD (27, 51, 73, 96).

1.3.4 Pathogenesis

The pathogenesis of NALFD is a very complex interaction which is still not completely understood. A well-established model that displays the progress in a simplified manner is the so called "two hit model" (27, 50, 110) (Figure 8).

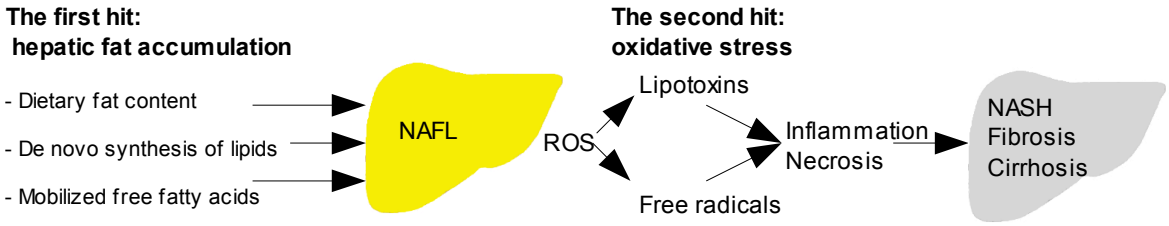


Figure 8: Pathogenesis of NAFLD, the "two hit model"

ROS = Reactive oxigen species

1.3.4.1 Pathogenesis of NAFL

Imbalances of the lipid metabolism and an increase in free fatty acids (FFA) are considered to be the "first hit". When the amount of lipids transported to the liver exceeds

the storage capacity of the hepatocytes, macrovesicular steatosis and NAFL are the consequences (27, 79).

There are mainly three sources of hepatic fat: (i) dietary fat content, (ii) de novo synthesis and (iii) circulating FFA mobilized from fat deposits.

FFA are the origin of about 60 % of hepatic fat. Fatty acids are mobilized from fat deposits even when food intake is within the normal range. The amount of FFA correlates with the body fat mass. Hereby, the visceral adipose tissue (VAT) mass plays a more important role than the total fat mass, since the lipolytic activity of the VAT mass is very high (50). However, the correlation between body fat and FFA is not linear. There seems to be some sort of metabolic compensation by the adipose tissue, leading to suppression of the mobilization of fatty acids to a certain degree (96).

There is a significant association between NAFLD and insulin resistance (IR) (54, 90), impaired glucose tolerance and T2DM (110). Insulin suppresses lipolysis and increases lipogenesis. Peripheral IR enhances lipolysis (96) and increases FFA delivery to the liver.

According to this theory, liver injury is the consequence of the metabolic imbalances. This theory is supported by the fact that NASH reoccurs frequently after liver transplantation (27). Peripheral IR, as the first threat, may be the cause not only for NAFLD, but also for T2DM, hypertriglyceridemia, obesity and hypertension. This is consistent with the fact that it is significantly associated with two or more features of the metabolic syndrome (79).

On the other hand, it has been discussed by several authors whether the cause-and-effect relationship might be the other way round and IR may be caused by hepatic steatosis. It has been shown in several animal experiments that steatosis may occur even prior hepatic and peripheral IR. Steatosis may lead to hepatic IR and, as a consequence hepatic glucose production will not be suppressed. Subsequently, blood glucose levels increase and in consequence this may lead to peripheral IR (27).

Whatever the reason for hepatic fat accumulation, which mainly consists of triglycerides (TG), the consequence is NAFL. TG per se are not hepatotoxic. However, their hepatic accumulation reflect the increased exposure of the liver to potentially toxic FFA. There are mechanisms to cope with these metabolic imbalances, but once they become insufficient the fatty liver becomes susceptible for further "hits" (50).

1.3.4.2 Pathogenesis of NASH, fibrosis and cirrhosis

The "second hit" is oxidative stress which is characterized by an imbalance between the production of reactive oxygen species (ROS) and the capacity of the antioxidant protective system in favor of the former. Several pathways may play a role in the overproduction of ROS in NASH including mitochondria, peroxisomes, cytochrome P-450, myeloperoxidase and nitric oxide synthase as the sources of ROS. When lipids are peroxided, the resulting lipotoxins and ROS may harm the liver and cause NASH, either by evoking an inflammatory response via proinflammatory cytokines or by affecting the hepatocytes directly by causing necrosis. There are physiological mechanisms to replace injured hepatocytes, but once their capacity is exceeded, hepatic stellate cells are activated to myofibroblasts and start to generate excessive matrix to replace the necrotic hepatocytes. This is the origin of fibrosis. Once the repair exceeds a certain threshold also cirrhosis will occur. The hepatic myofibroblasts may also stimulate progenitor cells to proliferate. These cells subsequently attract inflammatory cells via chemokines. Again, inflammation and NASH are the results (79, 132).

1.3.5 Natural history and prognosis

In the large spectrum of NAFLD, steatosis is always the first recognizable feature (96). Subsequently, there is a stepwise transformation of NAFL to NASH, fibrosis and cirrhosis (110) (Figure 9).

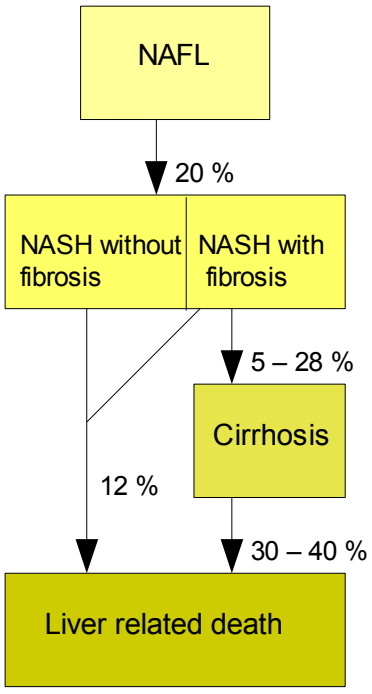


Figure 9: Natural history of NAFLD

For a long time, simple steatosis has been considered to be relatively benign and non-progressive (79, 96). However, evidence is emerging that this does not hold true. Shifflet et al. (110) showed a progression to NASH in 20 %. According to the Single Topic Conference of the AASLD in 2002, the progression of mild NAFL may be rare, but any amount of hepatic fat may sensitize the liver for further injuries (81). Mager et al. (73) confirmed that in particular in the pediatric population simple steatosis might not be entirely benign.

NASH occurs either isolated or in combination with hepatic fibrosis (79). Fibrosis itself may progress to cirrhosis in about 5-28 % within 10 years (27, 79, 86, 110). Progression to cirrhosis is also possible in childhood (73) and according to the AASLD, about 80 % of "cryptogenic cirrhosis" may be caused by NAFLD (81). About 12 % of patients with NASH and/or fibrosis (27) and 30-40 % of patients with cirrhosis (86, 110) decompensate and die due to liver disease. The AASLD estimates a 5-10 year survival of 59-67 %. However, the estimation of AASLD includes death from secondary disorders (81).

1.3.6 Diagnosis

The diagnosis of NAFLD is challenging and a completely satisfying answer to this problem has not been found yet.

The best possible diagnostic tool for NAFLD would have, at the same time, a high sensitivity (thus a great proportion of actual NAFLD positives would be correctly identified as such) and a high specificity (thus a great proportion of NAFLD negatives would be correctly identified as such). False positive test results (diagnosis of NAFLD in NAFLD negatives) and false negative test results (missed diagnosis of NAFLD in NAFLD positives) jeopardize the usefulness of a diagnostic tool. High sensitivity and specificity lead to a high predictive value which can be illustrated by the area under the receiver operating characteristic (ROC) curve.

The ROC curve is a graphical plot of the true positive results (sensitivity) versus the fraction of false positive results (1-specificity) for different cut-off values. The values of the area under the ROC curve (AUC) are useful to compare competing diagnostic tests (Figure 10). An AUC value of 1 indicates that the diagnostic test is both perfectly sensitive and specific. An AUC value of 0.5 indicates that the predictive value of the diagnostic test is not better than chance and therefore useless. An AUC value between 0.8 and 0.9 indicates a good predictive value and an AUC value above 0.9 an excellent predictive value of the diagnostic test (118, 129).

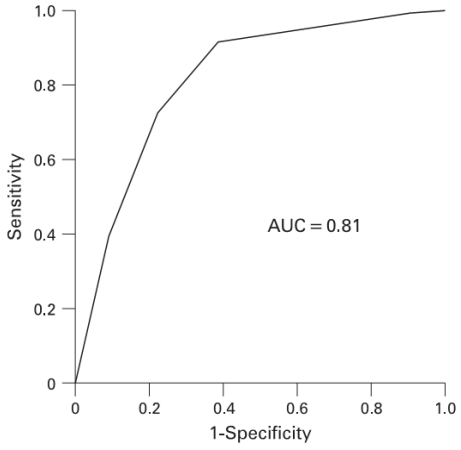


Figure 10: Area under the ROC curve from Harrison et al. (39)

Most diagnostic methods for NAFLD lack sensitivity or specificity and they are not able to distinguish between simple steatosis and inflammation or fibrosis. Therefore, biopsy is still the gold standard despite of its invasivity and high costs. There are lots of biomarkers and diagnostic indices under investigation and some of them are approximating liver biopsy in accuracy. However, further research on this topic is required.

1.3.6.1 Clinical findings

Most NAFLD patients, including children, are asymptomatic (79, 80, 96). Many patients are obese (2, 73), and especially in adults there may be other symptoms related to the metabolic syndrome, in particular to T2DM (2) and hypertension as well as to hyperuricemia and also to the polycystic ovarian syndrome (81).

Only in few adults and children with NAFLD there may be some sort of fatigue and hepatic symptoms such as pain in the right upper quadrant or hepatomegaly (80,96). In 33-50 % of the children with NASH also acanthosis nigricansas may be present (8,80). This dermatological marker of hyperinsulinism is characterized by a hyperpigmentation of the skin, which is usually found in body folds such as groin, neck and axilla (55).

In case of NAFLD-related cirrhosis the symptoms are similar to those of cirrhosis due to other causes: ascites, splenomegaly, bruising, jaundice (96), gastrointestinal bleeding from portal hypertension, muscle weakness and hepatic encephalopathy (27).

1.3.6.2 Biopsy as the gold standard

The definite diagnosis of NAFLD and the discrimination between NAFL, NASH, fibrosis and cirrhosis is not yet possible without liver biopsy, which remains the gold standard for diagnosis (14, 86). However, there is no consensus on which histological scoring system should be used. A common one, the NAS, has been presented in 1.3.1.2. (Histological features, p. 6).

There are no generally recognized guidelines on when to perform a biopsy (81). For several reasons, it is not applicable as a routine screening tool (61). First of all, the biopsy is a very cost and time consuming procedure (61). Also the clinical risk even in experienced hands is not negligible (96). The diagnostic value is limited by sampling variability and inter-rater variability. Given that a biopsy covers only a very small percentage of the liver, it does not necessarily represent the pathology of the liver as a whole.

In a study of Ratziu et al. (100), 51 patients have been evaluated by two biopsies and in 24 % of the cases steatosis would have been missed if only one biopsy had been taken.

1.3.6.3 Imaging procedures

The most common imaging procedures are ultrasonography, computed tomography (CT), and magnetic resonance imaging (MRI). They vary in their diagnostic power, but in general they are rather focused on measuring the degree of fat accumulation (17), and none of them is able to distinguish between simple steatosis and NASH. Even for the detection of cirrhosis their sensitivity is only low (27).

Ultrasonography is relatively inexpensive and easy to use. Increased echogenity compared to spleen or kidney indicates hepatic fat accumulation (110) (Figure 11).

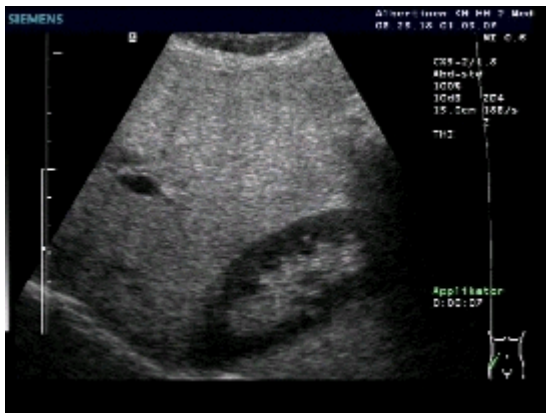


Figure 11: Fatty liver in ultrasonography
from Sonographie Atlas (114)

However, this "bright" scan is very unspecific, and steatosis, fibrosis and cirrhosis may not be distinguished (27, 79). Besides, its diagnostic value is operator-dependent (27). Overall, in normal weight patients the sensitivity is about 89 % and the specificity 93 %. In morbidly obese patients both sensitivity (49 %) and specificity (75 %) are significantly decreased (96).

The CT shows steatosis in a low liver attenuation. There is a correlation of the liver-to-spleen attenuation ratio and the severity of steatosis. The sensitivity is about 93 % and the positive predictive value in patients with a prevalesnce of steatosis of more than 33 % is 76 % (110). The CT is considered to be the most accurate imaging procedure for the diagnosis of liver steatosis (79).

The MRI is more sensitive than ultrasonography for detecting low degrees of steatosis (27). While it performs well in quantifying the liver fat content, it is not useful for routine examination due to its high costs (96).

Transient elastography is a more recent non-invasive diagnostic tool. It estimates liver stiffness by measuring the wave velocity of pulse-echo ultrasound waves. However, steatosis may counterbalance fibrosis and the inter-observer variability in assessing fibrosis is high (86). Its diagnostic value in determining the amount of hepatic fat is also poor (59).

1.3.6.4 Anthropometry

Anthropometric data is very easy to obtain. Data for their predictive value are inconsistent (Table 1). According to the AASLD, obesity is predictive for hepatic fibrosis (81). The waist circumference (WC) may even have a better predictive value than the BMI (56), given that visceral fat has a higher lipolytic activity (50). However, this has been denied by Cheung et al. (20), and according to Ishibashi et al. (48), WC is thought to be more susceptible to gender differences than to the actual VAT mass. Kotronen et al. (63) suggested that WC is a strong predictor not only for NAFLD but also for an elevated cardiovascular risk. A limitation of WC is that it is often measured at different sites and neither the exact way of measuring nor the cut-off value have been standardized yet (74).

Table 1: Anthropometry and associations with NAFLD

Authors, year	Variables	Study subjects	Associated conditions	Significance levels
Alavian et al., 2009 (5)	**BMI** **WC**	966 children with or without NAFLD, diagnosed by ultrasonography	NAFLD	P<0.001 P<0.001
Brunt et al., 2009 (12)	**BMI**	278 adults with or without NASH, confirmed by biopsy	Portal chronic inflammation	P<0.0001
	BMI	205 children with or without NASH, confirmed by biopsy	Portal chronic inflammation	NS
Manco et al., 2008 (74)	**BMI** **WC**	197 children with NAFLD, confirmed by biopsy	Fibrosis	NS P<0.0001
Patton et al., 2008 (89)	**BMI**	176 children with NAFLD, confirmed by biopsy	NASH Fibrosis	NS NS
Cheung et al., 2007 (20)	**BMI**	123 adults with or without NAFLD, confirmed by biopsy	Steatosis Lobular inflammation Hepatocyte ballooning Fibrosis Mallory hyaline	NS P<0.05 NS NS NS
	WC		Steatosis Lobular inflammation Hepatocyte ballooning Fibrosis Mallory hyaline	NS P<0.05 NS NS NS

Authors, year	Variables	Study subjects	Associated conditions	Significance levels
de Piano et al., 2007 (91)	**BMI** **WC**	43 children/adolescents with or without NAFLD diagnosed by ultrasonography	NAFLD	P≤0.05 NS
Yoneda et al., 2007 (136)	**BMI**	100 adults with NAFLD, thereof 71 with NASH, confirmed by biopsy	NASH (compared to steatosis) Advanced fibrosis (compared to mild fibrosis)	NS NS
Iacobellis et al., 2006 (45)	**BMI**	69 children with NAFLD, confirmed by biopsy	Fibrosis	P=0.01
Lee et al., 2006 (66)	**BMI** **WC**	150 adults with or without NAFLD, diagnosed by ultrasonography	NAFLD	P<0.001 P<0.001
Yesilova et al., 2005 (135)	**BMI**	81 male adults with and without NAFLD, confirmed by biopsy	NAFLD	NS

1.3.6.5 Serum markers

The serum markers that are most commonly used for the diagnosis of NAFLD are liver enzymes and metabolic markers. The diagnostic value of alanine transaminase (ALT), aspartat transaminase (AST) and γ-glutamyltransferase (GGT) will be discussed below. Serum levels of alkaline phosphatase are related to the activity of osteoblasts and thus the diagnostic value for NAFLD in growing children is limited (13).

Metabolic markers that have been investigated extensively in patients with NAFLD are fasting blood glucose, fasting serum insulin, total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol and triglycerides (TG).

In the last years also less commonly used markers such as hyaluronic acid (HA), endothelin, ferritin and others have become topics of interest. Unfortunately, no single biochemical test has been found that is sensitive and specific enough to be used in clinical practice for the diagnosis or subsequent monitoring of NAFLD (14).

Therefore, diagnostic indices, which are composed of several variables, have been developed. They are superior in their diagnostic value compared to single variables and will be discussed later.

1.3.6.5.1 Liver enzymes

The liver enzymes most commonly used for laboratory diagnosis of hepatic injury are the transaminases AST and ALT, GGT, glutamat-dehydrogenase and alkaline phosphatase (37). Their serum activities are elevated in various types of liver disease. The degree of elevation varies with the type of disease and it correlates with the amount of injured hepatic tissue (13). In general, the sensitivity of liver enzymes is high. Their specificity, however, is low and their power to distinguish between different types of liver disease is poor (109). When elevated activities start to decrease this may indicate either a recovery or a beginning liver failure. In cirrhosis, serum levels of AST and ALT may be elevated or normal (13).

1.3.6.5.1.1 GGT, AST, ALT and the ratio of AST to ALT

The enzyme **GGT** is mostly membrane-bound, but small amounts can also be found in the cytosol. The enzyme is nearly ubiquitous. High concentrations are present in the liver and within the liver, concentrations are highest in the bile canaliculi and the bile duct epithelium of the periportal zone. There is also some activity in the lipocytes in the space of Disse (13).

However, the highest specific activity of GGT is not found in the liver but in the kidney, where it is 25 times higher than in the liver. GGT is also located in pancreas, brain, lung, small intestine, spleen, mamma and prostate gland, but in contrast to alkaline phosphatase it is not present in bones, and in contrast to AST and ALT also not in erythrocytes (36).

Despite the fact that GGT is ubiquitous, elevations of its serum activities are very specific for a hepatic origin (13, 70). Serum activities start to rise when the enzyme activity is increased due to enhanced synthesis and/or when membrane fragments are released into the circulation. The half-life of GGT is 4.1 days (13).

As GGT is the most sensitive marker for liver injuries and hepatic bile duct injuries (13, 70), its serum activities are elevated in nearly all kinds of liver and bile diseases (70). According to Gressner et al. (36), in 95 % of all hepatobiliary diseases an elevation of serum GGT activities can be observed. Normal serum GGT activities rule out acute hepatic diseases. However, false positive results are very common and only one out of four persons with raised serum GGT activities actually has a hepatic disease (70).

In chronic hepatic diseases serum GGT activities may stay within the normal range. Mild elevations are observed in patients with virus hepatitis without further complications, chronic abuse of alcohol and liver congestion. Elevations are higher in chronic active hepatitis, alcoholic hepatitis, alcoholic cirrhosis, primary biliary cirrhosis, HCC, hepatic metastases and acute and chronic pancreatitis. Highest serum GGT activities are observed in intrahepatic or posthepatic cholestasis as well as in toxic liver injury (36).

Elevations of the transaminases **AST** and **ALT** are very common. In the United States they are present in about 8 % of the population. In most cases the etiology remains unknown. In children, 12 % of the idiopathic isolated elevations may be of genetic origin. Serum AST and ALT activities may increase after physical exercise. There are intra-individual fluctuations of 10-30 % that limit their diagnostic value (70). Serum levels of AST and ALT activities depend on the amount of these enzymes and their specific activity (13), as well as on the degree of hepatocellular injury (109) and the clearance from the bloodstream (70). The half-life of ALT is 47 hours and that of AST is 17 hours (13, 36). In contrast to GGT, the synthesis of the aminotransferases is never enhanced by liver diseases (70), but the hepatic transaminase activity *de*creases when the liver is injured. *Serum activities* of transaminases, however, *in*crease due to the hepatocellular injury (13).

ALT is present mainly in liver and kidney (70), but also in heart and skeletal muscles, pancreas, spleen, lung and erythrocytes (36). Its specific activity is highest in the liver and the elevation of serum ALT activities is relatively specific for a hepatic origin (36, 70). Within the liver, there exist two isoenzymes: the cytosolic ALT (85 %) and the mitochondrial ALT (15 %) (36). However, the specific activity of the mitochondrial isoenzyme is very low and its serum activities do not increase even in cases of severe hepatic injury (13). Serum activities of the cytosolic isoenzyme exceed the upper limit of normal range only when at least one out of 750 hepatocytes is injured. In 15 % of the patients with liver disease no elevation of serum ALT activities has been observed (70).

Even though the absolute amount of ALT is lower than the absolute amount of AST, its proportion present in the liver is greater and its increase is more specific for hepatic injury (109). Given that a great percentage of ALT is present in the cytosol, it increases earlier and in milder diseases than AST which exists mainly in the mitochondria (36).

Mild increases in serum ALT activities are observed in myocardial infarction, pancreatitis, primary liver tumor and hepatic metastases. Higher serum activities are found in mononucleosis infectiosa, liver cirrhosis, chronic hepatitis and liver congestion. High serum activities may also be found in routine screening due to obesity, T2DM, alcohol abuse, hepatic drug reaction, circulatory failure, α_1-antitrypsin-deficiency and hemochromatosis. Very high serum activities indicate acute hepatitis or acute toxic liver injury (36).

AST also exists in two isoforms, but in contrast to ALT the mitochondrial isoenzyme prevails with 80 %. The highest specific activity of AST is present in myocardium, liver, skeletal muscle and kidney. The serum AST activity increases when any of these tissues are injured (36, 70, 109). Therefore, the specificity of AST is low. Whenever serum AST activities are elevated, hepatic origin must be confirmed by a parallel increase in serum GGT or ALT activities (36).

In general, serum AST activities increase later than those of ALT. Therefore, serum AST activity elevations indicate more severe hepatic injury. The increase in serum AST activity is low in cirrhosis, myocarditis and mononucleosis infectiosa. A higher increase can be observed in myocardial infarction, trauma, muscular dystrophy, liver congestion, acute pancreatitis, pulmonary embolism, renal infarction and stroke. Serum activities are highest in acute hepatitis and acute toxic liver injury (36).

The **ratio of AST to ALT (AAR)** is also called de Ritis ratio. As described above, serum AST and ALT activities are not affected equally by the different types and degrees of liver disease. Therefore, the AAR may provide additional information (Table 2). However, the diagnostic value of the AAR is limited by the fact that half-lifes of AST (17 hours) and ALT (47 hours) differ substantially (36).

Table 2: The AAR and possible diagnoses according to Gressner et al. (36)

AAR	Possible diagnoses
<0.7	Acute and persistent virus hepatitis, toxic hepatitis, infectious mononucleosis, fatty liver including NAFL, obstructive icterus, cholestasis
~0.7	Cholestatic form of virus hepatitis, chronic NASH, chronic active hepatitis, reactive hepatitis, cholangitis
>0.7	Necrotic virus hepatitis, toxic hepatitis, cirrhosis, liver congestion, acute intoxication
>>0.7	Decompensated cirrhosis, HCC, hepatic metastases, myocardial infarction, skeletal muscular disease

In general, values <1 may indicate a mild and reversible inflammatory disease and values >1 are predictive for necrosis (36, 70). The AAR may also be used to differentiate between acute and chronic diseases. In acute liver diseases, both AST and ALT rise to values 30-80 times the upper limit of the normal range. Serum transaminase activities may also rise up to 2,000 U/L and even higher. Generally, serum AST activities increase even stronger than serum ALT activities, and thus the AAR is >1. In chronic liver diseases, serum ALT activities usually remain elevated, while serum AST activities may stay within the normal range. In this case the AAR is <1 (70).

The AAR has also been used to rule in or rule out alcohol abuse as the cause of liver injury. An AAR <1 makes a disease unrelated to alcohol more likely, while an AAR >1 is characteristic for alcoholic hepatitis (13). An AAR >2 may indicate alcoholic hepatitis and cirrhosis (109).

When cirrhosis develops and liver parenchyma is reduced, serum ALT activities decrease more rapidly than serum AST activities. Therefore, the AAR increases and values are >1. The sensitivity of an AAR >1 to predict cirrhosis has been shown to be about 32-83 % and the specificity 75-100 % (70).

Unfortunately, cut-off values have not been re-approved for several years (36) and similar data for AAR in the pediatric age range are not available at all.

1.3.6.5.1.2 Associations between liver enzymes and NAFLD

In NAFLD, elevations of serum ALT and AST activities are usually only modest (79) and tend to be higher in NASH than in NAFL (131). In general, serum GGT activities are lower than the serum AST and ALT activities. However, also an isolated elevation of serum GGT activities is possible. Serum ALT activities may increase to 1.5-2 times of the upper limit of the normal range while serum AST activities usually increase less (70). An elevation of serum transaminase activities to more than twice the upper limit of the normal range may be predictive for septal and bridging fibrosis (86). The AAR is usually <1 and increases with severity of liver injury. An AAR >1 may indicate advanced fibrosis or cirrhosis (2, 66) and disease progression (70).

NAFLD is often diagnosed in asymptomatic persons with raised serum transaminase activities during routine screening (27). In 1997, Tazawa et al. (120) confirmed the clinical significance of serum ALT tests as a simple diagnostic tool for NAFL. However, today the diagnostic value of transaminases is controversially discussed (96) (Table 3). According to

Wong et al. (137), serum ALT activities correlate poorly with metabolic and histological variables in NAFLD patients. Serum transaminase activities in adults and children may be normal despite histologically significant disease (27, 28, 75, 81), and the entire spectrum of NAFLD may be present in persons with normal serum ALT activities (132). According to Farrell et al. (27), 80 % of patients with steatosis may have serum transaminase activities within the normal range, and in 20 % of the liver donors who died of sudden death with normal serum transaminase activities, NAFL may have been present.

The diagnostic value of transaminases is limited by the fact that their serum activities fluctuate in individuals with liver disease (131, 134), as well as in healthy persons (134). Even in NAFLD patients who do have elevated serum transaminase activities there may be episodes in which their serum activities are within the normal range.

Wieckowska et al. (131) tried to improve sensitivity and specificity of serum ALT activities by changing the cut-off values of the normal range. However, they found ALT to be *either* specific *or* sensitive, but never both. This is consistent with Wong et al. (134) who confirmed that there is no appropriate cut-off value, neither for the diagnosis of NAFLD nor to distinguish between mild and severe NAFLD.

Also in the pediatric population, the diagnostic value of serum AST and ALT activities is limited. According to Manco et al. (75), normal serum ALT activities may be present in 21-23 % of children and adolescents with histologically confirmed NAFLD and even in 60 % of those with histologically confirmed fibrosis. Also inflammation, necrosis and fibrosis may be present in children with normal serum ALT activities (75).

In conclusion, the power of *absolute* serum transaminase activities to diagnose NAFLD is low. However, *changes* in serum transaminase activities over time may be a more reliable indicator of progression or regression of NAFLD (117).

According to Wong et al. (134), changes in serum ALT activities are associated with changes in the histological scoring system NAS. Several interventional and observational studies reported the impact that dietary restriction and exercise may have on serum AST and ALT activities. Two of these (121, 122) also demonstrated that the decrease in serum AST and ALT activities during weight reduction was accompanied by an improvement of liver histology (Table 4).

Table 3: Liver enzymes and their associations with NAFLD

Authors, year	Variables	Study subjects	Associated conditions	Significance levels
Brunt et al., 2009 (12)	**ALT**	278 adults with or without NASH, confirmed by biopsy	Chronic portal inflammation	NS
	ALT	205 children with or without NASH, confirmed by biopsy	Chronic portal inflammation	NS
Alavian et al., 2008 (5)	**ALT** **AST** **AAR**	966 children with or without NAFLD, diagnosed by ultrasonography	NAFLD	P<0.001 NS P<0.001
Patton et al., 2008 (89)	**AST** **ALT** **GGT**	176 children with NAFLD, confirmed by biopsy	NASH	P<0.0001 P=0.002 P<0.0001
	AST **ALT** **GGT**		Fibrosis	P=P=0.0003 NS P=0.001
Imhof et al., 2007 (47)	**AST** **ALT** **GGT**	378 children with or without NAFLD, diagnosed by ultrasonography	NAFLD	P<0.05 P<0.05 P<0.05
Yoneda et al., 2007 (136)	**AST** **ALT**	100 adults with NAFLD, 71 of which with NASH, confirmed by biopsy	NASH (compared to steatosis)	P=0.0128 P=0.0808
	AST **ALT**		Advanced fibrosis (compared to mild fibrosis)	NS NS
Bonnefont-Rousselot et al., 2006 (11)	**AST** **ALT** **GGT**	64 adults with or without NAFL, confirmed by biopsy	NAFL	NS NS P=0.047
Iacobellis et al., 2006 (45)	**AST** **ALT** **AAR**	69 children with NAFLD, confirmed by biopsy	Fibrosis	NS NS P=0.05
Lee et al., 2006 (66)	**AST** **ALT** **AAR** **GGT**	150 adults with or without NAFLD, diagnosed by ultrasonography	NAFLD	P<0.001 P<0.001 P<0.001 P<0.001
Yesilova et al., 2005 (135)	**AST** **ALT**	81 male adults with and without NAFLD, confirmed by biopsy	NAFLD	P<0.001 P<0.001

Table 4: Influence of lifestyle on serum transaminase activities and liver pathology

Authors, date	Study population	Study type	Trial period	Lifestyle factors	Significant amelioration of			
					AST l	ALT	Liver ultra-sonography	Liver histology
Reinehr et al., 2009 (102)	152 obese children with NAFLD, suspected by ultrasonography	1 year interventional and 1 year observational	2 years	Diet, exercise and behavior therapy	yes	yes	yes	-[1]
Gasteyger et al., 2007 (32)	43 obese men without known hepatic disease	6 weeks interventional and 60 weeks observational	66 weeks	Diet and exercise	yes	yes	-	-
	104 obese women without known hepatic disease				no	no		
Wang et al., 2007 (128)	76 obese children with NAFLD, suspected by ultrasonography	interventional and untreated control group	1 month	Diet	yes	yes	no	-
Nobili et al., 2006 (84)	84 children and adolescents with NAFLD, confirmed by biopsy	interventional	12 months	Diet and exercise	yes	yes	yes	-
Huang et al., 2005 (44)	23 adults with NASH, confirmed by biopsy	interventional	12 months	Diet	no	no	-	yes
Suzuki et al., 2005 (116)	348 male adults with elevated serum ALT activities of unknown origin	observational	12 months	Diet	-	yes	-	-
Hickman et al., 2004 (42)	31 adults with hepatic steatosis of different origin, confirmed by biopsy	interventional	15 months	Diet and exercise	-	yes	-	-
Ueno et al., 1997 (121)	25 obese adults with NAFL, confirmed by biopsy	interventional	3 months	Diet and exercise	yes	yes	-	yes
Uslan et al., 1997 (122)	25 obese adults with fatty liver, confirmed by biopsy	interventional	3 months	Diet and exercise	yes	yes	-	yes
Tazawa et al., 1996 (119)	73 obese children with elevated serum transaminase activities of unknown origin	observational	3 months	Diet	yes	yes	-	-
Vajro et al., 1994 (124)	9 obese children with elevated serum transaminase activities of unknown origin, ultrasonography performed in 7 patients, NAFLD suspected in 6. Biopsy performed in 1 patient, NASH confirmed	interventional	12 month	Diet and exercise	-	yes	yes	1 of 1

[1]The dash (-) indicates that the particular parameter has not been investigated in the study.

Furthermore, 5,237 men without serum ALT activity elevations and without ultrasonographic findings of NAFLD at baseline were followed over a period of 2.5 years (16). Of these, 18.8 % developed NAFLD. Elevated serum ALT activities were identified as independent predictors for the incidence of NAFLD. Kim et al. (58) studied 2,895 adults over a period of five years and demonstrated that individuals who developed NAFL showed more pronounced weight gain and a higher increase in serum activities of AST, ALT and GGT and a higher increase in fasting blood glucose concentrations than those who did not develop NAFL. In both studies the liver was evaluated by ultrasonography.

Most of these studies showed that decreases in serum transaminase activities during lifestyle modification are accompanied by an amelioration of hepatic steatosis. However, the relationship between serum transaminase activities and inflammation and fibrosis is less consistent (15).

In addition, there is a strong relationship between serum ALT activities and the features of the metabolic syndrome (19). A study on 37,085 adults showed that elevated serum ALT activities correlate with death from cardiovascular disease or T2DM (139).

Despite the diagnostic limitations of liver enzymes, they have been recommended by several authors as a screening tool for NAFLD. In addition, ultrasonography should be performed (30, 105).

1.3.6.5.2 Metabolic markers

1.3.6.5.2.1 Fasting blood glucose

Fasting blood glucose concentrations, determined 8-10 hours after food intake, give accurate information about the glucose metabolism. An elevated fasting blood glucose concentration is predictive for low insulin secretion, peripheral IR and low insulin sensitivity of the liver. It is also useful for the detection of an impaired carbohydrate metabolism due to hepatitis, pancreatitis, acromegaly, Addison's disease etc.

Fasting blood glucose concentrations increase with age and decrease with muscular activity. Interestingly, glucose concentrations at rest do not depend on physical fitness. There are great inter-individual differences in normal concentrations and the range of reference values is broad (70).

1.3.6.5.2.2 Serum lipids and lipoproteins

Serum lipids play a major role in atherogenesis and they are strongly associated with coronary heart disease. Both healthy diet and physical activity may have a positive impact on their plasma concentrations (26, 53, 70) (Table 5).

Table 5: Impact of diet and exercise on the lipid profile

	TG	Cholesterol	HDL	LDL
Healthy diet	↓	↓	↑	↓
Exercise	↓	↓	↑	↓

Triglycerides (TG) are the most common naturally occurring lipids. They consist of a backbone of glycerin that is esterified with three fatty acids (70). TG store energy and they are the physiological substrate with the greatest density of energy (9 kcal/g) (36). They also act as transport vehicles carrying energy from the gut and liver to peripheral tissues (109). They are transported mainly in the very low-density lipoproteins (VLDL) and in chylomicrons (70).

TG are either taken up from food or formed endogenously. Normal serum concentrations vary between individuals (70). Hypertriglyceridemia is caused mainly by alimentation but can also be caused by genetic disorders (36). Secondary hypertriglyceridemias can be caused by liver disease, nephropathy, hypothyroidism and pancreatitis. Laboratory analysis does not differentiate between primary and secondary hypertriglyceridemia (70).

Elevated serum TG concentrations indicate an increased risk of atherosclerosis. The risk increases exponentially when serum LDL concentrations are also elevated (70). Of all serum lipids, TG have the strongest association with NAFLD (81).

Cholesterol is synthesized ubiquitously in the body. It is an essential part of membranes and lipoproteins and also a precursor for the synthesis of steroid hormones and bile acids. Cholesterol is transported to the liver where it is secreted into the bile. It occurs partly as free cholesterol (25-40 %) and about 60-75 % are esterified with fatty acids. In routine laboratory analysis there is no differentiation. In blood plasma, cholesterol is transported mainly in LDL (66 %) and to a lesser extent in HDL (25 %) and VLDL (70).

Cholesterol is partly formed endogenously and partly ingested with food. Serum concentrations are dependent on age, gender, diet, physical activity and smoking habits, and there are great inter-individual differences. Several genetic disorders lead to high serum cholesterol concentrations. Elevated serum cholesterol concentrations indicate an increased risk of atherosclerosis and coronary heart disease. However, as there are great inter-individual differences in normal serum concentrations and as atherosclerosis is a multifactorial disease, the prognostic value for a given individual is only low (70).

HDL transports the extrahepatic cholesterol back to the liver where it is either metabolized or secreted (69). Low serum HDL concentrations are associated with atherosclerosis and coronary heart disease, and high serum HDL concentrations have a protective effect. Serum concentrations decrease with smoking and increase with physical exercise. They also depend on gender and age (70).

LDL is very rich in cholesterol. LDL carries cholesterol, that has been synthesized in the liver, to peripheral tissues (69). Of all serum lipids, LDL has the highest association with atherosclerosis. The incidence of myocardial infarction increases exponentially with increasing serum LDL concentrations. Serum LDL concentrations are increased in familial hypercholesterolemia (70). A healthy diet and exercise may lower serum LDL concentrations (53).

1.3.6.5.2.3 Associations between metabolic markers and NAFLD

Several authors assume that metabolic markers may correlate better with liver histology than the liver enzymes. Fracanzani et al. (28) compared 395 NAFLD patients with elevated serum ALT activities to 63 NAFLD patients with normal serum ALT activities. They came to the conclusion that normal serum ALT activities are not a valuable criterion to rule out NAFLD and that the diagnostic value of metabolic markers may be superior. Also Wong et al. (134) suggest that fasting blood glucose concentrations and the presence of T2DM may predict NASH and that fasting blood glucose, age and IR may predict fibrosis.

According to the Asia-Pacific Working Party on NAFLD, the latter should be suspected in subjects with metabolic risk factors and/or characteristic changes in hepatic ultrasonography (15). Data on the association between metabolic markers and NAFLD show inconsistent results (Table 6).

Table 6: Metabolic markers and their associations with NAFLD

Authors, year	Variables	Study subjects	Associated conditions	Significance levels
Alavian et al., 2009 (5)	**Fasting glucose** **Fasting insulin** **TG** **HDL** **LDL** **Total cholesterol**	966 children with or without NAFLD, diagnosed by ultrasonography	NAFLD	NS P<0.001 P<0.001 NS P<0.001 P<0.001
Brunt et al., 2009 (12)	**Insulin level**	278 adults with or without NASH, confirmed by biopsy	Chronic portal inflammation	P=0.001 P<0.0001
	Insulin level	205 children with or without NASH, confirmed by biopsy	Chronic portal inflammation	NS NS
Manco et al., 2008 (74)	**HDL** **TG** **Glucose**	197 children with NAFLD, confirmed by biopsy	Fibrosis	NS NS NS
Patton et al., 2008 (89)	**Fasting glucose** **Fasting insulin**	176 children with NAFLD, confirmed by biopsy	NASH NASH	NS NS NS
de Piano et al., 2007 (91)	**Fasting glucose** **Total cholesterol** **TG** **HDL** **LDL**	43 children/adolescents with or without NAFLD, suspected by ultrasonography	NAFLD	NS P=0.003 P=0.03 NS NS
Puri et al., 2007 (97)	**TG**	27 adults with or without NAFL/NASH, confirmed by biopsy	NAFL NASH	P<0.05 P<0.05
Yoneda et al., 2007 (136)	**HDL** **LDL** **TG**	100 adults with NAFLD, 71 of which with NASH, confirmed by biopsy	NASH (compared to steatosis)	NS NS NS
	HDL **LDL** **TG**		Advanced fibrosis (compared to mild fibrosis)	NS NS NS
Bonnefont-Rousselot et al., 2006 (11)	**Fasting glucose** **TG** **HDL** **LDL** **Total cholesterol**	64 adults with or without NAFL, confirmed by biopsy	Steatosis	P=0.006 P=0.014 P=0.01 NS NS
Iacobellis et al., 2006 (45)	**Total cholesterol** **TG** **Fasting glucose** **Fasting insulin**	69 children with NAFLD, confirmed by biopsy	Fibrosis	NS NS NS NS
Lee S. et al., 2006 (66)	**Total cholesterol** **TG** **HDL** **LDL** **Fasting glucose**	150 adults with or without NAFLD, suspected by ultrasonography	NAFLD	P<0.01 P<0.001 P<0.001 NS P<0.01
Yesilova et al., 2005 (135)	**HDL** **TG**	81 male adults with and without NAFLD, confirmed by biopsy	NAFLD	P<0.001 P<0.001

1.3.6.5.3 Markers of oxidative stress, inflammation and apoptosis

1.3.6.5.3.1 Malondialdehyde, interleukin-6, C-reactive protein, tumor necrosis factor-alpha, adiponectin and vitamin E

Malondialdehyde (MDA) is a marker of oxidative stress. It is formed as a result of the peroxidation of polyunsaturated fatty acids with three ore more conjugated double bounds. Increased levels are found in chronic inflammatory diseases and may indicate tissue injury (70).

Vitamin E is a fat-soluble vitamin. It occurs in green vegetables as well as in nuts vegetable oils and is absorbed by the upper small intestine. It has antioxidative effects and prevents polyunsaturated fatty acids in cell membranes and lipoproteins from peroxidation. Vitamin E also has immunomodulatory, anti-inflammatory, anti-thrombotic, anti-atherogen and neuro-protective effects. Symptomatic vitamin E deficiency is observed very rarely. Symptoms are hemolysis and neuromuscular dysfunctions. Low plasma concentrations are found in exocrine pancreatic insufficiency and cholestasis with subsequent fat malabsorption, parenteral nutrition without adequate vitamin E supplementation and long-term low-fat diets. (36).

Interleukin-6 is a proinflammatory cytokine which can be secreted by two different types of cells: (i) endothelial and epithelial cells, which are stimulated by hypoxia, trauma, cardiac insufficiency and muscular trauma and (ii) immunocompetent cells like monocytes and macrophages, which are stimulated by the contact with bacteria or bacterial toxins. While the stimulation of endothelium and epithelium leads to an isolated increase in IL-6, the immunocompetent cells also secrete other cytokines such as tumor necrosis factor-alpha (TNF-α). Therefore, in acute inflammatory diseases IL-6 and TNF-α concentrations are both increased. In chronic inflammatory diseases, the diagnostic power of IL-6 is only poor, and leukocytosis and elevations of C-reactive protein concentrations may be more useful diagnostic markers of inflammation (70).

The **C-reactive protein (CRP)** is the marker that is most commonly used for the diagnosis of inflammatory diseases. It is an acute phase protein produced in the liver. Its production is induced by proinflammatory cytokines like IL-6 and others. Serum CRP concentrations rise within six hours and reach their maximum after 48 hours. The half-live of CRP is 48 hours.

Normal serum CRP concentrations have an extremely high inter-individual variability (30-60 %) and correlate negatively with physical fitness and positively with smoking. Elevated serum CRP concentrations are observed in different kinds of inflammatory diseases (sepsis, acute and chronic inflammatory diseases), as well as in necrosis (malignant tumors, surgery, extreme exercise, trauma). However, a normal serum CRP concentration does not rule out mild local inflammation. The degree of the elevation of CRP depends on the inflammatory activity, the mass of inflamed or injured tissue and on the capacity of the liver to synthesize CRP. In general, bacterial infections lead to a greater increase than viral infections, and serum CRP concentrations may be used for the differentiation (70).

TNF-α is a cytokine which is secreted by the adipose tissue as well as by monocytes, macrophages, lymphocytes, granulocytes, astrocytes and smooth muscle cells. It is an acute phase protein and a very sensitive marker for an excessive inflammatory response. Its concentrations are increased in acute and chronic inflammatory diseases, traumata, autoimmune diseases and cardiac insufficiency (36, 70, 112).

Adiponectin is an opponent of TNF-α. It is secreted by the adipose tissue and has anti-inflammatory and anti-fibrotic effects. It also reduces body fat and ameliorates IR (112).

Cytokeratin-18 (CK-18) is an intracellular protein expressed by many cells of epithelial origin, including hepatocytes. It is released into the blood by cellular necrosis and apoptosis (126).

1.3.6.5.3.2 Association between markers of oxidative stress/inflammation/ apoptosis and NAFLD

Several markers of oxidative stress, inflammation and apoptosis are associated with NAFLD (Table 7). Inflammatory markers like adiponectin and TNF-α are used to predict not only NASH, but also fibrosis. In particular the imbalance of adiponectin and TNF-α may be associated with NASH. Serum CRP concentrations tend to be higher in NASH and fibrosis than in NAFL (86), but earlier suggestions that it might be valuable for the diagnosis of NAFLD have not been confirmed (7, 27). CK-18 may be able to predict the severity of liver disease and may indeed be a useful marker (27). Yesilova et al. (135) demonstrated an association between MDA and NAFLD.

Table 7: Markers of oxidative stress, inflammation and apoptosis and their associations with or predictive values for NAFLD

Authors, year	Variables	Study subjects	Associated conditions	Significance levels/ predictive value
Jarrar et al., 2008 (49)	**TNF-α** **IL-6**	95 adults with or without NAFLD, confirmed by biopsy	NAFLD	P=4.32096e-13 P=0.005
	TNF-α **IL-6**		NASH, compared to steatosis	P=0.0007 NS
Vos et al., 2008 (126)	**CK-18**	62 children, 14 of which with NAFL, suspected by ultrasonography and 6 with NASH, confirmed by biopsy	NAFLD	P<0.001
Yoneda et al., 2007 (136)	**CRP**	100 adults with NAFLD, 71 of which with NASH, confirmed by biopsy	NASH, compared to steatosis Advanced fibrosis, compared to mild fibrosis	P=0.0048 P=0.0384
Yesilova et al., 2005 (135)	**MDA**	81 male adults with and without NAFLD, confiremed by biopsy	NAFLD	P<0.001
Anty et al., 1996 (7)	**CRP**	46 severely obese adults with or without NASH, confirmed by biopsy	NASH	not predictive

1.3.6.5.4 Others

There are also some other markers of inflammation and fibrosis under investigation (Table 8). For those variables, which are not only associated with NAFLD, but actually have a positive predictive value, AUC-values are provided instead of P-values.

Table 8: Different markers and their associations with or predictive values for inflammation or fibrosis

Authors, year	Variables	Study subjects	Associated conditions	Significance levels/ predictive values
Degertekin et al., 2007 (24)	**Endothelin-1**	92 adults, 52 of which with NAFLD, confirmed by biopsy and and 40 of which with NAFL, suspected by ultrasonography	NASH (compared to steatosis)	P<0.01
Yoneda et al., 2007 (136)	**Ferritin** **HA**	100 adults with NAFLD, 71 of which with NASH, confirmed by biopsy	NASH (compared to steatosis)	P=0.0450 P=0.0478 NS P=0.0010
	Ferritin **HA**		Advanced fibrosis (compared to mild fibrosis)	NS P=0.0010
Lydatakis et al., 2006 (72)	**HA** **Laminin**	50 adults with NASH, confirmed by biopsy	NASH with fibrosis (compared to NASH without fibrosis)	P<0.001 P<0.001
Suzuki et al., 2005 (115)	**HA**	79 adults with NAFLD, confirmed by biopsy	Different stages of fibrosis	AUC=0.67-0.92
Yoneda et al., 2009 (137)	**Ferritin**	106 adults with or without NAFLD, confirmed by biopsy	NASH (compared to steatosis)	P=0.006 AUC=0.732

1.3.6.6 Diagnostic indices

Diagnostic indices consist of serum markers and anthropometric variables, which are entered into an algorithm to finally give a single value. Based on this value, the presence of the different features of NAFLD can be estimated. There are different indices for the prediction of NAFL, NASH and fibrosis. In addition to the variables that have been presented above, some diagnostic indices also include albumin, platelet count, blood pressure or less common variables like transforming growth factor-β, aminoterminal peptide of pro-collagen III, type IV collagen propeptide, α_2-macroglobulin, laminin, matrix metallo-proteinases or tissue inhibitors of metalloproteinases (27, 86).

There are lots of diagnostic indices under investigation. Their diagnostic performance depends on the variables included. The indices that include less common variables are limited by the lack of their availability and standardization. In recent years some indices with excellent performance have been developed, but none has been validated prospectively for its ability to monitor changes. Further studies are needed to decide which diagnostic index is the one that is most capable to replace liver biopsy (17).

1.3.6.6.1 Indices for NAFL

To predict steatosis, the Fatty Liver Index (FLI) and the SteatoTest have been developed.

1.3.6.6.1.1 The Fatty Liver Index

The FLI is a simple algorithm that was developed by Bedogni et al. (9) to predict steatosis in the general population. In total they studied 496 adults, 216 with and 280 without liver diseases of different origin, diagnosed by ultrasonography. To identify the most potent predictors of steatosis among 13 variables (gender, age, ethanol intake, AST, ALT, GGT, BMI, WC, sum of four skin folds, glucose, insulin, TG and total cholesterol) they used stepwise logistic regression analysis. The FLI was developed based on the four best performing variables: TG, BMI, GGT and WC.

Calculation of the FLI is done as follows:

$$FLI = \frac{e^{0.953 * \ln(TG) + 0.139 * BMI + 0.718 * \ln(GGT) + 0.053 * WC - 15.745}}{1 + e^{0.953 * \ln(TG) + 0.139 * BMI + 0.718 * \ln(GGT) + 0.053 * WC - 15.745}} * 100$$

The values of the FLI range from 0 to 100. For adults, FLI-values <30 rule out and FLI-values ≥60 rule in fatty liver. The same authors (9) showed a very good performance of the FLI in the study population with an AUC-value of 0.85. So far, the FLI has not been used in other populations. The advantage of the FLI is that it consists of variables that are readily available and easy to obtain.

1.3.6.6.1.2 The SteatoTest

The SteatoTest was designed by Poynard et al. (94). It was applied in 310 adults with different liver diseases and detected steatosis with an accuracy that was similar to that of the FLI (0.79-0.86). It consists of the routine variables GGT, ALT, total bilirubin, BMI, glucose, TG and total cholesterol and the less commonly used variables α_2-macroglobulin, haptoglobin and apolipoprotein A I. Given that these markers are not readily available in smaller laboratories, the clinical value of the test is limited.

1.3.6.6.2 Indices of inflammation and NASH

There are several indices to predict inflammation and NASH with AUC-values ranging from 0.69 to 0.91 (Table 9).

Table 9: Diagnostic indices for NASH

Authors, year	Name of the methods	Variables	Study subjects	Predictive values (AUC)
Poynard et al., 2006 (93)	**NashTest**	Age, gender, height, weight, TG, cholesterol, α_2-macroglobulin, apolipoprotein AI, haptoglobin, GGT, ALT, ALT and total bilirubin	160 adults with NAFLD, confirmed by biopsy	0.69-0.79
Younossi et al., 2008 (138)	**NASH Diagnostics**	Cleaved and intact CK-18, resistin and adiponectin	101 adults with NAFLD, confirmed by biopsy	0.91
Palekar et al., 2006 (88)	-	Age, gender, BMI, AST, AAR and HA	80 adults with NAFLD, confirmed by biopsy	0.76
Shimada et al., 2007 (111)	-	Adiponectin, HOMA-IR[1] and serum type IV collagen 7S	85 adults with NAFLD, confirmed by biopsy	Separately for the 3 variables: 0.77, 0.76, 0.76

[1]Homeostasis model assessment insulin resistance (HOMA-IR) = [(fasting glycemia (mmol/L) * fasting insulinemia (μIU/L)] / 22.5

1.3.6.6.3 Indices for fibrosis

There are also many diagnostic indices to predict fibrosis (Table 10). The AUC-values range from 0.76 to 0.99. The FibroTest showed excellent results in a meta-analysis including a total of 30 studies (92). The ELF Test has also been validated for the pediatric population.

Table 10: Diagnostic indices for fibrosis

Authors, year	Name of the methods	Variables	Study subjects	Predictive for	Predictive values (AUC)
Nobili et al., 2009 (85)	**Enhanced Liver Fibrosis (ELF) test**	HA, amino-terminal propeptide of type III collagen and tissue inhibitor of metalloproteinase 1	112 children and adolescents with NAFLD, suspected by ultrasonography	Different stages of fibrosis	0.90-0.99
Guha et al., 2008 (38)	**Enhanced Liver Fibrosis (ELF) test**	HA, amino-terminal propeptide of type III collagen and tissue inhibitor of metalloproteinase 1	196 adults with NAFLD, confirmed by biopsy	Different stages of fibrosis	0.82-0.93
	Simple panel	Age, BMI, T2DM/impaired fasting glucose, AAR, platelet count and albumin			0.79-0.89
Harrison et al., 2008 (39)	**BARD score**	BMI, AAR and T2DM	827 adults with NAFLD, confirmed by biopsy	Different stages of fibrosis	0.81-0.83
Angulo et al., 2007 (6)	**NAFLD Fibrosis Score**	Age, BMI, hyperglycemia, AAR, platelet count and albumin	733 adults with NAFLD, confirmed by biopsy	Advanced fibrosis	0.84
Ratziu et al., 2006 (100)	**FibroTest**	Bilirubin, GGT, haptoglobin, α_2-macroglobulin and apolipoprotein A1, corrected for age and gender	170 adults with NAFLD, confirmed by biopsy	Different stages of fibrosis	0.81-0.92

1.3.7 Therapy

Currently, there are no approved therapies for NAFLD (86). Due to its strong association with overweight, weight reduction appears to be the most promising strategy. However, there is still very little empirical evidence on the effects that weight loss may have on liver pathology (4, 21). Kim et al. (58) analyzed 2,895 persons over five years and showed that weight loss may ameliorate steatosis diagnosed by ultrasonography. Liver biopsy was not performed. That IR may be improved by weight reduction has been shown in several studies (40, 86, 98, 112).

Weight loss can be achieved by lifestyle modification, pharmacological therapy, surgery or a combination thereof. Lifestyle modification including dietary restriction or exercise or a combination of both is the gold standard therapy (140) and should always be the first option. If weight loss cannot be achieved by these interventions, pharmacological therapy should be considered. For morbidly obese and for those at great risk of fibrosis, bariatric surgery may be indicated (4).

In addition, NAFLD patients should be screened for IR, dyslipidemia and hypertension, and these conditions should be treated appropriately (1, 15).

1.3.7.1 Lifestyle modification

Lifestyle modification includes dietary restriction and exercise. It not only reduces weight but also improves the lipid profile and other metabolic markers (26, 70).

1.3.7.1.1 Multidisciplinary approach

Given that lifestyle modification is not achieved easily, the majority of obese subjects has major difficulty in losing weight (98). The compliance in obesity intervention programs is poor and even under trial conditions the dropout is about 30-41 % (1). This is the reason why a multidisciplinary approach is required. The team should include physicians, dietitians, psychologists and physical activity coaches. Behavior and cognitive strategies are recommended to improve motivation and compliance of the participants and to encourage them to achieve a lifestyle modification that will be sustainable (10, 127).

1.3.7.1.2 Dietary restriction

As energy intake is significantly higher in patients with NAFLD, the reduction of food intake may be beneficial for the liver. Table 4 (Influence of lifestyle on serum transaminase activities and liver pathology, p. 24) displays several studies and their effects on serum transaminase activities and liver pathology. There are three studies that also demonstrated beneficial effects of lifestyle changes on liver histology. Recently, Huang et al. (44) showed that liver histology of NASH patients correlates with the extent of weight loss during a 12-month dietary intervention program. Large prospective randomized clinical studies on the effects of weight loss on liver histology are still lacking (40).

So far, there is no general agreement, neither on the amount of recommended weight loss nor on the ideal dietary composition in NAFLD.

The American Gastroenterological Association recommends a weight loss of 1-2 pounds/week which equals about 450-900 g/week. A weight reduction of 10 % should be targeted if the BMI exceeds 25 kg/m² (112). According to Harrison et al. (40), this may improve liver histology including steatosis and inflammation as well as the metabolic variables. Suzuki et al. (116) showed that also a weight reduction of only 5 % may have similar effects. They observed a decrease in TG, fasting blood glucose and blood pressure as well as an increase in serum HDL cholesterol concentrations.

Evidence suggests that major and/or rapid weight loss may lead to metabolic imbalances (35) and increase the risk for exacerbation of liver injury (112, 140). While steatosis may improve, inflammation and fibrosis may progress and worsen the prognosis significantly (40, 77). According to Oh et al. (86), for adults a weight loss of more than 1.6 kg/week has been associated with exacerbation of NASH. Adams et al. (1) assume that a low energy diet of 388 kcal/day may be associated with an increase in portal inflammation and thus should be avoided.

In terms of the composition of the food there are also no general recommendations. The American Dietetic Association, the American Heart Association and the National Heart, Lung and Blood Institute each developed different guidelines (140). There is a great body of evidence suggesting that high carbohydrate diets are associated with inflammation (33, 113, 140). Therefore, some dietary recommendations may not be beneficial but could even contribute to liver injury (113).

There are three studies that showed a significant histological improvement of NAFLD during dietary intervention programs (Table 11). In two of them a percentage of energy derived from carbohydrates at or below 50 % has been recommended. This is in contrast to the current recommendations of the DGE that are an energy intake from carbohydrates above 50 % (25).

Table 11: Different dietary regimes with improvement of liver histology and comparison to the dietary intake in the MODUL Program

Author, year	**Distributions of macronutrients**		
	Fat	**Protein**	**Carbohydrate**
Ueno et al. 1997 (121)	30 %	20 %	50 %
Huang et al. 2005 (44)	35-40 %	15-20 %	40-45 %
Uslan et al. 2007 (122)	15 %	25 %	60 %
MODUL Program - as targeted - as achieved	 25 % 30 %	 20 % 20 %	 55 % 50 %

As for adults, also for obese children and adolescents a weight reduction of 10 % is recommended. The weekly weight loss should be about 0.5 kg. For the pediatric population only few studies investigated the effects of lifestyle interventions and none of them monitored the histological response (77). Recently, Reinehr et al. (102) conducted a

longitudinal study on 187 obese children with NAFLD diagnosed by ultrasonography. Thee children were studied during one year of lifestyle modification including diet, exercise and behavior therapy. BMI, transaminases and sonographic signs of NAFLD improved significantly. Liver histology remained unknown.

1.3.7.1.3 Exercise

Physical activity has beneficial effects on components of the metabolic syndrome, such as T2DM, obesity, dyslipidemia and IR, all of which are risk factors for NAFLD (15, 98). Also elevated serum transaminase activities decrease with regular physical activity (67). Suzuki et al. (116) showed that even moderate exercise that did not reduce body weight reduced visceral fat and decreased IR.

There is no general agreement on the recommendations of type and extent of exercise. Just as for dietary advices, individual needs and physical fitness must be taken into account (140). Harrison et al. (40) recommended moderate exercise, preferably a combination of aerobic and restrictive exercise, expending 400 calories, 3-4 times a week. Grattagliano et al. (35) suggested that aerobic activity should be gradually increased.

Effects of physical exercise that occur independently from dietary restriction are difficult to assess in human studies since there are no randomized controlled trials addressing this issue (15). According to Rafiq et al. (98), especially aerobic exercise may prevent steatosis independently of weight loss. Chen et al. (18) conducted a study comparing the effects of an exercise-only program with the effects of a diet-plus-exercise program. He found beneficial effects on anthropometric variables, IR and ultrasonographic findings in both groups, but the degree of improvement in the diet-plus-exercise program was higher.

1.3.7.1.4 Combined dietary restriction and exercise

There is a great body of evidence supporting the beneficial effects of a combination of dietary restriction and exercise in the management of NAFLD (Table 4: Influence of lifestyle on serum transaminase activities and liver pathology, p. 24). Recently, Nobili et al. (84) demonstrated on 84 children and adolescents with histologically confirmed NAFLD that a diet and exercise lead to a significant improvement of ultrasonographic findings of NAFLD. Two additional studies (121, 122) also documented the effects of dietary-plus-exercise programs on liver histology.

1.3.7.2 Pharmacological therapy

If sufficient weight loss is not achieved by lifestyle modification, pharmacological therapy is recommended. There are many different drugs under investigation, each of which is targeting a different aspect of the pathogenesis of NAFLD. Pilot studies showed promising results, but randomized controlled trials are lacking (35) and further investigations are needed to define the appropriate dosage and duration of treatment, as well as safety and tolerability (86).

1.3.7.2.1 Amelioration of insulin resistance

The strong association between IR and NAFLD has been discussed above. There are two different groups of drugs interfering with IR: anti-obesity drugs and insulin sensitizers.

1.3.7.2.1.1 Anti-obesity drugs

Orlistat is a gastrointestinal lipase inhibitor. The gastrointestinal lipase is responsible for the breakdowns of TG in the intestine (99) and its blockade leads to a reduction of fat absorption by 30 % (4).

Sibutramine is a serotonin and norepinephrine reuptake inhibitor that increases satiety (86).

Both orlistat and sibutramine may decrease body weight, IR, serum ALT and GGT activities, as well as steatosis, hepatic inflammation and fibrosis. Orlistat additionally may improve the lipid profile. However, it is limited by gastrointestinal side effects, particularly if patients do not adhere to the prescribed diet (4, 86). According to Vullampachi et al. (127), the degree of both weight loss and histological improvement that may be achieved by orlistat is not superior to that achieved with dietary restriction alone.

1.3.7.2.1.2 Insulin sensitizers

The thiazolidinediones (TZD) **pioglitazone**, **troglitazone** and **rosiglitazone** evoke a redistribution of FFA by decreasing the storage in the liver and increasing the deposition in the subcutis. This may lead to an amelioration of IR. TZD may also have anti-inflammatory effects and increase adiponectin levels. Despite these findings, also a worsening of inflammation has been observed and the effects that TZD may have on liver fibrosis still require further investigation (4).

To achieve long-lasting results, treatment with TZD may need to be life-long. As an unwanted effect TZD may increase body weight. Other side effects include peripheral edema and osteoporosis. While pioglitazone seems to have a beneficial effect on the cardiovascular system, rosiglitazone is associated with an increased cardiovascular risk and congestive heart disease (86, 96). TZD also have hepatotoxic effects and should not be used in any active liver disease with serum ALT activities of more than 2.5 times the upper limit of the normal range. However, there are only few reports of hepatic dysfunction associated with troglitazone (110).

Biguanides like **metformin** improve IR by decreasing hepatic glucose production and increasing glucose uptake by skeletal muscles. They also increase fatty acid oxidation and suppress lipogenesis. Unlike TZD, biguanides cause weight loss. However, the effects on NAFLD are inconsistent (4, 35, 52, 86) and generally disappointing. Based on currently available data, biguanides are not recommended as monotherapy for NAFLD (52), while a combination with insulin or pioglitazone may have beneficial effects (96).

Metformin is associated with elevated lactate levels but no case of lactic acidosis has been reported. Apart from gastrointestinal intolerance it appears to be well tolerated. Long-term safety still needs to be evaluated. It should be used with caution in patients with renal insufficiency or congestive heart disease (86).

Exedin-4 (nateglidine) improves insulin sensitivity by stimulating growth of pancreatic β-cells and insulin release from the pancreas. The improvement of liver histology and biochemical variables has been demonstrated in animal studies as well as in a small pilot study on five patients with NASH (86).

1.3.7.2.2 Improvement of the oxidant-antioxidant balance

As oxidative stress plays an important role in disease progression, antioxidants have been investigated.

Vitamin E contributes to the detoxification of free radicals. Some studies showed promising results on liver histology, but these results need to be proven in randomized, double-blind placebo-controlled trials (41). Nobili et al. (82) showed that changes in serum transaminase activities during an obesity intervention program were not significantly different in the presence versus absence of vitamin E.

A small pilot study with 10 patients investigated the effects of **betaine**, a metabolite of choline. Biochemical and histological improvement was shown, but larger controlled studies need to be performed (86).

1.3.7.2.3 Blockade of TNF-α

TNF-α has proinflammatory effects and may also increase IR. Therefore, it may be targeted to avoid NAFL disease progression to NASH. The TNF-α blocker **pentoxifylline** has been tested in three studies, two of which showed decreases in serum transaminase activities (112). The third and most recent one also demonstrated histological improvement (106).

Also **adiponectin**, an antagonist of TNF-α, has been investigated. It reduces body fat and FFA and reduces IR. It also has anti-inflammatory effects (112).

1.3.7.2.4 Lipid-lowering drugs

Given that imbalances of lipid metabolism are associated with NAFLD, several lipid altering agents have been considered for treatment. They might not only have an effect on NAFLD but also on associated comorbidities including the metabolic syndrome. Data in the literature appear promising but further investigation is warranted.

Atorvastatin, **pravastatin** and **rosuvastatin** belong to the pharmacological group of hydroxymethylglutaryl-CoA reductase inhibitors, commonly known as statins. They exert a cholesterol-lowering effect (99). Studies on the impact on liver histology are limited, but existing data provide evidence that they may have beneficial effects (4), in particular on steatosis and fibrosis (86). The well known potential risk for hepatotoxic side effects of statin therapy is not increased in NAFLD patients (86). Therefore, they can safely be used for treatment of NAFL and NASH, and mildly elevated transaminase activities should not prevent their use (4, 96). Riley et al. (103) demonstrated on 71 NAFLD patients the safe and effective use of statins.

Fibrates have failed to show beneficial effects so far (96). In some studies a decrease in serum transaminase activities has been observed (86, 110), but liver histology did not always improve (110).

Probucol is an agent that lowers serum LDL cholesterol concentrations and also has some antioxidant properties. A small pilot study has shown normalization of serum transaminase activities and reduction of histological signs of steatohepatitis.

1.3.7.2.5 Others

Many other agents are being investigated. They will be mentioned briefly.

Currently, **rimonabant**, a cannabinoid-type 1 receptor blocker, has attracted attention. Cannabinoid-type 1 (CB1) receptors in the central nervous system stimulate the synthesis of fatty acids in hepatocytes and increase lipogenesis (86). Stimulation of CB1 receptors increases appetite (99). Therefore, antagonists may lead to a reduced food intake (86). It may also have beneficial effects on hepatic steatosis and fibrosis (86, 127). Steatosis, hepatomegaly and dyslipidemia decreased while adiponectin levels increased in a rat model of obesity (4). Recently, rimonabant has been shown to be effective in reducing weight (14). Randomized clinical trials are needed.

Angiotensin II-receptor inhibitors are another promising group of drugs. Angiotensin II (ATII), a component of the renin-angiotensin system, activates hepatic stellate cells and causes matrix production. Animal studies have demonstrated a decrease in fibrosis and in a small pilot study, the ATII-receptor inhibitor losartan showed a decrease in serum transaminase activities and in serum markers of fibrosis.

Probiotics may have beneficial effects, given that bacterial overgrowth may play a role in the pathogenesis of NAFLD. Bacteria are able to produce ethanol. Ethanol itself is hepatotoxic and it also increases the intestinal permeability of lipopolysaccharides and other gut bacterial products that may have hepatotoxic effects (86).

Ursodeoxycholic acid is an agent that has been approved for therapy of primary biliary cirrhosis. It reduces the proportion of hydrophobic bile acids which contribute to oxidative stress. A pilot study in 1996 showed histological amelioration of steatosis and inflammation. However, these results have not been confirmed by other studies (112).

1.3.7.3 Bariatric surgery

In morbidly obese patients (BMI >35), lifestyle modification and pharmacological treatment may not lead to a sufficient weight reduction. In those cases, bariatric surgery may be indicated in order to achieve a substantial, long-term weight reduction (86).

The first bariatric surgery has been performed 42 years ago and approximately 250,000 bariatric surgeries are currently performed in the United States per year. Subsequently to bariatric surgery, weight loss is achieved by a reduction of food intake and/or a reduction of nutrient absorption, depending on whether a *restrictive* or a *malabsorptive* surgery method has been performed (see below). Furthermore, the excision of parts of the

gastrointestinal mucosa also has an important impact on the release of gastric peptides that influence food intake, energy expenditure, gut motility and appetite. Thus, weight loss is the result of both anatomic and neurohumoral changes (125). In general, also liver histology improves significantly and the risk of adverse side effects on the liver is very low (127).

1.3.7.3.1 Surgical methods

Today, bariatric surgery is performed laparoscopically as a minimal invasive procedure. There are many different surgical methods and each one has its advantages and disadvantages. Decision should be made on the basis of current body weight, comorbidities and target weight loss. There are only few controlled trials comparing different techniques. Therefore, there are no general guidelines regarding the preferable method.

The most common **restrictive procedures** in the United States and in Europe are laparoscopic adjustable gastric banding and sleeve gastrectomy. Both procedures lead to a reduction of food intake by a reduced stomach size. In gastric banding, a device is placed around the stomach and in sleeve gastrectomy, a portion of the stomach is surgically removed. These are the least invasive methods, but they are technically very complex. An associated complication is gastroesophageal reflux.

Malabsorptive procedures do not only reduce the stomach size but also the intestinal passage. The reduced intestinal passage leads to malabsorption and subsequently to additional weight loss. Adverse side effects of malabsorptive procedures are diarrhea, electrolyte disturbances and malnutrition. In some cases liver failure has been reported. Today, most malabsorptive procedures have already been abandoned. Currently, the so called Roux-en-Y gastric bypass is the malabsorptive method which is most commonly performed in the United States. This method is least likely to cause nutritional side effects (125).

1.3.7.3.2 Benefits and side effects

All of these procedures may lead to a weight reduction of 50 % and more. A growing body of evidence also suggests that liver histology improves, but randomized controlled trials are lacking (4, 110). There is also an amelioration of T2DM, hypertension, dyslipidemia, obstructive sleep apnea, depression and an improvement of quality of

life (86), as well as an improvement of gastroesophageal reflux, degenerative joint disease, venous stasis, urinary incontinence and infertility.

Most common side effects are malabsorption, nutritional deficiencies and diarrhea. However, advanced techniques allow for better control of these side effects (86).

As discussed above, rapid weight loss may aggravate NAFLD (4). This may also be the case when weight loss is a result of bariatric surgery. The risk is highest in the early postoperative period (125) and in few cases even liver failure has been reported (112). Fortunately, such dramatic cases are very rare and mortality from bariatric surgery has been reduced steadily. Nevertheless, the risk has to be taken seriously and research on this topic is still required to predict which patients are at risk to develop liver failure (125).

As bacteria may contribute to the progress of NAFLD, also bacterial overgrowth in the blind loop may worsen the outcome of bariatric surgery. Metronidazole, a drug of the class of nitroimidazole antibiotics, may decrease this risk (125).

1.3.7.4 Liver transplantation

Cirrhosis and HCC may require liver transplantation (112). In the United States, NAFLD is estimated to be the cause for about 1 % of the liver transplantations. However, given that there are many cases of "cryptogenic" cirrhosis that have never been diagnosed as NAFLD, the real number of transplantations due to NAFLD might be underestimated (80). Graft surgery may be complicated by comorbid conditions like obesity and others. Unfortunately, fibrosis, steatosis and steatohepatitis may reoccur in the transplanted organ (112). Therefore, during the follow up, weight, IR, T2DM and dyslipidemia need to be monitored and well controlled (80).

1.3.7.5 Therapeutic options for children

In childhood NAFLD, slow and consistent weight loss has been shown to be effective. Most findings are based on the evolution of serum transaminases or ultrasonography (80) (Table 4: Influence of lifestyle on serum transaminase activities and liver pathology, p. 24). Recently Nobili et al. (83, 84) and Reinehr et al. (102) conducted obesity intervention programs on children with ultrasonographically diagnosed NAFLD and improvements of the ultrasonographic findings were observed. The ideal rate and degree of weight loss has not yet been established, but currently 0.5 kg/week are recommended. A diet with a low glycemic index seems to be preferable to a low fat diet. Controls of blood glucose and

serum lipids are obligatory. In addition, moderate exercise is recommended. It is very important to support the child and its family and a family-based behavioral intervention may be the most successful type of intervention (77, 80).

When serum transaminase activities are elevated in overweight or obese children, weight loss should always be the first intervention. If high levels persist even after a weight reduction of 10 %, liver biopsy should be considered to exclude other reasons for steatosis or steatohepatitis (77).

Pharmacological agents have hardly been investigated in children. Vitamin E and ursodeoxycholic acid are commonly used and atorvastatin has been used successfully to treat hyperlipidemia (80). Nobili et al. (82, 83) showed that vitamin E has no impact on the histological outcome. As in adults, also in children surgery and transplantation should be considered in cases of cirrhosis or hepatic decompensation (77).

2 Study aims

The aim of the present study was to investigate the impact of the obesity intervention program called MODUL on the liver. Markers related to liver function, as well as to the pathogenesis of NAFLD and disease progression to NASH were identified according to current literature. The Fatty Liver Index (FLI) was identified as the best performing marker for the prediction of hepatic steatosis (9). For the first time, the FLI has been applied in an obesity intervention program for children and adolescents to monitor changes related to NAFL.

3 Study design

This is a retrospective analysis of data collected in the obestiy intervention program MODUL as far as they were relevant to the aims of the present study.

4 Material and Methods

The material used for this study was the database established within the MODUL Program.

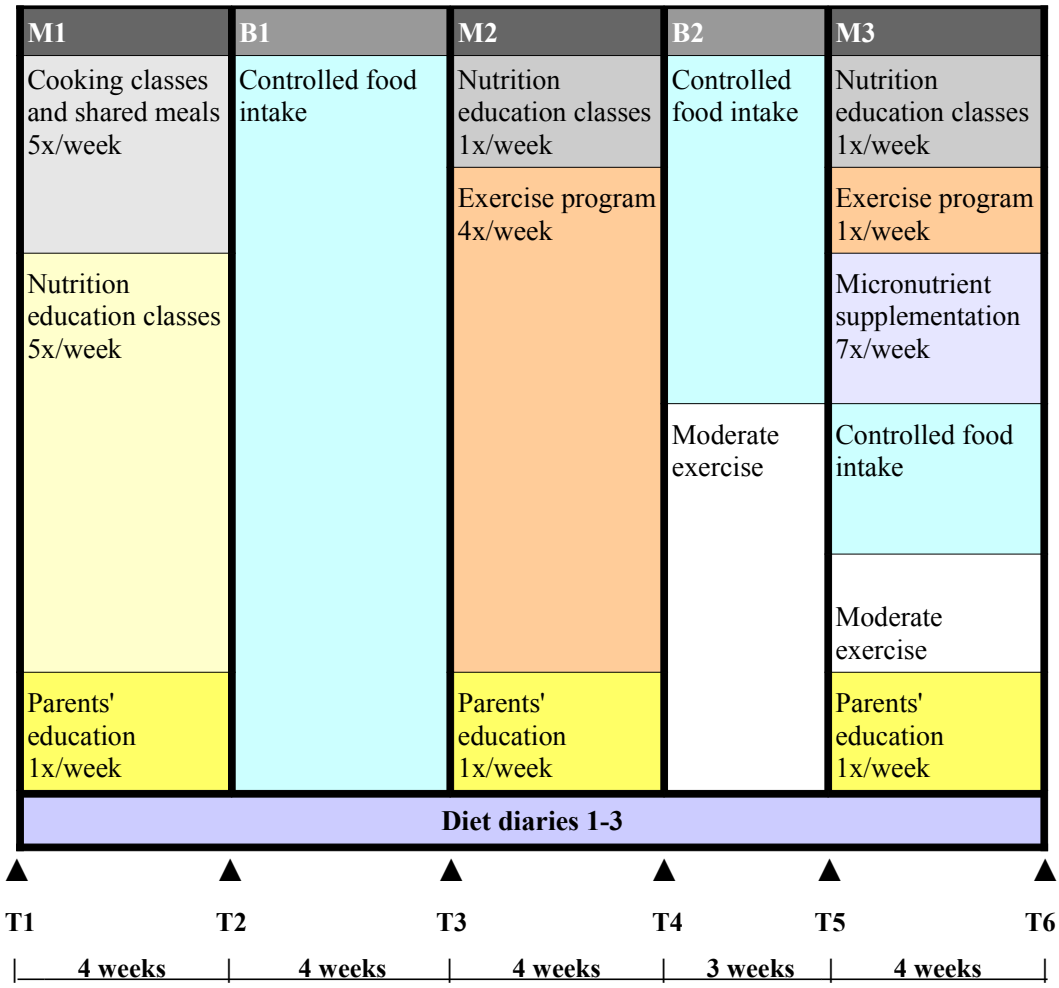
4.1 General information on the MODUL Program

The MODUL Program is an interventional program designed for overweight and obese children and adolescents to gradually and sustainably lose weight and improve body composition, fat distribution and metabolic variables. MODUL stands for "Mitmachen Ohne Diät - Ultraleicht" which may be translated as "Join us without diet - ultra easy". The program was developed by two students of the academy of dietetics, Eva Hütter and Martina Kandlbauer, within the scope of their masters thesis. In the MODUL Program, weight loss was achieved mainly by a controlled food intake and a moderate exercise program. A major focus was directed on nutritional education. The intention was not to achieve a rapid weight reduction, but to teach the participants how to adopt and enjoy healthy eating in daily life. The program integrated also the parents.

Anthropometric and biochemical markers were taken six times during the course of the MODUL Program to gain insight into nutritional and oxidant-antioxidant status and to monitor the effects of the intervention. Also total food intake was recoreded prior to the program and during the program, and the plasma concentrations of vitamins and micronutrients were detemined. In case of deficiencies, supplements were applied.

4.2 Structure of the MODUL Program

The MODUL team that worked with the participants consisted of two physicians, two students of dietetics, a nutrition pedagogue, three sports scientists and sports pedagogues, a student of nutritional sciences and an administrative assistant. The program was coordinated by the Human Nutrition & Metabolism Research and Training Center (HNMRC) Graz, Institute of Molecular Biosciences, Karl Franzen's University Graz, which was the recruiting center.



T1 - T6: Time points of measurements
M1 - M3: Modules
B1 - B2: Intervention breaks

Figure 12: Structure of the MODUL Program

Prior to the program the participants were requested to record their daily food intake during five days. This was necessary to assess dietary habits and to determine changes in dietary intake as a result of the MODUL Program.

The MODUL Program itself consisted of three modules (M1 to M3) of four weeks each (Figure 12). Between the modules there were two intervention breaks: B1 (4 weeks) and B2 (3 weeks). During the intervention breaks participants were asked to maintain the adopted lifestyle changes. The program took place Monday through Friday from 4 to 6 pm. Parents met for two hours per week. Measurements of anthropometric and biochemical markers were performed before and after each module. In total, there were six time points of measurement (T1-T6). The participants kept diet diaries throughout the whole program.

4.2.1 Dietary intervention

According to the recommendations of the DGE (Deutsche Gesellschaft für Ernährung - German Association for Nutrition), normal weight 12-15-year-old girls need about 2,200 kcal/day and normal weight boys of the same age group 2,700 kcal/day (25). During the MODUL Program a reduction of 200-800 kcal/day was recommended and the target energy intake was calculated to be 1,893 kcal/day for both girls and boys. The target relative fat intake was decreased in favor to the *relatively increased* intake of proteins and carbohydrates. The target *total* intake of all three components was decreased.

The participants of MODUL did not strictly follow the recommendations of the program concerning food intake. Therefore, the actual food intake and the actual distribution of macronutrients were different from the target ones (Figures 13 and 14).

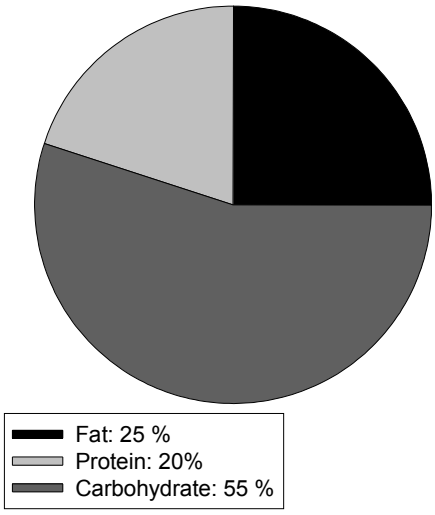


Figure 13: Target distribution of macronutrients in the program

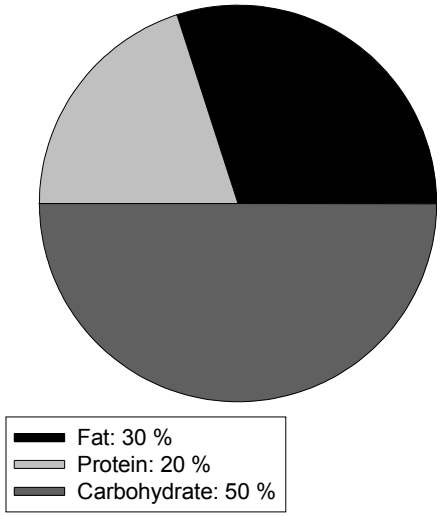


Figure 14: Achieved distribution of macronutrients in the program

Five meals per day were scheduled and the total energy intake was distributed among breakfast (25 %), cold dish (25 %), hot dish (15 %) and two types of snacks (10 and 15 %, respectively). For each type of the meal, a collection of recipe cards (Figure 15 and 16) was provided. On each card, the corresponding percentage of recommended daily energy intake was printed. The composition of the recipes was in agreement with the recommendations of the DGE (25).

When selecting the dishes, the preferences of the age group were taken into account. Detailed recipes were printed on the back of the cards and in a small booklet. The participants were free to choose from the different cards but were supposed to not exceed the daily maximum of 100 %. Each combination of the five differently colored types of cards ensured a daily dietary intake in accordance with the recommended energy intake and a balanced distribution of fat, protein and carbohydrate. Using the card system as recommended, the daily intake would cover approximately 70 g of fat, 80 g of protein and 290 g of carbohydrate.

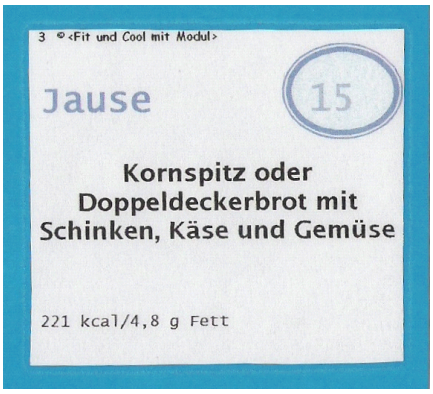


Figure 15: Example of a recipe card with percentage of daily energy intake printed in the upper right corner

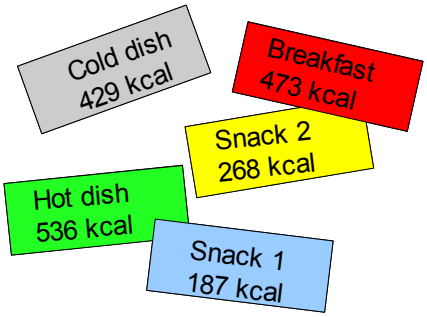


Figure 16: Card system to control food intake

During the Module 1 of the program, the hot dish was prepared on-site by a group of 12-13 participants under the supervision of a nutrition pedagogue. The portions were sized strictly following the recommendations on the cards and no additional servings were allowed. Cooking started at 4 pm and the shared meal with all participants took place in the dining room at 5 pm.

4.2.2 Nutrition education classes

While one half of the group was busy working in the kitchen, the other half attended the nutrition education classes. These two groups rotated daily. The main topics of the nutrition education classes included basic knowledge about nutrition and healthy foods, as well as healthy ways of food preparation. It was a major concern to not only transfer theoretical knowledge, but also to apply the knowledge in hands-on exercises and games. Another important issue was also to provide psychological support as many overweight and obese children suffer from poor self-esteem.

4.2.3 Exercise program

The exercise program was adapted to the needs and preferences of the participants of the MODUL. Sports classes were scheduled two hours per day four times per week and included swimming, water aerobic, endurance and strength training, as well as dexterity training and games. The intention was to spark interest in physical activity. The participants were encouraged to integrate physical activity in their daily lifes. Fitness was evaluated before and after M2.

4.2.4 Micronutrient supplementation

In M3 a low-dose vitamin E supplement was applied, since plasma concentrations had dropped considerably and for one boy they were below the normal range for children and adolescents.

4.2.5 Intervention breaks

During the intervention breaks B1 and B2 the participants were requested to autonomously adhere to the dietary recommendations and to maintain the lifestyle changes acquired during the previous module(s).

4.2.6 Parents' education

Each Friday, parents were educated from 4 to 6 pm. They were instructed in the same topics as their children and there was also enough time to discuss questions and concerns.

4.3 Study participants

A total of 25 motivated overweight and obese boys (n=11) and girls (n=14) with BMI >95th centile were enrolled in the program (Table 12).

Inclusion criteria were the age between 12 and 15 years and a BMI above the 95th centile for age and gender. Each participant had a physical check-up and in case of any acute or chronic inflammatory disease or other medical disorder (allergies etc.) he or she was not included in the study. Other exclusion criteria were the intake of any dietary supplement, participation in any other study during the last four weeks before the MODUL Program and current participation in any other obesity intervention program or any diet

under medical supervision. Written informed consent was obtained from the children and adolescents, as well as from their parents or legal representatives.

Table 12: Baseline characteristics of the study population

	Sex	**Age (years)**	**Weight (kg)**		**BMI (kg/m²)**		**WC (cm)**		**AST (U/L)**	**ALT (U/L)**		**GGT (U/L)**	**TG (mmol/L)**	**Chol[1] (mmol/L)**
ID number														
1	f	12.5	84.0		32.8		100		29	50		19	2.11	3.05
2	m	13.0	60.0		27.0		77.0		27	30		20	1.73	4.32
3	f	13.3	85.0		34.1		87.0		18	16		18	1.21	4.09
4	f	13.3	85.0		32.8		87.0		23	27		14	1.43	4.76
5	f	14.4	105		40.3		109		17	19		17	0.855	3.80
6	f	12.8	100		32.7		97.0		20	27		16	1.15	5.30
7	f	12.3	75.0		29.9		92.0		24	20		13	0.650	3.83
8	f	14.7	82.0		31.3		86.0		23	26		19	1.65	3.83
9	m	12.5	66.0		28.8		83.0		30	28		16	0.547	4.22
10	m	12.0	87.0		35.3		101		30	42		37	1.17	3.59
11	m	13.4	84.0		31.4		101		28	31		17	1.08	4.58
12	m	12.4	86.0		30.5		87.0		32	42		16	1.54	5.12
13	f	12.4	89.0		31.9		89.0		17	15		12	1.63	5.59
14	m	13.3	63.0		27.6		84.0		28	35		14	2.94	6.80
15	m	13.0	70.5		24.5		84.0		40	58		20	0.524	3.70
16	m	12.2	105		35.7		100		35	44		38	1.09	4.29
17	m	13.4	77.0		29.2		90.0		22	27		22	2.98	4.45
18	f	14.7	67.0		26.7		77.0		26	26		18	1.32	4.40
19	m	12.9	61.0		23.8		77.0		20	17		11	1.01	3.80
20	f	12.1	61.5		28.3		75.0		27	22		9	1.09	3.98
21	f	12.2	74.0		27.0		85.0		26	33		14	1.33	5.28
22	f	11.6	56.0		26.8		79.0		28	21		12	0.889	4.22
23	f	14.5	93.5		32.0		89.5		23	32		19	1.84	4.65
24	f	13.5	95.0		29.7		85.5		21	20		18	0.536	4.29
25	m	13.5	122		40.1		114		22	17		17	3.04	4.89
Mean ± SD[2]	**11m 14f**	**13.0 ± 0.855**	**3.19 ± 2.15**		**30.8 ± 4.18**		**89.4 ± 10.2**		**25.4 ± 5.58**	**29.0 ± 11.1**		**17.8 ± 6.70**	**1.42 ± 0.720**	**4.43 ± 0.775**
Upper limit of normal range			**f**	**m**	**f**	**m**	**f**	**m**		**f**	**m**			
			64.7[3,4] 70.4[6] 74.8[7] 77.4[8]	64.6[3,4] 73.7[6] 81.7[7] 87.0[8]	25.5[3,4] 26.3[6] 27.0[7] 27.5[8]	25.4[3,4] 26.3[6] 27.0[7] 27.5[8]	71.7[3,5] 73.6[6] 75.2[7] 76.6[8]	72.8[3,5] 75.3[6] 77.8[7] 80.0[8]	43[9]	35[9]	45[9]	38[9]	3.89[9]	5.18[9]

[1]Cholesterol, [2]standard deviation, [3]for 12-year-olds, [4]reference values according to Kromeyer-Hauschild et al. (64), [5]reference values according to Fredriks et al. (29), [6]for 13-year-olds, [7]for 14-year-olds, [8]for 15-year-olds, [9]reference values according to the Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz.

During M1 and at T1 and T2, all 25 participants were present. Two participants dropped out of the program during B1, one due to a fracture of the leg, the other one due to family problems. Another two dropouts at a later stage were also due to family problems. A total of 21 participants finished the program and a complete data set was available from 20 participants (Table 13).

Table 13: Number of participants and dropouts

	T1	T2	T3	T4	T5	T6
Number of participants	25	25	23	22	21	21
Anthropometric data available	25	25	23	22	21	21
Biochemical data available	25	25	23	22	21	20

4.4 Study variables

Before and after each module anthropometric assessment took place and blood samples were taken in the morning after an overnight fast. The examination of each participant always took place at the same hour. A comprehensive panel of laboratory analyses has been determined and will be presented elsewhere. Given that the present study is focused on the effects that participation in the MODUL Program may have on the liver, evaluations were restricted to variables that may provide such information. These included:

1) Food intake during the intervention program (Table 14)

Table 14: Variables of food intake

Food intake
Total energy intake (kcal) Energy intake from fat (%) Energy intake from proteins (%) Energy intake from carbohydrates (%)

2) The liver enzymes AST, ALT and GGT and the AAR calculated from the former (Table 15)
3) Metabolic markers constituting risk factors of NAFLD, including biomarkers of lipid and glucose metabolism, inflammation and oxidative stress (Table 15)
4) The FLI, combining variables (BMI, WC, GGT, TG) identified as those with the highest predictive values for fatty liver according to Bedogni et al. (9)

Table 15: Anthropometric and biochemical markers identified in the literature as risk factors for NAFLD

Liver disease	Risk factors and markers	References
NAFL	Fat accumulation (overweight and obesity)	50, 81, 50
	Body weight (absolute value and z-score) BMI (absolute value and z-score) WC (absolute value and z-score) Total adipose tissue mass VAT mass SAT mass	 5, 12, 20, 45, 56, 66, 79, 91 5, 20, 56, 66, 74 79, 50
	Imbalanced lipid metabolism	15, 28, 81
	Total cholesterol LDL HDL TG	5, 91, 66 5 11, 66, 135 5, 11, 66, 81, 91, 97, 134, 135
	Imbalanced glucose metabolism	11, 15, 28, 54, 81, 90
	Glucose	11,66
NASH and fibrosis	Inflammation	79,13
	CRP IL-6	86, 136 49
	Oxidant-antioxidant status	79, 132
	Malondialdehyde (MDA) α-Tocopherol Ratio of α-tocopherol to cholesterol (TCR)	135

4.5 Data collection

4.5.1 Dietary intake

The participants were asked to record their food intake during five days prior to the start of the program, using standardized food balances. The participants also kept diet diaries throughout the whole program. Each day the card ID of the selected recipe card was recorded, as well as any drinks and additional food. The macro- and micronutrient content was calculated by the dietitians using the food composition database software program EWP, which is based on the German Bundeslebensmittelschlüssel with some specific Austrian additions (Dato Denkwerkzeuge, Wien).

4.5.2 Anthropometry

Anthropometric measurements were performed and recorded by the same physician throughout the program. Weight, height and WC were measured according to standardized procedures. Measurement values were interpreted on the basis of age- and sex-specific centiles. Body weight was compared to the reference values of Kromeyer-Hauschild et al. (64), BMI was compared to those of Prader et al. (95) and Kromeyer-Hauschild et al. (64) and WC was compared to those of Fredriks et al. (29). Z-scores of body weight, BMI and WC were calculated. An infra-red-based device (Lipometer, patent by R. Möller, Medical University of Graz) was used for measuring the SAT thickness at 15 defined body sites. Total adipose tissue mass and VAT mass were then calculated by the software program.

4.5.3 Clinical chemistry and biochemistry

Clinical chemistry routine analyses were determined in the laboratories of the Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz.

Vitamin E (α- and γ-tocopherol) concentrations were determined by high performance liquid chromatography (HPLC) in the research laboratory of the HNMRC (3). Malondialdehyde concentrations were determined by HPLC in the laboratories of the Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz (62).

Reference values for α-tocopherol and TCR were taken from Winklhofer-Roob et al. (133) and other laboratory reference values were taken from the institute for medical and chemical laboratory diagnostic of the university hospital (LKH) Graz.

4.6 Statistical analysis

Changes over time were analyzed using one way repeated measures analysis of variance (One Way RM ANOVA). In case of statistically significant difference ($P<0.005$) all pairwise multiple comparison procedures were used to isolate the group or groups that differed from the others. The Holm-Sidak Test was chosen as it can be used for pairwise comparison and is more powerful than the Tukey and Bonferroni tests. It is recommended as the first line procedure for most multiple comparison testing. All statistical analyses were performed using SigmaPlot 11.0. A P-value of 0.05 was chosen to determine

statistical significance. The graphs displayed in all figures were created using SigmaPlot 11.0. Box-and-whisker plots have been used to display the distribution of data. The box represents 50 % of the data and the bottom and top of the box are the 25th and 75th percentile. The band near the middle of the box is the median. The ends of the whiskers represent the 3rd and the 97th percentile. Outliers are presented as individual dots.

5 Results

5.1 Food intake

Food intake was monitored in four different periods of time. The first time period included the five days prior to the MODUL Program. During the course of the program, three subsequent time periods were evaluated separately.

5.1.1 Energy intake

Prior to the program the energy intake was 2,182 ± 555 kcal/day (Figure 17). During the program, the mean energy intake was considerably below the target energy intake of 1893 kcal/day and averaged 1,188 ± 306 kcal/day.

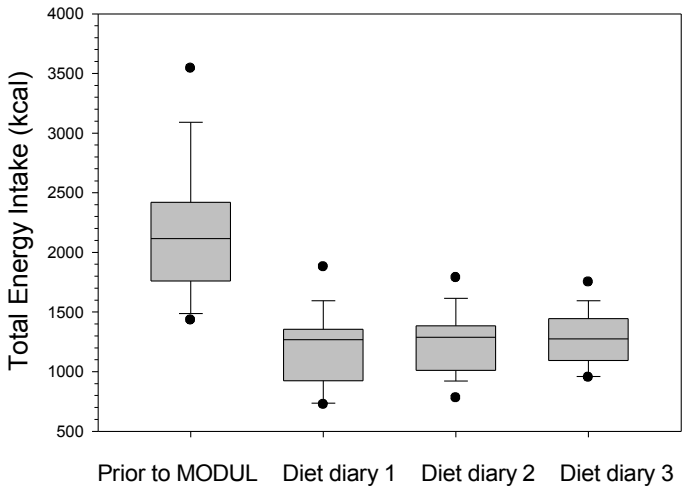


Figure 17: Total energy intake during the program

5.1.2 Distribution of macronutrients

Prior to the program, the energy intake derived from fat was 37 ± 5.3 % of the total energy intake. During the program, it decreased to 29 ± 2.9 % (Figure 18). However, the recommended level of 25 % was not reached. The energy intake derived from proteins was 15 ± 2.43 % before the program. During the program, it increased to 20 ± 2.0 % of the total energy intake and matched the recommended 20 % (Figure 19). The energy intake derived from carbohydrates did not match the recommended 55 %. It was 47 ± 6.38 % before the program and 50 ± 3.3 % during the program (Figure 20).

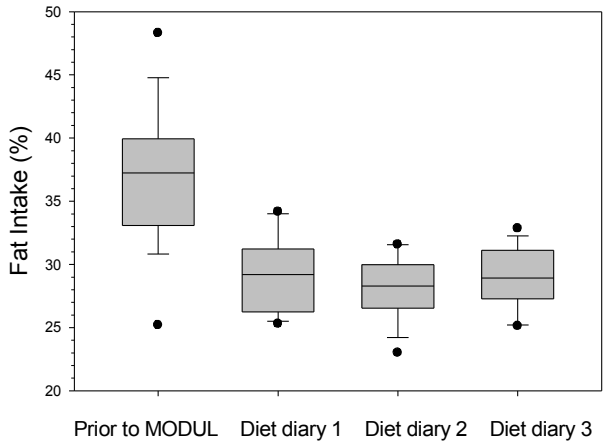


Figure 18: Energy derived from fat before and during the program expressed as % of total energy intake

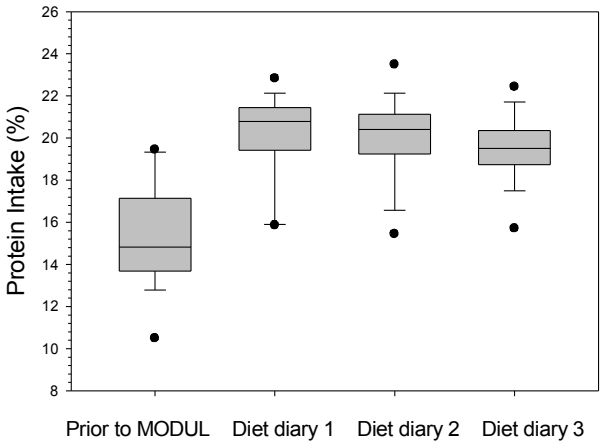


Figure 19: Energy derived from proteins before and during the program expressed as % of total energy intake

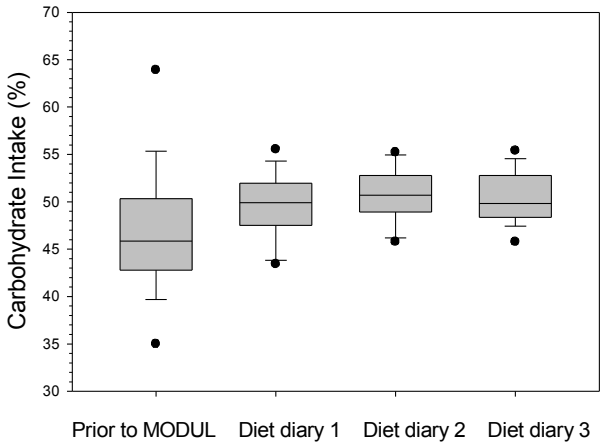


Figure 20: Energy derived from carbohydrates before and during the program expressed as % of total energy intake

5.2 Overview of anthropometry and biochemistry

Table 16 gives an overview of the study results. Results of anthropometric and biochemical variables are presented as mean ± standard deviation (SD) at the six time points (T1-T6). Reference values are provided if available. With the exception of HDL cholesterol and IL-6, all variables showed statistically significant changes during the program. Subsequently, each variable will be discussed separately.

Table 16 a: Overview of the study results

	Reference values		T1	T2	T3	T4	T5	T6	P-value
	Girls	**Boys**							
Body weight (kg)	3rd - 97th centile	3rd - 97th centile	81.3 ± 16.5	78.2 ± 15.8	76.0 ± 15.0	74.2 ± 14.9	72.6 ± 14.7	72.2 ± 14.7	P<0.001
12-year-olds	29.0-55.7[1]	28.8-49.9							
13-year-olds	32.4-61.2	31.1-58.3							
14-year-olds	35.9-65.5	34.5-65.4							
15-year-olds	38.8-69.8	39.5-72.3							
12-year-olds	30.3-64.7[2]	29.4-64.6							
13-year-olds	34.8-70.4	32.7-73.7							
14-year-olds	39.1-74.8	37.6-81.7							
15-year-olds	42.3-77.4	43.3-87.0							
Body weight (z-score)	± 2.00		2.01 ± 0.714	1.88 ± 0.727	1.81 ± 0.764	1.72 ± 0.789	1.67 ± 0.801	1.65 ± 0.820	P<0.001
BMI (kg/m²)	3rd - 97th centile	3rd - 97th centile	30.8 ± 4.18	29.5 ± 4.13	28.5 ± 4.37	27.8 ± 4.40	27.0 ± 4.25	26.7 ± 4.25	P<0.001
12-year-olds	14.5-25.5[2]	14.5-25.4							
13-year-olds	15.0-26.3	15.0-26.3							
14-year-olds	15.7-27.0	15.5-27.0							
15-year-olds	16.2-27.5	16.0-27.5							
BMI (z-score)	± 2.00		2.44 ± 0.464	2.28 ± 0.518	2.12 ± 0.591	1.96 ± 0.662	1.83 ± 0.652	1.77 ± 0.670	P<0.001
WC (cm)	overweight/ obesity	overweight/ obesity	89.4 ± 10.2	87.6 ± 9.78	85.9 ± 9.38	84.1 ± 9.40	82.5 ± 9.63	81.5 ± 9.52	P<0.001
12-year-olds	<71.7/83.6[3]	<72.8/84.1							
13-year-olds	<73.6/85.9	<75.3/86.9							
14-year-olds	<75.2/87.7	<77.8/89.3							
15-year-olds	<76.6/89.1	<80.0/91.4							
WC (z-score)	± 2.00		2.93 ± 1.23	2.71 ± 1.19	2.51 ± 1.17	2.27 ± 1.17	2.09 ± 1.20	1.97 ± 1.19	P<0.001
Total adipose tissue mass (kg)	-		29.3 ± 8.72	29.4 ± 8.30	26.0 ± 7.87	23.8 ± 9.24	22.8 ± 8.77	21.4 ± 8.08	P<0.001

[1]Reference values accoring to Prader et al. (95), [2]reference values according to Kromeyer-Hauschild et al. (64), [3]reference values according to Fredriks et al. (29).

Table 16 b: Overview of the study results

	Reference values		T1	T2	T3	T4	T5	T6	P-value
	Girls	Boys							
SAT mass (kg)	-		25.6 ± 7.20	25.9 ± 6.78	22.8 ± 6.40	20.9 ± 8.18	20.0 ± 7.68	18.5 ± 6.77	P<0.001
VAT mass (kg)	-		3.70 ± 1.94	3.48 ± 1.92	3.22 ± 1.96	2.97 ± 1.72	2.79 ± 1.71	2.88 ± 1.80	P<0.001
AST (U/L)	<43[4]		25.4 ± 5.58	24.4 ± 4.55	23.4 ± 5.18	23.4 ± 4.49	22.2 ± 3.48	21.1 ± 5.29	P<0.001
ALT (U/L)	<35[4]	<45[4]	29.0 ± 11.1	24.8 ± 9.46	21.7 ± 5.15	19.9 ± 5.63	18.3 ± 5.23	17.5 ± 4.45	P<0.001
AAR	-		0.938 ± 0.209	1.06 ± 0.269	1.10 ± 0.170	1.22 ± 0.248	1.26 ± 0.228	1.24 ± 0.290	P<0.001
GGT (U/L)	<38[4]		17.8 ± 6.70	14.0 ± 5.81	12.6 ± 5.03	13.4 ± 5.94	13.3 ± 4.43	11.8 ± 4.43	P<0.001
Cholesterol (mmol/L)	<5.18[4]		4.43 ± 0.775	3.64 ± 0.645	3.77 ± 0.712	3.58 ± 0.659	3.69 ± 0.714	3.75 ± 0.587	P<0.001
HDL cholesterol (mmol/L)	>1.04[4]		1.39 ± 0.346	1.29 ± 0.247	1.36 ± 0.335	1.31 ± 0.313	1.32 ± 0.280	1.30 ± 0.246	P=0.073
LDL cholesterol (mmol/L)	<4.14[4]		2.40 ± 0.656	1.91 ± 0.596	1.98 ± 0.676	1.88 ± 0.606	2.01 ± 0.656	2.03 ± 0.452	P<0.001
TG (mmol/L)	<3.89[4]		1.42 ± 0.720	0.992 ± 0.401	0.876 ± 0.432	0.747 ± 0.490	0.673 ± 0.417	0.765 ± 0.600	P<0.001
α-Tocopherol (µmol/L) 12-year-olds 14-year-olds	 14.5-28.5[5] 15.0-29.5		 23.7 ± 5.06	 18.2 ± 3.20	 18.8 ± 3.18	 18.3 ± 3.08	 19.4 ± 4.05	 24.6 ± 4.89	P<0.001
TCR (µmol/mmol)	5th - 95th centile 4.29-6.76[5]		5.40 ± 0.935	5.06 ± 0.910	5.07 ± 0.846	5.18 ± 0.764	5.30 ± 0.725	6.61 ± 1.40	P<0.001
CRP (mg/L)	<8.00[4]		3.33 ± 3.08	2.04 ± 2.03	1.44 ± 1.42	2.33 ± 2.38	2.47 ± 2.84	1.66 ± 1.87	P=0.002
Glucose (mmol/L)	3.05-6.11[4]		5.29 ± 0.366	5.03 ± 0.416	5.11 ± 0.407	5.09 ± 0.403	4.95 ± 0.340	5.13 ± 0.643	P=0.030
IL-6 (ng/L)	-		2.86 ± 1.53	2.41 ± 1.51	2.13 ± 1.35	2.79 ± 3.74	3.34 ± 5.05	2.27 ± 1.21	P=0.674
MDA (µmol/L)	-		0.461 ± 0.0720	0.446 ± 0.0605	0.387 ± 0.0475	0.422 ± 0.0926	0.470 ± 0.0922	0.415 ± 0.0615	P<0.001
FLI	in adults <30[6]		45.3 ± 26.3	31.2 ± 22.9	27.0 ± 23.2	23.6 ± 24.6	20.1 ± 21.7	20.6 ± 23.2	P<0.001

[4]Reference values accoring to the Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz, [5]reference values according to Winklhofer-Roob et al. (133), [6]reference value according to Bedogni et al. (9).

5.2.1 Anthropometry

5.2.1.1 Body weight and z-score of body weight

Baseline: At the start of the MODUL Program, the weight of the study participants ranged from 60.0 to 122 kg. The mean ± SD of the body weight was 81.3 ± 16.5 kg. The z-score at T1 ranged from 1.02 to 3.71 with a mean ± SD of 2.01 ± 0.714.

Effects of the MODUL Program: Table 17 gives an overview of the weight loss during the MODUL Program.

Table 17: Overview of the weight loss during the MODUL Program

Time period	Mean weight loss	Number of participants				
		Total	Weight loss ≤ 0.5 kg/week	Weight loss between 0.5 and 1 kg/week	Weight loss ≥ 1.0 kg/week	Weight gain (Individual participants)
T1-T6	0.56 kg/week	21	10	9	1	1 (A)
M1	0.80 kg/week	25	4	8	11	2 (A,B)
B1	0.57 kg/week	23	12	4	6	1 (C)
M2	0.47 kg/week	22	11	6	3	2 (A,D)
B2	0.37 kg/week	21	14	4	2	1 (D)
M3	0.12 kg/week	21	16	0	0	5 (A,C,E,F,G)

A-G: Individual participants

All participants except one (the participant A) achieved a weight reduction during the MODUL Program. The participant A put on weight during M1, M2 and M3. The participant B dropped out after T3.

Changes in body weight and z-score of body weight reached statistical significance ($P<0.001$) (Figures 21, 22, 23). At T6, the body weight ranged from 49.5 to 106 kg and the mean weight ± SD was 72.2 ± 14.7 kg. The difference of the means was greatest during M1 (3.19 kg). It decreased gradually and was 2.36 kg during B1, 2.01 kg during M2, 1.24 kg during B2 and 0.476 kg during M3. All Pairwise Multiple Comparison Procedures (Holm-Sidak method) showed statistically significant differences ($P<0.05$) during M1, M2 and B1. Changes were not significant during B2 ($P=0.080$) and M3 ($P=0.506$). However, between T4 and T6 statistical significance was reached again.

At T6, the z-score of body weight ranged from 0.440 to 3.38 and the mean ± SD was 1.65 ± 0.820. Changes were statistically significant during M1 and B1. Changes were also statistically significant at all time points compared to baseline and to T2 as well as during the time periods T3-T5 and T3-T6. Changes did not reach statistical significance during M2, B2 and M3.

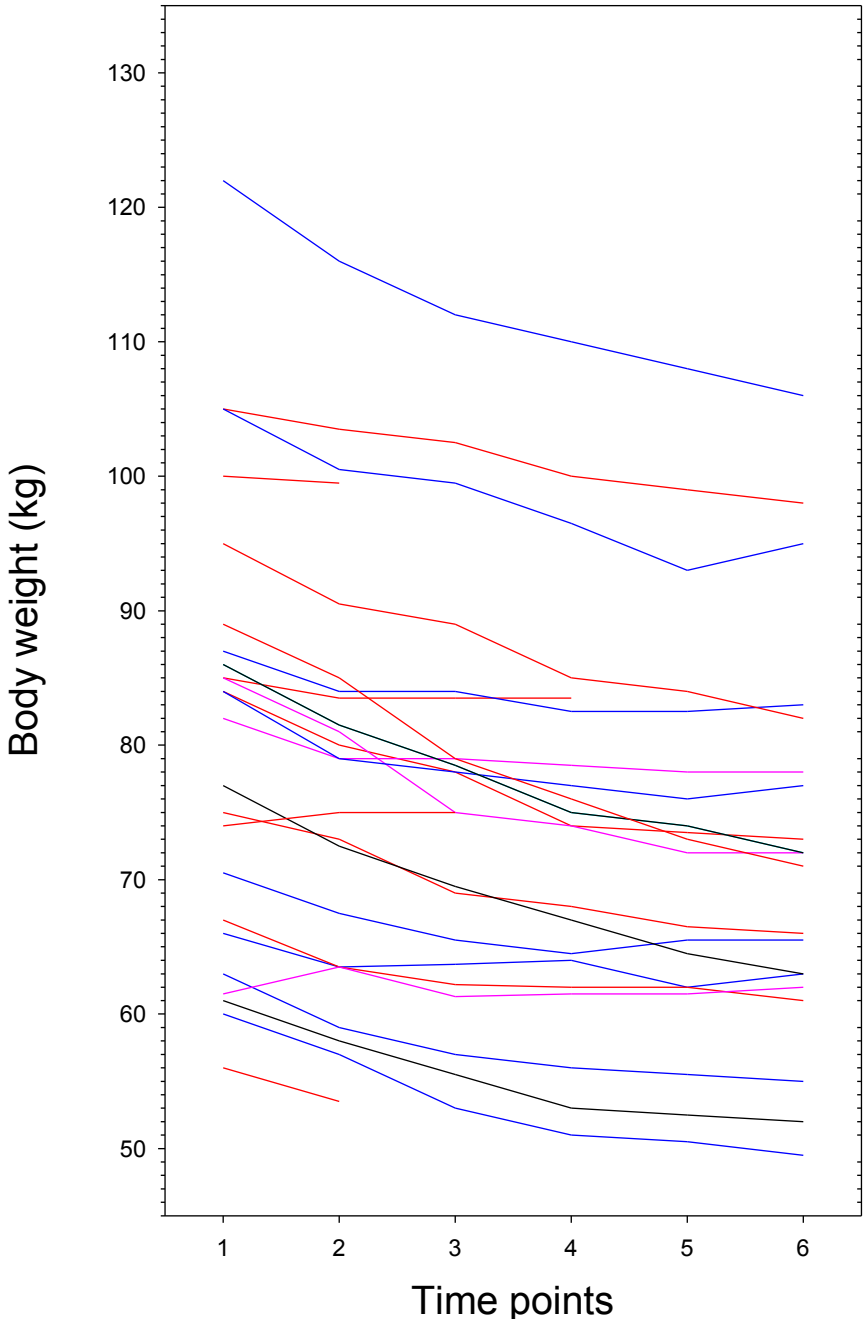


Figure 21: Individual changes in body weight during the program

(Boys are presented in black and blue and girls in red and pink)

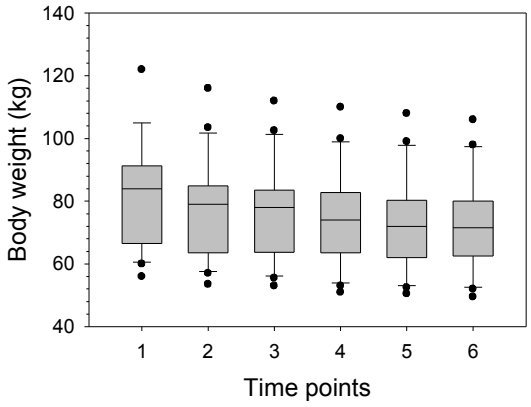


Figure 22: Changes in body weight during the program

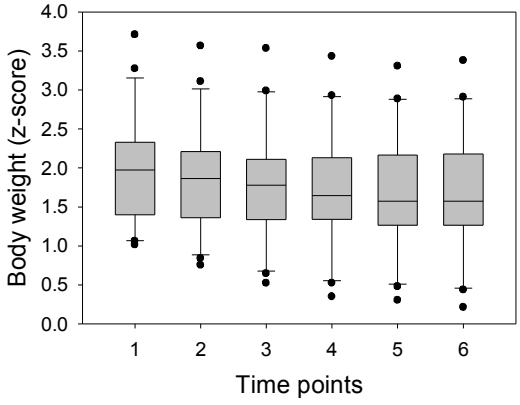


Figure 23: Changes in z-score of body weight durng the program

5.2.1.2 BMI and z-score of BMI

Baseline: At T1, the BMI ranged from 23.8 to 40.3 kg/m². The mean ± SD was 30.8 ± 4.18 kg/m². At this time point, the z-score ranged from 1.46 to 3.37 with a mean ± SD of 2.44 ± 0.464.

Effects of the MODUL Program: Changes in BMI and z-score of the BMI were statistically significant (P<0.001) (Figures 24, 25). At T6, the BMI ranged from 20.2 to 36.5 kg/m² and the mean ± SD was 26.7 ± 4.25 kg/m². The z-score ranged from 0.456 to 3.04 and the mean ± SD was 1.77 ± 0.670. Changes reached statistical significance during M1, B1, M2 and B2, while statistical significance was not reached during M3.

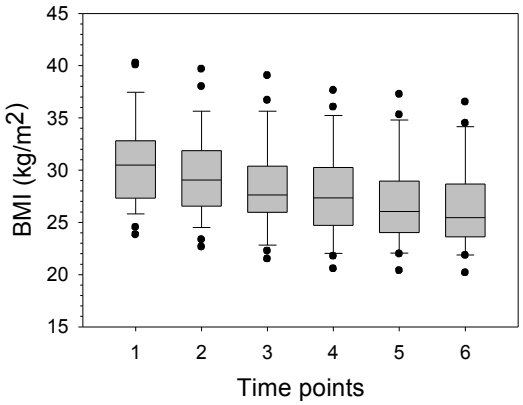


Figure 24: Changes in z-score of BMI during the program

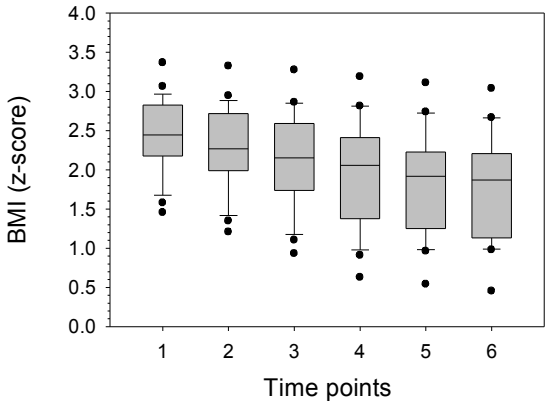


Figure 25: Changes in BMI during the program

5.2.1.3 Waist circumference and z-score of waist circumference

Baseline: At the start of the program, the WC ranged from 77.0 to 114 cm. The mean ± SD was 89.4 ± 10.2 cm. At this time point, the z-score ranged from 1.15 to 5.74 with a mean ± SD of 2.93 ± 1.23.

Effects of the MODUL Program: Changes in WC and z-score of the WC were statistically significant (P<0.001) (Figures 26, 27). At T6, the WC ranged from 69.0 to 104 cm and the mean ± SD was 81.5 ± 9.52 cm. The z-score ranged from 0.35 to 4.54 and the mean ± SD was 1.97 ± 1.19. Changes in WC reached statistical significance during all time periods except M3. Changes in the z-score of WC reached statistical significance during all time periods without exception.

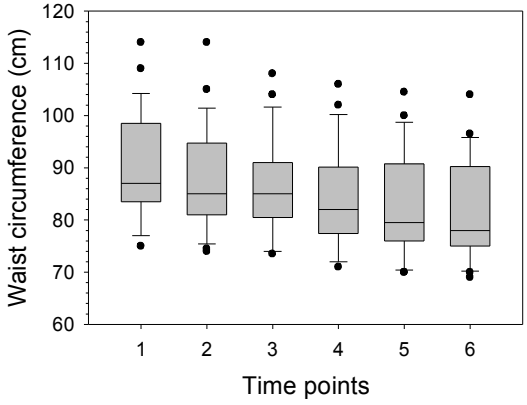


Figure 26: Changes in WC during the program

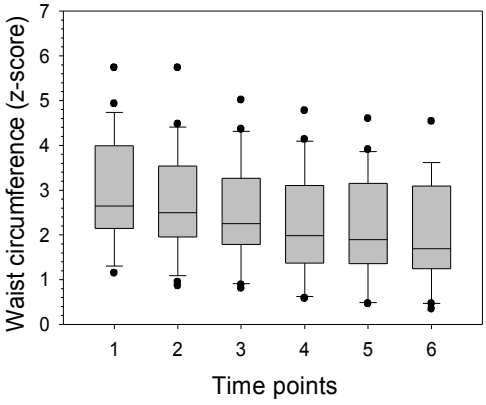


Figure 27: Changes in z-score of WC during the program

5.2.1.4 Total adipose tissue mass, subcutaneous adipose tissue (SAT) mass and visceral adipose tissue (VAT) mass

Baseline: At T1, the total adipose tissue mass ranged from 16.3 to 53.5 kg. The mean ± SD was 29.3 ± 8.72 kg. The SAT mass ranged from 13.6 to 44.6 kg and the mean ± SD was 25.6 ± 7.20 kg. The VAT mass ranged from 1.15 to 8.85 kg. The mean ± SD was 3.70 ± 1.94 kg.

Effects of the MODUL Program: Changes in total adipose tissue mass, SAT mass and VAT mass reached statistical significance (P<0.001) (Figure 28, 29, 30). At the end of the MODUL Program, the total adipose tissue mass ranged from 8.65 to 38.9 kg and the mean ± SD was 21.4 ± 8.08 kg. At the same time point, the SAT mass ranged from 8.25 to

34.0 kg and the mean ± SD was 18.5 ± 6.77 kg. The VAT mass ranged from 0.300 to 7.10 kg and the mean was 2.88 ± 1.80 kg. All Pairwise Multiple Comparison Procedures (Holm-Sidak method) showed statistically significant differences ($P<0.05$) for total adipose tissue mass and for SAT mass during the time periods T1-T3, T1-T4, T1-T5 and T1-T6, as well as during the time periods T2-T3, T2-T4, T2-T5, T2-T6, T3-T5 and T3-T6. Changes in SAT mass also reached statistical significance in the time period T4-T6. Changes in VAT mass were, compared to baseline, statistically significant at all time points except for T2 and also in the time periods T2-T4, T2-T5 and T2-T6.

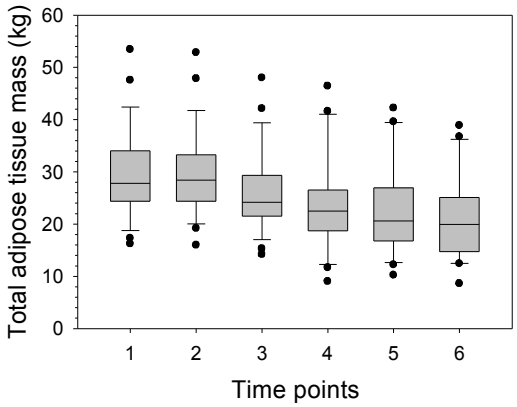


Figure 28: Changes in total adipose tissue mass during the program

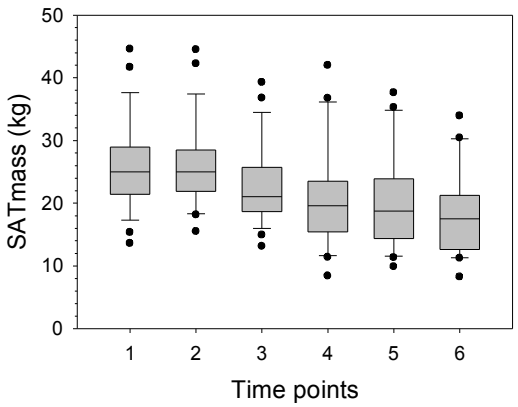


Figure 29: Changes in SAT mass during the program

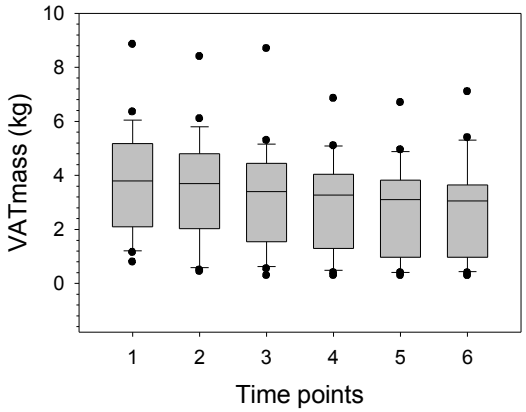


Figure 30: Changes in VAT mass during the program

5.2.2 Biochemistry

5.2.2.1 Liver enzymes

5.2.2.1.1 AST

Baseline: At T1, serum AST activities ranged from 17 to 40 U/L. The mean ± SD was 25.4 ± 5.58 U/L. Serum AST activities of all participants were within the normal range.

Effects of the MODUL Program: During the MODUL Program the serum AST activities decreased significantly (P<0.001) (Figure 31) but changes showed some fluctuations. At T6, All Pairwise Multiple Comparison Procedures (Holm-Sidak method) showed that changes in AST activities were statistically significant in the periods T1-T5 and T2-T6. At T6, serum AST activities ranged from 13 to 32 U/L and the mean ± SD was 21.1 ± 5.29 U/L. All values were within the normal range. The difference of the means between two successive midpoints ranged from 0.123 to 1.35 U/L.

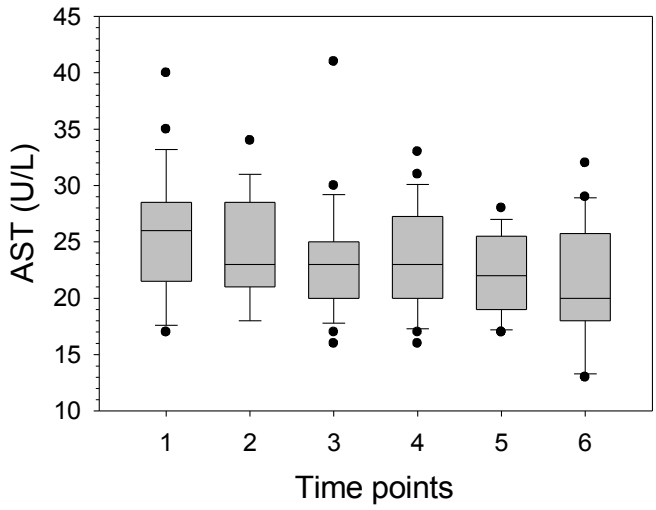


Figure 31: Changes in serum AST activities during the program

5.2.2.1.2 ALT

Baseline: At T1, serum ALT activities ranged from 15 to 58 U/L and the mean ± SD was 29.0 ± 11.1 U/L. Serum activities of one girl and one boy exceeded the normal range.

Effects of the MODUL Program: Serum ALT activities decreased significantly ($P<0.001$). There was a continuous decrease in ALT activities over the entire study period (Figure 32, 33). At T6, serum ALT activities ranged from 10 to 23 U/L and the mean ± SD was 17.5 ± 4.45 U/L. Serum activities of all participants were within the normal range. The difference of the means was greatest during M1 (4.16 U/L) and decreased gradually to 0.903 U/L during M3. All Pairwise Multiple Comparison Procedures (Holm-Sidak method) showed that changes in serum ALT activities reached statistical significance in the time periods T1-T3, T1-T4, T1-T5 and T1-T6, as well as in the time periods T2-T4, T2-T5 and T2-T6.

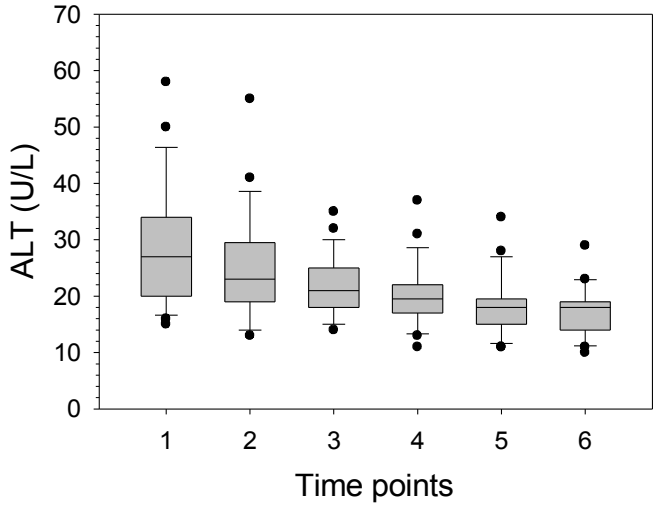


Figure 32: Changes in serum ALT activities during the program

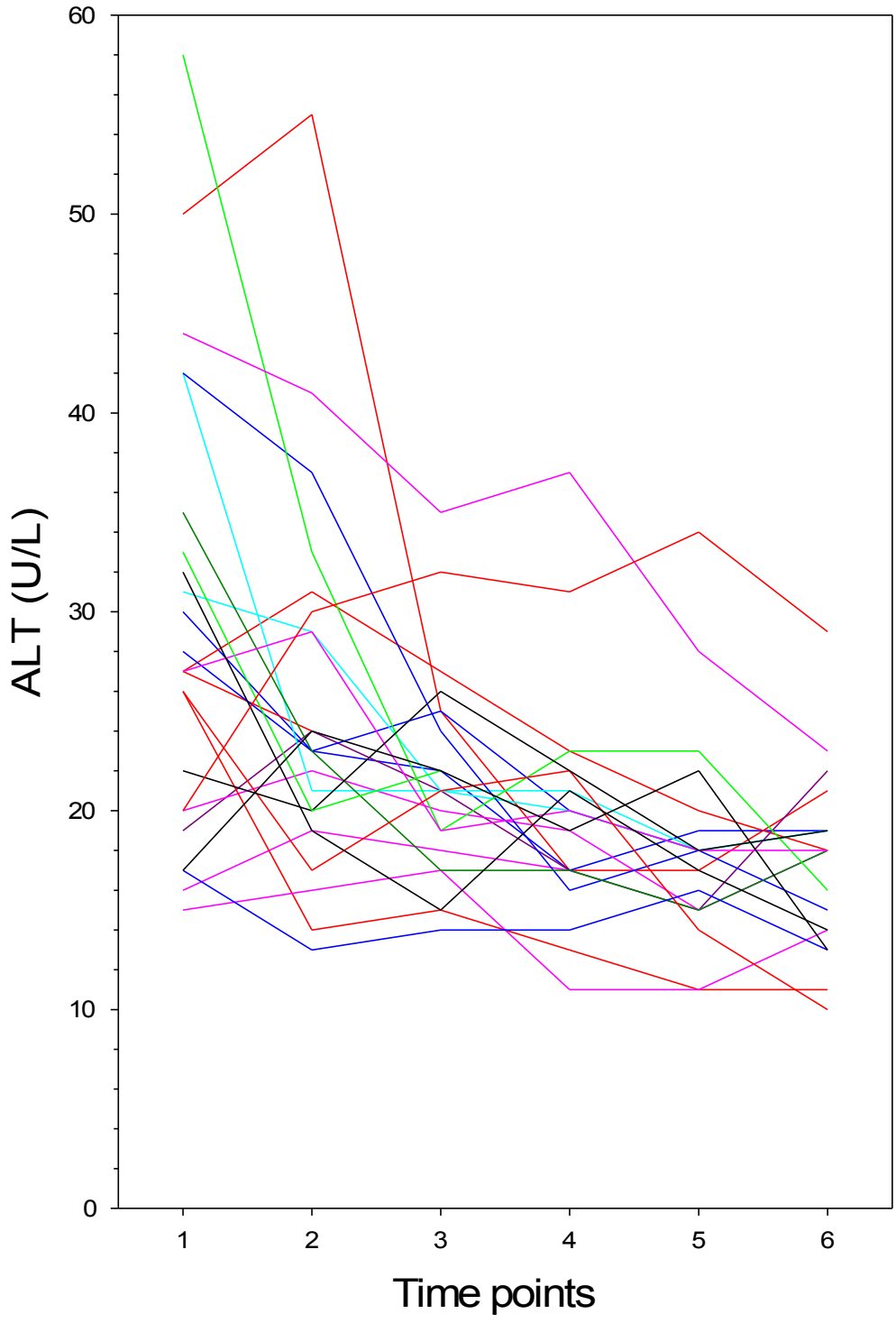


Figure 33: Individual changes in serum ALT activities during the program

5.2.2.1.3 AAR

Baseline: At T1 the AAR ranged from 0.580 to 1.33 and the mean ± SD was 0.938 ± 0.209. AAR values ≥1 were present in ten participants and AAR values <1 in 15 participants.

Effects of the MODUL Program: changes in AAR were statistically significant (P<0.001) (Figure 34). In the course of the program, the AAR increased gradually and at T6 values ranged from 0.862 to 1.80. The mean ± SD at this point was 1.24 ± 0.290. AAR values <1 were present in only three participants. All Pairwise Multiple Comparison Procedures (Holm-Sidak method) showed that changes in AAR were statistically significant in the same periods as changes in serum ALT activities.

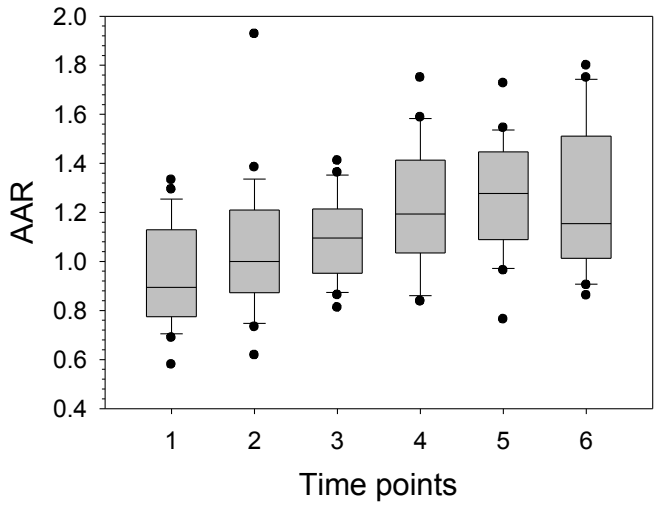


Figure 34: Changes in AAR during the program

5.2.2.1.4 GGT

Baseline: Before the start of the MODUL Program, serum GGT activities ranged from 9 to 38 U/L and the mean ± SD was 17.8 ± 6.70 U/L. Serum activities of all participants were within the normal or high normal range.

Effects of the MODUL Program: Changes in GGT activities were statistically significant (P<0.001) (Figure 35) but they showed some fluctuations. Changes in GGT activities were, compared to baseline, statistically significant at all time points. An additional significant difference was observed in the time period T2-T6. The mean ± SD decreased during M1 and B1 and reached its lowest level at T3 (12.6 ± 5.03 U/L). It reincreased during M2 to 13.4 ± 5.94 U/L and during B2 to 13.3 ± 4.43 U/L. During M3 it decreased again and at T6, the mean ± SD was 11.8 ± 4.43 U/L. At this point, serum GGT activities ranged from 6 to 24 U/L. All values were within the normal range.

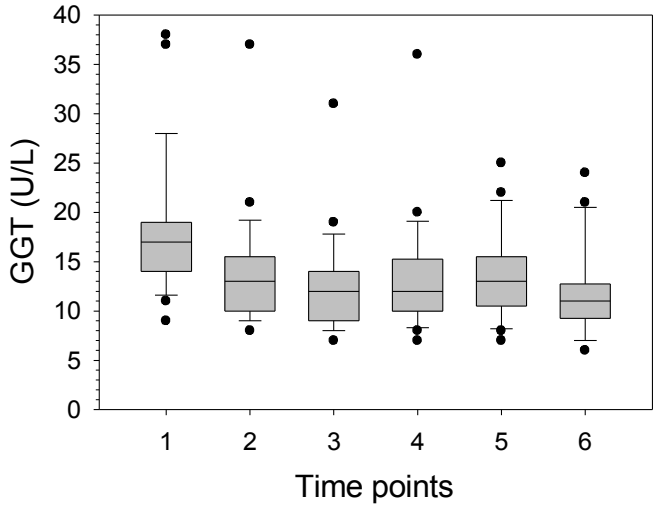


Figure 35: Changes in serum GGT activities during the program

5.2.2.2 Lipid profile

Except for HDL cholesterol, all variables of the lipid profile changed significantly (P<0.001).

5.2.2.2.1 Total cholesterol

Baseline: At T1, serum cholesterol concentrations ranged from 3.05 to 6.80 mmol/L. The mean ± SD was 4.43 ± 0.775 mmol/L. Cholesterol concentrations exceeded the normal range in three participants.

Effects of the MODUL Program: Changes in total cholesterol concentrations reached statistical significance (P<0.001) (Figure 36). At T6, cholesterol concentrations ranged from 2.84 to 5.46 mmol/L and the mean ± SD was 3.75 ± 0.587 mmol/L. The cholesterol concentration of one participant exceeded the upper limit of the normal range. The most pronounced decrease was observed during M1 when the difference of the means was 0.795 mmol/L. In all other time periods (B1, M2, B2, M3), the differences of the means were smaller and remained between 0.064 and 0.161 mmol/L. Compared to baseline, changes in total cholesterol concentrations were statistically significant at all time points.

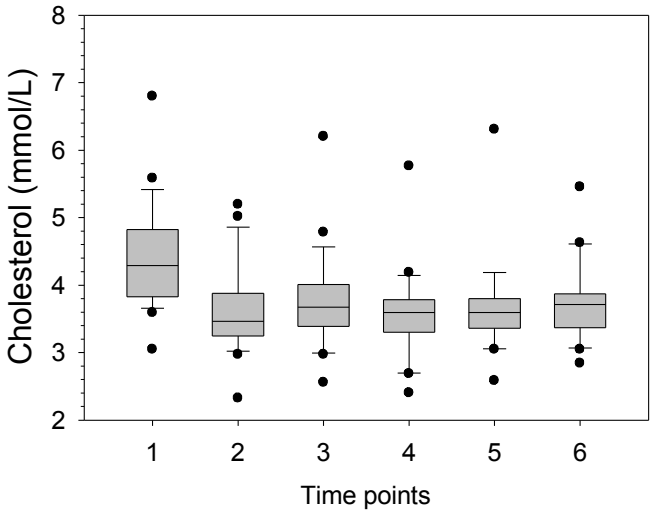


Figure 36: Changes in serum cholesterol concentrations during the program

5.2.2.2.2 HDL cholesterol

Baseline: At the start of the program, serum HDL cholesterol concentrations ranged from 0.855 to 2.05 mmol/L. The mean ± SD was 1.39 ± 0.346 mmol/L. The HDL cholesterol concentrations of all participants but one were within the normal range.

Effects of the MODUL Program: Changes in serum HDL cholesterol concentrations did not reach statistical significance (P=0.073) (Figure 37). At T6, serum HDL cholesterol concentrations ranged from 0.907 to 1.74 mmol/L and the mean ± SD was 1.30 ± 0.246 mmol/L. The spread of the values was similar at T1, T3, T4, T5 and T6 but considerably smaller at T2, which could possibly be explained by a regression to the mean during M1.

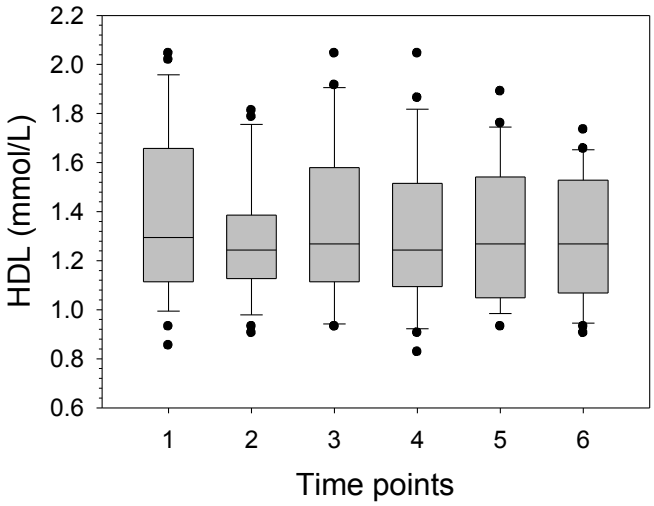


Figure 37: Changes in serum HDL cholesterol concentrations during the program

5.2.2.2.3 LDL cholesterol

Baseline: At the start of the MODUL Program serum LDL cholesterol concentrations ranged from 1.24 to 4.33 mmol/L. The mean ± SD was 2.41 ± 0.656 mmol/L and serum LDL cholesterol concentrations of all participants but one were within the normal range.

Effects of the MODUL Program: Changes in serum LDL cholesterol concentrations reached statistical significance (P<0.001) (Figure 38). At T6, LDL concentrations of all participants were within the normal range. The values ranged from 1.42 to 3.34 mmol/L. At this time point, the mean ± SD was 2.03 ± 0.452 mmol/L. Compared to baseline, changes in serum LDL cholesterol concentrations were statistically significant at all time points.

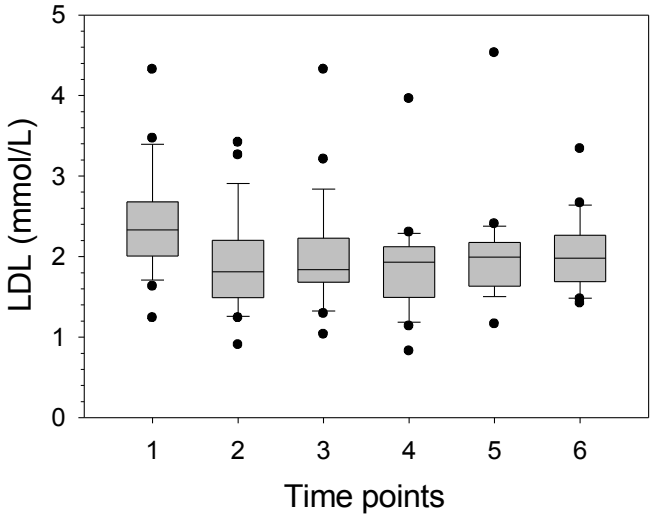


Figure 38: Changes in serum LDL cholesterol concentrations during the program

5.2.2.2.4 Triglycerides

Baseline: At T1, serum TG concentrations ranged from 0.524 to 3.04 mmol/L. The mean ± SD was 1.42 ± 0.720 mmol/L. Serum TG concentrations of all participants were within the normal or high normal range.

Effects of the MODUL Program: Changes in serum TG concentrations reached statistical significance (P<0.001) (Figure 39). At T6, TG concentrations ranged from 0.400 to 2.28 mmol/L and the mean ± SD was 0.765 ± 0.600 mmol/L. Serum concentrations of all participants were within the normal range. Compared to baseline, changes in serum TG concentrations were statistically significant at all time points. An additional significant difference was observed in the time period T2-T5.

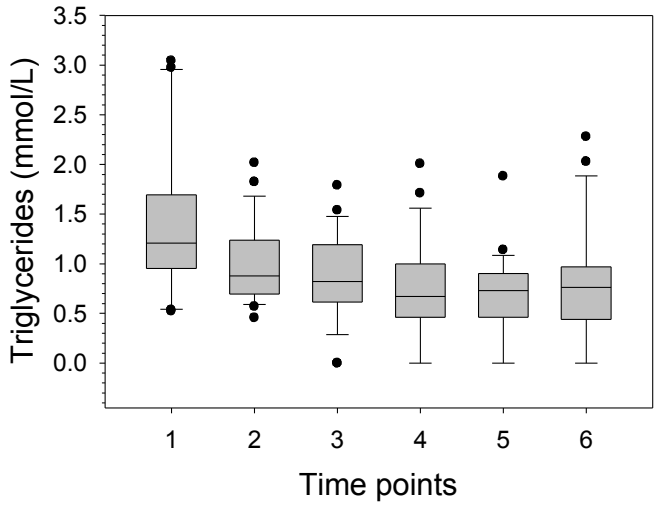


Figure 39: Changes in serum TG concentrations during the program

5.2.2.3 Glucose

Changes in fasting blood glucose concentrations reached statistical significance (P=0.030).

Baseline: At T1, blood glucose concentrations ranged from 4.72 to 6.05 mmol/L and the mean ± SD was 5.29 ± 0.366 mmol/L. The blood glucose concentrations of all participants were within the normal range.

Effects of the MODUL Program: Changes in glucose concentrations reached statistical significance (P=0.030) (Figure 40). At T6, blood glucose concentrations ranged from 3.39 to 6.49 mmol/L and the mean ± SD was 5.13 ± 0.643 mmol/L. The glucose concentration of one participant exceeded the upper limit of the normal range. All Pairwise Multiple Comparison Procedures (Holm-Sidak method) showed that changes were statistically significant in the period T1-T5.

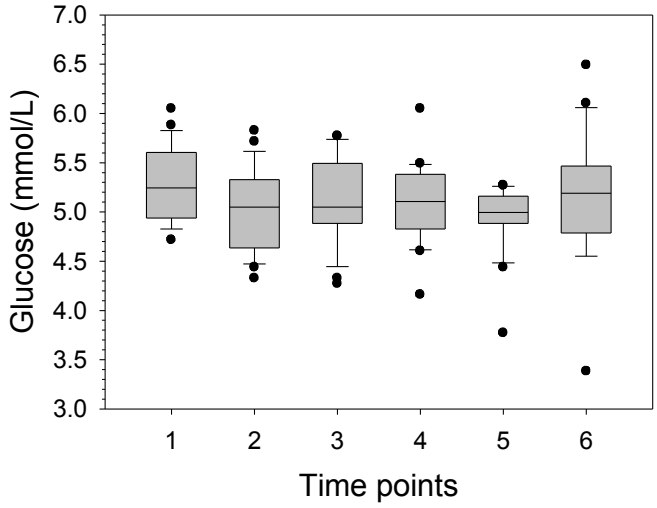


Figure 40: Changes in fasting blood glucose concentrations during the program

5.2.2.4 Biochemical markers of inflammation

While changes in serum CRP concentrations were statistically significant (P=0.002), changes in serum IL-6 concentrations did not reach statistical significance (P=0.674).

5.2.2.4.1 CRP

Baseline: At T1, serum CRP concentrations ranged from 0.750 to 13.0 mg/L and the mean ± SD was 3.33 ± 3.08 mg/L. The CRP concentrations of two participants exceeded the upper limit of the normal range (10.4 and 13.0 mg/L).

Effects of the MODUL Program: Changes in serum CRP concentrations reached statistical significance (P=0.002) (Figure 41) but there were great fluctuations. All Pairwise Multiple Comparison Procedures (Holm-Sidak method) showed that changes were statistically significant in the periods T1-T3 and T1-T6. The mean was greatest at T1 and decreased during M1 and B1. It reached its lowest concentration at T3 (1.44 ± 1.42 mg/L) and reincreased during M2 to 2.33 ± 2.38 mg/L and during B2 to 2.47 ± 2.84 mg/L. During M3, it decreased again and at T6, serum CRP concentrations ranged from 0.750 to 7.50 mg/L. At this time point, the mean ± SD was 1.66 ± 1.87 mg/L and all values were within the normal range.

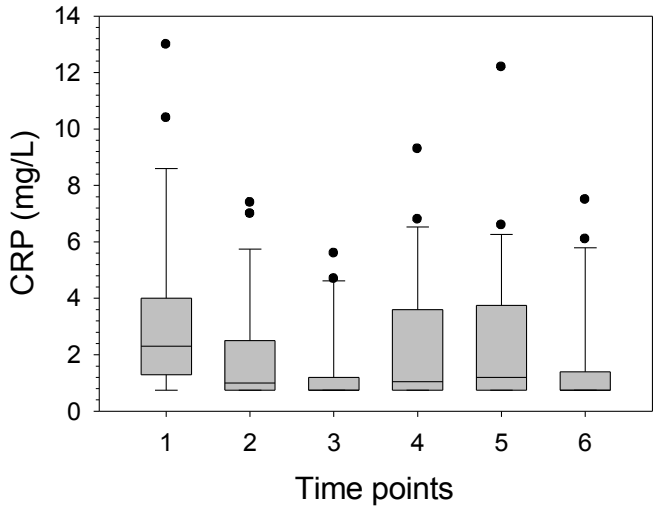


Figure 41: Changes in serum CRP concentrations during the program

5.2.2.4.2 IL-6

Baseline: At T1, serum IL-6 concentrations ranged from 0.500 to 6.90 ng/L and the mean ± SD was 2.86 ± 1.53 ng/L.

Effects of the MODUL Program: Changes in serum IL-6 concentrations did not reach statistical significance (Figure 42). The mean remained between 2.13 and 3.34 ng/L during the whole program. At T6, serum IL-6 concentrations ranged from 0.500 to 4.80 ng/L and the mean ± SD was 2.27 ± 1.21 ng/L.

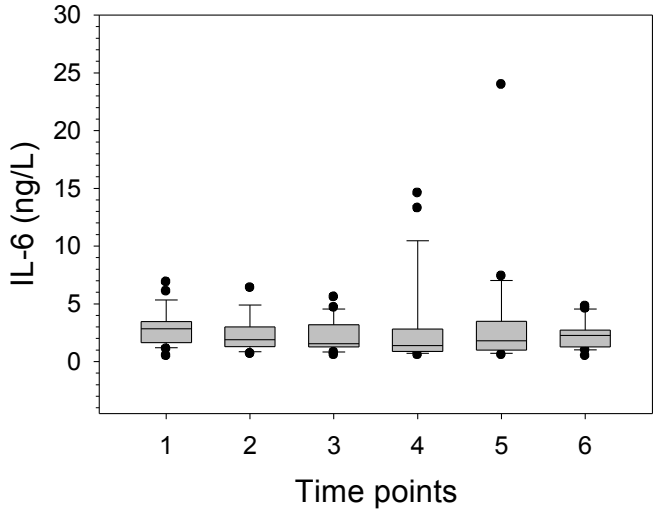


Figure 42: Changes in serum IL-6 concentrations during the program

5.2.2.5 Biochemical markers of the oxidant-antioxidant status

Changes in all observed markers of the oxidant-antioxidant status reached statistical significance (P<0.001).

5.2.2.5.1 MDA

Baseline: At T1, plasma MDA concentrations ranged from 0.360 to 0.650 µmol/L and the mean ± SD was 0.461 ± 0.0720 µmol/L.

Effects of the MODUL Program: Changes in plasma MDA concentrations reached statistical significance (P<0.001) (Figure 43) but there were great fluctuations. All Pairwise Multiple Comparison Procedures (Holm-Sidak method) showed that changes were

statistically significant in the periods T1-T3 (significant decrease) and T3-T5 (significant increase).

The mean ± SD decreased during M1 and B1 and reached its lowest concentration at T3 (0.387 ± 0.0475 µmol/L). It reincreased during M2 and B2 and reached its greatest value at T5 (0.470 ± 0.0922 µmol/L). During M3 it decreased again. At T6, the mean ± SD was 0.415 ± 0.0615 µmol/L and plasma MDA concentrations ranged from 0.290 to 0.540 µmol/L.

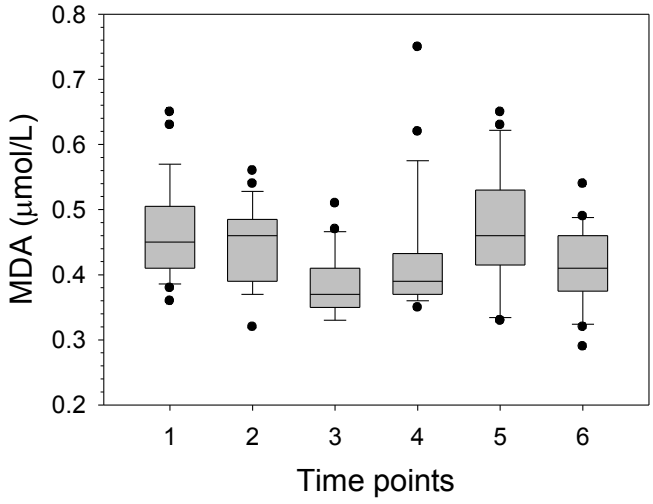


Figure 43: Changes in plasma MDA concentrations during the program

5.2.2.5.2 Vitamin E

Given that during M3 a supplement of α-tocopherol (100 mg/d) was applied, the period before supplementation (T1 to T5) and the period after supplementation (T5 to T6) will be discussed separately. Changes in both time periods reached statistical significance ($P<0.001$) (Figure 44).

5.2.2.5.3 α-Tocopherol

Baseline: At T1, plasma α-tocopherol concentrations ranged from 17.9 to 44.3 µmol/L and the mean ± SD was 23.7 ± 5.06 µmol/L. Except for the outlier at 44.3 µmol/L, all values were within the normal range.

Effects of the intervention from T1 to T5: Before supplementation, changes in plasma α-tocopherol concentrations reached statistical significance (P<0.001). At T5, α-tocopherol concentrations ranged from 13.8 to 33.4 µmol/L and the mean ± SD was 19.4 ± 4.05 µmol/L. The plasma α-tocopherol concentration of one boy was below the lower limit of the normal range and that of another boy exceeded the upper limit of the normal range. The greatest difference of the means (5.55 µmol/L) was observed during M1. During B1, M2 and B2, the difference of the means was smaller and ranged from 0.398 to 1.14 µmol/L. All Pairwise Multiple Comparison Procedures (Holm-Sidak method) showed that the plasma concentrations off all time points were significantly different compared to baseline (T1).

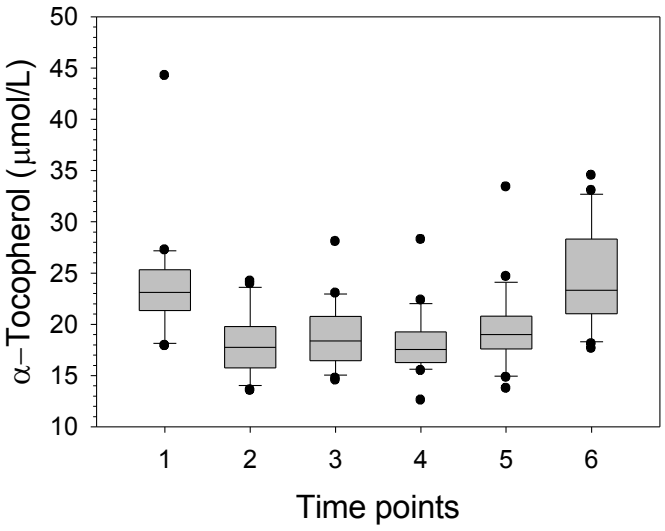


Figure 44: Changes in plasma α-tocopherol concentrations during the program

Effects of the intervention during M3: After supplementation changes in plasma α-tocopherol concentrations reached statistical significance (P<0.001). At T6 values ranged from 17.7 to 34.5 µmol/L and the mean ± SD was 24.6 ± 4.89 µmol/L. The plasma α-tocopherol concentrations of five participants exceeded the normal range. Compared to T6, plasma concentrations at all the other time points were statistically significant.

5.2.2.5.4 Ratio of α-tocopherol to cholesterol (TCR)

Baseline: At T1, the TCR ranged from 4.17 to 8.45 µmol/mmol and the mean ± SD was 5.40 ± 0.935 µmol/mmol. The TCR of one participant was below the lower limit of the normal range and the TCR of two participants exceeded the upper limit of the normal range.

Effects of the intervention from T1 to T5: Before supplementation, changes in TCR reached statistical significance (P<0.001) (Figure 45). At T5 the TCR ranged from 4.47 to 7.34 µmol/mmol and the mean ± SD was 5.30 ± 0.725 µmol/mmol. At this time point, the TCR of one participant exceeded the upper limit of the normal range. All Pairwise Multiple Comparison Procedures (Holm-Sidak method) showed that changes were statistically significant in the time period T1-T3.

Effects of the intervention from T5 to T6: After supplementation, changes in TCR reached statistical significance (P<0.001). At T6 values ranged from 4.71 to 9.68 µmol/mmol and the mean ± SD was 6.61 ± 1.40 µmol/mmol. At this time point, the TCR values of seven participants exceeded the upper limit of the normal range.

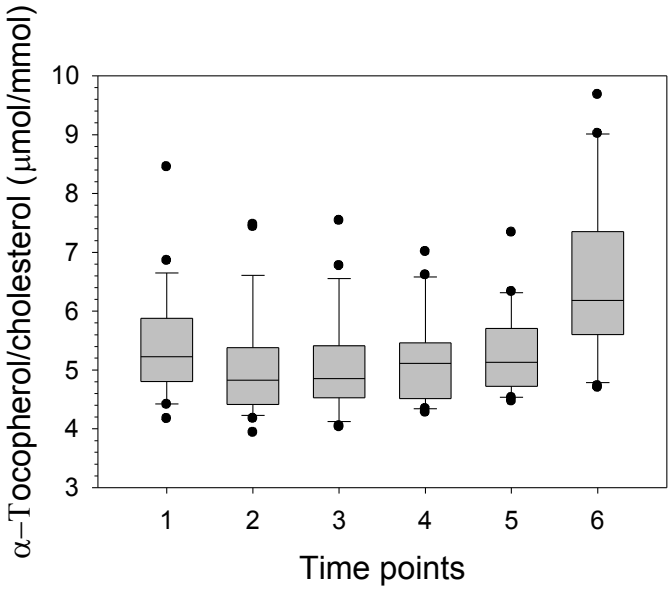


Figure 45: Changes in TCR during the program

5.3 The Fatty Liver Index

Baseline: At the start of the MODUL Program, the FLI ranged from 8.67 to 96.2 and the mean ± SD was 45.3 ± 26.3 (Figure 46). In 10 participants the FLI was <30, in 9 the FLI was ≥30 and ≤60 and in 6 participants the FLI was >60 (Table 18).

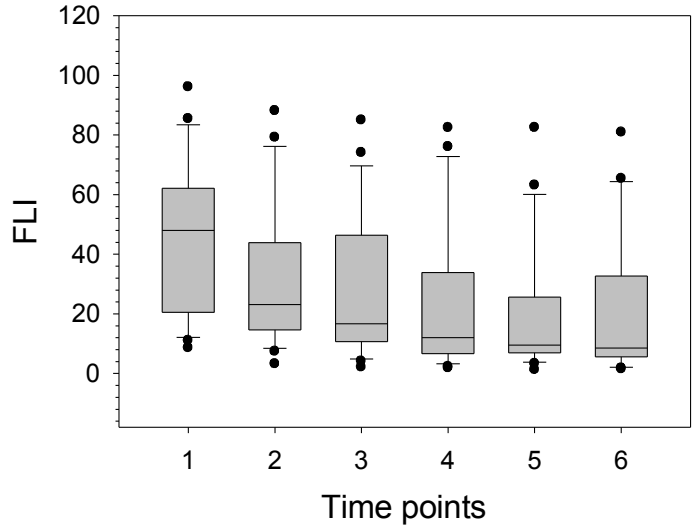


Figure 46: Changes in the FLI during the program

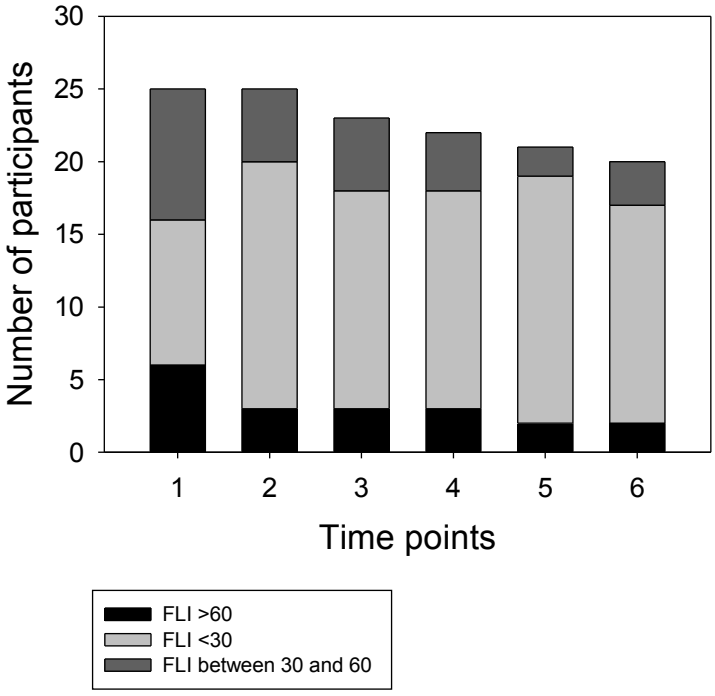


Figure 47: Cumulative number of participants with 3 categories of FLI at the different time points

Effects of the MODUL Program: Changes in the FLI were statistically significant during the program (P<0.001). At the end of the program (T6), the FLI ranged from 1.56 to 81.0 and the mean ± SD was 20.6 ± 23.2. The difference of the means was greatest during M1 (14.0). It decreased gradually and was smallest during M3 (0.165). All Pairwise Multiple Comparison Procedures (Holm-Sidak method) showed statistically significant differences (P<0.05) between the FLI values at all time points compared to baseline. Additional significant differences were observed in the time periods T2-T4, T2-T5, T2-T6 and T3-T5 and T3-T6 (Figures 47, 48).

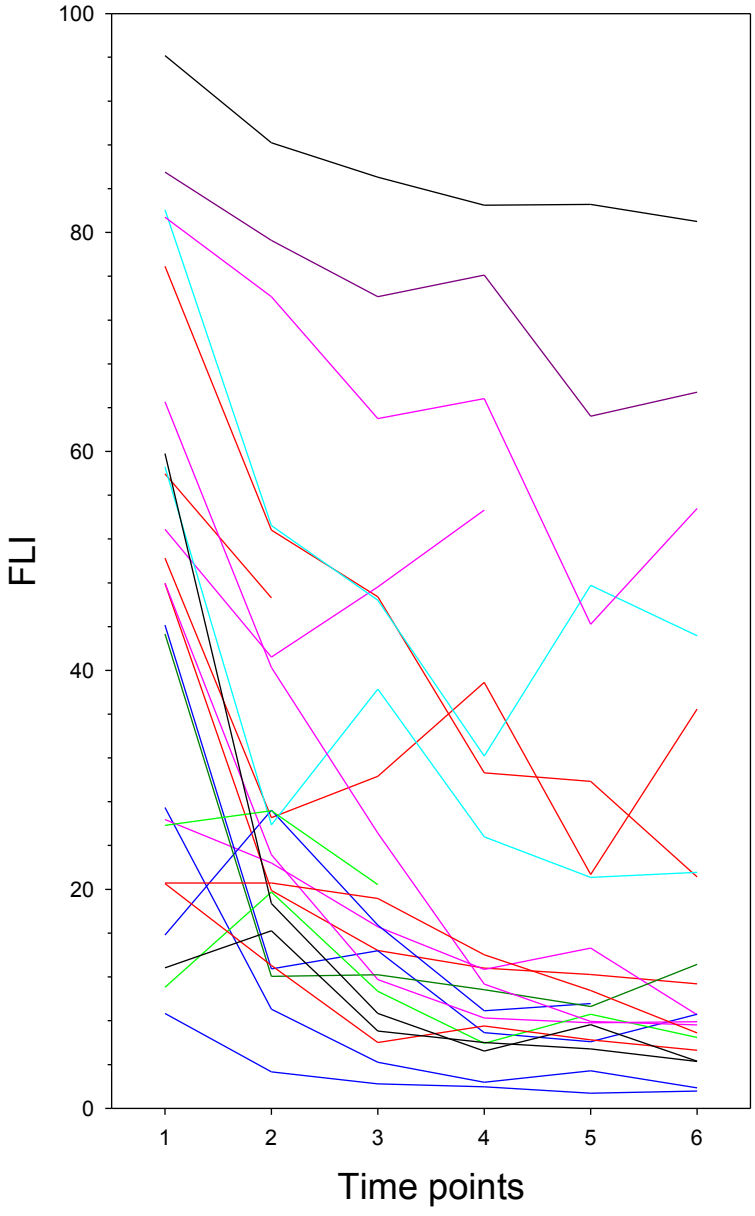


Figure 48: Individual changes in FLI during the program

While at T1 only 40 % of the participants had FLI values <30 (that are likely to rule out the presence of NAFL), this percentage was almost doubled at T6. The percentage of participants who had FLI values >60 (that are likely to rule in the presence of NAFL) decreased from 24 % to 10 % (Table 18).

Table 18: Number and percentage of the participants at risk for NAFL according to the FLI

	T1	T2	T3	T4	T5	T6	T1	T2	T3	T4	T5	T6
FLI	**n**						**%**					
<30	10	17	15	15	17	15	40	68	65	68	81	75
30-60	9	5	5	4	2	3	36	20	22	18	9.5	15
>60	6	3	3	3	2	2	24	12	13	14	9.5	10
Total	25	25	23	22	21	20	100	100	100	100	100	100

6 Discussion

In this study, overweight and obese children and adolescents, aged 12 to 15 years, with BMI >95th age- and sex-specific centile, were studied during participation in the obesity intervention program MODUL. The main interest was to investigate the impact that weight reduction may have on the liver and on NAFLD. Therefore, energy intake and distribution of macronutrients were monitored along with the changes in liver enzyme activities and the serum or plasma concentrations of other variables that are closely related to the pathogenesis of NAFL and disease progression to NASH. These variables included anthropometric variables, the lipid profile and fasting glucose concentrations, as well as markers of inflammation and oxidant-antioxidant status. Moreover, for the first time, the FLI, a predictior of hepatic steatosis (9) was applied in a pediatric population and changes over the observation period were evaluated. This index is composed of the four variables BMI, WC, TG and GGT and has been applied for the prediction of hepatic steatosis.

During the MODUL Program a statistically significant decrease (P<0.001) of the FLI was observed. The proportion of children and adolescents with normal FLI (<30) increased from 40 % at the beginning of the program to 75 % at the end of the program. On the other hand, the proportion of those with FLI >60, which indicates the presence of NAFL, decreased from 24 % to 12 % within four weeks and further to 10 % at the end of the

19-week observation period. These results indicate that after successful weight reduction the number of participants for whom NAFL can be ruled out had almost doubled, while the number of those with suspected NAFL (FLI >60) had decreased by more than 50 %. It needs to be noted that the applied cut-off values have been established for adults and pediatric cut-off values are still not available. Considering that the upper limits of the normal ranges of the four FLI variables in children and adolescents are equal to or below the corresponding ones in adults, the proper cut-off values of the FLI are expected to be smaller than those used in the present study. This would imply that the percentage of overweight and obese children and adolescents with an FLI that is indicative of NAFL may have been even higher. In any case, the fact that the beneficial effect of weight loss on FLI occurred within a period of only four weeks needs to be particulary emphazised and is considered promising.

Weight loss during the program reached statistical significance and averaged 9.67 kg. For children, a weight loss of 0.5 kg/week is recommended (77). According to Kim et al. (58), even a very modest weight reduction may be beneficial. In contrast, a rapid weight loss has been shown to have adverse effects on NALFD (112, 140). For adults a weight loss between 0.4 and 0.9 kg/week is recommended (112), while a weight loss of 1.6 kg/week has been associated with disease progression (86). For children, however, there is no agreement on the cut-off value for appropriate versus too rapid weight loss. Thus, during the 19 weeks of the MODUL Program, a weight loss of 9.5 kg (2.0 kg during each intervention period and B1 and 1.5 kg during B2) would be desirable. Whether this reduction can be achieved by the MODUL Program *as it was designed* cannot be evaluated as the participants did not strictly follow the recommendations of the program concerning food intake. In fact, the daily energy intake was less than the recommended 1,893 kcal/day and averaged only 1,181 kcal. Thus the weight loss was more pronounced than intended.

Given that in our study many adolescents lost more than 0.5 kg/week and some even more than 1.0 kg/week, adverse effects on the liver in some participants cannot be ruled out. Especially during M1 and B1, weight loss may have been too fast. Therefore, participants of obesity intervention programs should receive detailed information about the negative impact of rapid weight loss. This may contribute to better compliance and thus better results.

Regarding the composition of the diet there are no general recommendations for NAFLD patients. However, three interventional studies demonstrated significant effects of

diet only or a combination of diet and exercise on liver histology. In Table 11 (Different dietary regimes with improvement of liver histology and comparsion to the diatary intake in the MODUL Program p. 36) the distribution of macronutrients in these studies has been compared to the distribution of macronutrients in the MODUL Program. The fraction of carbohydrates recommended in the MODUL Program was lower than in the study by Uslan et al. (122) but higher than in those by Ueno et al. (121) and Huang et al. (44). Ryan et al. (104) showed that a greater decrease in serum ALT activities is achieved by a diet containing 40 % of carbohydrates and 45 % of fat compared to a low fat diet containing 60 % of carbohydrates and 25 % of fat. In consideration of these findings, the distribution of macronutrients during the MODUL Program is likely to have exerted beneficial effects on liver pathology. However, it needs to be determined whether a lower fraction of carbohydrates may be preferable.

During this study, different anthropometric and biochemical variables changed significantly. The only exceptions were HDL cholesterol and IL-6 which did not show significant changes.

Of the variables under investigation, BMI, WC and TG decreased significantly ($P<0.001$) during the MODUL Program. Interestingly, changes followed a similar pattern and paralleled each other. The decline did not show any fluctuations or transient elevations. The difference of the means of all three variables was greatest during M1 and decreased during the course of the program with changes being smallest during M3. Published evidence is suggesting that elevated BMI (20, 45) and WC (20, 74) are strongly associated with advanced states of NAFLD. As visceral fat has a very high lipolytic activity (50) the diagnostic power of the WC for NALFD is expected to be superior to that of the BMI (56). However, the associations of these variables to simple steatosis and their performance as a diagnostic tool in the course of an obesity intervention program has not sufficiently been investigated.

In terms of lipid profile evidence is suggesting best performance for the prediction of NAFLD for TG (5, 11, 97, 135). In the participants of the MODUL Program serum TG concentrations decreased significantly ($P<0.001$). They increased again during M3 but only by 0.0178 mmol/L. Also total cholesterol and LDL cholesterol concentrations decreased significantly, while HDL cholesterol concentrations did not change significantly.

Our findings that body weight, BMI, WC, total cholesterol and TG decreased significantly during the intervention are consistent with the results of de Piano et al. (91),

who showed significant changes in the same variables, as well as in SAT mass, fasting glucose and the homeostasis model assessment insulin resistance (HOMA-IR) during an obesity intervention program of 12 weeks.

The participants of the MODUL Program also showed statistically significant decreases in the serum activities of the liver enzymes AST, ALT and GGT ($P<0.001$). Even though the absolute values of liver enzyme activities have only poor diagnostic power to predict NAFLD as shown in Table 3 (Liver enzymes and their associations with NAFLD, p. 23), the changes in enzyme activities over a period of time are considered to be indicative of the progression or regression of NAFLD (16, 121, 122, 134). Our findings are consistent with those of Reinehr et al. (102), Nobili et al. (84), Wang et al. (128), Suzuki et al. (116), Hickman et al. (42), Ueno et al. (121), Uslan et al. (122) and Tazawa et al. (119), all of whom showed a significant decrease in serum liver enzyme activities during lifestyle modification. Ueno et al. (121) and Uslan et al. (122) additionally monitored liver histology and showed that decreasing serum enzyme activities were accompanied by an improvement of histological findings. Furthermore, Kim et al. (58) analyzed a population of 2,895 subjects over five years and showed that those subjects who developed NAFLD within five years of follow-up had significantly higher levels of body weight, AST, ALT, GGT, TG and fasting glucose already at baseline. The degree of increase in AST, ALT and GGT was associated with the development of NAFLD. Therefore, elevated serum liver enzyme activities, even within the normal range, are a condition that should be taken seriously, in particular when they last for a long time. Even if liver histology may not have been altered yet, a decrease in serum liver enzyme activities may indicate a beneficial effect. In this study, we showed that in overweight and obese children and adolescents elevations of serum liver enzyme activities are reversible and that the decrease can be achieved within a short period of time.

It attracted our attention that changes in activities of ALT, AST and GGT did not follow a similar pattern. While ALT decreased in parallel to BMI, WC and TG, the changes in AST and GGT showed fluctuations and a transient increase at T4 and T5. Interestingly, a similar pattern was also observed for CRP and MDA concentrations. Whether a causal relationship exists that may explain the similar patterns of changes in AST, GGT, CRP and MDA, needs to be investigated.

During the MODUL Program the AAR increased significantly ($P<0.001$). This can be explained by the fact that serum ALT activities decreased faster compared to the serum

AST activities. The mean AAR was <1 only at T1. At the end of the program, only three of the 20 adolescents had AAR values <1. These findings are surprising, given that in general the AAR is <1 even in patients with NAFL (79, 23, 70) and the AAR increases with the severity of liver damage. An AAR >1 may indicate advanced fibrosis (86) or cirrhosis (27, 88, 70) and in NAFLD, an increasing AAR may indicate disease progression (70). Harrison et al. (39) developed a scoring system to distinguish simple fatty liver from more advanced liver disease and they considered an AAR >0.8 to be a risk factor for advanced liver disease. Thus, the current findings would indicate a worsening of NAFLD. This seems unlikely, given that the FLI, AST and ALT as well as all other observed variables (except IL-6 and HDL cholesterol) decreased significantly during the MODUL Program. However, the results are still to be taken seriously. On the other hand, it cannot be ruled out that the cut-off value of the AAR might not be suitable for children and adolescents. As the AAR is not commonly used in today's practice and cut-off values have not been re-approved for several years (36), further research on this topic is needed.

In conclusion, participation of overweight and obese children and adolescents in the obesity intervention program MODUL has beneficial effects on obesity-related NAFLD as evidenced by a significant decrease in the FLI as a non-invase predictior of NAFLD. Significant reductions could be observed in body weight, BMI, WC, total fat mass, SAT mass, VAT mass, TG, GGT, AST, ALT, and, for the first time, also in the FLI. It should be noted that the effects of weight loss may occur within a period of only four weeks. Similar programs are recommended for overweight and obese children and adolescents to treat overweight and obesity and prevent obesity-related comorbidities including NAFLD.

Curriculum vitae

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Sprachen

Englisch	Verhandlungsfähig in Wort und Schrift
Französisch	Sehr gut in Wort und Schrift
Portugiesisch	Gut in Wort und Schrift
Spanisch	Gut in Wort und Schrift

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