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Regular paper

Obesity, lipid profiles and oxidative stress in children after liver transplantation

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Purpose. In adult liver transplant recipients, coronary artery disease and congestive heart failure are significant cause of morbidity and mortality. This may be attributed to the long-term immunosuppressive treatment, mostly with calcineurin inhibitors and steroids, which in long-term may be associated with hyperlipidemia, oxidative stress and cardiovascular complications. Since such data for children is sparse, the aim of this study was to assess the lipid and oxidative stress markers after pediatric liver transplantation (LTx). Method. We performed prospective analysis of 74 children, at the median age of 7.9 (2.8–11.6) years, 3.2 (1.2–4.3) years after LTx. We assessed the BMI Z-scores, cholesterol fractions (LDLc, HDLc, VLDLc), triglicerides, apolipoproteins (ApoAI, ApoB, ApoE), LCAT, insulin resistance by HOMA-IR and markers of oxidative stress and atherosclerosis: glutathione (GSH), glutathione peroxidase (GPx), asymmetrical dimethyl arginine (ADMA) and oxidized low-density lipoprotein (oxyLDL). At baseline, the results were compared with a healthy age-and-sex matched control group. After 3.1±0.3 year follow-up we repeated all investigations and compared them with the baseline results. RESULTS. At the baseline, we investigated 74 patients 3.2 (1.2–4.3) years after LTx, at the median age of 7.9 (2.8–11.6) years. The prevalence of overweight or obesity (BMI >85th percentile) was 23% and was more common in girls (24% *vs* 20%). Fourteen patients had TCH >200 mg%, 9 patients had LDLc >130 mg% and TG were at normal levels in all patients. Compared to the controls, there were no significant differences in lipid profiles but we found decreased GSH (p<0.001) and GPx (p<0.001) which play role as an antioxidant defense. OS markers were higher in the study group: ADMA (p<0.001), and oxyLDL (p<0.0001). Insulin resistance by HOMA-IR was increased in the study group (p=0.0002) but fasting glucose remained within normal ranges in all patients. After 3.1-year follow-up, the BMI >95th and >85Th percentile was present in 8% and 14% respectively. ADMA and oxyLDL decreased, whilst GSH and GPx increased when compared to the baseline. There was also significant decrease in apoB and Lp(a). Conclusion. Children after LTx had normal lipid profiles when compared to controls, however there is a tendency for hypercholesterolemia and obesity, which may play a role in cardiovascular complications in the future. Some markers of oxidative stress were increased after LTx, however further investigations are required to establish its clinical significance.

Key words: reactive oxygen species, apolipoprotein, atherosclerosis, cholesterol

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Abbreviations: ADMA, asymmetrical dimethyl arginine; ApoAI, ApoB, ApoE, apolipoproteins AI, B,E; TG, triglyceride; HDL, cholesterol high-density lipoprotein; GSH, glutathione; GPx, glutathione peroxidase; LCAT, lecithin: cholesterol acyltransferase; LDLc, low-density lipoprotein; LTx, liver transplantion; oxyLDL, oxidized low-density lipoprotein; PTMS, post-transplant metabolic syndrome; TC, total cholesterol; VLDLc, cholesterol very low-density lipoprotein

BACKGROUND

Advances in medical management and surgical techniques resulted in ongoing improvement in the outcomes of pediatric liver transplantation (LTx) and currently we may expect 5-year survivals in over 85% of patients (Buculvas *et al.,* 2009).

Along with the overall success, we've observed the shift from graft related causes of morbidity toward non-immune complications of immunosuppressive drugs. (Lucey MR *et al.,* 2005).

Diabetes, arterial hypertension and dyslipidemia contribute to the post-transplant metabolic syndrome (PTMS), atherosclerosis and cardiovascular events, which are the leading cause of mortality in adults (Neal *et al.,* 2004; Satapathy *et al.,* 2011).

Dyslipidemia and reactive oxygen species are highly involved in the pathogenesis of atherosclerosis. Poly-unsaturated fatty acids occur as a major part of low density lipoproteins (LDL) in the blood, and oxidation of these lipid components in LDL plays a vital role in atherosclerosis, which progresses slowly during a person's lifetime and typically begins before adulthood (Singh *et al.,* 2005). Long-lasting subclinical development of coronary artery disease requires reliable methods of early selection of the risk groups. A classic lipid profile is currently supplemented by apolipoproteins and oxidative stress markers, like oxidized LDL cholesterol. The problem of PTMS and CV complications is recognized in pediatric transplant population (Nobili *et al.,* 2013) but current evidence is limited and the majority of come from adult centers.

We have efficient ways of treatment, including pharmacological therapies and lifestyle modifications, therefore early detection of subclinical changes is of major importance. In this study we raised the question whether children after LTx are at higher risk of lipid disturbances and oxidative stress.

METHODS

Study group. After approval by Institutional Ethical Board we performed prospective observational study of 74 children (54 females) at median age of 7.9 (2.8–11.6) who underwent LTx between 1996 and 2004. The indication for transplantation was biliary atresia (52 patients, 70%), acute liver failure (7 patients, 9.4%), progressive familial intrahepatic cholestasis – PFIC (5 patients, 6.7%) and Alagille syndrome (4 patients, 5.4%), autoimmune hepatitis (3 patients, 4.0%), hepatocellular carcinoma – HCC (2 patients, 2.7%) and oxalosis (1 patient, 1.3%). Living donor transplantation was performed in 52 (70%) and deceased donor transplantation in 22 (29%) of patients. In all patients, the graft function remained stable during the study period and at least 6 months before enrolment, with normal liver function tests and no significant graft-related issues. There were also no changes in immunosuppressive regiments for at least 6 months before enrolment. All patients received calcineurin inhibitors, tacrolimus (n=65) or cyclosporine (n=9) in combination with mycophenolate mofetil (n=23) and/or steroids (n=5).

At baseline, the results were compared with healthy age-matched control group. After 3.1±0.3 year follow-up, we repeated investigations in the study group and compared them with the baseline results. Patients who developed deterioration of liver functions and/or required significant change in immunosupressive regiment during follow-up were withdrawn from observations.

Control group. As a control group we used age and sex matched group of 62 children (39 females) at the mean age of 8.3±3.1 years. Healthy children were voluntarily recruited for a research project entitled "Evaluation of risk factors for atherosclerosis in patients after liver transplantation" with the registration number 1977/P01/2007/32. None of the participants was taking vitamin and/or antioxidant supplements for at least 8 weeks prior to the beginning of the study.

Laboratory assessment. We determined parameters of lipid metabolism: total cholesterol (TCH), total triglycerides (TG), high density lipoproteins (HDL), low density lipoproteins (LDL), very low density lipoproteins (VLDL) and apolipoproteins AI, B and E (ApoAI, ApoB, ApoE). HDL and LDL lipoproteins were obtained with precipitation methods and the VLDL lipoproteins were isolated using the method of density gradient ultracentrifugation, and then in each of the isolated fractions cholesterol concentration was measured. ApoA1 and ApoB were determined by polyclonal immunoturbitymetric goat antibodies. ApoE and Lpa (lipoprotein a) concentrations were determined by immunoelectrophoresis in agarose gels with specific antibodies. For determination of glutathione (GSH) and glutathione peroxidase (GPx), 0.5 ml of whole blood was sampled and centrifuged for 10 min at 3000 rpm/min to distract plasma. Then red blood cells were washed four times with 0.9% NaCl solution, vortexed after each wash for 10 min at 3000 r/min. Deionized water was added to erythrocytes up to the volume of 2.0 ml and left at 4°C for 15 min. Then, hemolyzed erythrocytes were diluted 25-fold with 0.01 M phosphate buffer, pH 7.0 to 100-fold final dilution. The activity of glutathione peroxidase (GPx) and glutathione (GSH) concentrations in the whole blood hemolysate was determined based on the method described by Paglia and Valentine. Enzyme activity was expressed in units per g of Hb (U/g Hb) and in units per liter of plasma (U/l). One unit of enzyme activity was expressed as 1 μmol oxidized within 1 min of NADPH for 1 g of Hb, or 1 liter of plasma. The reaction was carried out at a temperature of 37°C and the other parameters' optimum for the enzyme. Asymmetric dimethylarginine (ADMA) and oxidized-LDL (oxyLDL) concentrations were measured as reliable biochemical markers of atherosclerosis**.** Enzyme Immunoassay ELISA (DRG International Inc. NJ, USA) was used to determine serum ADMA and oxidized-LDL (oxyLDL) concentrations. The absorbance of tested samples was measured at a wavelength of 450 nm, with wavelength correction at 540 nm and 570 nm, using an automatic reader (Biotek ELx800) and analyzed by a computer software version 5 gene.

HOMA-IR was a measure of insulin resistance and was calculated from fasting (glucose mmol/l × insulin mU/L)/22.5. Values >3.16 are considered as insulin resistant in a pediatric population.

Lecithin-cholesterol acyltransferase (LCAT) – was measured with a colorimetric enzymatic method. 100 μl of fresh plasma was pipetted into six tubes. To each tube, 100 μl of the LCAT reagent was added, and the mixtures in three tubes were preincubated at 37°C for 40 min to inhibit the LCAT reaction, and the other three tubes were preincubated at 4°C for 40 min. This method uses stable liposomes composed of lecithin and free cholesterol as a substrate and measures LCAT activity by the decrease in the free cholesterol during a 37°C incubation. Then, 1000 μL of a cholesterol reagent were added to each tube, and the mixtures were incubated at 37°C for 10 min to allow equilibrium of the tracer cholesterol with unlabeled plasma lipoproteins. This was immediately followed by reading absorbance at 505 nm.

Statistical analysis. Statistical analysis was assessed by using GraphPad Prism 7.0 for mac (GraphPad Software, Inc CA, USA). Descriptive data are presented as means or medians as appropriate. Comparisons between groups were calculated by using Mann-Whitney *U* test or Wilcoxon test. Statistical significance was defined as p value <0.05.

RESULTS

Baseline assessment

At the baseline, we investigated 74 patients 3.2 (1.2–4.3) years after LTx, at the median age of 7.9 (2.8–11.6) years. Liver tests were normal in the study group (ALT 42±20 IU, AST 38±18 IU, GGTP 36±17 IU). The prevalence of overweight or obesity (BMI >85th percentile) was 23% and was more common in girls (24% *vs* 20%). Obesity (BMI >95th percentile) was present in 5% of patients and only in girls (Fig. 1). Fourteen patients had TCH >200 mg %, 9 patients had LDLc >130

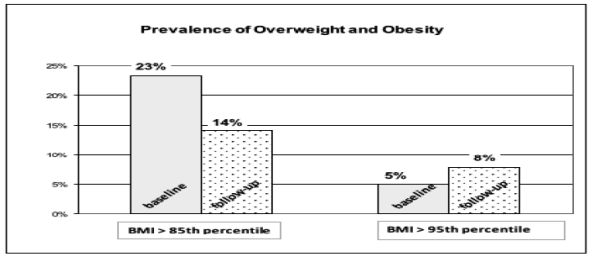

Figure 1. Prevalence of overweight and obesity

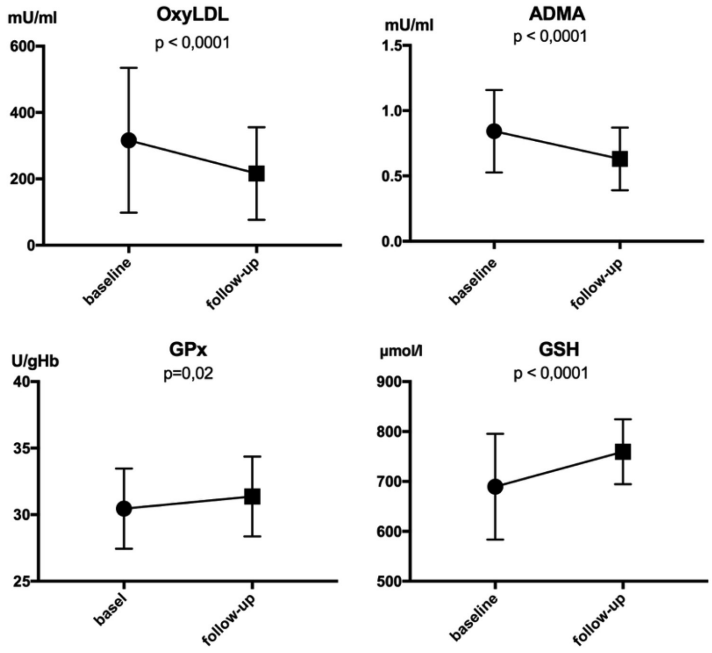

Figure 2. Correlation between oxidative stress parameters measured at baseline and after 3-year follow-up
GSH, glutathione; GPx, glutathione peroxidase; ADMA, asymmetrical dimethyl arginine; oxyLDL, oxidized low-density lipoprotein.

Table 1. Comparison of lipids and oxidative stress markers between study group at baseline and control and between baseline and follow-up

	Study group	Control	*p*	Follow-up	*p*
Age median/range	7.9 (2.8-13.4)	8.1 (3.3-11.3)	0.43	10.8 (5.3-16.3)	–
BMI	16.81±0.26	17.32±2.24	0.13	17.58±0.42	0.13
HOMA-IR	2.7±0.8	2.0±0.1	0.0002	2.6±0.8	0.74
TCH (mg/dl)	159.6±35.5	154.8±38.2	0.33	154.8±48.2	0.19
TG (mg/dl)	62.9±17.8	68.7±27.7	0.18	65.6±17.2	0.29
Lp (a) (mg/dl)	14.7±5.8	13.3±5.2	0.36	12.6±5.2	<0.0001
LCAT	133.8±42.8	128.5±23.2	0.71	140.1±47.2	0.71
apoA1 (g/l)	1.45±0.19	1.43±0.14	0.62	1.46±0.19	0.90
apoB (g/l)	0.88±0.40	0.92±0,23	0.59	0.81±0,33	0.01
apoE (mg/dl)	12.4±3.4	12.6±4.3	0.73	12.4±2.7	0.92
LDL (mg/dl)	96.2±31.7	92.2±26.1	0.35	92±17.1	0.22
VLDL (mg/dl)	12.6±5,4	12.1±3.1	0.50	13.0±3.5	0.50
HDL (mg/dl)	50.8±9.8	47.7±7.7	0.16	49.5±7,2	0.11
oxyLDL (mU/ml)	316.4±218.3	124.6±20.4	0.0004	216.2±139.6	<0.0001
ADMA (nmol/l)	0.84±0.31	0.29±0.17	<0.0001	0.63±0.24	<0.0001
GPx (U/gHb)	30.4±3.0	32.6±1.6	0.0004	31.3±3.0	0.02
GSH (mmol/ml)	689.5±66,2	782.4±17.2	0.0004	759.5±64.9	<0.0001

mg % and TG were at normal levels in all patients. There were no differences between the study group and controls (Table 1). OS markers were increased in the transplant group. There were significant differences in GSH ($p<0.001$), GPx ($p<0.001$), ADMA ($p<0.001$), and oxyLDL ($p<0.0001$). Insulin resistance by HOMA-IR was increased in the study group ($p=0.0002$) but fasting glucose remained within the normal range in all patients. Exclusion of overweight/obese patients from the study group did not affect results.

Follow-up assessment

The observation after 3.1±0.3 years was accomplished in 70 patients. BMI >95th and >85th percentile was present in 8% and 14%, respectively (Fig. 1). We found significant changes in the OS parameters. Both markers of OS had decreased: ADMA 0.84±0.31 *vs.* 0.63±0.24 μmol/l and oxyLDL: 316±218 *vs* 216±139 mU/ml, with $p<0.001$, whilst markers of defense mechanisms had increased: GSH 689±105 *vs* 779±64 μmol/l $p<0.001$, GPx 30.4±3.0 *vs* 31.3±3.0 U/gHb, with $p<0.01$ (Fig. 2). There was also significant decrease in apoB and Lp(a). Out of 14 patients with high TCH and 9 patients with high LDLc at baseline, only one had TC >200 mg % and LDL >130 mg % at the end of the follow-up (Table 1). Three patients had BMI z-score >95 percentile.

DISCUSSION

The majority of liver transplant recipients require lifelong immunosuppression, mainly based on calcineurin inhibitors (CNI), or with steroids which are associated with hypertension, hyperlipidemia, diabetes mellitus and increased risk of CV events. (D'Avola *et al.,* 2017; Llado *et al.,* 2006).

In adults, coronary artery disease and congestive heart failure are leading causes of non-graft related death (Neal DA *et al.,* 2004). Therefore, adolescents after liver transplantation make a special target group for cardiovascular risk factors screening.

In our study we were able to demonstrate that children after transplantation have increased laboratory markers of oxidative stress, including oxyLDL and ADMA which may be linked to atherosclerosis. Interestingly, all markers of oxidative stress had decreased over time. The reason for this observation is unclear, however, it may reflect some process of adaptation to long-term immunosuppression or gradual recovery from severe oxidative stress during early post-transplant period. Ischemia-reperfusion injury, frequent infections and vascular complication are regarded as possible sources of ROS. Interestingly, classic lipid profiles were not significantly disturbed in the study group which may indicate a necessity to introduce new biochemical investigations. Our patients also had higher incidence of insulin-resistance than controls but remained within normal range of values.

Approximately 5% to 8% of our patients had BMI >95th percentile which is less than reported in previous reports, where obesity was observed in 20% of transplanted children (Sundaram *et al.,* 2012).

One of the long-term consequences of OS is atherosclerosis. Atherosclerosis, the main cause of coronary artery disease (CAD), is an inflammatory disease in which immune mechanisms interact with metabolic risk factors to initiate, propagate, and activate lesions in the arterial tree (Hannson *et al.,* 2005). There is also significant role of reactive oxygen species (ROS), which are mainly produced by vascular cells, and are implicated in pathogenic mechanisms of atherosclerosis, dyslipidemia and diabetes (Singh *et al.,* 2015; Steven *et al.,* 2017).

Imbalance between free radical formation and antioxidant defense system may lead to oxidative stress and transplant recipients are considered vulnerable mostly due to immunosuppressive drugs (Steven *et al.,* 2011). Garnot and coworkers (2002) assessed oxidative status in 23 children, 1.5–12 years after liver transplantation, receiving calcineurin inhibitors (14 CsA, 9 TAC) and compared them with age-matched healthy controls. There was no correlation between CNI administration and increased oxidative damage or plasma antioxidant capacity. Christians and coworkers (2004) investigated the effect of combination of calcineuryn inhibitors (CsA or TAC) with mTOR inhibitors on energy production and ROS generation in perfused rat brain slices. Everolimus and sirolimus demonstrated different effects – everolimus ameliorated cyclosporine-induced mitochondrial dysfunction, whereas sirolimus increased CsA's toxicity by inhibition of compensatory anaerobic glycolysis. In the *in vitro* study on cultured human endothelial cells it was demonstrated that mycophenolate acid (MPA) inhibits endothelial O_2 formation by inhibition of NADPH oxidase, whereas CNI increased its activity. Other study showed that methylperednisolone and MMF induced minor changes in the endothelial function in comparison with CsA, SRL and TAC (Trapp *et al.,* 2005).

The study presented here has some limitations. First, we were not able to measure organ damage by measurement of carotid intima-media thickness and it requires further studies. For most measured parameters, normal values for age are not established as yet, and we did not observe the study group during follow-up.

In conclusion, children after LTx had normal lipid profiles when compared to controls, however, there was a tendency for hypercholesterolemia and obesity, which may play a role in cardiovascular complications in the future. Some markers of oxidative stress are increased after LTx, however further investigations, including assessment of subclinical organic damage, are required to establish its clinical significance.

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