Lathosterol and Noncholesterol Sterols in Routine Use for the Differentiation and Monitoring of Dietary and Drug Induced Treatment of Hypercholesterolemias in Children and Adolescents

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Abstract: *Aims*: The authors discuss their 15 years of experience with use of noncholesterol sterols (NCS) when diagnosing heterozygous familial hypercholesterolemia (HFH) and the dietary and drug treatment of children and adolescents when lathosterol (Lat) and desmosterol (Des) as cholesterol synthesis precursors, and campesterol (Cam) and sitosterol (Sit) as cholesterol absorption precursors are included.

Patients and Methods: 38 children and adolescents (6-18 yrs) with HFH proven by molecular genetic testing of LDL-cholesterol receptor deficit; 107 children patients with clinical and laboratory symptoms of other hypercholesterolemias; 84 healthy school-age children as a control group. Routine lipid spectrum scan—total cholesterol (TCh), LDL-Ch, HDL-Ch, TAG, with additional apo A1, apo B, Lp (a), LDL-receptors, apo E polymorphism; Lat, Des, Cam and Sit in the plasma—was established by means of GC/MS.

Results: The HFH patients on a low cholesterol diet (LCHD) who come to our lipid outpatient clinic have elevated levels of Lat and Des, unlike patients with alimentary hypercholesterolemia (p<0, 001). Lat and Des levels are high following interruption of medical treatment during long vacations or when drug treatment is neglected. Administration of statins only in sufficiently high therapeutic doses reduces Lat and Des (p<0, 001). Compensatory elevation of Cam and Sit occurs only in few pediatric patients. Ezetimibe decreases Cam and Sit more efficiently than Lat or Des. Combination of statin with ezetimibe is most efficient in decrease of not only TCh but also Lat and Des, as well as Cam and Sit.

Conclusions: Extending the laboratory spectrum by precursors of cholesterol synthesis and absorption improves the differential diagnosis of HFH and makes monitoring and/or treatment of children and adolescents more precise.

Keywords: Noncholesterol sterols, lathosterol, desmosterol, campesterol, sitosterol, phytosterols, heterozygous familial hypercholesterolemia, statins, ezetimibe, dietary and drug treatment.

1. INTRODUCTION

All patients suffering from inherited hypercholesterolemias are burdened by premature cardio- or cerebrovascular events. Early identification of these inherited diseases allows administration of proper lifestyle and drug therapy as early as in childhood to prevent cardiovascular or cerebrovascular complications [1-4]. There exist 2 types of disorders characterized by marked elevation of total cholesterol (TCh) and LDL-cholesterol (LDL-Ch): (1) familial hypercholesterolemias resulting from deficient LDL receptor proteins that cannot a) bind LDL (binding defects), b) internalize LDL (internalizing defects), c) disrupt the normal recycling of the LDLR (recycling defects), and (2) familial ligand defect of B-100 (FDB) hypercholesterolemia with missence mutation R3500Q.

It is a well-known fact that heterozygous familial hypercholesterolemia (HFH) with autosomal dominant mode of inheritance (OMIM 143890) cannot be successfully treated by even a strict low-cholesterol diet (LCHD), and requires differentiated drug therapy. Recently, it has been finally accepted that pediatric HFH (boys from the age of 10 years and girls after first menstruation) require the same intensity of medical therapy as adults [5-10].

However, what are our options for early distinction between the so called "alimentary hypercholesterolemia" (AH) caused by overeating and an unhealthy lifestyle, which is most frequently encountered in our lipid clinic, and the most severe forms of HFH? The clinical symptoms in children being not yet as sufficiently developed as in adults, we can only use typical laboratory findings from the lipid spectrum supported by the molecular genetic examination of LDL-cholesterol receptors. Over the past 15 years, we have included noncholesterol sterols (NCS) in the testing, such as surrogate of cholesterol synthesis precursors and surrogate for cholesterol absorption in the testing.

Following the introduction of compulsory nation-wide selective screening for hyperlipoproteinemia in the

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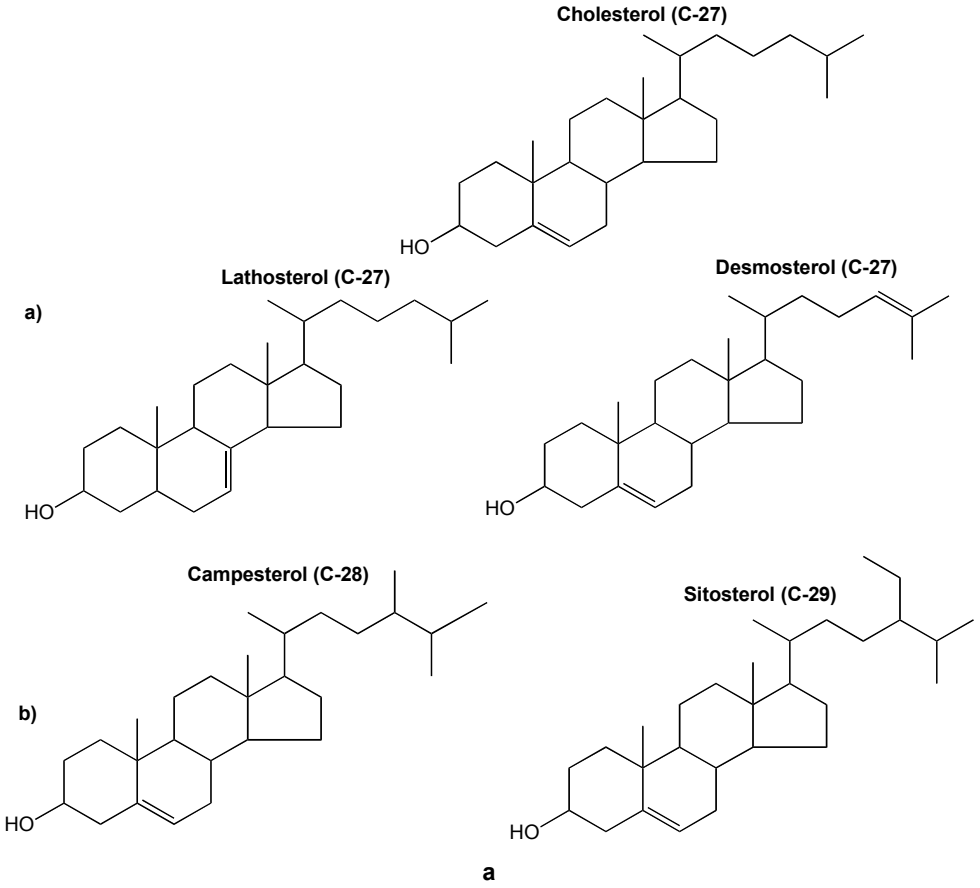
E-ISSN: 1929-5634/14



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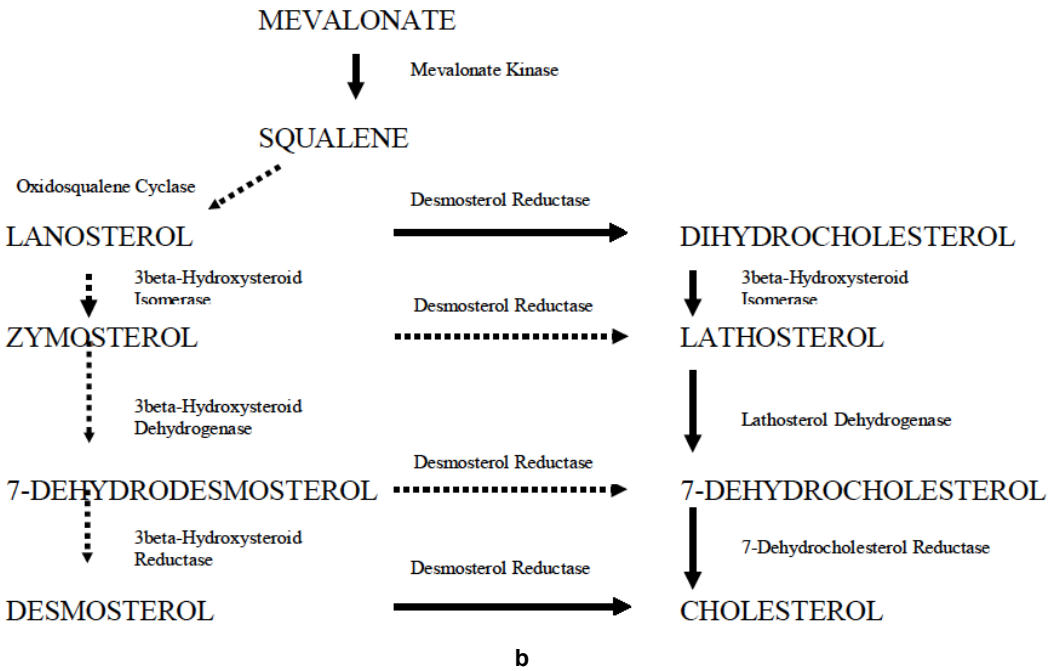

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Figure 1: a) Structures of cholesterol, its most important precursors and some phytosterols.
b) Simplified metabolism of cholesterol synthesis.

Czech Republic for children from high-risk families in their fifth and thirteenth year of life (since 1994), it is not only children of patients operated on in our hospital with a surgically documented risk of CVD who come to our metabolic clinic for further examination, but primarily children who have been identified with a range of different hypercholesterolemia symptoms in pediatric primary care settings [11]. These symptoms

are subsequently differentiated and the required therapy is determined depending on the results—either LCHD in cases of AH or drug treatment in cases of HFH. Encouraged by the pioneering work of Hamilton, Miettinen, Kempen, Jones, Gylling [12-20], and the promising latest research outcomes [21-23], we have attempted to combine the standard lipid marker examination with the most easily available markers of cholesterol synthesis precursors—lathosterol (Lat) and desmosterol (Des)—, and the cholesterol absorption markers—campesterol (Cam) and beta-sitosterol (Sit)— for better hypercholesterolemia differentiation. (for NCS structures and simplified metabolism scheme see Figure **1a**, **b**).

From a large cohort of pediatric and adolescent patients classified into groups according to the etiology of their hypercholesterolemia, we have till now observed important diagnostic and therapeutic dependencies sometimes similar to observations of Noto *et al*. or Hedman *et al*., which are presented below [24, 25].

2. PATIENTS DEFINITION AND METHODS

Out of 1,000 patients regularly attending our lipid clinic, the following were selected for the study:

- 38 statin and ezetimibe treated children (10-18 yrs) with a positive family history of CVD (Framingham Risk Score 10-20%) and the molecular genetically proved deficit of LDL-receptor mutations, specifically in p.Gly592Glu (8 patients), p. Cys200Tyr (2 patients), p. Pro424_Asn425ins32 (2 patients), p. Gly218del (2 patients), p. Arg4+6Trp (1 patient), p. Asp266Glu (1 patient), p. Gly396Alafsx54 (1 patient), p. Ser177Leu (1 patient), p. Trp 666X (l patient), p. His690ThrfsX19 (1 patient), p. Cys143Arg (1 patient), p. Asp227Glu (1 patient), p. Gly149Cys (1 patient), p. Gly176Val (1 patient), p. Ala299Val (1 patient), Ala 232Asn27+del (1 patient); and with the familial ligand-defective apoB 100 (FDB) 3500W mutation (1 homozygote patient and 11 heterozygote patients);
- 107 statin, ezetimibe or LCHD treated children of the same age with metabolic findings resembling HFH, where molecular genetic analysis has not yet confirmed LDL-R deficiency (familial combined hyperlipidemia, polygenic hypercholesterolemia);
- 84 healthy school-age children and adolescents (6-18 years old) from schools in Prague area as a control group;
- 37 children of the same age with confirmed “alimentary hypercholesterolemia”, frequently accompanied by variously presented hypertriglyceridemia and obesity;
- 35 randomly selected healthy blood donors (18-60 years old) as a control group for NCS of healthy adults.

The basic characteristics of the cohorts investigated are shown in Table **1**.

The LCHD was defined as 1992 NCEP-Ped Step II diet (<300 mg/d cholesterol in natural food) [26]. The “Alimentary Hypercholesterolemia” was determined on the basis of a lipid spectrum assessment, pedigree analysis, low risk of CVD in the family, and evaluation of a submitted two-week food journal, which was analyzed for nutrient content and cholesterol intake (mg/d) by the Ostrasoft Co. (1992) software programme [27]. According to recommendations by Rifkind, deJong, and Wiegman [5, 6, 28], individual cholesterol tolerance was respected so as for TCh blood levels not to increase over 4.8 mmol/l.

Medical treatment involves the use of simvastatin—Zocor (MSD) in individual doses of 5-20 mg/d—, which has been successfully combined with ezetimibe—

Table 1: Basic Characteristic of Investigated Groups of Healthy Children, Healthy Adults and Child Patients Suffering from Hypercholesterolemias

	Age (yrs)	Women/Men	Skin Fold (mm)	BMI	TCh (mmol/l)	Apo-B (g/l)
48 Children on LCHD	10 to 18	25/23	5.5/10.2/8.9		6.7	1.86
38 Children on Statins	10 to 18	25/23	5.5/10.2/8.9		5.4	1.56
84 Healthy Children	6 to 18	52/32	4.2/8.5/7.5		3.7	1.12
35 Healthy Adults	20 to 65	12/13		28.1	5.2	1.52

Ezetrol (MSD) in individual doses of 5-10 mg/d— over the past 10 years [5, 6, 9, 10]. During the two-month summer vacations, when the patients are usualy not available for monitoring and drug therapy, the drug treatment is often insufficiently observed, and the only remaining treatment is LCHD.

Monitoring of the diet and drug treatment is ensured by biochemical and immunological testing of lipid markers at 3-6 month intervals (depending on tha patient's compliance); levels of NCS, lipid soluble vitamins, sex hormones and trace elements in 6-month intervals. Height and weight are recorded using a software programme, secondary signs of sex maturation are registered according to Tanner and Goldstein [29] on each visit to the clinic. Performed annually, skinfold measurements are taken by the Best caliper, and IMT is measured using a VigmedSound device.

The routine biochemical and classical lipid spectrum (TCh colorimetric assay, HDL immunoassay, LDL direct immunoassay, TAG enzymatically) is determined using a Synchron Beckman Coulter LX 20; haematological spectrum by means of an Advia 120 Bayer; apo Al and apo B nephelometrically using an Immage; apo E polymorphism is measured by molecular genetic analysis; sexual hormone levels are determined with the IRMA method using a Stratec SR300; trace elements using a GT-AAS Varian; lipid soluble vitamins by means of HPLC with UV detection; vitaminD_3 with the RIA technique using a Multicrystal Berthold LB211; vitamin B6 using a Chromsystem; holotranscobalamin using an Abbot AXIM; and beta-carotene by means of HPLC.

The following DNA analyses of genes for LDL-receptors and FDB (familial ligand-defective ApoB) are performed: direct DNA analysis, sequential analysis of exon 4 and 5, and analysis of deletions and duplications of the LDL-R gene and the entire region using the Multiplex Ligation Probe Amplification method, carried out through the goodwill of the Genetic Laboratory, Centre of Cardiovascular and Transplantation Surgery, Brno (MedPed project Czech National Coordinator, MUDr. T. Freiberger, PhD.) [30]. PCSK9 genes are not sequenced. The first degree relatives of HFH probands with identified gene mutations undergo DNA sequencing regardless of the LDL-cholesterol plasma levels.

Plasma NCS were determined first by GC following Phillips *et al*. [31], later using the Theunissen [32] modification by means of a GC/MS Finnigan Mat 120B: internal standard epicoprostenal, CV <10%. The values of detectable lanosterol are very low without any possible diagnostic use. According to our practical experience and a longitudinal evaluation of the NCS levels, Des essentially follows in lower concentrations the Lat values, and Sit follows Cam values in a similar way: we have retained only these two markers in routine testing over the recent years and found them to be sufficient to register both cholesterol synthesis and phytosterol absorption.

The overall lipid extraction from the serum samples was performed using the Folch method [44]. First, the inner standard of epicholesterol (5 µg) was added to the serum sample (0.9 ml). Methanol (0.9 ml) was added, and the whole mixture was agitated for 15 minutes. The extraction was done using chloroform (2 x 0.9 ml) while adding water solution of NaCl. The mixture was then centrifuged (at 200 g) for 10 minutes.

The lipid saponification was performed using the modified Thompson and Merola method [45]. Following evaporation of chloroform ethanol with 3% pyrogalol (8ml) was added to the lipid extract sample. The preparation was briefly mixed by hand, and KOH (0.5 ml, 1.28g/ml) solution was added. The saponification (30 min. at 88ºC) was performed following mixing and sonification of each sample. Cyclohexan (20 ml) and distilled water (12 ml) were added to each sample and the content was agitated. After centrifugation (at 200 g) the upper layer of cyclohexan was transferred into a vial, and evaporated under a nitrogen stream (approx. 65ºC).

Another step in the preparation of samples for GC/MS was their extraction derivatization using methanol-methyl chloroformate-pyridine. The reason was derivatization of sterols while separating the neutral lipid fraction from polar lipids. The derivatization was performed using the modified Hušek Method [46].

The extracted compounds were then analysed on the Focus GC with ITQ 700 ion trap mass spectrometer (manufactured by Thermo Electron Corporation) heated to 200°C. TR-1 column (30 m length, 0.25 mm internal diameter, 0.25 µm film of dimethyl polysiloxane) was used for separation. The ion source was operated in the electron ionisation mode (70 eV, 20 µA) and the mass spectra were obtained using the ion trap operating in a SIM scan mode.

For expression of NCS serum values in this paper absolute values are used in µmol/l, as well as in µmol/l

divided by total cholesterol in mmol/l x 100 as ratios, which according to Miettinen and Kempen better express the true NCS balance [12, 13].

The study was approved by the Na Homolce Hospital Ethical Committee as part of Project No. NA 7452-3 supported by the Internal Grant Agency of the Czech Ministry of Health (IGA MZ CR).

Statistical Analysis

Statistical analysis was performed using MedCalc Version 8.1.1. 1 (Belgium). The basic statistical parameters included mean or median calculation (95% CI), number of elements in sample, maximum and minimum values, and relevant standard deviations. The normal distribution of the set was tested using the D'Agostino-Pearson test. For graphical comparison of patient groups Box-and-Whisker plots were used with the median and 25-75 percentil.

Paired t-test was used to compare the two independent means of NCS measured values and lipid metabolites during treatment. The difference was significant with values <0.05.

3. RESULTS

The individual cohorts of the children and adolescents under survey are basically characterized in Table **1**. The average NCS results for the group of 84 healthy school-age children used as reference values are given in Table **2**. Values for the healthy subjects are dominated by Cam, followed by Sit, while Lat and Des levels are lower. Despite the slightly lower levels in the youngest children, there is not much variance in the overall values between the healthy children and healthy adults.

Table 2: Reference Values of Noncholesterol Sterols in 84 Healthy Children and 25 Healthy Adults (µmol/l)

	Lathosterol	Desmosterol	Campesterol	Sitosterol
6-8yrs				
girls	4.0+/-0.8	2.6+/-0.5	9.9+/-1.2	7.4+/-0.9
boys	3.7+/-0.9	2.2+/-0.6	9.7+/-1.4	7.5+/-1.1
8-12yrs				
girls	6.0+/-0.6	3.2+/-0.4	11.0+/-1.0	7.7+/-0.7
boys	6.0+/-0.6	3.3+/-0.4	9.7+/-0.9	7.2+/-0.7
12-15yrs				
girls	7.2+/-0.5	3.8+/-0.3	10.5+/-0.7	7.0+/-0.5
boys	6.1+/-0.5	3.5+/-0.3	9.6+/-0.8	7.0+/-0.6
15-18yrs				
girls	5.7+/-0.8	3.1+/-0.5	10.1+/-1.2	5.8+/-0.9
boys	6.3+/-1.3	3.3+/-0.8	16.0+/-2.0	7.4+/-1.5
adults 18-65yrs				
women	6.4+/-0.7	4.2+/-0.4	10.7+/-1.0	8.7+/-0.8
men	7.4+/-1.1	3.9+/-0.6	9.1+/-1.6	7.7+/-1.2

Table 3: Average Plasmatic Levels of NCS in Healthy Children Compared with LCHD Treated Patiens Suffering from HFH

		Lat	Des	Cam	Sit
	Number	µmol/l	µmol/l	µmol/l	µmol/l
Healthy children					
girls	52	4.8+/-0.4	2.8+/-0.2	9.3+/-0.7	6.2+/-0.5
boys	32	4.6+/-0.5	2.4+/-0.3	9.4+/-0.9	6.4+/-0.6
Children on lowcholesterol diet (300 mg/d)					
girls	25	11.1+/-1.2	5.3+/-0.7	11.7+/-1.9	7.3+/-1.4
boys	23	7.7+/-1	4.5+/-0.6	11.6+/-1.7	9.2+/-1.2
t-test		p<0.0001	p<0.0001	p<0.0001	p<0.0001

Children with HFH who have been on longitudinal LCHD, with which they often arrive at the outpatient lipid clinic, have higher levels of Lat and Des; Cam and Sit are also increased because the mothers usually enrich the LCHD with plant sterol margarines (Table **3**, Figure **2a**, **2b**).

In the group of children with documented LDL-R mutations, the increase in Lat and Des is greater than in the group of children with hypercholesterolemia where LDL-receptor deficiency has not been confirmed. Statins administrated in effective doses reduce TCh as well as Lat and Des levels (Figure **3**). Alterations of both in absolute values correlate with the TCh lowering response ($p<0.001$). It is only when statin doses are too low that the Lat level persists or falls slowly

Doses of statin can be individually set for each patient according to the intensity of its Lat levels decrease. Compensatory increase of Cam and Sit has

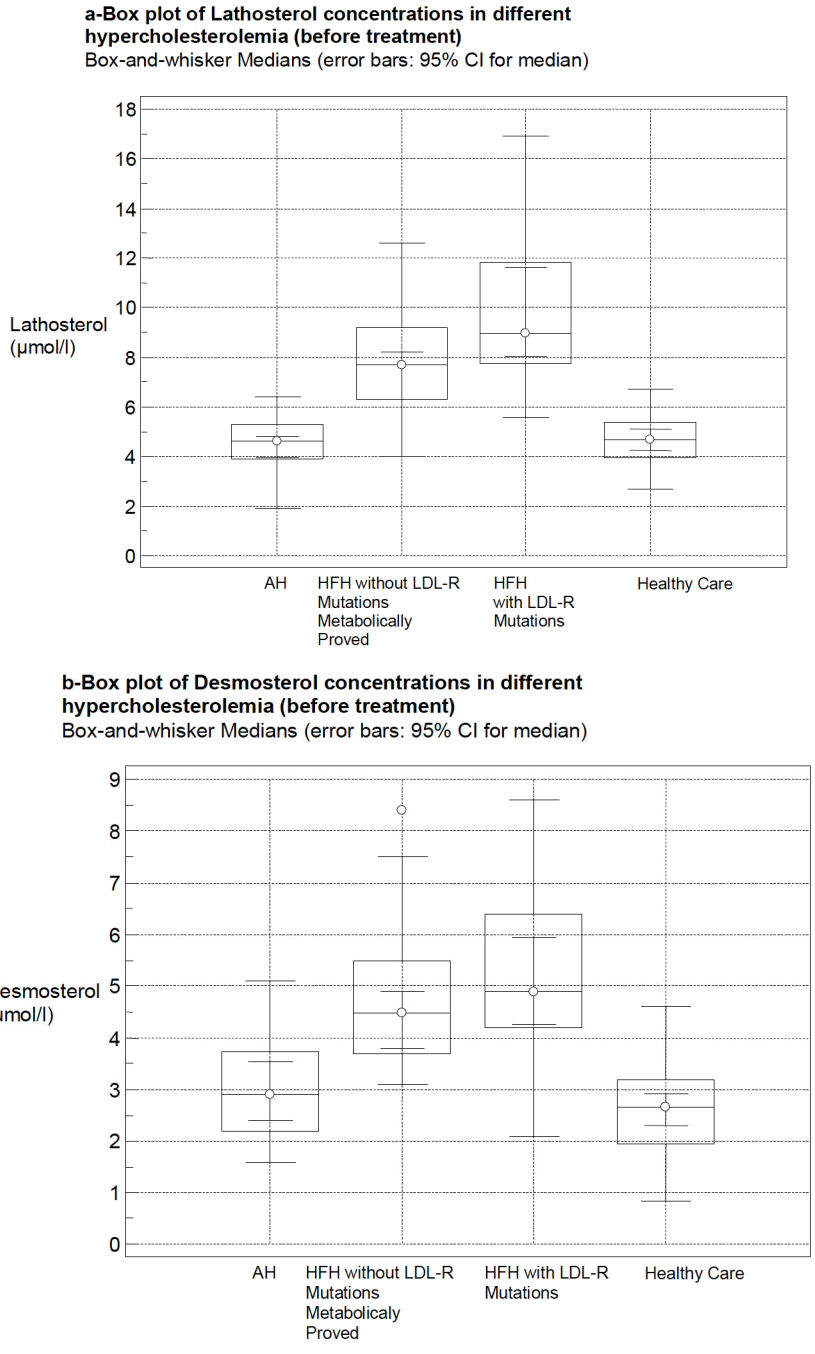


Figure 2: a) Box plot of lathosterol concentrations in different hypercholesterolemias (before treatment).

b) Box plot of desmosterol concentrations in different hypercholesterolemias (before treatment).

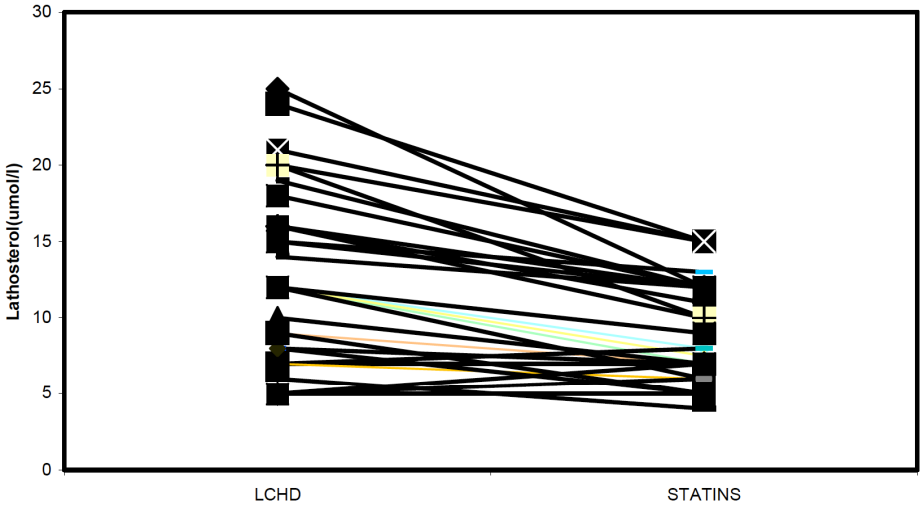
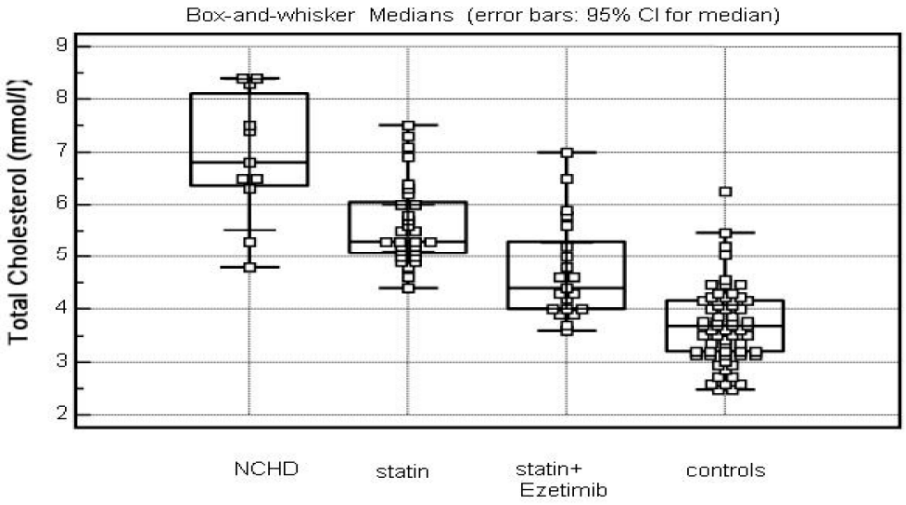


Figure 3: Lathosterol decrease after interruption of LCHD and introduction of statin therapy in patients with HFH.



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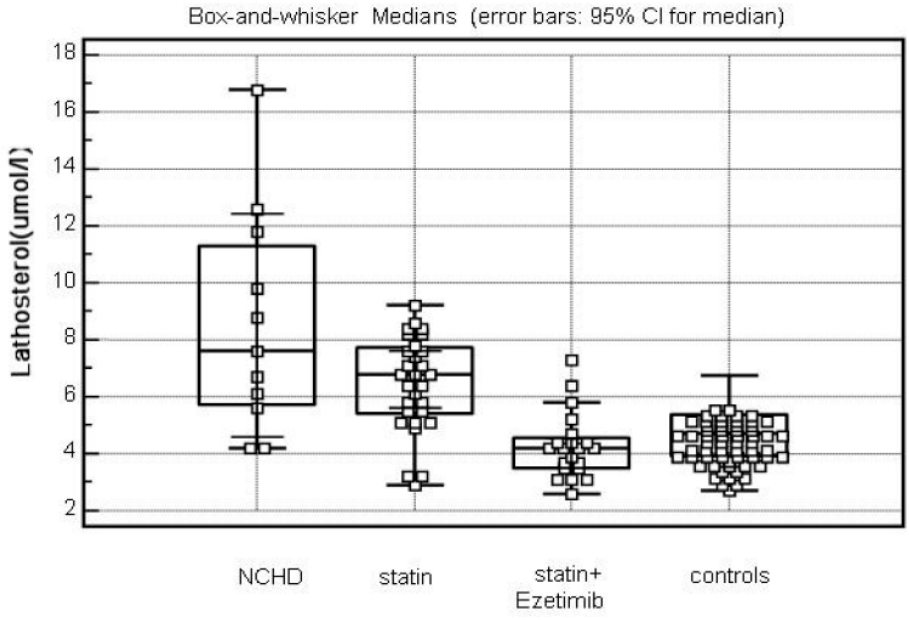

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(Figure 4). Continued.

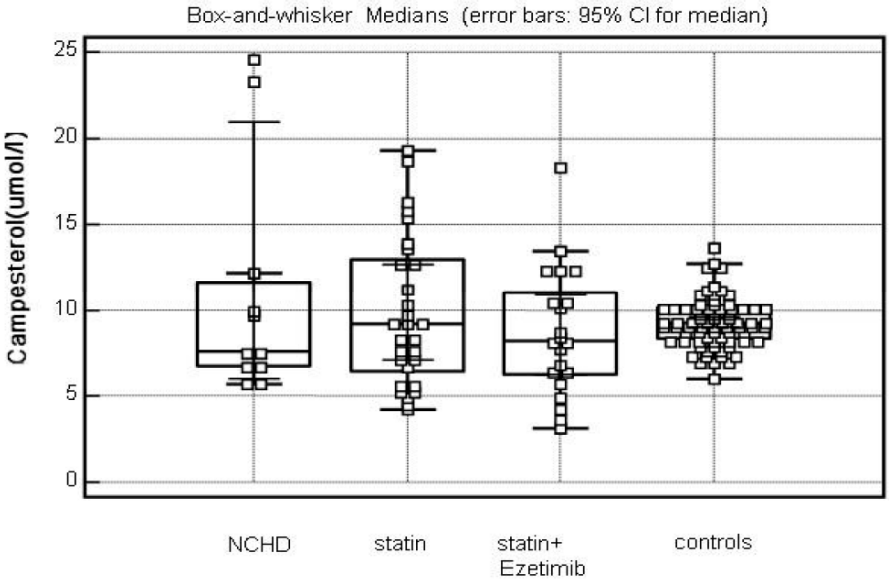

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Figure 4: a) Box plot of cholesterol concentrations in hypercholesterolemias according to the type od dietary or medical treatment.
b) Box plot of lathosterol concentrations in hypercholesterolemias according to the type of dietary or medical treatment.
c) Box plot of campesterol concentrations in hypercholesterolemias according to the type of dietary or medical treatment.

only been observed in few patiens. Combined treatment of statin with ezetimibe has to date produced the most effective decrease in both Lat and Des along with decreased TCh, while the Cam and Sit are also reduced (Figure **4a**, **4b**, **4c**).

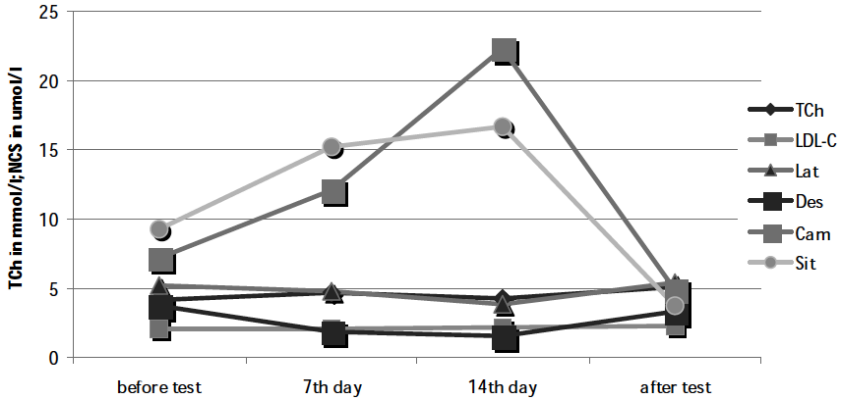

Figure 5: Loading test with phytosterol enriched margarine in a stabilized HFH patient.

Table 4: Noncholesterol Sterols in (a) 37 Children with AH and (b) 38 Children with HFH

NCS	a) Median (+/-SD)	b) Median (+/-SD)	(Significance (p<)
Lat (µmol/l)	4.6 +/- 0.93	8.95 +/- 2.7	0.0001
Lat/TChx100	92.0 +/- 18.6	129 +/- 63.2	0.0011
Cam (µmol/l)	8.6 +/- 2.5	7.7 +/- 5.1	0.3786
Cam/TChx100	168 +/- 47.5	117 +/- 103	0.0211
Des (µmol/l)	2.9 +/- 0.89	4.9 +/- 1.69	0.0011
Des/TChx100	92+/-18	59+/-22	0.1469
Sit (µmol/l)	6.3 +/- 2.4	6.0 +/- 3.6	0.6898
Sit/TChx100	104+/-49	74+/-45	0.025

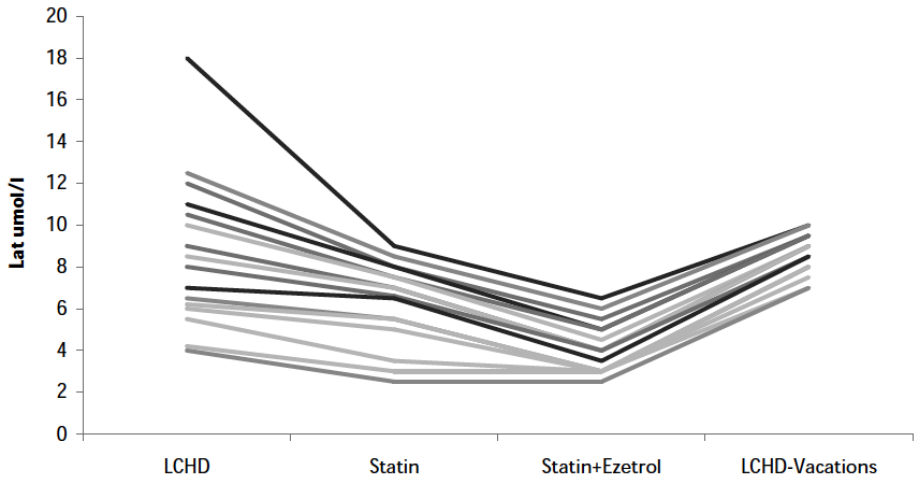

Figure 6: Monitoring of lathosterol levels in children with HFH on LCHD and differentiated drug treatment when carefully monitored also during vacations.

Ingesting plant sterol-enriched margarines such as Flora Pro Active (Unilever) 20g/d raises Cam and Sit levels and the intensity of dietary supplementation can be controlled in this way (Figure **5**).

Children with AH do not have elevated levels of Lat, Des compared to HFH (Table **4**, Figure **2a**, **2b**).

In 30 child patients we could observe the "typical changes" in plasmatic Lat levels where the LCHD and targeted drug therapy were applied as shown in the graph in Figure **6**.

Our explanation is that during vacations the children are often without proper parental control for drug treatment, and thus usually only the LCHD is observed, which results in immediate Lat level increase.

Example of typical curves for Lat and other NCS during dietary and medical treatment in a 12-year homozygous female patient with FDB are shown in Figure **7**.

4. DISCUSSION

Every doctor working in a lipid clinic certainly has experience with the failure of LCHD in the treatment of HFH even when the parents comply with this treatment. We have all also encountered strict mothers or grandmothers who apply even stricter LCHD (200mg/d) than necesssary (leading more often than not to protein deficiency) trying to protect their children from the risk of the vascular failure they have experienced among their closest relatives, and whose attempts result in reduced growth as indicated by the children's growth

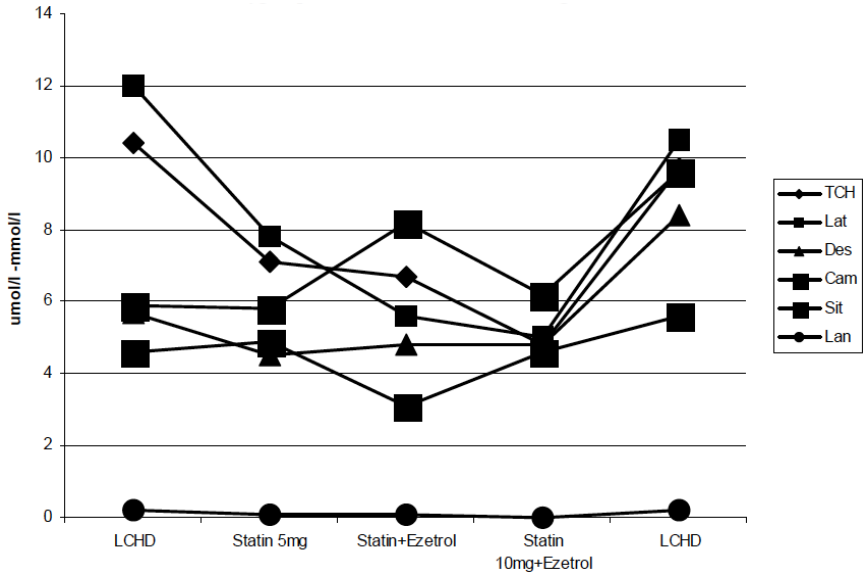

Figure 7: Monitoring of NCS plasmatic levels in a homozygous patient with FDB mutation during dietary and drug treatment.

curves. On the other hand, we see gullible mothers, and especially grandmothers, accompanying typically "overfed children" with TCh and TAG levels of 8-12 mmol/l, who only follow television advertising or clamorous leaflets offering "guaranteed" dietary products from domestic and foreign companies. We have therefore enlarged the range of markers for the differential diagnosis of hypercholesterolemias in order to simplify the system as much as possible and avoid harming the children.

A lot has been publicized about metabolism, physiological and pathobiochemical findings of NCS, esp. in connection with CVD, but little research is still available on the practical experience with its daily use [21, 23, 25]. In our decision-making concerning treatment in our clinic we have been so far most helped by Lat levels. The compensatory increase of Cam and Sit in statin therapy postulated by Miettinen, Descamps, Sudhop, Santosa, Hedman and al. [17, 18, 21, 22, 33, 34] have been observed only in few patients because the doses of statin used in combined therapy with ezetimibe in our children were mostly below 20 mg/d. However, some children cannot be clearly classified as belonging to any of the above mentioned groups based upon their first metabolic evaluation, and they maintain enormously high Lat values or high Cam values during various types of treatment. These are probably the types of patients described a long time ago by Katan *et al*. as "hyperresponders or hyporesponders" [35].

However, we do agree with the recommendation of Miettinen *et al*. that effective statin therapy is mainly observed in the "synthetizer" type patients with high levels of Lat and Des, whereas ezetimibe treatment is more effective in the "absorber" type patients with high Cam and Sit. [17-19, 21, 23]. The combination of ezetimibe and statin is the most effective and least intrusive form of medical treatment, which fully corresponds with Sudhop's observations [36]. LCHD continues to be reserved primarily for AH, as well as for patients with mixed dyslipidemia, where no molecular genetic examination is yet available or the lipid laboratory spectrum does not allow for successful differentiation.

The possible correlation between low cholesterol synthesis of Lat and high absorption of Cam as seen by Weingartner and other authors in patiens proving the high risk of CVD could not be assessed in our children patiens with HFH [17, 37, 38, 39].

It should be noted that phytosterols are also atherogenic substances and their increased intake is just as harmful to our health as high levels of cholesterol [40, 41].

Given the existence of around 1,000 known mutations of LDL-Rs with different functional capacities for cholesterol internalization (from 5 to 70%) and also given the proven influence of other risk factors, such as high Lp (a) >1000 mg/l, or adverse polymorphism of apo E 3/4 and 4/4, it is not at all surprising to find such a wide range of hypercholesterolemic patients with diverse reactions to any treatment. The sample size of patients appearing too small to give such cathegorical conlusions, however, it will be necessary in the future to increase the number of patients, complete the methodology, compare practical experience, standardize the testing and verify the observations of different authors so that the discovered value of NCS can really be of practical daily use to clinic doctors, as recommended by MacKay , Thompson *et al*. [39, 42, 43].

5. CONCLUSION

Evaluation of NCS in the lipid outpatient clinic is of practical use:

- in the differentiation of familial heterozygous hypercholesterolemia and alimentary hypercholesterolemia;
- in determining the size of therapeutic statin doses;
- in monitoring effective medication (monotherapy or combined therapy);
- for checking whether the patient is actually taking the recommended medication and for verification of the actual intake of food enriched with plant sterol esters.

CONFLICT OF INTERESTS

All te authors declare that there is no conflict of interest that could be perceveid as prejudicing the impartiality of the research reported.

ACKNOWLEDGEMENTS

Dr. Hyanek reports receiving research support from Thermo Fisher Scientific s.r.o., Prague.

ABBREVIATIONS

ApoB	=	apolipoprotein B
AH	=	alimentary hypercholesterolemia
Cam	=	campesterol
CVD	=	cardiovascular disorders
Des	=	desmosterol
FDB	=	familial ligand- defective apoB 100
HFH	=	heterozygous familial hypercholesterolemia
GC/MS	=	gas chromatography/mass spectrometry
IMT	=	intima media thickness
Lat	=	lathosterol
LCHD	=	low-cholesterol diet
LDL-Ch	=	low density lipoprotein cholesterol
Lp (a)	=	lipoprotein (a)
NCEP	=	National Cholesterol Education Program (USA)
NCS	=	non-cholesterol sterols
LDL-R	=	LDL-cholesterol receptor
Sit	=	sitosterol
TAG	=	triacylglycerols
TCh	=	total cholesterol

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Received on 31-01-2014 Accepted on 05-03-2014 Published on 31-03-2014

DOI: http://dx.doi.org/10.6000/1929-5634.2014.03.01.1