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Effects of single nucleotide polymorphisms and haplotypes of the *SLCO1B1* gene on the pharmacokinetic profile of atorvastatin in healthy

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Received December 16, 2014, accepted January 19, 2015

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Pharmazie 70: 1–9 (2015) *doi: 10.1691/ph.2015.4899*

OATP1B1 is an influx transporter known to mediate the uptake of various endogenous compounds and xenobiotics. Several sequence variations have been discovered in the *SLCO1B1* gene encoding OATP1B1. The aim of this study was to investigate the effects of *SLCO1B1* polymorphisms on the pharmacokinetics of atorvastatin in healthy volunteers of Macedonian origin. Twenty three participants, genotyped for *SLCO1B1* c.388A > G, c.521T > C, c.571T > C, c.597C > T, c.1086C > T, c.1463G > C and c.*439T > G polymorphisms using TaqMan allelic discrimination assay, ingested a single 80 mg dose of atorvastatin. The plasma concentrations of atorvastatin were measured for 48 h using Tandem Liquid Chromatography-Mass Spectrometry, LC-MS-MS, and the peak plasma concentration (C_{max}), time to peak plasma concentration (T_{max}), elimination half-life ($t_{1/2}$), constant rate of elimination (k_{el}), mean residence time (MRT, expo), volume of distribution (Vd/kg), clearance (CL/kg), area under curve $AUC_{0\text{-}48h}$ and $AUC_{0\text{-}\infty}$ were determined. Our data confirmed that the *SLCO1B1* gene is highly polymorphic, with a frequency of the c.521T > C single-nucleotide polymorphism (SNP) being the lowest (app. 15%) and of all other SNPs alleles above 40%. Exceptions were c.1463G > C and c.1086C > T SNPs for which variant alleles were not identified. The strongest correlation was observed between the c.521T > C and c.571T > C SNPs pair. The haplotype analysis revealed 10 different haplotypes, with **1J/*1K/*1L* being the dominant, with a frequency of app. 40%. The haplotype **15/*16/*17*, containing both variant alleles of the functionally most distinguished SNPs, c.388A > G and c.521T > C, occurred with a frequency of 13%. However, **15/*16/*17* homozygotes were not identified in the study group. In this study, no significant differences in the k_{el}, $t_{1/2}$, C_{max}, T_{max}, $AUC_{0\text{-}48h}$, $AUC_{0\text{-}\infty}$, MRT expo, Vd and CL between the carriers of different c.388A > G, c.597C > T and c.*439T > G genotypes were observed. Subject with a variant allele C in the c.521T > C SNP, c.521CC genotype, had markedly higher values for C_{max} and $AUC_{0\text{-}48h}$, 140% and 67%, respectively, in comparison with the carriers of the c.521TT genotype. Also, the carriers of the variant allele C at c.571T > C SNP, c.571 CC genotype, had 55% and 43% lower mean C_{max} and $AUC_{0\text{-}48h}$ in comparison with the carrier of c.571TT. These differences lacked statistical significance due to the size of the sample. In addition, no significant differences in the pharmacokinetic parameters of atorvastatin between the **15/*16/*17* heterozygotes and **15/*16/*17* non-carriers were observed. In conclusion, this extensive analysis of the effect of *SLCO1B1* polymorphisms on the pharmacokinetic profile of atorvastatin showed that c.521T > C and c.571T > C SNPs may affect the inter-individual response to atorvastatin. Additional studies, with a large sample size, are needed to confirm this finding.

1. Introduction

As 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, statins are widely prescribed for the treatment of dyslipidemia. Their use also reduces the risk of vascular events and even the mortality rate. Therefore, statins in conjunction with lifestyle changes are proposed as a first line treatment in the primary and secondary prevention of cardiovascular disease (Expert panel on detection, evaluation, and treatment of high blood cholesterol in adults 2001).

Among statins, atorvastatin is widely used in the treatment of hypercholesterolemia to reduce cardiovascular morbidity and mortality in high risk patients (Pasanen et al. 2007). Compared to other statins, it causes greater reductions of cholesterol and triglyceride levels (Jones et al. 1998). However, despite its clinical efficacy, great variability in the clinical response exists and

the reason for this phenomenon is still not fully understood**.** There is evidence that even when the same dose is administered, the LDL cholesterol serum concentrations can vary largely, from reduction of only 6% to nearly 62% (Rodrigues et al. 2011). Apart from intrinsic factors (i.e. genetic variations), variations in the drug response was shown to be related to extrinsic factors, such as compliance, time of administration, concomitant medication, and dietary intake (Thompson et al. 2002; Magee et al. 2010).

Much attention is paid to genetic factors affecting inter-individual differences in clinical response and there is clear evidence that adverse effects such as myalgia, myositis, myopathy and even rhabdomyolysis, although rare, are often associated with increased plasma concentrations caused by a hereditary difference in Statin pharmacokinetics (Mangravite et al. 2006; Superko et al. 2012; Postmus et al. 2012; Brunham et al. 2012; Srecko et al. 2012, Zhou et al. 2013a, b; Pasanen et al. 2006b). Several studies in this area point to single-nucleotide polymorphisms (SNPs) in the gene encoding multiple organic anion transporting polypeptide 1B1 (OATP1B1), *SLCO1B1*, as responsible for the variability of atorvastatin response (Rodrigues et al. 2011; Pasanen et al. 2007; Thompson et al. 2005; Lee et al. 2010; Lau et al. 2007). OATP1B1, expressed on the sinusoidal membrane of human hepatocytes, is responsible for the transport of atorvastatin and its uptake from the sinusoidal blood into hepatocytes where atorvastatin not only manifests its pharmacodynamic effect but is metabolized as well (Rodrigues et al. 2011; Hubacek et al. 2012).

SNPs 388A > G (**1b*, rs2306283) and 521T > C (**5*, rs4149056) that encode alanine substitution of valine at amino acid 174 (p.Val174Ala), and amino acid change at position 130 (p.Asn130Asp), respectively, are considered as the most prevalent *SLCO1B1* variants relevant for the variability of drug response. The c.388A > G (p.Asn130Asp) variant has been associated with increased OATP1B1 transport activity of several substrate drugs *in vitro* (Jiake et al. 2011; Michalski et al. 2002; Kameyama et al. 2005) unlike the c.521T > C variant for which reduced OATP1B1 transport activity *in vitro* and increased plasma concentrations of several substrate drugs in the human body were observed (Kalliokoski et al. 2010; Niemi 2007; Pasanen et al. 2007).

Recent data indicate that the two variants 388A > G and 521T > C are in linkage disequilibrium (LD) and exist in different *SLCO1B1* haplotypes; AT (**1A*, reference haplotype), GT (**1B*), AC (**5*) and GC (**15*), for c.388A > G and c521T > C, respectively (Pasanen et al. 2006). Haplotypes **15* and **5* have been consistently associated with a decreased transport activity, while controversial results have been reported for **1B* haplotype (Kameyama et al. 2005). In a study of Mwinyi et al. (2004), *SLCO1B1*1B* c.388G/c.521T carriers had a higher transport activity than the carriers of wild-type *SLCO1B1*1A* c.388A/c.521T. It was also demonstrated that the *SLCO1B1*17* haplotype (g.-11187G > A, c.388G > A and c.521T > C) was associated with increased plasma concentrations of pravastatin in humans (Niemi et al. 2004), while enhanced response to fluvastatin was confirmed for **14* haplotype (c.388G-c.463A-c.521T) (Couvert et al. 2008). Similarly, in the study of Pasanen et al. (2007), the $AUC_{0\text{-}48h}$ of atorvastatin was higher in the c.521TC heterozygotes with **17* and **16* haplotypes than in the carriers of **15* haplotype. Also, Lee et al. (2010) reported significantly higher $AUC_{0\text{-}\infty}$ for atorvastatin in **15/*15* carriers than in carriers with **1A/*15*, **1B/*15*, **1A/*1A*, **1A/*1B* and **1B/*1B* haplotypes. It was also suggested that the *SLCO1B1*15* allele may be associated with individual differences in the $AUC_{0\text{-}\infty}$ of atorvastatin.

In our previous work (Daka et al., *submitted for publication*) we characterized, for the first time, the diversity of the *SLCO1B1* gene in 266 subjects of Macedonian ethnicity and within the study, the distribution of *SCLO1B1* alleles was determined at 7 variant sites (c.388A > G, c.521T > C, c.571T > C, c.597C > T, c.1086C > T, c.1463G > C and c.*439T > G). Our data confirmed that *SLCO1B1* is highly polymorphic in this ethnic group and that several variants appear at a high frequency. In addition, 20 different haplotypes were detected, of which the haplotype **1J/*1K/*1L* containing the variant allele C at position c.571 and referent alleles in other SNPs was the most frequent. Knowing that *SCLO1B1* polymorphism may markedly affect the pharmacokinetics of several statins, including atorvastatin, the aim of this study was to investigate the effects of variant *SLCO1B1* genotypes and haplotypes on the pharmacokinetics of atorvastatin in human subjects of Macedonian ethnicity. Together with the effects of the relevant c.388A > G and c.521T > C SNPs, the effects of three other SNPs (c.571T > C, c.597C > T and c.*439T > G), present in the studied population at high frequencies, were also evaluated. At the best of our knowledge, no data for the effects of these three SNPs on atorvastatin pharmacokinetics are available, although their variant alleles can be found in many haplotypes.

2. Investigations and results

2.1. Genotype and haplotype analysis

Data for the distribution of genotypes and allele frequencies of *SLCO1B1* polymorphisms among 23 healthy volunteers are presented in Table 1.

The observed genotypes and allelic frequencies of *SLCO1B1* polymorphisms did not show significant deviations from the Hardy-Weinberg equilibrium ($p > 0.05$). All SNPs, except c.1463G > C and c.1086C > T occurred at an allele frequency higher than 15%. Variant alleles of *SLCOB1* c.1463G > C and c.1086C > T polymorphisms were not identified in the study group. The frequency of the c.521T > C variant allele SNP was the lowest (15.2%), while the frequencies of all other SNPs alleles were above 39%, with a frequency of c.571T > C variant allele being the highest (app.70%).

LD analysis of the single SNPs, quantified by r^2 and D' values, showed that the most strongly correlated ($r^2 \geq 0.33$) SNPs pair was c.597C > T/c.388A > G ($r^2 = 0.571$, $D' = 0.792$). The c.521T > C showed the strongest correlation with c.571T > C ($r^2 = 0.410$, $D' = 1.000$) followed by c.597C > T ($r^2 = 0.306$, $D' = 1.000$). Another SNPs pair showing a significant association was c.388A > G/c.*439T > G ($r^2 = 0.309$, $D' = 0.636$). The correlation of the most active SNPs pair, c.388A > G/c.521T > C, was relatively weaker compared to the other SNPs pairs, with $r^2 = 0.137$ and $D' = 0.700$.

The haplotype analysis of the study group revealed ten different haplotypes (Table 2. Two haplotypes were designated as new. Seven haplotypes occurred at a frequency greater than 3%. The most common haplotype in the study group **1J/*1K/*1L* had a frequency of 40.5%. The variant allele G at positions c.388 and c.*439 existed in five haplotypes, with a frequency between 2% and 13%, while the variant allele C at position c.571 existed in four haplotypes that occurred with frequencies between 5.5% and 40.5%. The frequencies of the haplotypes that included the variant allele T at c.597, six haplotypes, ranged from 2% to 13% and the same was observed for the frequencies of the two haplotypes that contained the variant allele C at c.521. Both variant alleles G and C of the functionally most distinguished SNPs, c.388A > G and c.521T > C, respectively, were present in one haplotype, **15/*16/*17*, with a frequency of 13% (Table 2).

Table 1: Distribution of genotype and allele frequencies of SLCO1B1 polymorphisms

	Observed frequency n (%)	Expected frequency by HWE (%)	&p value
Position‡/ dbSNP ID			
c.388A > G/rs2306283			
AA	8 (34.8)	37.0	
AG	12 (52.2)	47.6	0.647874
GG	3 (13.0)	15.3	
G allele	18 (39.1)		
A Allele	28 (60.9)		
c.521T > C rs4149056			
TT	17 (73.9)	2.3	
CT	5 (21.7)	25.8	0.450052
CC	1 (4.3)	71.9	
C allele	7 (15.2)		
T allele	39 (84.8)		
c.571T > C/rs4149057			
CC	10 (43.5)	48.4	
CT	12 (52.2)	42.3	0. 265625
TT	1 (4.3)	9.3	
C allele	32 (69.6)		
T allele	14 (30.4)		
c.597C > T/rs229107			
CC	9 (39.1)	39.7	
CT	11 (47.8)	46.6	0.899371
TT	3 (13.0)	13.7	
C allele	29 (63.0)		
T allele	17 (37.0)		
c.1086C > T/rs57040246			
CC	23 (100)		
CT	0		
TT	0		
T allele	0		
c.1463G > C/rs59502379			
CC	0		
CG	0		
GG	23 (100)		
C allele	0		
c.*439T > G/rs4149087			
GG	5 (21.7)	20.8	
GT	11(47.8)	49.6	0.862216
TT	7(30.4)	29.5	
G allele	21(45.7)		
T allele	25(54.3)		

‡The positions of SNPs are given in relation to the NCBI reference sequences NM_006446.2 (cDNA; c.) with the first nucleotide of the ATG first codon set to 1 and the nucleotide 5' of ATG set to -1. The position of c.*439 is given with the first nucleotide 3' of the stop codon (TAA) set to *1.

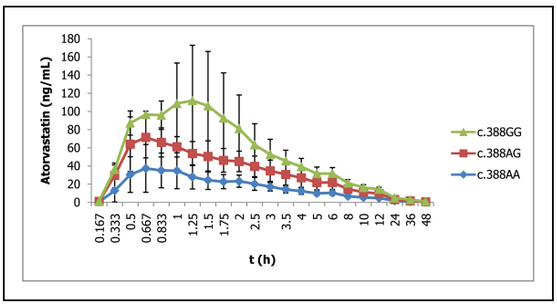

Fig. 1: Mean (± SD) plasma concentrations of atorvastatin after single 80 mg oral dose of atorvastatin in 23 healthy volunteers in relation to the SLCO1B1 c.388A > G SNP: (♦) c.388AA subjects with genotype (n = 8); (■) c.388AG genotype (n = 12); and (▲) c.388GG genotype (n = 3).

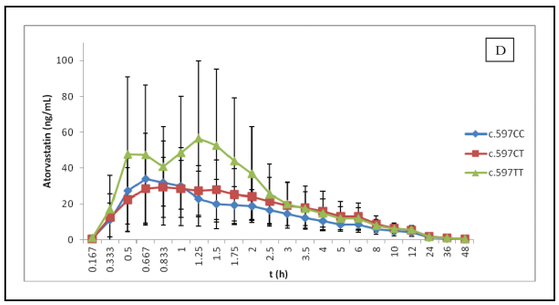

Fig. 4: Mean (± SD) plasma concentrations of atorvastatin after single 80 mg oral dose of atorvastatin in 23 healthy volunteers in relation to the SLCO1B1 c.597C > T SNP: (♦) c.597CC subjects with genotype (n = 9); (■) c.597CT genotype (n = 11); and (▲) c.597TT genotype (n = 3).

2.2. Effects of SLCO1B1 SNPs on the pharmacokinetics of atorvastatin

The association between the *SLCO1B1* genotypes and the pharmacokinetic profile of atorvastatin is presented in Figs. 1–5 and Table 3. Considering the kel, $t_{1/2}$, C_{max}, T_{max}, $AUC_{0\text{-}48h}$, $AUC_{0\text{-}\infty}$, MRT $_{expo}$, Vd and CL of atorvastatin, statistically no significant differences in these parameters were observed between the carriers of different c.388A > G, c.597C > T and c.*439T > G genotypes. However, noticeable differences in C_{max}, T_{max}, $AUC_{0\text{-}48h}$, $AUC_{0\text{-}\infty}$, Vd and CL of atorvastatin between the carriers of c.521CC and c.521TT genotypes and between the c.571TT and c.571CC genotypes were observed. The effects of c.521CC and c.571TT genotypes on these pharmacokinetic parameters could not be effectively analyzed due to the low number of subjects homozygous for the c.521C and c.571T alleles.

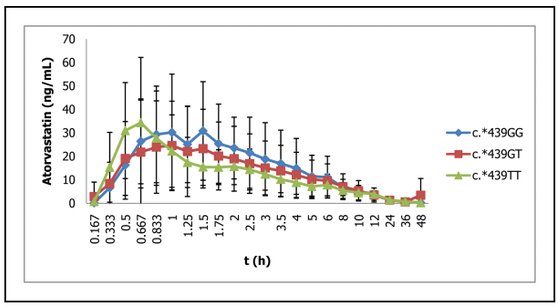

Fig. 5: Mean (± SD) plasma concentrations of atorvastatin after single 80 mg oral dose of atorvastatin in 23 healthy volunteers in relation to the SLCO1B1 c.*439G > T SNP: (♦) subjects with c.*439GG genotype (n = 5); (■) c.*439GT genotype (n = 11); and (▲) c.*439TT genotype (n = 7).

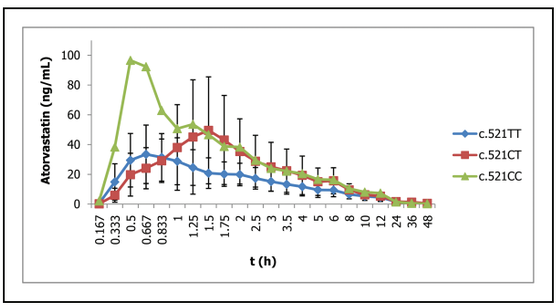

Fig. 2: Mean (± SD) plasma concentrations of atorvastatin after single 80 mg oral dose of atorvastatin in 23 healthy volunteers in relation to the SLCO1B1 c.521T > C SNP: (♦) c.521CC subjects with genotype (n = 1); (■) c.521CT genotype (n = 5); and (▲) c.521TT genotype (n = 17).

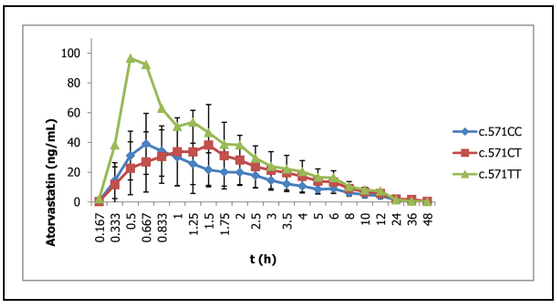

Fig. 3: Mean (± SD) plasma concentrations of atorvastatin after single 80 mg oral dose of atorvastatin in 23 healthy volunteers in relation to the SLCO1B1 c.571C > T SNP: (♦) c.571CC subjects with genotype (n = 10); (■) c.571CT genotype (n = 12); and (▲) c.571TT genotype (n = 1).

In the subjects with the *SLCO1B1* c.388GG genotype (n = 3), the mean C_{max} was app. 45% and 59% higher than the mean C_{max} in the subjects with the c.388AA (n = 8) and c.388AG (n = 12) genotypes, p = 0.702 (Table 3). Similarly, a slightly higher mean value for the $AUC_{0\text{-}48h}$ in the subjects with the c.388GG genotype was detected, 15% and 13% higher in comparison with the $AUC_{0\text{-}48h}$ in the carriers of c.388AA and c.388AG genotype, respectively, p = 0.977.

Considering the pharmacokinetic parameters of atorvastatin in carriers of different c.521T > C genotypes, the mean C_{max} and $AUC_{0\text{-}48h}$ were app. 42% higher in the carriers of the c.521CT genotype (n = 5) compared to the carriers of the c.521TT genotype (n = 17), p = 0.157 and p = 0.112, respectively. C_{max} and $AUC_{0\text{-}48h}$ of atorvastatin in c.521CC genotype were app. 140% and 67%, respectively, higher than the mean C_{max} and $AUC_{0\text{-}48h}$ in the carriers of the c.521TT genotype and app. 69% and 18% higher than the mean values of the corresponding parameters in the subjects with the c.521CT genotype, p = 0.057 and 0.138, respectively. Despite the fact that a subject with c.521CC genotype had markedly higher values for C_{max} and $AUC_{0\text{-}48h}$ in comparison with the subjects carrying c.521CT and c.521TT genotypes, these differences lack statistical significance due to the size of the sample.

Similar findings were observed when comparing the pharmacokinetic parameters of atorvastatin between the carriers of different c.571T > C genotypes. Namely, the C_{max} in the c.571TT genotype was app. 120% higher than the mean C_{max} in the c.571CC and app. 114% higher than the mean C_{max} in c.571CT carriers, p = 0.164. In addition, the $AUC_{0\text{-}48h}$ of a c.571TT carrier was app. 75% and 27% higher than the mean $AUC_{0\text{-}48h}$ of the c.571CC and c.571CT carriers, respectively, p = 0.149.

The mean C_{max} in the carriers of the c.597TT genotype (n = 3) was app. 88% and 74% higher than the one in the c.597CT (n = 11) and c.597CC carriers (n = 9), respectively, p = 0.134.

Table 2: Alignment and frequencies of SLCO1B1 haplotypes in the study group

SLCO1B1 haplotype	c.388A>G	c.521T>C	c.571T>C	c.597C>T	c.1086 C>T	c.1463 G>C	c.*439 T>G	Haplotypes found		
Reference	A	T	T	C	C	G	T	N	%	95%Cl
**1J/*1K/*1L*[a]			C					18	40.5	0.425–2.355
**15*[a]*/*16*[b]*/*17*[c]	G	C		T			G	6	13.0	0.295–3.390
**1J/*1K/*1L*[a]			C				G	6	12.6	0.290–3.451
**18*[d]/New[e]	G		C	T			G	5	10.9	0.268–3.733
**1B/*1F*[a]/New[h]	G						G	3	6.5	0.190–5.266
**18*[g]/New[f]	G		C	T				2	5.5	0.167–5.986
**1A*[a]/New[h]*/*1E*[i]								1	3.4	0.105–9.500
**20*[j]*/*21*[k]/New[h]	G			T				1	2.7	
New		C		T				1	2.2	
New				T			G	1	2.2	

The gray boxes denote reference sequence

a The name includes the presented sequence of the SNPs investigated in the actual study and referent alleles of the additional SNPs investigated in the study of Pasanen et al. (2006) (at positions g.-11187, g.-11110, g.-10499, c.411, c.463 and c.1929)

[b,c,d,g,i,j,k] The haplotype name includes a sequence of the SNPs investigated in the actual study and referent alleles in other SNPs investigated in the cited study (Pasanen et al. 2006), except at the following positions b: g.-10499; c: g.-11187; d: c.411 and c.463; g: c.411 and c.463; i: g.-11110; j: c.1929 and k; g.-11187 and c.1929, where variant alleles exist.

[e,h,f] The haplotype is assigned as new by Pasanen et al. (2006), having the same sequence of the SNPs investigated in the actual study and referent alleles at other SNPs investigated in the cited study (Pasanen et al. 2006), except at the following positions e: g.-11110, c.411 and c.463; h: g.-11187 and f. c.411, c.463 and c.1929, where variant alleles exist.

Insignificantly higher mean value for the mean $AUC_{0\text{-}48h}$ in the carriers of the c.597TT genotype was observed in comparison with the mean values of the corresponding parameter in the c.597CT and c.597CC carriers (15% and 31%, respectively, p = 0.611) (Table 3).

As mentioned previously, there was no statistically significant association between the pharmacokinetic parameters of atorvastatin and c.*439T>G SNP. The difference in C_{max} between the carriers of different *SLCO1B1* c.*439T>G genotypes was between 13% and 20% (p = 0.603), while for the mean $AUC_{0\text{-}48h}$, differences of app. 30% were observed (p = 0.371).

In addition, we investigated the effects of the *SLCO1B1* haplotypes found in our study group (with a frequency above 5.5%) on the pharmacokinetic parameters of atorvastatin (Table 4). Participants were assigned to one of two groups based on their *SLCO1B1* genotype: **15/*16/*17* non-carriers (n = 13) and **15/*16/*17* heterozygotes (n = 5). **15/*16/*17* homozygotes were not identified in the study. **15/*16/*17* haplotype was chosen as a carrier of the variant c.521C allele, known to affect the pharmacokinetics of statins. Thus, the group 1 (n = 13) included: **1J/*1K/*1L/*1J/*1K/*1L* (n = 6), **1J/*1K/*1L/*18/new* (n = 3), **1J/*1K/*1L/*18/new* (n = 1), **1J/*1K/*1L/*1B/*1F/new* (n = 1), **1J/*1K/*1L/*1B/*1F/new* (n = 1) and **1B/*1F/new/18/new* (n = 1), containing (c.521TT, c388GG + AG + AA, c.571CC + CT, c.597CC + CT and c.*439TT + GT + GG). In the group 2 (n = 5), **1J/*1K/*1L/*15/*16/*17* (n = 2), **1J/*1K/*1L/*15/*16/*17* (n = 1), **15/*16/*17/*18/new* (n = 1), **15/*16/*17/*18/new* (n = 1) were included, containing (c.521CT, c.388AG + GG, c.571CT, c.597TT + CT and c.*439GG + GT). No significant differences in the pharmacokinetic parameters of atorvastatin between the **15/*16/*17* heterozygotes and **15/*16/*17* non-carriers were observed (Table 4). However, the mean value of C_{max} was higher in the group of **15/*16/*17* heterozygotes (app. 22%, p = 0.375) as well as the mean values of $AUC_{0\text{-}48h}$ (app. 37%, p = 0.136) and $AUC_{0\text{-}\infty}$ (app. 38%, p = 0.127).

3. Discussion

The search for potent and efficacious inhibitors of the enzyme HMG-CoA reductase in the 1980 s resulted in the discovery and development of atorvastatin (Roth et al. 2002). It is still among the top 20 prescribed medicines in the USA (www.drugs.com/stats/top100/sales) and top 10 in the Republic of Macedonia, with the highest sales (www.fzo.org.mk). There is evidence for a large variability in the individual response and occurrence of adverse effects, such as myopathy, and even rhabdomyolysis. As one of the reasons for adverse effects, hereditary differences in atorvastatin pharmacokinetics have been pointed out, including the effects of *SLCO1B1* SNPs on atorvastatin pharmacokinetics (Pasanen et al. 2007). Thus, the aim of this study was to compare the effects of different *SLCO1B1* SNPs and their haplotypes on the pharmacokinetic profile of atorvastatin in 23 subjects of Macedonian origin. At the best of our knowledge, this study is the first that has investigated the effects of the *SLCO1B1* c.571T>C, c.597C>T and c.*439G>T on the pharmacokinetic parameters of atorvastatin and the first one in which the effects of the most active SNPs, c.388A>G and c.521T>C SNPs, were evaluated in the Macedonian subjects as well.

Our data confirmed that *SLCO1B1* is highly polymorphic and that several variants appear at a high frequency. These results are in consistency with the allele frequencies for the same SNPs found in Caucasians of different ethnic background (Pasanen et al. 2008; Mwinyi et al. 2004; 2008) and in our previous study, in which genotype distribution and allele frequencies of *SLCO1B1* polymorphisms in a large sample of 266 subjects of Macedonian origin were evaluated (Daka et al. *submitted for publication*). The results for the association between the SNPs pairs as well as the results pointing to the frequencies of the most common *SLCO1B1* haplotypes present in the study group, **IJ/*IK/*IL* followed by **15/*16/*17*, containing the variant alleles at positions c.388A>G, c.521T>C, c.597C>T and c.*439T>G were very similar to those of our previous study (Daka et al. *submitted for publication*). Thus, we consider the group of 23 subjects as a representative sample of the Macedonian population.

Current knowledge suggests that the *SLCO1B1* polymorphisms may have particularly important effects on the pharmacokinetic profile of atorvastatin and the studies are mainly focused on the influence of c.521 T>C polymorphism. In this study, higher C_{max}, $AUC_{0\text{-}48h}$, $AUC_{0\text{-}\infty}$, MRT_{expo} and lower T_{max}, Vd and CL for atorvastatin in *SLCO1B1* c.521CC and c.571TT homozygotes were observed in comparison with the other genotype groups of the c.521T>C and c.571T>C SNPs. Same results

Table 3: Pharmacokinetic parameters of a single 80 mg oral dose of atorvastatin in relation to SLCO1B1 polymorphisms in the study group

SLCO1B1 genotype	C_{max} (ng mL^{-1})	T_{max} (h)	$t_{1/2}$ (h)	AUC_{0-48h} (ng h mL^{-1})	$AUC_{0-\infty}$ (ng h mL^{-1})	$Vd_{area/kg}$ (L kg^{-1})	$CL_{area/kg}$ (L h^{-1} kg^{-1})	MRT_{expo} (h)
c.388A > G								
c.388AA (n = 8)	46.33 ± 24.17	0.69 (0.5–1.00)	9.63 ± 2.84	205.42 ± 52.20	209.64 ± 52.93	82.98 ± 38.43	5.91 ± 2.10	13.89 ± 4.10
c.388AG (n = 12)	42.16 ± 20.25	0.75 (0.5–1.5)	9.66 ± 2.37	208.37 ± 104.71	212.84 ± 106.54	94.10 ± 49.53	6.83 ± 3.66	13.93 ± 3.42
c.388GG (n = 3)	66.94 ± 48.55	0.87 (0.5–1.25)	8.05 ± 0.92	235.97 ± 174.49	241.90 ± 179.93	72.65 ± 47.78	6.53 ± 4.86	11.61 ± 1.32
c.521T > C								
c.521TT (n = 17)	40.20 ± 18.62	0.68 (0.5–1.0)	9.40 ± 2.20	188.79 ± 77.67	192.71 ± 79.08	94.71 ± 45.27	6.95 ± 3.07	13.57 ± 3.18
c.521CT (n = 5)	57.11 ± 30.51	1.02 (0.5–1.5)	9.55 ± 3.7	267.79 ± 113.31	274.88 ± 115.82	67.94 ± 37.14	5.16 ± 3.25	13.78 ± 5.34
c.521CC (n = 1)	96.62	0.5	10.86	315.61	319.28	56.09	3.58	15.67
c.571T > C								
c.571CC (n = 10)	43.84 ± 21.90	0.63 (0.5–1.00)	9.39 ± 2.51	179.93 ± 63.96	183.37 ± 65.00	98.32 ± 48.83	7.24 ± 3.42	13.55 ± 3.63
c.571CT (n = 12)	45.18 ± 21.83	0.87 (0.5–1.5)	9.22 ± 2.39	249.15 ± 109.23	254.94 ± 111.21	73.39 ± 39.45	5.50 ± 2.72	13.30 ± 3.45
c.571TT (n = 1)	96.62	0.5	10.86	315.61	319.28	56.09	3.58	15.67
c.597C > T								
c.597CC (n = 9)	44.10 ± 23.70	0.68 (0.5–1.0)	9.65 ± 2.69	199.90 ± 51.76	204.08 ± 52.22	85.49 ± 38.19	6.04 ± 2.01	13.92 ± 3.88
c.597CT (n = 11)	40.89 ± 12.51	0.79 (0.5–1.5)	9.24 ± 2.38	228.76 ± 117.26	233.79 ± 119.43	86.33 ± 52.65	6.45 ± 3.83	13.33 ± 3.43
c.597TT (n = 3)	76.83 ± 38.37	0.75 (0.5–1.25)	8.99 ± 1.75	262.51 ± 131.67	267.69 ± 134.85	67.13 ± 35.11	5.55 ± 3.83	12.97 ± 2.53
c.*439T > G								
c.*439GG (n = 5)	42.02 ± 21.10	0.93 (0.5–1.5)	8.52 ± 1.75	195.26 ± 94.03	199.00 ± 94.88	83.45 ± 36.35	6.70 ± 2.65	12.29 ± 2.52
c.*439GT (n = 11)	50.45 ± 26.23	0.77 (0.5–1.25)	9.85 ± 2.08	252.12 ± 113.09	257.71 ± 115.32	82.67 ± 52.64	5.81 ± 3.85	14.21 ± 3.00
c.*439TT (n = 7)	44.59 ± 23.11	0.57 (0.5–0.67)	9.22 ± 2.08	193.53 ± 59.09	197.46 ± 60.09	84.81 ± 40.36	6.37 ± 2.28	13.30 ± 3.00

Data are presented as mean ± SD; tmax data are represented as the median (range). AUC0-∞, area under the plasma concentration-time curve from 0 h to infinity; AUC0-48 h, area under the plasma concentration-time curve from 0 to 48 h; Cmax, peak plasma concentration; t1/2, elimination half- life; Tmax, time to Cmax; Vdarea/kg, weight-adjusted volume of distribution; Cl_{area}/kg, weight-adjusted clearance.

were also obtained in the study of Pasanen et al. (2007), in which subjects with *SLCO1B1* c.521CC genotype (n = 4) had significantly larger (144%) mean AUC_{0-48h} than the subjects with c.521TT (reference) genotype (n = 16) and 61% larger than the subjects with c.521TC genotype (n = 12). These findings support the idea that impaired (reduced) OATP1B1 function could theoretically reduce the clearance of atorvastatin, because of the decreased entry into the liver, the main site of atorvastatin metabolism and elimination (Pasanen et al. 2007). Several clinical studies have also demonstrated that subjects, who are carriers of *SLCO1B1* 521C allele, had increased plasma concentrations of other OATP1B1 substrates (e.g. pravastatin and repaglinid) compared to the subjects who were wild type homozygotes (Aquilante et al. 2008; Kivisto et al. 2007; Niemi et al. 2005; Kalliokoski et al. 2008). To our knowledge, no data for the effect of the c.571T > C SNP on the pharmacokinetics of atorvastatin have been reported so far. Two studies, involving healthy Chinese volunteers, report for lack of significant effect on simvastatin (n = 12) and pitavastatin (n = 17) pharmacokinetics by *SLCO1B1* c.571T > C. The same was obtained for the *SLCO1B1* c.597C > T (Chou et al. 2013 a,b).

In the previous studies it was reported that the *SLCO1B1*15* variant (c.388G and c.521C) reduced the transport activity of OATP1B1 and had an important effect on the atorvastatin systemic exposure and elimination (Nizhizato et al. 2010; Lee et al. 2010; Rodrigues et al. 2011; Pasanen et al. 2007). Therefore, one expected that the group of **15/*16/*17* heterozygotes, which included subjects with c.521CT genotype (group 2), would have

Table 4: Pharmacokinetic parameters of a single 80 mg oral dose of atorvastatin in relation to SLCO1B1 haplotypes in the study group.

Group of SLCO1B1 haplotypes	N°	C_{max} (ng mL^{-1})	T_{max} (h)	$t_{1/2}$ (h)	$AUC_{0\text{-}48h}$ (ng h mL^{-1})	$AUC_{0\text{-}\infty}$ (ng h mL^{-1})	Vd_{area}/kg (L kg^{-1})	CL_{area}/kg (L h^{-1} kg^{-1})	MRT_{expo}(h) (h)
Group 1: **1J/*1K/*1L[a]/*1J/*1K/1L[a]*	13	43.8 ± 21.2	0.7(0.5−1.00)	9.5 ± 2.3	198.0 ± 85.3	201.9 ± 86.5	92.9 ± 48.4	6.7 ± 3.2	13.7 ± 3.3
**1J/*1K/*1L[a]/*18[d]/new[e]*									
*1J/*1K/*1L[a]/*18[d]/new[e]*									
**1J/*1K/*1L[a]/*1B/*1F[v]/new[h]*									
**1J/*1K/*1L[a]/*1B/*1F[v]/new[h]*									
**1B/*1F[v]/new[h]/*18[e]/new[f]*									
Group 2: **15[u]/*16[b]/*17[c]/*18[d]/new[e]*	5	53.6 ± 27.6	1.02 (0.5−1.50)	9.1 ± 3.3	271.4 ± 98.5	278.3 ± 100.6	62.9 ± 34.1	4.9 ± 2.9	13.1 ± 4.8
**15[u]/*16[b]/*17[c]/*18[g]/new[f]*									
**15[u]/*16[b]/*17[c]/*1J/*1K/*1L[a]*									
**15[u]/*16[a]/*17[u]/*1J/*1K/*1L[a]*									
P value		**0.375	∞ 0.163	∞0.336	**0.136	**0.127	**0.214	**0.178	**0.566

N-number of subjects; ∞p value evaluated with Mann-Whitney Test. **p value evaluated with independent t test.

larger $AUC_{0\text{-}48h}$ for atorvastatin compared to the $AUC_{0\text{-}48h}$ of the **15/*16/*17* non-carrier group that included subjects with c.521TT (group 1). So, the larger mean $AUC_{0\text{-}48h}$ and $AUC_{0\text{-}\infty}$ and lower Vd and CL, observed in the study of the group of *15/*16/*17* heterozygotes, can be explained with the decreased activity of c.521C variant allele present in this group.

In conclusion, this study provides an valuable analysis of the effect of *SLCO1B1* variant genotype and haplotype distribution on the pharmacokinetic profile of atorvastatin in individuals of Macedonian origin. *SLCO1B1* c.521T>C and c.571T>C SNPs investigated in this study affected the pharmacokinetic parameters of atorvastatin. The lack of statistically significant association between these *SLCO1B1* polymorphisms and the pharmacokinetic parameters of atorvastatin may be due to the size of the sample and low number of individuals homozygous for the rare c.521C variant allele included in the study. Additional studies, with a larger sample size are needed to confirm the influence of the *SLCO1B1* polymorphisms on atorvastatin pharmacokinetics.

4. Experimental

4.1. Subjects, genotyping and haplotype analysis

A total of 23 young Caucasian male healthy volunteers (average age 22 ± 4, BMI 26.33 kg/m^2, Macedonian ethnic origin) selected based on their medical history, physical examination, 12-led electrocardiogram (ECG) and routine clinical laboratory tests and urinary drug screen participated in the study. All participants were non-smokers. Subjects were not allowed to take any alcohol and grapefruit-containing foods or xanthine-containing beverages 48 h before and 96 h after the drug administration. The subjects did not receive any other medication at least 2 weeks prior to the atorvastatin administration. Subjects with a history of a significant allergic reaction to any drug, an immunosuppressive condition, malignancy within the past 5 years, significant blood loss within 8 weeks prior to the initiation of the study, diseases of the gastrointestinal tract, liver, kidneys or any other conditions known to interfere with the absorption, distribution, metabolism or elimination of drugs were excluded from the study. Individuals with active drug or alcohol abuse in the past or consumption of large quantities of coffee or tea were also excluded. Serology screening tests for hepatitis B and C and HIV, urine drug screening and alcohol breath tests were negative for all subjects. No concomitant diseases were reported in any of the subjects as well as previous uptake of medications.

The participants were allocated into groups according to the genotype and haplotype data for *SLCO1B1* SNPs (Table 1 and Table 2). For genotyping, three mL venous blood samples drawn with ethylenediamintetraacetic acid (EDTA) as anticoagulant were collected and stored at 4 °C prior to DNA isolation. DNA isolation was performed at the Center for Biomolecular Pharmaceutical Analyses, UKIM-Faculty of Pharmacy, Republic of Macedonia, using Qiagen Qiamp DNA Blood kit (Qiagen Gmbh, Hilden, Germany) according to the manufacturer's protocol. The samples were kept at -20 °C until further analysis. The *SLCO1B1* SNPs to be genotyped were selected on the basis of literature data (Rodrigues et al. 2011; Pasanen et al. 2006; Santos et al. 2012; Xu et al. 2007) and a previous study in which 266 subjects were included (Daka et al. *submitted for publication*). Thus, the following variants in the *SLCO1B1* gene were analyzed: c.388A>G (Asn130Asp, rs2306283), c.521T>C (Val174Ala, rs4149056), c.571T>C (Leu191Leu, rs4149057), c.597C>T (Phe199Phe, rs2291075), c.1086C>T (Tyr362Tyr, rs57040246), c.1463G>C (Gly488Ala, rs59502379), c.*439T>G (rs4149087) using TaqMan allelic discrimination assay (Applied Biosystems, Foster City, CA, USA). Polymerase chain reaction was performed on the Real-Time PCR system Mx3005P (Stratagene, La Jolla, CA, USA) using TaqMan genotyping protocols (TaqMan®Drug Metabolizing assay; Applied Biosystems Foster City, Ca, USA) in total volume of 12.5 μL under the following conditions: one cycle of 2 min at 50 °C, one cycle of 10 min at 95 °C, and 50 cycles of 15 s at 92 °C and 1 minute at 60 °C.

The study samples alleles and genotype frequencies were estimated with a gene counting method. The agreement with Hardy-Weinberg Equilibrium (HWE) of the observed genotypic distribution for the *SLCO1B1* gene was tested with the Chi-square test. Linkage disequilibrium (LD) for each pair of SNPs was quantified (correlation r^2 and *D'* values) to find haplotypes in the study group. For the analysis of LD, haplotype construction and genetic association at polymorphism loci, SHEsis software platform was used (http://analysis2.bio-x.cn/myAnalysis.php) (Shi et al. 2005). The haplotypes are presented with their previously assigned names, as cited in the

study of Pasanen et al. (2006) in which allelic frequencies at 11 variant sites were determined (g.11187G > A, g.11110T > G, g.10499A > C, c.388A > G, c.411G > A, c.463C > A, c.521T > C, c.571T > C, c.597C > T, c.1929A > C and c.*439T > G). Considering that five of these SNPs and two other SNPs have been analyzed in the present investigation, one haplotype has several names and there are haplotypes that we designated as new.

4.2. Study design

The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and the International Conference on Harmonization (ICH) Note for Guidance on Good Clinical Practice (GCP) (CPMP/ICH/135/95) and with applicable local legal requirements. All participants received oral and written information and gave a written informed consent before entering the study. The final clinical trial protocol as well as the informed consent and other information that required pre-approval were reviewed and approved by the Independent Ethics Committee according to the specifications outlined in the applicable regulations. After a night fasting, the participants received a single dose of 80 mg atorvastatin (from the same producer), orally with 200 ml water at 08:00. Standard warm meal was served four hours after the ingestion of atorvastatin and a standard light meal after 7 and 10 hours. Blood samples (5-10 ml each) were drown before the administration of the agent and 0.167, 0.333, 0.5, 0.667, 0.833, 1, 1.25, 1.5, 1.75, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 10, 12, 24, 36 and 48 h after the placement of atorvastatin in tubes that contained EDTA and stored on ice immediately after sampling. Within 30 min from the blood sampling, the plasma was separated and stored at -70 °C until analysis.

4.3. Determination of plasma drug concentrations

Tandem Liquid Chromatography/Mass Spectrometry LC-MS-MS (Shimadzu LC-30 paired with Nexera Shimadzu LCMS 8030 triple quadruple mass spectrometer ESI interface) was used to determine the concentration of atorvastatin in the samples, with a method previously described by Pasanen et al. (2007). In brief, chromatography was performed on a Symetry C8 column (50 x 2.1 mm, 3.5 μm, Waters, Milford, Massachusetts, USA) using a gradient of 10 mmol/L ammonium acetate (pH 4.95) and acetonitrile. The ion transitions monitored were mass-to-charge ratio (m/z) 559 to m/z 250. Data processing was carried out with software Rapidtrace V 2.0.

4.4. Pharmacokinetics and statistical analysis

The pharmacokinetic parameters were calculated with the non-compartment analysis of serum concentration curve *versus* time using PK Solutions 2.0 software (copyrighted © 1997-2009, Summit Research Services, USA). C_{max}, time to C_{max} (T_{max}), elimination half-life ($t_{1/2}$), constant rate of elimination (k_{el}), mean residence time (MRT, expo), volume of distribution (Vd/kg), clearance (CL/kg) and area under curve $AUC_{0\text{-}48h}$ and $AUC_{0\text{-}\infty}$ were calculated for atorvastatin. The terminal log-linear part of each concentration-time curve was identified visually, and the elimination rate constant (k_{el}) was determined from ln-transformed data with a linear regression analysis. The $t_{1/2}$ was calculated with the equation $t_{1/2} = \ln 2/k_{el}$. The AUC values were calculated according to the combination of the linear and long-linear trapezoidal rules, with extrapolation to infinity, when appropriate, by dividing the last measured concentration by k_{el}.
The results for the pharmacokinetic parameters are expressed as mean ± SD in the text and tables. All data were analyzed with the statistical program IBM SPSS 19.0. The pharmacokinetic variables of atorvastatin between the genotype groups of *SLCO1B1* SNPs were compared using the analysis of variance and independent t test. Homogeneity of variance was tested using Levene's Test of Equality. Post hoc testing was done with the Games-Howell test. Compatibility of the residuals with normal distribution was assessed with the Shapiro-Wilk test. When appropriate, the data were logarithmically transformed before the analysis or analyzed with the Kruskall-Wallis test with a posteriori testing with the Mann-Whitney U-test. Differences were considered statistically significant when p was below 0.05.

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