Supplemental Digital Content for:

Cerivastatin *in vitro* metabolism by CYP2C8 variants found in patients experiencing rhabdomyolysis

Rüdiger Kaspera[1], Suresh B. Naraharisetti[1], [2,4]Bani Tamraz, Tariku Sahele[1], Matthew J. Cheesman[5], [2,3,4]Pui-Yan Kwok, Kristin Marciante[6], Susan R. Heckbert[7,9], Bruce M. Psaty[6,7,8,9], and Rheem A. Totah[1*].

[1]Department of Medicinal Chemistry, University of Washington, Seattle, WA, USA; [2]Cardiovascular Research Institute, [3]Department of Dermatology, [4]Institute for Human Genetics, University of California at San Francisco, CA, USA; [5]School of Biomedical Sciences, The University of Queensland, St. Lucia, Brisbane, Australia; [6]Cardiovascular Health Research Unit, Department of Medicine, [7]Department of Epidemiology, [8]Department of Health Services, University of Washington, Seattle, WA, USA; [9]Group Health Research Institute, Group Health Cooperative, Seattle, WA, USA.

Table of content:

5. **Kinetic evaluation of cerivastatin metabolism by CYP3A4 supersomes**
6. **Characterization data for kinetic evaluation of microsomes from the human liver tissue bank at the University of Washington (K_m/V_{max})**
7. **Calculations of haplotypes for CYP2C8 within the patient population**

1. Patient selection:

Seventeen United States based attorneys and one attorney from Canada helped recruit rhabdomyolysis case participants. The attorneys made initial contact with potential participants by mailing letters describing the study to their clients. The attorneys telephoned each letter recipient to obtain permission to release clients' contact information to study staff. Study staff then contacted the clients who released their contact information to explain the study, answer questions, and obtain consent. Buccal mouthwash samples were extracted using either a Quiagen or Puregene extraction kit. The DNA was aliquoted and stored at -70 °C.

2. PCR and Sequencing Method:

DNA samples were PCR amplified using the primers listed in table S1 representing 9 amplicons designed to target 9 coding regions, the intron-exon bounderies, promoter and the UTR regions of the CYP2C8 gene (reference gene UCSF). PCR primers were designed using Primer 3 (http://frodo.wi.mit.edu) and evaluated by *in silico* annealing to expected location in the genome and by gel electrophoresis ensuring one binding site. PCR conditions are as follows for all the amplicons: 4 ng of genomic DNA (1 µl of water) was incubated in a 10 µl reaction composed of 1 µl of Buffer, 0.7 µl of $MgCl_2$ (50mM), 0.4 µl of dNTP (2.5mM), 0.03 µl of Taq polymerase, 4.87 µl of water, 1 µl of Forward primer (1µM), 1 µl of Reverse primer (1µM) (Buffer, and enzyme are from Invitrogen Corporation, Carlsbad, CA) with cycling conditions of denaturation at 95°C for 2 min followed by 35 cycles of 92°C for 10 sec, annealing at 60°C for 20 sec, and extension at 72°C for 1 min. At the end of the 35 cycles, the reaction mixture was held at 72°C for 10 min before being cooled to 4°C until the next step. The 10 µl PCR product was purified by incubation with 0.4 µl of PCR Clean-up Reagent (PerkinElmer Life Sciences,

Inc., Boston, MA) and 3.6 µl of PCR Clean-Up Dilution Buffer (PerkinElmer) at 37°C for 1 hour followed by enzyme inactivation at 90°C for 15 minutes. The purified PCR product was sequenced using ABI PRISM BigDye terminator sequencing Version 3.1 on an ABI Prism 3730xl DNA analyzer (Applied Biosystems, Inc., Foster City, CA). The 12 µl sequencing reaction was composed of 2.5 µl of purified PCR product, 4.5 µl of sequencing primer (1µM), 1 µl BigDyeV3.1, 2 µl of 5X buffer, and 2 µl water. Cycling conditions were 96°C for 2 min, 25 cycles of 96°C for 15 sec, 50°C for 1 sec, 60°C for 4 minutes. After sequencing, the DNA sequence files were imported into and aligned with SEQUENCHER 4.6 (Gene Codes Corporation, Ann Arbor, MI) for variant analysis. No secondary sequencing peaks were observed in sequencing reactions confirming primer specificity.

Table S1: Primer for PCR amplification of DNA samples from rhabdomyolysis patient population.

		Primer Sequence		Amplicon
Name	**Covers**	**P1**	**P2**	**Size (bp)**
Amplicon 1	Pro,3'UTR	GCACCAGGACCACAAAAGGT	GCCAGCTGTGGTGTAAGTGG	1040
Amplicon 2	Ex 1, 3'UTR	CACACACTAAATTAGCAGGGAGTG	TTCAGAGGGAGTATTTTGCTTTACAA	379
Amplicon 3	Ex 2, 3	GGCACATCACAGGCCATCTA	CCTACACCCTATGAACCAACACA	890
Amplicon 4	Ex 4	GGTCCCCAACTTTTCTCTTCC	ACCCCTTGCACTTCTGATGG	795
Amplicon 5	Ex 5	TTTCCCTTCAAAATGGACATGA	TGAAACCTTTCTTCTGTTCCACA	743
Amplicon 6	Ex 6	ATGAAGGCCATTGCCAGAAG	TGCTGGCTCTCCTTACCACA	846
Amplicon 7	Ex 7	TGGCTGGTTGTACTTCTGGAC	TGCATGAACATGTTAAGTCTTTCC	651
Amplicon 8	Ex 8	CTTCAAATGTGATTGGAAAGCTC	GGAGCTCTTGGGTGCCTTAG	623
Amplicon 9	Ex 9, 5'UTR	ATGGAAACTCAAAATGGCAAAA	TCCTCACCCTCTTCTCCTTTG	1000

Table S2: SNP genotyping data and corresponding minor allele frequencies obtained from patient population (alleles are reported in CYP2C8 sequence on the positive strand; the original manuscript reports the negative strand in accordance to literature).

NCBI	location	allele	Caucasian		African American	
			with gemfibrozil coadmin., n=118	without gemfibrozil n=46	With gemfibrozil coadmin., n=4	without gemfibrozil n=2
rs or ss			MAF		MAF	
ss86217925	Pro	AT/--	0.089	0.087	0	0
ss86217924	Pro	A/T	0	0	0.125	0
ss86217923	Pro	A/C	0.004	0.011	0	0
rs7909236	Pro, *1B	G/T	0.174	0.130	0.250	0
rs17110453	Pro, *1C	A/C	0.114	0.087	0.125	0.250
rs7912549	Pro	A/G	0.178	0.185	0.500	0.250
ss179319940	Exon 1, N56S	T/C	0.004	0.011	0	0
ss179319939	Intron 2	A/T	0.004	0.011	0	0
ss179319938	Intron 2	C/T	0.004	0	0	0
rs2275622	Intron 2	C/T	0.271	0.272	0.125	0.250
rs3216029	Intron 2	-/A	0.199	0.196	0.250	0.250
rs11572079	Intron 2	A/G	0.013	0.011	0	0
rs11572080	Exon 3 *3	C/T	0.097	0.098	0	0
rs11572081	Exon 3 K160K	C/T	0.004	0	0	0
rs11572082	Intron 3	C/G	0.097	0	0	0
rs2185571	Intron 3	C/T	0.308	0.378	0	0
rs11572090	Intron 3	G/A	0.010	0.013	0	0
rs3752988	Intron 3	T/C	0.307	0.278	0.250	0.250
rs11572091	Intron 3	A/-	0.068	0.054	0.125	0
ss86217922	Intron 3	T/A	0.008	0.011	0	0
rs7098376	Intron 3	A/T	0.644	0.598	0.500	0
rs11572093	Intron 4	C/T	0.352	0.402	0	0
rs1058930	Exon 5, *4	G/C	0.042	0.043	0	0
ss179319937	Intron 5	T/C	0.004	0.011	0	0
rs11572105	Intron 5	G/T	0.047	0.043	0	0
rs1536430	Intron 5	C/T	0.013	0.022	0	0
rs11188154	Intron 5	G/C	0.098	0.098	0	0
rs11572155	Intron 5	T/C	0.060	0.043	0.125	0
ss179319936	Intron 6	A/G	0.004	0.011	0	0
rs1891071	Intron 6	C/T	0.657	0.685	0.250	0.250
rs11188150	Exon 7, H353H	G/A	0.004	0	0	0
rs2275620	Intron 7	A/T	0.716	0.685	0.250	0.250
rs10509681	Exon8, *3	T/C	0.097	0.098	0	0
ss86217921	Exon 8, M426V	T/C	0.004	0	0	0
rs11572170	Intron 8	G/T	0.047	0.043	0	0
rs1934951	Intron 8	C/T	0.153	0.130	0.125	0.250
ss107123045	Intron 8	G/A	0.017	0.011	0.125	0.250
rs11572177	Intron 8	C/T	0.581	0.543	0.875	0
ss86217920	Exon 9, V472fsL494	T/-	0.004	0.011	0	0
rs1058932	3'-UTR	G/A	0.153	0.130	0.125	0.250

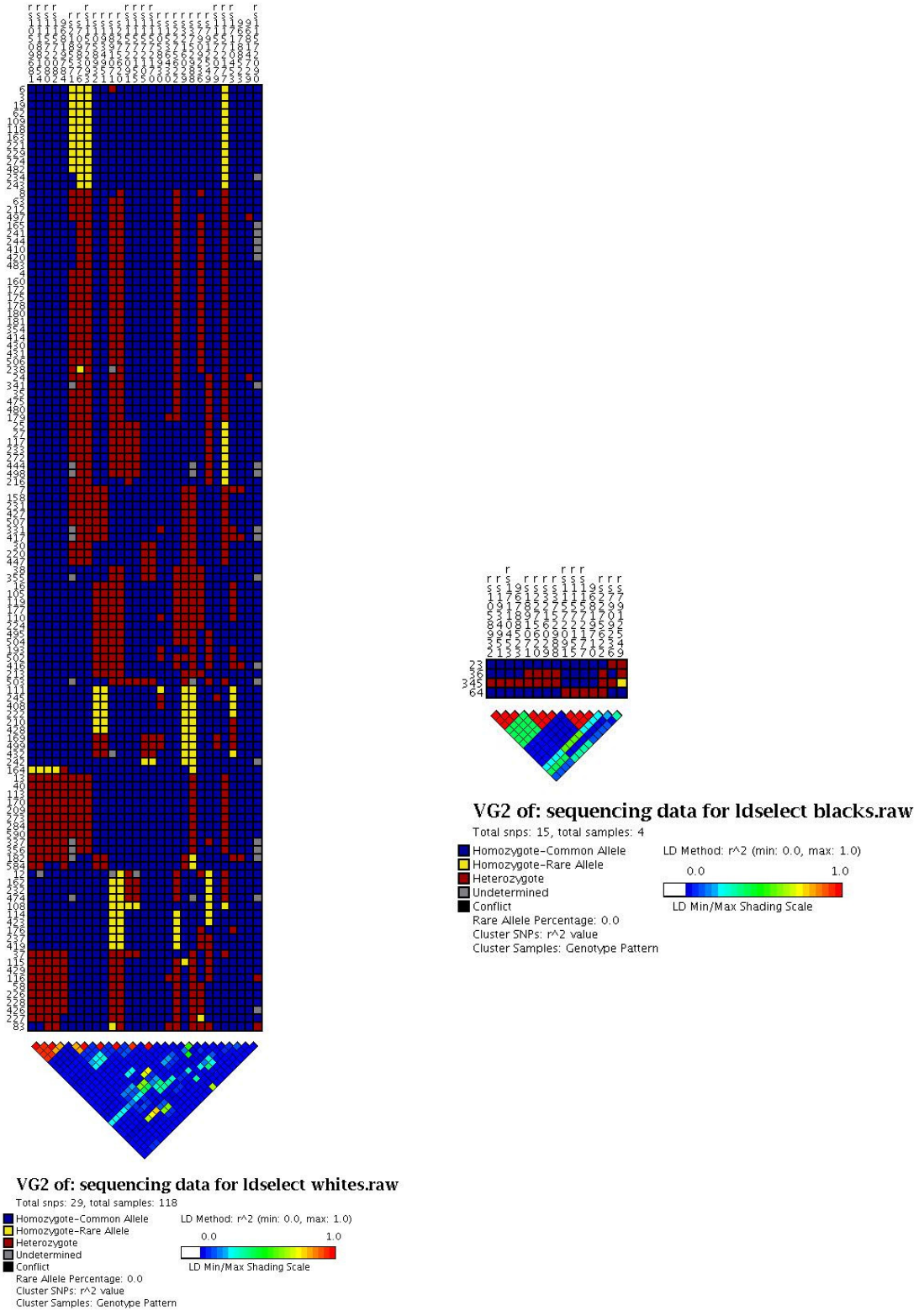

Figure S1: Distribution of SNPs in patient population (homozygous/ heterozygous alleles) with corresponding linkage.

3. PCR amplification of DNA from University of Washington liverbank:

The primers for PCR amplification of the ss86217925 (-TA) containing DNA constituted of the forward primer: CAAAAAATAGGAGACTTAGCCC and reverse primer: AAACTGAATTAGCAGGGAGTG. Primer Express-predicted amplicon size is ~300bp and PCR reactions were performed at the following conditions: 0.5 µl 10 mM dNTPs (200 uM final), 0.5 µl Pfu HotStart Turbo DNA polymerase (Stratagene #600320), 0.5 µl primer 2C8, 10 µM stock-500 nM final concentration, 0.5 µl primer 2C8, 10 µM stock-500 nM final concentration, 1 µl template DNA, 20.5 µl Dnase/Rnase free ddH_20, Cycling Conditions were 95˚C, 2 min, (95˚C, 10 sec, 56˚C, 20 sec, 72˚C, 3 min) 35 cycles, 72˚C, 10 min, 4˚C hold. The assay has given readable DNA sequence with input DNA ranging from 20-200 ng Following PCR, amplicons were purified using Qiamp PCR purification kit (Qiagen #28104) protocol and sequenced with above mentioned primer.

4. Generation of novel CYP2C8 cDNA by site directed mutagenesis

CYP2C8 variant expression vectors were constructed using the Quickchange II XL site directed mutagenesis kit and using pCWori+-*2C8*1* as a template with the primers of table S3. The PCR ran at 50 mU *Pfu* Turbo Polymerase per mL, and cycling conditions of 95° for 1 minutes followed by 18 cycles of 95° for 50 seconds, 60° for 50 seconds, 68° for 8 minutes followed by a final 7 min extension at 68°. For *V472fsL494*, the reading frame for the HIS-tag was realigned through a subsequent PCR reaction. Template was digested with Dpn1. All genes were sequenced entirely to confirm the introduction of the desired mutations and the absence of other undesired mutations as a result of PCR.

Table S3: Primer for site directed mutagenesis:

CYP2C8 variant	Forward and Reverse primer
N56S:	5′:ACATCTGCAAATCTTTCACCAGTTTCTCAAAAGTCTATGGTCC
	5′: GGACCATAGACTTTTGAGAAACTGGTGAAAGATTTGCAGATGT
A426V:	5′:TTTAAGAAAAGTGACTACTTCGTGCCTTTCTCAGCAGGAAAAC
	5′: GTTTTCCTGCTGAGAAAGGCACGAAGTAGTCACTTTTCTTAAA
V472fsL4	5′:AAGAACCTCAATACTACTGC_GTTACCAAAGGGATTGTTTC, deleted A at _
94 (1):	5′:GAAACAATCCCTTTGGTAAC_GCAGTAGTATTGAGGTTCTT, deleted T at _
V472fsL4	5′:ATCTGCTTCATCCCTGTCTGAAGAATGCTCGTCGACCCATCATCATC
94 (2):	5′:GATGATGATGGGTCGACGAGCATTCTTCAGACAGGGATGAAGCAGAT
	(add of 3'-UTR and align of 6xHIS)

5. Kinetic evaluation of cerivastatin metabolism by CYP3A4 supersomes

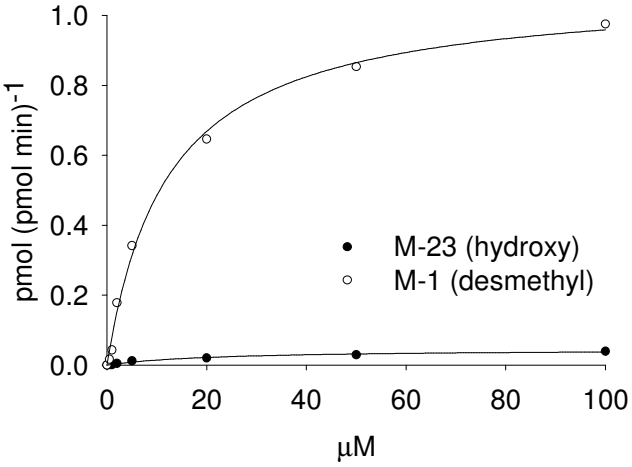

Figure S2: Characterization of cerivastatin metabolism catalyzed by CYP3A4 supersomes (20 pmol/mL).

6. Characterization data for kinetic evaluation of microsomes from the human liver tissue:

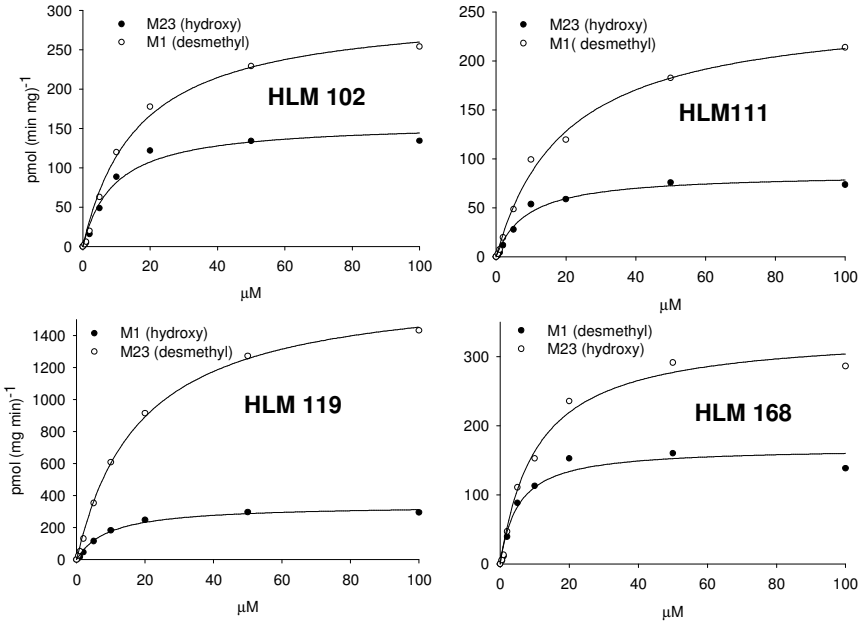

Figure S3: Characterization of Human Liver Microsomes genotyped as *CYP2C8*1/*1* (HLM 102 and 111) and **1/*3* (119 and 168).

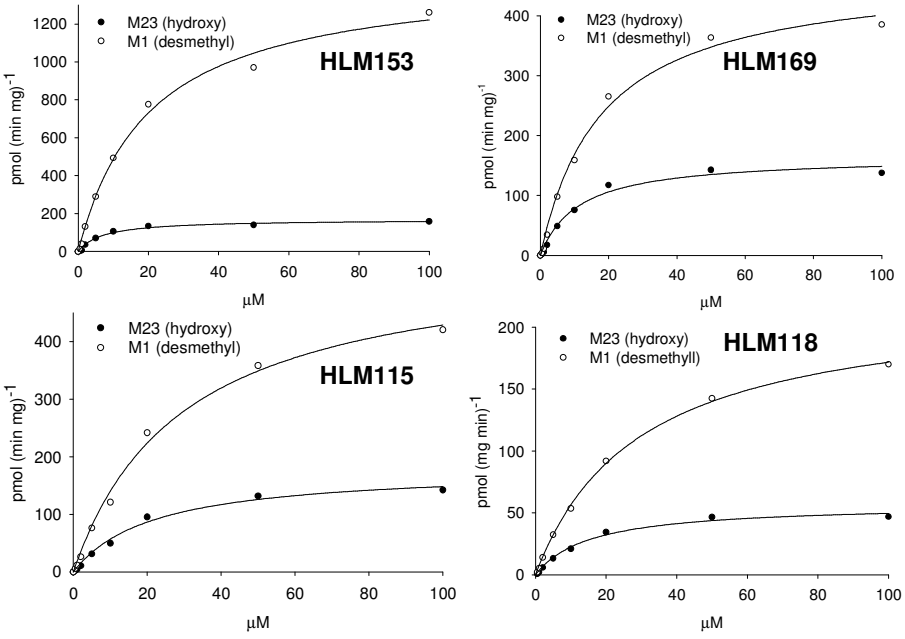

Figure S4: Characterization of Human Liver Microsomes of genotyped as *CYP2C8*3/*3* (HLM 153 and 159) and **1/*4* (HLM 115 and 118).

Table S4: kinetic data for Human liver microsomes from Figure S3 and S4.

	Hydroxyl (M23)		Desmethyl (M1)	
Liver (genotype)	K_m [μM]	V_{max} [pmol min^{-1} mg^{-1}]	K_m [μM]	V_{max} [pmol min^{-1} mg^{-1}]
102 (*1/*1)	9.3 ± 1.4	157 ± 7	16.3 ± 1.7	302 ± 11
111 (*1/*1)	8.7 ± 1.4	85 ± 4	19.8 ± 2.4	255 ± 11
119 (*1/*3)	9.3 ± 1.3	341 ± 14	18.6 ± 1.2	1723 ± 38
168 (*1/*3)	6.8 ± 0.9	233 ± 10	13.3 ± 1.2	463 ± 17
153 (*3/*3)	7.1 ± 1.3	169 ± 9	20.1 ± 3.1	1468 ± 82
169 (*3/*3)	11.3 ± 1.4	165 ± 6	18.1 ± 1.5	474 ± 14
115 (*1/*4)	22.8 ± 2.5	186 ± 8	30.7 ± 2.7	568 ± 22
118 (*1/*4)	15.6 ± 2.1	57 ± 3	29.4 ± 1.4	222 ± 4

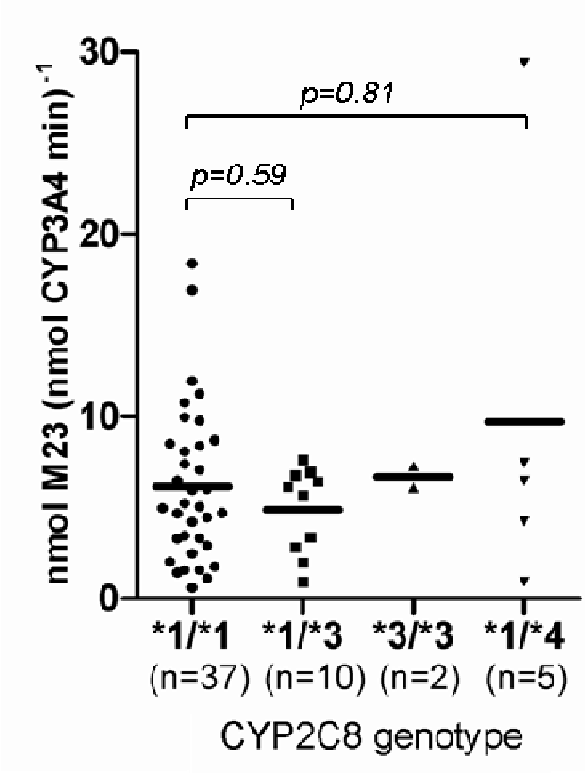

Figure S5: Cerivastatin metabolite formation normalized to CYP3A4 content (pmol/mg) and categorized according to liver bank CYP2C8 genotype (A: M23 formation, the bar displays the median of each data set, assays performed at 10 μM cerivastatin and 0.2 mg mL^{-1} protein concentration and 5 min incubation time). No statistical significant differences were observed ($p>0.05$, Mann Whitney test).

7. Calculations of haplotypes for CYP2C8 within the patient population

Table S4: CYP2C8 haplotype distribution in white patient population (n=118). Haplotypes were determined by using tagSNPs and characteristic additional SNPs and displayed if they have a frequency >2%. Others were classified as rare.

refSNP	*rs1058932*	*rs1934951*	*rs10509681*	*rs2275620*	*rs11188154*	*rs1058930*	*rs2185571*	*rs11572082*	*rs11572080*	*rs3216029*	*rs2275622*	*rs7909236*	*rs17110453*	*ss86217925*	
			CYP2C8*3			CYP2C8*4			CYP2C8*3			CYP2C8*1B	CYP2C8*1C	CYP2C8 del -AT	Frequency
Location	Int	Int	Exon	Int	Int	Exon	Int	Int	Exon	Int	Int	Exon	Exon	Pro	
A	G	C	T	T	G	G	**T**	C	C	-	C	G	A	AT	0.297
B	G	C	T	**A**	G	G	C	C	C	-	**T**	**T**	A	AT	0.159
B2	G	C	T	**A**	G	G	C	C	C	-	**T**	G	A	AT	0.099
C1	**A**	**T**	T	T	G	G	C	C	C	**A**	C	G	**C**	AT	0.059
C2	**A**	**T**	T	T	G	**C**	C	C	C	**A**	C	G	**C**	AT	0.038
C3	G	C	T	T	G	G	C	C	C	**A**	C	G	A	AT	0.042
C4	**A**	**T**	T	T	G	G	C	C	C	**A**	C	G	A	AT	0.044
D	G	C	**C**	T	**C**	G	C	**G**	**T**	-	C	G	A	**-**	0.076
E	G	C	T	**A**	G	G	C	C	C	-	**C**	G	A	AT	0.069
W[1]	G	C	T	T	G	G	C	C	C	-	C	G	A	AT	0.050
rare															0.067

[1]This haplotype does not show any similarity to reported haplotype but is more abundant than 2%, thus this haplotype was arbitrarily classified as W.