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**Title page**

# Pharmacogenetics of Human Carbonyl Reductase 1 (*CBR1*) in Livers from Black and White Donors

Vanessa Gonzalez-Covarrubias, Jianping Zhang, James L. Kalabus, Mary V. Relling, and Javier G. Blanco

Department of Pharmaceutical Sciences, The State University of New York at Buffalo, Buffalo, NY 14260, USA (V. G-C, J.Z, J.L.K, and J.G.B)
Department of Pharmaceutical Sciences, St. Jude Children's Research Hospital, Memphis, TN 38105, USA (M.V.R)

## Running title page

### a) Running Title

Pharmacogenetics of Human Carbonyl Reductase 1 in Liver

### b) Corresponding author

Javier G. Blanco, PhD
Department of Pharmaceutical Sciences, The State University of New York at Buffalo; 545 Cooke Hall, Buffalo, NY 14260-1200
Telephone: 716-645-2854 ext. 545
Fax: 716-645-3693
Email: jgblanco@buffalo.edu
URL: http://pharmacy.buffalo.edu

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### d) Abbreviations

CBR1, carbonyl reductase 1; CBR3, carbonyl reductase 3; AIM, ancestry informative markers; 3'-UTR, 3'-untranslated region; SNP, single nucleotide polymorphism; HPLC-MS/MS, high-performance liquid chromatography and tandem mass spectrometry; doxol, doxorubicinol, NQO1, NAD(P)H:quinone oxidoreductase.

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## Abstract

Carbonyl reductase 1 (CBR1) reduces the anticancer drug doxorubicin into the cardiotoxic metabolite doxorubicinol. We documented the hepatic expression of CBR1 in samples from white and black donors. Concordance between ethnicity and geographical ancestry was examined with ancestry informative markers. Livers from blacks and whites showed similar *CBR1* mRNA levels (*CBR1* $mRNA_{blacks}$ = 4.8 ± 4.3 relative fold vs. *CBR1* $mRNA_{whites}$ = 3.6 ± 3.6 relative fold, p = 0.217). CBR1 protein levels did not differ between both groups ($CBR1_{blacks}$= 8.0 ± 3.4 nmol/g cytosolic protein vs. $CBR1_{whites}$= 9.0 ± 4.6 nmol/g cytosolic protein, p = 0.347). The *CBR1* 3'-UTR polymorphism 1096G>A was detected in DNA samples from whites (*p* = 0.875, *q* = 0.125), and livers with homozygous G/G genotypes showed a trend towards higher *CBR1* mRNA levels compared to samples with heterozygous G/A genotypes (*CBR1* 1096G>$A_{(G/G)}$ = 4.1 ± 4.1 relative fold vs. *CBR1* 1096G>$A_{(G/A)}$ = 3.0 ± 2.5 relative fold, p = 0.266). *CBR1* 1096G>A genotype status was associated with CBR1 protein levels (p = 0.030) and CBR activity expressed as the rate of synthesis of doxorubicinol (p = 0.028). Our findings warrant further studies to evaluate the impact of *CBR1* 1096G>A genotype status on the variable pharmacodynamics of anthracycline drugs.

## Introduction

Carbonyl reductase 1 (CBR1) is a cytosolic short-chain dehydrogenase that catalyzes the two-electron reduction of relevant pharmacological substrates such as the antipsychotic haloperidol and the anticancer anthracyclines doxorubicin and daunorubicin (Forrest and Gonzalez, 2000; Rosemond and Walsh, 2004; Matsunaga et al., 2006). CBR1 reduces doxorubicin into its main circulating C-13 alcohol metabolite doxorubicinol by using the cofactor NADPH (Wermuth, 1981; Forrest et al., 1990). On average, 16-45% of the total doxorubicin is eliminated as doxorubicinol and the remainder of the drug is eliminated unchanged. Other minor metabolites such as 7-deoxy aglycones are synthesized by cytochrome P450 reductase and circulate in plasma at low concentrations (1-2% of the parent drug) (Speth et al., 1988; Joerger et al., 2005). The pharmacodynamics of doxorubicin in different cancer settings is variable, and the development of anthracycline-related cardiotoxicity in some patients hampers the clinical utilization of the drug. Interestingly, the metabolite doxorubicinol synthesized by CBR1 activity plays a key role during the pathogenesis of anthracycline-related cardiotoxicity. Doxorubicinol exerts cardiotoxicity by a combination of mechanisms including inhibition of $Ca^{+2}/Mg^{+2}$-ATPase in the sarcoplasmic reticulum and inactivation of the cytoplasmic aconitase/iron regulatory protein-1 complex (Olson et al., 1988; Minotti et al., 2004). Olson et al. reported that an overall 40-50% reduction of Cbr1 protein levels in *Cbr1*$^{+/-}$ mice was sufficient to confer protection against anthracycline-related cardiotoxicity. *Cbr1*$^{+/-}$ animals treated with a single injection of doxorubicin (20 mg/kg, i.p.) showed lower plasma levels of doxorubicinol than wild type animals

(*Cbr1*$^{+/+}$). Furthermore, histopathological analyzes together with echocardiographical assessments demonstrated anthracycline-related cardiotoxicity in *Cbr1* $^{+/+}$ but not in *Cbr1* $^{+/-}$ mice (Olson et al., 2003).

The liver is the major organ for the metabolism of doxorubicin, and various reports including a very recent one by Kassner et al. have shown that CBR1 is the main source of hepatic doxorubicin reductase activity. The aldo-keto reductases AKR1A1 and AKR1B1 are expressed in liver and also catalyze the reduction of doxorubicin. However, AKR1A1 and AKR1B1 have 7- to 18-fold lower catalytic efficiencies for the reduction of anthracycline substrates as compared to CBR1 (Wermuth et al., 1986; Ohara et al., 1995; O'Connor et al., 1999; Rosemond and Walsh, 2004; Kassner et al., 2008). Thus, variable hepatic CBR1 expression may affect the unpredictable pharmacodynamics of doxorubicin. Therefore, the first aim of this study was to analyze the expression of CBR1 in a collection of liver tissue samples from white (n = 64) and black donors (n = 32). Towards this end, we documented *CBR1* mRNA and CBR1 protein levels by quantitative real time RT-PCR and immunoblotting with a polyclonal anti-CBR1 antibody, respectively.

Functional single nucleotide polymorphisms (SNPs) on *CBR1* may contribute to variable CBR1 activity. We have characterized the functional impact of a non-synonymous SNP on *CBR1* (*CBR1* V88I, rs1143663) that appears to be confined to individuals with African ancestry ($q$ = 0.014). *CBR1* V88I results in CBR1 protein variants (CBR1 V88 and CBR1 I88) with distinctive catalytic and thermodynamic properties (Gonzalez-Covarrubias et al., 2007). Further studies demonstrated that the anthracycline reductase activities of CBR1 V88 and CBR1 I88 are differentially

inhibited by the cardioprotectant flavonoid monoHER (Gonzalez-Covarrubias et al., 2008). A second non-synonymous SNP on *CBR1* (*CBR1* S131P, rs41557318) has been recently reported by the dbSNP database (build 129). In addition, Avramopoulos et al. identified a relatively common SNP on the *CBR1* 3'-untranslated region (*CBR1* 1096G>A, rs9024) (Avramopoulos et al., 1992). Thus, we investigated the presence of *CBR1* V88I, *CBR1* S131P, and *CBR1* 1096G>A in paired liver DNA samples; and we determined whether *CBR1* 1096G>A genotype status dictates variable hepatic CBR1 expression.

## Materials and Methods

### Human liver samples

The Institutional Review Board of the State University of New York at Buffalo approved this research. Demographic information (e.g. age, gender, and ethnicity) was obtained from medical records (Table S1 supplemental). Human liver tissues from black (n = 32) and white donors (n = 64) were processed at St. Jude Children's Research Hospital, and were provided by the Liver Tissue Procurement and Distribution System (NIH Contract N01-DK-9-2310), and by the Cooperative Human Tissue Network, respectively. Liver tissue samples were processed following standardized procedures to obtain cytosolic fractions, RNA, and DNA. DNA and RNA isolations were performed with phenol-chloroform extraction (n = 1, 1% of the total), Tri Reagent® (n = 7, 7% of the total; Molecular Research Center, Cincinnati, OH) and Qiagen DNA/RNA kits (n = 88, 92% of the total; Qiagen, Valencia, CA).

### Ancestry informative markers

One hundred and seventy-six autosomal genetic markers showing large differences in allele frequencies between populations with distinctive geographical ancestries were used as ancestry-informative markers (AIM). A subset of DNA samples from white and black donors were selected by blinded operators for AIM genotyping (whites, n = 49, 77% of the total, and blacks, n = 27, 84% of the total). AIM were genotyped by DNAprint Genomics (Sarasota FL). The results are reported as the estimated percentage of Sub-Saharan African, European, Native American, and East Asian ancestry (Kishi et al., 2007).

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### Hepatic *CBR1* mRNA expression

The expression of *CBR1* mRNA was analyzed in 23 total liver RNA samples from blacks (72% of the total) and 42 total liver RNA samples from whites (66% of the total. Figure S1 supplemental). RNA concentrations were measured with a NanoDrop™ spectrophotometer. Total RNA (100 ng) was reverse-transcribed and amplified by using one-step QuantiTect SYBR Green RT-PCR kits (Qiagen, Valencia, CA). RT-PCR reaction mixtures were incubated in a MX3050P thermal cycler equipped with proprietary software for data analysis (MxPro v3.00, Stratagene, La Jolla, CA). *CBR1* primers were: 5'-CTGATCCCACACCCTTTCAT-3' (forward), and 5'-TTAAGGGCTCTGACGCTCAT-3' (reverse). *β-actin* primers were: 5'-ACGGCTCCGGCATGTGCAAG-3' (forward), and 5'-TGACGATGCCGTGCTCGATG-3' (reverse). *CBR1* and *β-actin* (normalizer) mRNAs were amplified in parallel with the following cycling parameters: 50°C for 30 min (reverse transcription), 95°C for 10 min (Taq DNA polymerase activation), 40 cycles of 95°C for 15 s (denaturation), 51°C for 30 s (annealing), 72°C for 30 s (extension), and 78°C for 30 s (fluorescence collection). Standard curves for *CBR1* and *β-actin* mRNAs (20-fold dynamic range) were run in parallel to ensure accurate mRNA quantifications. In all cases, the regression coefficients of the standard curves were $r^2 \geq 0.94$. Amplification efficiencies for *CBR1* and *β-actin* mRNAs were comparable and ranged between 125 and 175%. Experimental samples and standards for calibration curves were analyzed in quadruplicate. The relative amount of *CBR1* mRNA in each liver sample was automatically calculated with the comparative quantitation algorithm by using individual *β-actin* mRNA levels as normalizers. *CBR1*

mRNA values were expressed relative to the normalized *CBR1* mRNA content of liver sample 237. Liver sample 237 was randomly selected for data normalization. (Blanquicett et al., 2002; Bustin, 2002; Zamber et al., 2003).

**Hepatic CBR1 protein expression**

Randomly selected liver cytosols from white (n = 28) and black (n = 28) donors were analyzed by quantitative immunoblotting (Figure S1 supplemental). Liver cytosols (150 µg) and recombinant CBR1 standards (0.05, 0.08, 0.10, 0.15, 0.20, and 0.30 µg) were heated in a boiling water-bath for 5 min. Recombinant CBR1 was obtained as described (Gonzalez-Covarrubias et al., 2007). Nano liquid chromatography coupled to triple quadrupole mass spectroscopy showed that the purity of CBR1 was ≥96%. Samples were supplemented with Laemmli buffer and 5% β-mercaptoethanol (Bio-Rad, Hercules, CA). Samples were loaded into 4-10% Precise-protein gels (Pierce, Thermo Scientific, Rockford, IL), and separated by electrophoresis at 90 V for 90 min in a Bio-Rad mini-cell apparatus. Proteins were transferred into Hybond-PVDF membranes (GE, Healthcare, Buckinghamshire, UK). Membranes were blocked at room temperature for one hour with StartingBlock T20 (Pierce, Thermo Scientific). After blocking, membranes were incubated for one hour with a specific polyclonal anti-human CBR1 antibody (1:2,000 dilution; Abcam Inc., Cambridge, MA) or with an anti-β-actin monoclonal antibody (1:5,000 dilution; Sigma-Aldrich, St. Louis, MO). Next, membranes were incubated for one hour with a secondary anti-IgG antibody conjugated with horseradish peroxidase (1:10,000 dilution, Sigma-Aldrich). Immunoreactive bands were visualized with the ECL Plus

Western blotting detection system (GE Healthcare, Piscataway, NJ). CBR1 band intensity values (pixel/mm$^2$) were quantified with a ChemiDoc XRS gel documentation system equipped with Quantity One software (Bio-Rad). Hepatic CBR1 levels were estimated by direct extrapolation from the calibration curves with recombinant CBR1. The limit of quantification was 0.02 µg and the limit of detection was 0.01 µg. Detection of CBR1 was linear (range: 0.05 – 0.30 µg, $r^2$ >0.85, CV = 9.5%). No immunoreactive bands were detected in immunoblotts of recombinant human CBR3 (purity ≥ 90%) probed with the polyclonal anti-human CBR1 antibody. CBR3 was obtained as described (Lakhman et al., 2005).

**Hepatic CBR activity**

Maximal CBR activities were measured in liver cytosols from whites (n = 64) and blacks (n = 32) with the substrate doxorubicin at 37°C. Validation experiments with liver cytosols from black and white donors showed that 400 µM of doxorubicin [S] ensured conditions of $V_{Max}$ and/or maximal CBR activity (zero order kinetics). Thus, maximal CBR activities (reaction rates) were directly proportional to the amount of cytosolic CBR1 enzyme. CBR activity with the substrate doxorubicin was linear within the following total protein concentration range: 0.2 - 5.0 mg/ml ($r^2$ = 0.99). Incubation mixtures (final volume: 1 ml) contained potassium phosphate buffer (0.1 M, pH 7.4), NADPH (200 µM, Sigma–Aldrich), and doxorubicin (400 µM, Sigma–Aldrich). Kinetic reactions were started by the addition of liver cytosols (100 µL, total protein concentration: 1.2 ± 0.6 mg/ml, range: 0.1 - 3.7 mg/ml). The oxidation rates of the NADPH cofactor were recorded at 340 nm (NADPH molar absorption coefficient 6220 $M^{-1}$ $cm^{-1}$) for 3.0 min at an acquisition speed of 5

readings/s (900 readings) in a Cary Varian Bio 300 UV-Vis spectrophotometer equipped with thermal control and proprietary software for enzyme kinetics analysis (Wermuth, 1981; Covarrubias et al., 2006). Enzymatic velocities ($V_0$) were automatically calculated by linear regression of the $\Delta_{Abs}/\Delta_{time}$ points ($r^2 \geq 0.95$). After the kinetic measurements, the reaction mixtures were immediately frozen at -70ºC for doxorubicinol quantification. Direct quantification of doxorubicinol with an HPLC-MS/MS assay adapted for human liver cytosols was performed in a subset of randomly selected reactions mixtures from whites (n = 40, 63% of the total) and blacks (n = 20, 63% of the total) (DiFrancesco et al., 2007). Doxorubicinol was extracted from the cytosolic reaction mixtures by solid phase extraction using reversed-phase sorbent cartridges (Waters Oasis HLB, Milford, MA). The HPLC system consisted of an autosampler, a degasser, and a LC pump (1100 series Agilent Technologies, Palo Alto, CA) coupled to an Applied Biosystems PE/Sciex API 3000 mass spectrometer (Applied Biosystems, Foster City, CA). The mass spectrometer was operated in the mixed-reaction-monitoring positive ion mode using a turbo ionspray interface. The desolvation temperature of the interface was 350°C and the ion current was 4000 V. Chromatographic separation was performed in a C18 2.1 x 30 mm column (Waters Symmetry, Milford, MA) protected by a C18 2.1 x 10 mm guard column (Waters Symmetry). Separation of doxorubicinol and doxorubicin was achieved at a flow rate of 250 µL/min using a mobile phase gradient of 75% mobile phase A (5 mM acetate buffer, pH 3.5; 5% methanol) and 25% mobile phase B (5 mM acetate buffer, pH 3.5; 95% methanol), with a transition to 25% mobile phase A and 75% mobile phase B in 8 min. The anthracycline daunorubicin

was used as an internal standard (240 ng/ml). The range of linearity for the quantification of doxorubicinol was 50 - 1000 ng/ml ($r^2$ >0.990). The inter- and intraday coefficients of variations were <15%, and the accuracy range for doxorubicinol was 107-112%. Quality control points were routinely prepared by spiking doxorubicinol (Toronto Research Chemicals, Toronto, Ontario, Canada) into pooled liver cytosols at the following final concentrations: 50 ng/ml, 100 ng/ml, 200 ng/ml, and 400 ng/ml. The matrix effects of plasma and liver cytosols were comparable (% recovery plasma = 99%, and % recovery cytosols = 103%). Cytosolic protein concentrations were determined with an assay based on Bradford's technique using bovine serum albumin as standard (Bio-Rad). Maximal hepatic CBR activities were expressed as doxorubicinol synthesis rates (nmol doxol/min.mg).

### *CBR1* genotyping

The *CBR1* V88I polymorphism (rs1143663, 391G>A) was analyzed with a validated assay for allelic discrimination with specific fluorescent probes as described (Gonzalez-Covarrubias et al., 2007). The *CBR1* 3'-UTR 1096G>A (rs9024, 1096G>A) and *CBR1* S131P (rs41557318, 520C>T) polymorphisms were investigated by allelic discrimination with fluorescent probes followed by real time PCR (Assays-by-designs, Applied Biosystems). Supplemental table 2 lists the nucleotide sequences of genotyping primers and probes. Genotyping reactions were performed according to the manufacturer's protocol in a Bio-Rad iQ5 thermal cycler (Bio-Rad). All genotyping runs included appropriate negative (no DNA template) and positive controls (DNA samples from the Coriell Institute with known *CBR1* V88I,

*CBR1* 1096G>A, and *CBR1* S131P genotypes). For quality control purposes, *CBR1* 3'-UTR 1096G>A was further investigated by direct sequencing on 33 *CBR1* full-length cDNA samples from black (n = 13) and white (n = 20) donors as described (Gonzalez-Covarrubias et al., 2007).

**Human lymphoblastoid cell lines**

Nine human lymphoblastoid cell lines (GM10853, GM10845, GM10857, GM10858, GM10860, GM17240, GM16654, GM16688, and GM16689) derived from individual donors with Chinese (n = 3) or European (n = 6) ancestries were purchased as live cultures from the Coriell Institute for Medical Research (Camden, NJ). Cultures were maintained in RPMI media (Invitrogen) supplemented with 2 mM L-glutamine and 15% fetal bovine serum (Invitrogen). Total RNA was extracted from lymphoblastoid cell cultures (1 X $10^6$ cells/ml) with Illustra RNA minispin kits according to the manufacturer's instructions (GE Healthcare). The relative amount of *CBR1* mRNA in each lymphoblastoid cell line was determined as described above. *CBR1* mRNA levels in lymphoblastoid cells were expressed relative to the normalized *CBR1* mRNA content of cell lines with *CBR1* 1096G>A homozygous G/G genotypes (cell lines: GM10857 and GM16688). Genomic DNA was extracted with Qiagen kits. Cell cultures (1 X $10^6$ cells/ml) were centrifuged at 3000 rpm for 15 minutes for the preparation of cytosolic fractions. The resulting pellets were treated with 2.0 ml of lysis buffer (320 mM sucrose, 10 mM potassium phosphate, 1 mM EDTA, 1mM tris(2-carboxyethyl)phosphine, pH 7.4) supplemented with protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Pellets were homogenized with a handheld tissue homogenizer (BioSpec Products, Inc. Bartlesville, OK). Cell

homogenates were sequentially centrifuged at 10,000 rpm (15 min), 15,000 rpm (30 min), and 45,000 (1 hour). Cytosols were obtained by collecting the supernatants from the last centrifugation step. Synthesis of doxorubicinol by lymphoblastoid cell lines was determined by incubating cytosolic fractions with the substrate doxorubicin (400 µM) and NADPH (200 µM) for 5 hours. Reactions were started by the addition of cytosols (volume: 600 µL, total protein concentration: 3 mg/ml). Doxorubicinol concentrations were determined as described above.

### Statistical analysis

Descriptive statistics (e.g. group means, variances, standard deviations, group ranges, and group percentiles) were computed with Microsoft Excel 2000 version 9.0 (Microsoft Office) and GraphPad Prism version 4.03 (GraphPad Software Inc., San Diego, CA). The D'Agostino & Pearson omnibus normality test was used to examine data normality with alpha levels <0.05. Unpaired Student's *t*-tests were used to compare population means of data sets normally distributed. The Mann–Whitney test was used to compare population means of data sets with non-normal distributions. In all cases, differences were considered to be statistically significant at $p<0.050$. Pearson's coefficient of correlation ($r_p$) was used to analyze data sets with normal distributions and Spearman's coefficient of correlation ($r_s$) was used for data sets with non-normal distributions.

## Results

### AIM in samples from black and white liver donors

First, we sought to examine the extent of concordance between self-reported ethnicity and geographical ancestry by genotyping 176 AIM in a sub-set of randomly

selected liver DNA samples from black (n = 27) and white (n = 49) donors (Figure S1 supplemental). Samples from black donors showed an average AIM score of 76 ± 13% for the Sub-Saharan African panel, whereas samples from white donors showed an average AIM score of 91 ± 8% for the European panel (Figure 1). Further analysis showed that the hepatic expression of *CBR1* (*CBR1* mRNA, CBR1 protein, and CBR activity) was similar whether stratifying by ethnicity or AIM-determined geographical ancestry (Table 1). Thus, the hepatic expression of *CBR1* was analyzed after stratifying samples by self-reported ethnicity.

### *CBR1* mRNA and CBR1 protein expression in liver tissues from black and white donors

Hepatic *CBR1* mRNA expression was analyzed in 23 total liver RNA samples from blacks and 42 total liver RNA samples from whites, respectively. The number of RNA samples represented 72% (blacks) and 66% (whites) of the total number of liver samples available for each ethnic category (Figure S1 supplemental). Hepatic *CBR1* mRNA levels varied widely in blacks (49-fold, *CBR1* $mRNA_{blacks}$ range: 0.4 – 19.6 relative fold) and whites (48-fold, *CBR1* $mRNA_{whites}$ range: 0.3 – 14.3 relative fold). Statistical comparisons demonstrated that the relative expression of hepatic *CBR1* mRNA was similar between samples from black and white donors (*CBR1* $mRNA_{blacks}$ = 4.8 ± 4.3 relative fold vs. *CBR1* $mRNA_{whites}$ = 3.6 ± 3.6 relative fold, p = 0.217; Figure 2).

A randomly selected subset of liver cytosols from blacks (n = 28) and whites (n = 28) was examined for CBR1 protein expression by immunoblot analysis (Figure

S1 supplemental). CBR1 was detected as a single band of approximately 30-kDa in all samples. β-actin expression was also examined to assess the integrity of each cytosolic sample. The expression of CBR1 varied by 8-fold in samples from blacks (range: 2.2 – 17.0 nmol/g cytosolic protein) and by 6-fold in samples from whites (range: 3.3 – 19.2 nmol/g cytosolic protein). CBR1 protein levels did not differ between blacks and whites ($CBR1_{blacks}$= 8.0 ± 3.4 nmol/g cytosolic protein vs. $CBR1_{whites}$= 9.0 ± 4.6 nmol/g cytosolic protein, p = 0.347; Figure 3). Correlation analysis showed no association between *CBR1* mRNA and CBR1 protein expression in samples from black ($r_p$ = 0.244, p = 0.305) and white ($r_p$ = 0.296, p = 0.150) liver donors (Figure S2 supplemental).

**CBR activities in liver cytosols from blacks and whites**

Hepatic CBR activities for the substrate doxorubicin were assessed by measuring the oxidation rates of the NADPH cofactor in liver cytosols from white (n = 64) and black liver donors (n = 32). Variable levels of CBR activity were observed in samples from whites (range: <0.1 - 10.6 nmol/min.mg) and blacks (range: 0.9 – 14.0 nmol/min.mg), respectively. Statistical comparisons demonstrated that maximal CBR activities were similar between both groups ($CBR_{blacks}$= 4.0 ± 2.5 nmol/min.mg vs. $CBR_{whites}$= 3.9 ± 2.2 nmol/min.mg; p = 0.820; Figure S3 supplemental). Direct quantification of the C-13 alcohol metabolite doxorubicinol was performed in a subset of randomly selected cytosolic reaction mixtures from whites (n = 40, 63% of the total) and blacks (n = 20, 63% of the total). There was a significant correlation between the values of maximal CBR activities obtained with the spectrophotometric assay and those measured by direct quantification of doxorubicinol (n = 60, $r_s$ =

0.270, p = 0.039; Figure S3 supplemental). Statistical comparisons showed that maximal CBR activities expressed as the rate of synthesis of doxorubicinol were similar between blacks and whites ($CBR_{blacks}$ = 4.2 ± 2.3 nmol doxol/min.mg vs. $CBR_{whites}$ = 3.9 ± 2.1 nmol doxol/min.mg, p = 0.610; Figure 4). Further correlation analyses showed no association between CBR1 protein and CBR activity in samples from white ($r_p$ = 0.203, p = 0.377) and black ($r_p$ = 0.055, p = 0.826) liver donors (Figure S4 supplemental). Similarly, there was no association between hepatic *CBR1* mRNA expression and CBR activity in samples from white ($r_p$ = 0.325, p = 0.070) and black ($r_p$ = 0.357, p = 0.175) donors (Figure S5 supplemental).

Regression analyses showed a significant negative correlation between maximal CBR activities expressed as the rate of synthesis of doxorubicinol and the donors' age in samples from whites ($r_p$ = -0.439, p = 0.006). Similar analyses in samples from black donors showed no correlation between CBR activity and age ($r_s$ = 0.008, p = 0.715; Figure 4). We also determined whether a relationship existed between age and cytosolic protein yield. Cytosolic protein yield was not significantly associated with age in samples from blacks ($r_p$ = 0.126, p = 0.325) and whites ($r_p$ = 0.231, p = 0.340), respectively.

### *CBR1* genotype-phenotype associations in liver samples from black and white donors

To pinpoint genetic determinants of hepatic CBR1 expression, paired liver DNA samples were genotyped for the non-synonymous SNPs *CBR1* V88I and *CBR1* S131P, and for the 3'-UTR SNP *CBR1* 1096G>A, respectively (Avramopoulos

et al., 1992; Gonzalez-Covarrubias et al., 2007). The variant alleles for *CBR1* V88I (A) and *CBR1* S131P (T) were absent in DNA samples from white and black liver donors. The *CBR1* 1096G>A polymorphism was detected in samples from whites but not in samples from blacks (Table 2). *CBR1* 1096G>A genotype distributions were in Hardy-Weinberg equilibrium ($\chi^2$ test, $p = 0.565$). Genetic surveys in small DNA human diversity panels from the Coriell Institute revealed that the *CBR1* 1096G>A polymorphism appears to be relatively common ($q > 0.12$) among individuals with distinctive geographical ancestries such as Chinese, Japanese and South East Asians (Table 2).

On average, relative *CBR1* mRNA levels tended to be higher in samples from white donors with *CBR1* 1096G>A homozygous G/G genotypes compared to samples from donors with heterozygous G/A genotypes, but the differences between genotype groups did not reach statistical significance (*CBR1* $mRNA_{1096G>A(G/G)}$ = 4.1 ± 4.1 relative fold vs. *CBR1* $mRNA_{1096G>A(G/A)}$ = 3.0 ± 2.5 relative fold, $p = 0.266$; Figure 5). Further analysis showed statistically significant differences in cytosolic CBR1 protein levels between both *CBR1* 1096G>A genotype groups (CBR1 $protein_{1096G>A(G/G)}$ = 10.2 ± 4.7 nmol/g cytosolic protein vs. CBR1 $protein_{1096G>A(G/A)}$ = 6.1. ± 2.8 nmol/g cytosolic protein, $p = 0.030$; Figure 6). In line, liver cytosols with *CBR1* 1096G>A homozygous G/G genotypes showed higher maximal rates of doxorubicinol synthesis (1.5-fold) compared to samples with heterozygous G/A genotypes (CBR $activity_{1096G>A(G/G)}$ = 4.4 ± 2.2 nmol doxol/min.mg vs. CBR $activity_{1096G>A(G/A)}$ = 2.9 ± 1.4 nmol doxol/min.mg, $p = 0.028$; Figure 7).

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The impact of the *CBR1* 1096G>A polymorphism was further investigated in cultures of lymphoblastoid cell lines with known *CBR1* 1096G>A genotype status. *CBR1* mRNA levels in lymphoblastoid cells were expressed relative to the normalized *CBR1* mRNA content of cell lines: GM10857 and GM16688 (*CBR1* 1096G>A G/G genotypes). Cell lines with *CBR1* 1096G>A homozygous G/G genotypes exhibited higher *CBR1* mRNA relative expression levels than cell lines with *CBR1* 1096G>A homozygous A/A genotypes (*CBR1* $mRNA_{1096G>A(G/G)}$ = 1.0 ± 0.1 relative fold vs. *CBR1* $mRNA_{1096G>A(A/A)}$ = 0.7 ± 0.2 relative fold, $p \approx 0.04$; Figure 5). Furthermore, cell lines with *CBR1* 1096G>A homozygous G/G genotypes synthesized 2-fold more doxorubicinol than cell lines with homozygous A/A genotypes (CBR $activity_{1096G>A(G/G)}$ = 44.2 ± 25.4 nmol doxol/min.mg vs. CBR $activity_{1096G>A(A/A)}$ = 22.0 ± 24.0 nmol doxol/min.mg, $p \approx 0.02$; Figure 7).

## Discussion

The reduction of carbonyl groups by hepatic CBR1 activity is an important step during the metabolism of clinically relevant drugs such as haloperidol (antipsychotic), doxorubicin (anticancer), dolasteron (antiemetic), and pentoxyfilline (hemorrheologic) (Rosemond and Walsh, 2004). In the other hand, differences in the average expression of drug metabolizing enzymes between ethnic groups with distinctive geographical ancestries may impact on drug response and toxicity (Wilson et al., 2001; Daar and Singer, 2005; Diczfalusy et al., 2008). Therefore, our first aim was to document the hepatic expression of *CBR1* in a cohort of liver tissue samples from white and black donors. Recent pharmacogenetic studies suggest that in certain cases, discrete population labels based on self-reported ethnicity and/or skin color may be inappropriate surrogates of geographical ancestry due to population admixture (Suarez-Kurtz et al., 2007a; Suarez-Kurtz et al., 2007b). Thus, we sought to examine 176 autosomal genetic markers of geographical ancestry to refine the stratification criterion. Stratification by ethnicity or AIM-determined geographical ancestry did not modify the average expression values of hepatic *CBR1* mRNA, CBR1 protein and CBR activity (Table 1). Therefore, in this group of samples, self-reported ethnicity appears to be an accurate indicator of geographical ancestry.

*CBR1* mRNA and CBR1 protein were detected in all liver samples. On average, *CBR1* mRNA expression varied by 48-fold (Figure 2). Further analysis showed that the hepatic expression of *CBR1* mRNA varied by 13- and 23-fold in samples from black and white donors after the elimination of outlier values located

above the 95$^{th}$ or below the 5$^{th}$ percentiles (blacks, n = 2, and whites, n = 3). Hepatic CBR1 protein expression varied by 7-fold, and CBR1 protein was detected as a single immunoreactive band after electrophoretic separation in 4-10% gradient polyacrylamide gels (Figure 3). Recently, Kassner et al. reported that CBR1 expression in liver cytosols varied by >70-fold. The 10-fold discrepancy between both studies may be due in part to different strategies for CBR1 immunodetection. That is, Kassner et al. performed electrophoretic separations by using 12% polyacrylamide gels, and CBR1 was detected as a triple band after immunoblotting due to the binding of 2-oxocarbonyl acids to lysine 239 (Wermuth et al., 1993; Kassner et al., 2008). We also found that hepatic doxorubicin reductase activity varied by 22-fold in samples from blacks and whites (Figure 4). In this case, the extent of variable CBR activity for the substrate doxorubicin is in agreement with the 22-fold value reported by Kassner et al. (Kassner et al., 2008). We observed a modest although significant correlation between CBR1 activities determined by UV-spectrophotometry vs. direct quantification of doxorubicinol (Figure S5 supplemental). The comparatively low specificity of UV-spectrophotometric methods in complex biological matrices (i.e. liver cytosols) may have impacted on the extent of correlation between both measurements of CBR1 activity (Tietz, 1999).

Recently, we reported that *CBR1* mRNA, CBR1 protein and CBR activity are concomitantly up regulated by strong ligands of the aryl hydrocarbon receptor (AHR) such as β-naphtoflavone and 2,3,7,8-tetrachlorodibenzo-p-dioxin (Lakhman et al., 2007). Here, we found no significant relationships between *CBR1* mRNA, *CBR1* protein and CBR activity levels in liver samples from white and black donors. Based

on these observations, it is possible to hypothesize that the hepatic expression of *CBR1* under “basal conditions” (i.e. absence of AHR ligands) may be regulated by factors that operate at various levels (e.g. transcriptional, and/or post transcriptional), whereas the expression of *CBR1* under “non-basal conditions” (i.e. presence of AHR ligands) would be primarily driven by AHR through coupled transcriptional-translational regulation.

The *CBR1* 1096G>A polymorphism was common in samples from white donors, and we pinpointed a significant association between *CBR1* 1096G>A genotype status and the hepatic expression of CBR1 protein. Furthermore, *CBR1* 1096G>A genotype status in whites was significantly associated with the maximal rates of synthesis of the cardiotoxic C-13 metabolite doxorubicinol (Figure 7). It should be noted that Kassner et al. did not detect associations between *CBR1* 1096G>A genotype status and doxorubicin reductase activity or CBR1 protein expression in 57 liver samples (Kassner et al., 2008). The reasons for this discrepancy are unclear, and highlight the need for confirmatory studies. Nevertheless, it is also interesting to note that the impact of the *CBR1* 1096G>A polymorphism on the maximal rates of synthesis of doxorubicinol was also apparent in cultures of lymphoblastoid cell lines. That is, cell lines with *CBR1* 1096G>A homozygous G/G genotypes synthesized two-fold more doxorubicinol per unit of time as compared to cell lines with homozygous A/A genotypes. This observation is limited by the small number of cell lines analyzed (n = 3 per each genotype combination), but it provides the rationale to test whether peripheral blood

lymphocytes would serve as surrogates to predict the impact of *CBR1* 1096G>A genotype status on variable hepatic CBR activity.

We found that on average, the hepatic expression of *CBR1* mRNA, CBR1 protein and CBR1 activity was similar in samples from black and white donors (Figures 2, 3 and 4). However, these findings need to be interpreted with caution by considering: a) sample size limitations, b) the potential functional impact of *CBR1* 1096G>A, and c) the distribution of the variant *CBR1* 1096 A allele among individuals with distinctive geographical ancestry (Table 2). For example, cytosols from whites with *CBR1* 1096G>A homozygous G/G genotype and the entire group of cytosols from black donors exhibited similar doxorubicinol synthesis rates (CBR activity $1096G>A_{(G/G)\text{-whites}}$ = 4.4 ± 2.2 nmol doxol/min.mg, n = 24 vs. CBR activity $1096G>A_{(G/G)\text{-blacks}}$ = 4.2 ± 2.3 nmol doxol/min.mg, n = 20, p = 0.826). Thus, the survey of larger sample sizes from white (i.e. more samples with the variant allele) and black donors may pinpoint differences in the hepatic expression of *CBR1* between both groups. Interestingly, clinical studies in pediatric cancer survivors treated with anthracyclines identified higher incidence of anthracycline-related cardiotoxicity among black patients (Krischer et al., 1997; Grenier and Lipshultz, 1998). Therefore, it is tempting to speculate that the comparatively higher risk of anthracycline-related cardiotoxicity among black cancer patients may be due in part to the scarcity of the variant allele (A) among individuals with African ancestry..

A pharmacokinetic study in patients receiving concomitant administration of doxorubicin and cyclophosphamide showed a significant negative correlation between age and the clearance of doxorubicin ($r_p$ = -0.46, p = 0.00037) (Li and

Gwilt, 2003). In addition, Joerger et al. detected a negative correlation between the clearance of doxorubicin and age by using a physiologically based pharmacokinetic model. The authors concluded that a 10-year increase in patient age led to a 9% decrease in doxorubicin clearance (Li and Gwilt, 2003; Joerger et al., 2007). In this study, we detected a significant negative correlation between age and maximal CBR activity in samples from whites ($r_p$ = -0.439, p = 0.006). Regression analysis without stratification by *CBR1* 1096G>A genotype demonstrated that age would account for approximately 16% of the variation in doxorubicin reductase activity (Figure 4). Further analysis after stratification by *CBR1* 1096G>A genotype status showed that maximal CBR activity was significantly associated with age only in the group of samples with *CBR1* 1096G>A homozygous G/G genotypes ($r_p$ = -0.505, p = 0.014; Figure S6 supplemental). Thus, age would account for 25% of the variation in doxorubicin reductase activity among subjects with homozygous G/G genotypes. In this group, samples from donors 1 - 25 years old showed 1.9-fold higher maximal rates of doxorubicinol synthesis compared to those from donors older than 55 years ($CBR_{1\text{-}25\ years\ old}$= 6.7 ± 1.5 nmol doxol/min.mg, n = 5 vs. $CBR1_{>55\ years}$= 3.0 ± 3.0 nmol doxol/min.mg, n = 5, p = 0.039). Age did not correlate with doxorubicinol synthesis rates in the group of samples with heterozygous G/A genotypes ($r_p$ = 0.054, p = 0.410; Figure S6 supplemental). These findings are intriguing and highlight the potential functional impact of the *CBR1* 1096G>A polymorphism. Recent studies on genes of pharmacogenetic relevance indicate that certain 3'-UTR polymorphisms affect the binding of specific microRNAs (miRNA) and are associated with differential gene expression (Mishra et al., 2007; Gow et al., 2008).

In consequence, we performed bioinformatics searches by using the PolymiRTS and miRBase databases to test whether the polymorphic *CBR1* 3'-UTR sequence constitutes a potential target for miRNA. The searches revealed that the presence of the A allele at the polymorphic position (5'-ACTAAT**A**TACTAC-3') creates a potential binding site for miR-656 (Glazov et al., 2008). Thus, we hypothesize that *CBR1* 1096G>A regulates the steady-state concentrations of hepatic *CBR1* mRNA levels through the binding of specific miRNA species such as miR-656 (He and Hannon, 2004).

Anthracycline C-13 alcohol metabolites circulate in plasma, and are devoid of significant tumor cell killing activity (Forrest and Gonzalez, 2000). Therefore, it will be of clinical relevance to explore whether *CBR1* 1096G>A genotype status influence: a) the therapeutic efficacy of anthracycline drugs, and b) the risk of anthracycline-related cardiotoxicity. Our studies support the notion that information on specific genetic determinants of variable CBR activity may assist to optimize anticancer therapy with anthracycline drugs.

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## Footnotes

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## Figure Legends

### Figure 1

AIM scores in samples from white (n= 49; panel A) and black liver donors (n= 27; panel B). Boxes indicate the $5^{th}$ and $95^{th}$ percentiles, horizontal bars indicate medians, and whiskers indicate the range after excluding outliers (circles).

### Figure 2

Hepatic *CBR1* mRNA expression in samples from white (n = 42) and black donors (n = 23). Relative *CBR1* mRNA levels were determined with the comparative quantitation method (see details in the text). Individual *β-actin* mRNA levels were used as normalizers. Samples and standards for calibration curves ($r^2$>0.94) were analyzed in quadruplicates. Each circle depicts the average of individual samples. Horizontal lines indicate group means (p = 0.217).

### Figure 3

Hepatic CBR1 protein expression in samples from white (n = 28) and black donors (n = 28; panel A). Each circle represents individual liver samples. Horizontal lines indicate group means (p = 0.347). Immunodetection of hepatic CBR1 and β-actin in human liver cytosols (panel B). Immunodetection of recombinant CBR1 standards (rCBR1) from a typical calibration curve (panel C).

**Figure 4**

Maximal CBR activities with the substrate doxorubicin in liver cytosols from white (n = 40) and black (n = 20) donors (panel A). Each sample was analyzed in duplicates. Each circle depicts the average of individual samples. Group means are indicated by horizontal lines (p = 0.610). Linear regression analyzes of age vs. maximal CBR activity in samples from white (panel B) and black liver donors (panel C).

**Figure 5**

Impact of *CBR1* 1096G>A genotype on the hepatic expression of *CBR1* mRNA (panel A). Each circle represents individual liver samples. Horizontal lines indicate group means (p = 0.266). Impact of *CBR1* 1096G>A genotype on the expression of *CBR1* mRNA in cultures of lymphoblastoid cell lines (panel B). Each circle represents the average of quadruplicate measurements of relative *CBR1* mRNA levels in individual cell lines ($p \approx 0.04$ for G/G vs. A/A).

**Figure 6**

Impact of *CBR1* 1096G>A genotype on hepatic CBR1 protein expression. Each circle represents individual liver samples. Horizontal lines indicate group means (p = 0.030).

**Figure 7**

Impact of *CBR1* 1096G>A genotype on maximal cytosolic CBR activities for the substrate doxorubicin (p = 0.028; panel A). Impact of *CBR1* 1096G>A genotype on

maximal cytosolic CBR activities in cultures of lymphoblastoid cell lines (panel B). Each circle represents the average of duplicate measurements of maximal CBR activities in individual cell lines ($p \approx 0.02$ for G/G vs. A/A).

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**Table 1.** Hepatic *CBR1* expression in samples stratified by ethnicity and AIM-determined geographical ancestry. Number of samples is indicated in parentheses.

| | Ethnicity | | AIM-determined geographical ancestry | | *P* (Black vs. Sub-S. African) | *P* (White vs. European) |
|---|---|---|---|---|---|---|
| | Black | White | Sub-S. African | European | | |
| *CBR1* mRNA (relative fold) | 4.8 ± 4.3 (23) | 3.6 ± 3.6 (30) | 4.8 ± 4.3 (23) | 3.9 ± 3.8 (33) | 1.000 | 0.728 |
| CBR1 protein (nmol/g cytosolic protein) | 8.0 ± 3.4 (28) | 9.0 ± 4.6 (28) | 8.1 ± 3.5 (25) | 9.9 ± 4.8 (20) | 0.897 | 0.522 |
| Maximal CBR activity (nmol doxol/min.mg) | 4.2 ± 2.3 (20) | 3.9 ± 2.1 (40) | 4.2 ± 2.3 (20) | 3.9 ± 2.2 (29) | 1.000 | 0.991 |

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**Table 2.** *CBR1* 1096G>A genotype distributions in liver donors and human DNA diversity panels.

| Population | N[a] | G/G | G/A | A/A | *p*[b] | *q* |
|---|---|---|---|---|---|---|
| Black liver donors | 32 | 32 | - | - | 1.000 | - |
| White liver donors | 64 | 48 | 16 | - | 0.875 | 0.125 |
| US African ancestry* | 69 | 67 | 2 | - | 0.990 | 0.010 |
| US European ancestry* | 50 | 38 | 11 | 1 | 0.870 | 0.130 |
| Indo-Pakistani* | 9 | 7 | 2 | - | 0.889 | 0.111 |
| Middle Eastern* | 10 | 2 | 8 | - | 0.900 | 0.100 |
| Africans north of the Sahara* | 7 | 6 | 1 | - | 0.929 | 0.071 |
| Africans south of the Sahara* | 9 | 9 | - | - | 1.000 | - |
| Chinese* | 10 | 6 | 3 | 1 | 0.750 | 0.250 |
| Japanese* | 10 | 3 | 6 | 1 | 0.600 | 0.400 |
| Mexican* | 10 | 9 | 1 | - | 0.950 | 0.050 |
| Asia Pacific* | 7 | 3 | 3 | 1 | 0.642 | 0.357 |
| South American Andes* | 10 | 6 | 3 | 1 | 0.750 | 0.250 |
| South East Asia* | 10 | 4 | 6 | - | 0.800 | 0.200 |

[a]N, number of DNA samples. [b]*p*, denotes the G allele, and *q* denotes the A allele. *DNA human diversity panels from the Coriell Institute.

# Figure 1

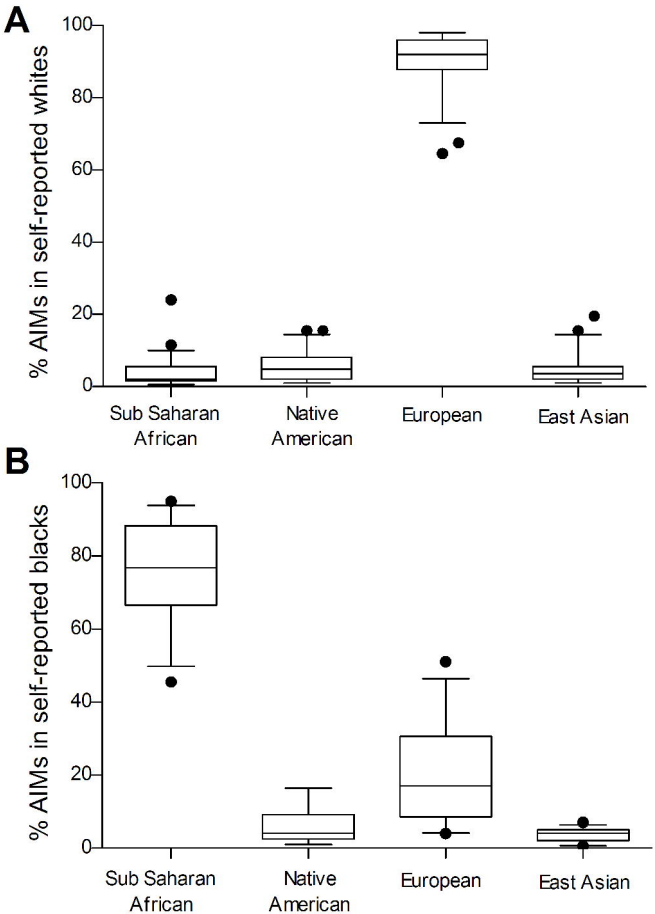

A
% AIMs in self-reported whites
100
80
60
40
20
0
Sub Saharan African
Native American
European
East Asian
B
% AIMs in self-reported blacks
100
80
60
40
20
0
Sub Saharan African
Native American
European
East Asian

**Figure 2**

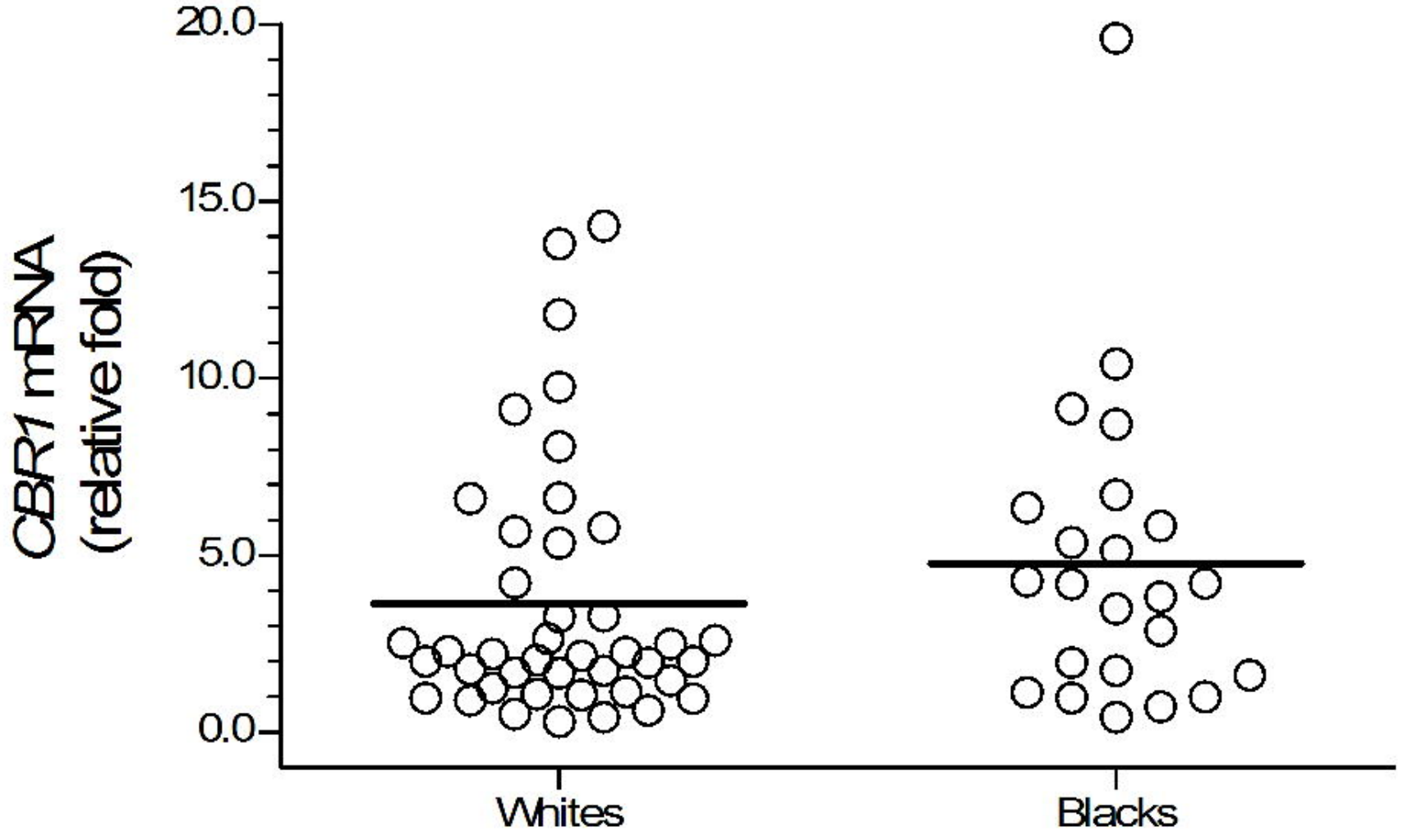


**Figure 3**

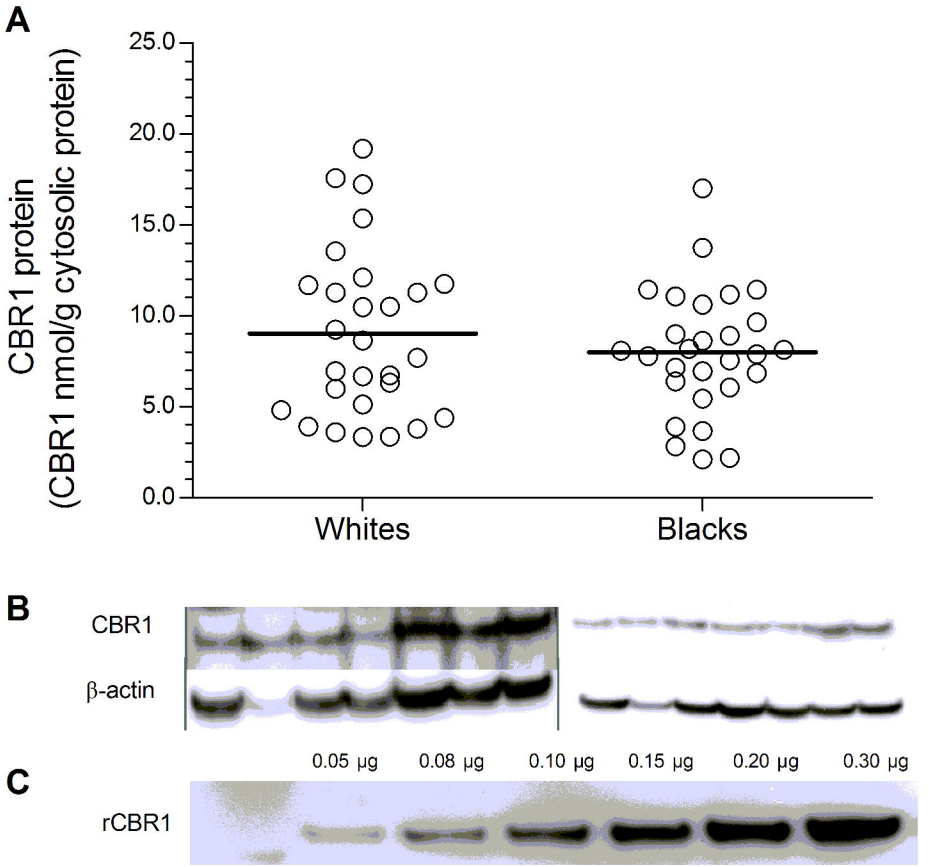

A
CBR1 protein
(CBR1 nmol/g cytosolic protein)
25.0
20.0
15.0
10.0
5.0
0.0
Whites
Blacks
B
CBR1
β-actin
C
0.05 µg
0.08 µg
0.10 µg
0.15 µg
0.20 µg
0.30 µg
rCBR1

**Figure 4**

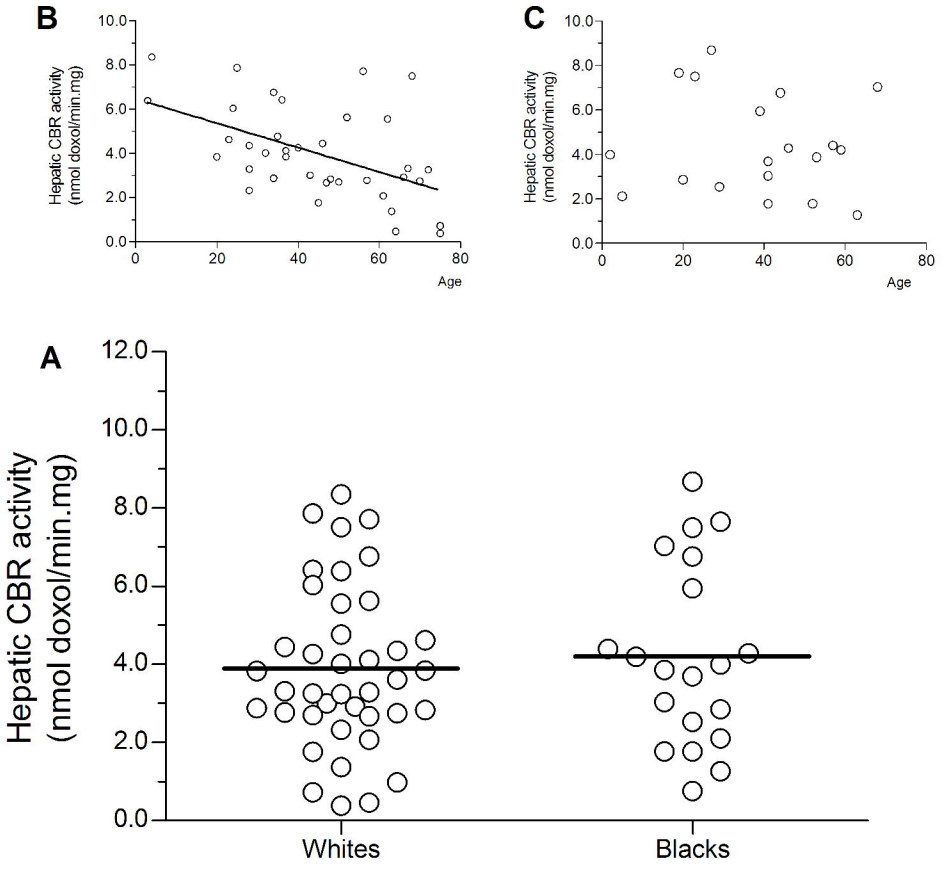

B
Hepatic CBR activity (nmol doxol/min.mg)
10.0
8.0
6.0
4.0
2.0
0.0
0
20
40
60
80
Age
C
Hepatic CBR activity (nmol doxol/min.mg)
10.0
8.0
6.0
4.0
2.0
0.0
0
20
40
60
80
Age
A
Hepatic CBR activity (nmol doxol/min.mg)
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Whites
Blacks

Figure 5

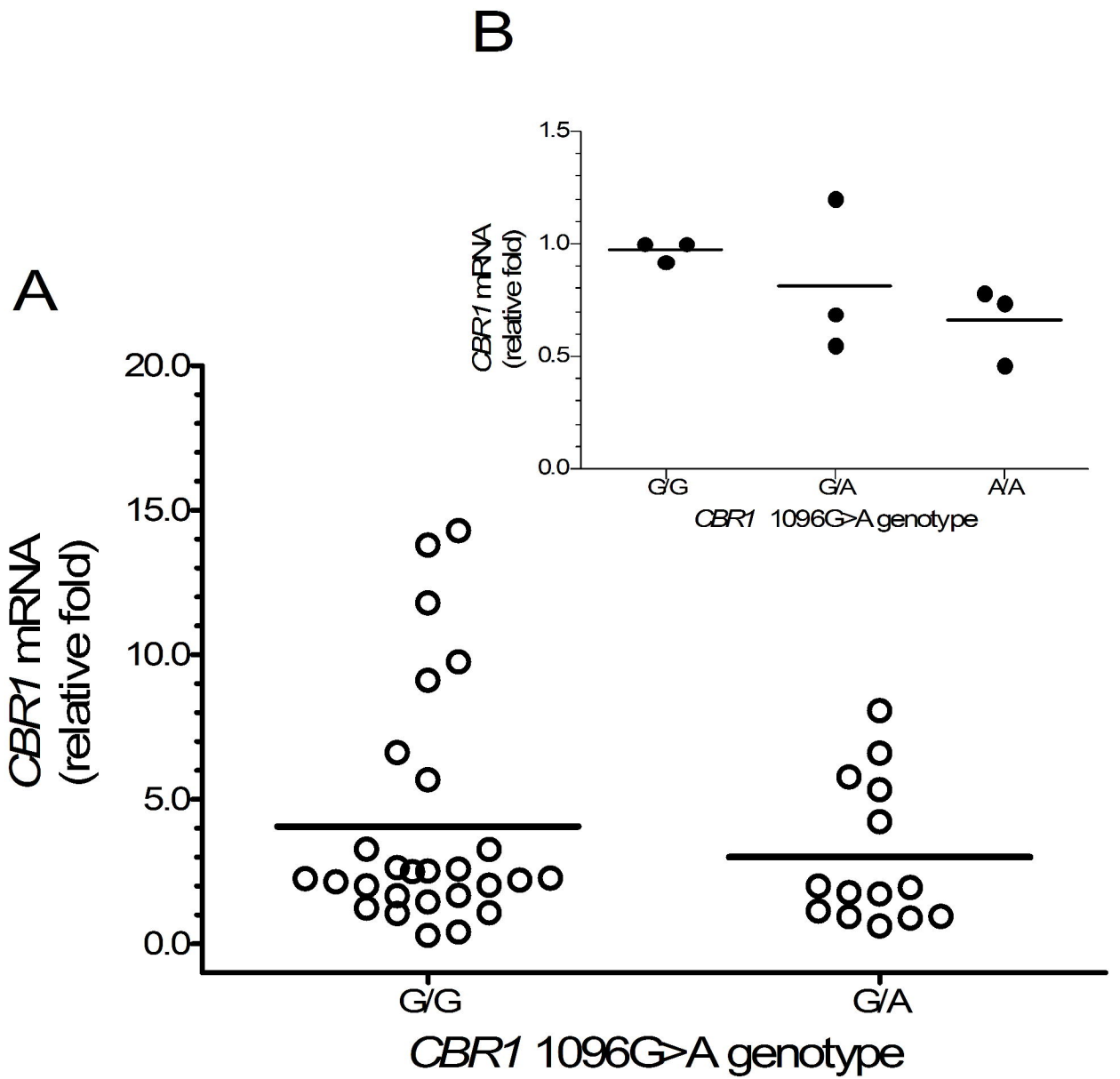

B
1.5
1.0
0.5
0.0
CBR1 mRNA (relative fold)
G/G
G/A
A/A
CBR1 1096G>A genotype
A
20.0
15.0
10.0
5.0
0.0
CBR1 mRNA (relative fold)
G/G
G/A
CBR1 1096G>A genotype

**Figure 6**

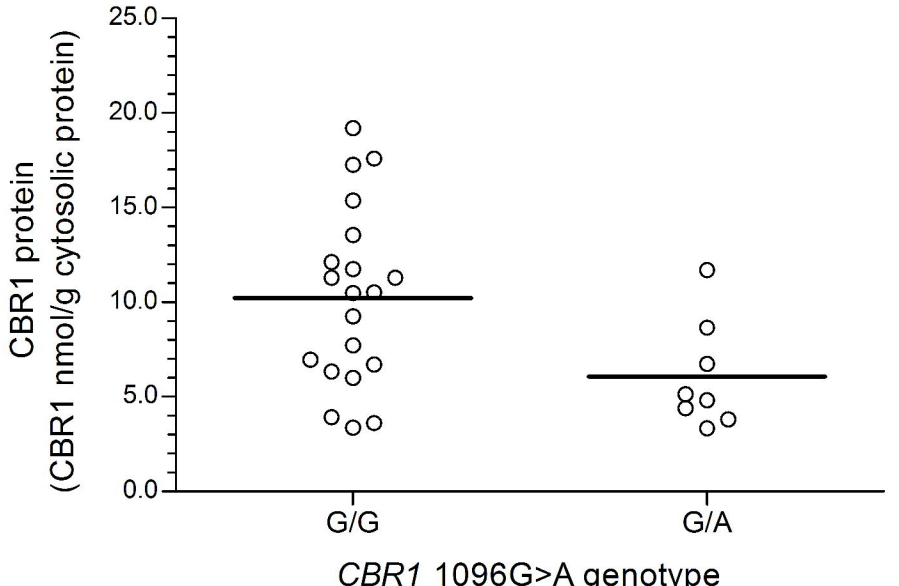
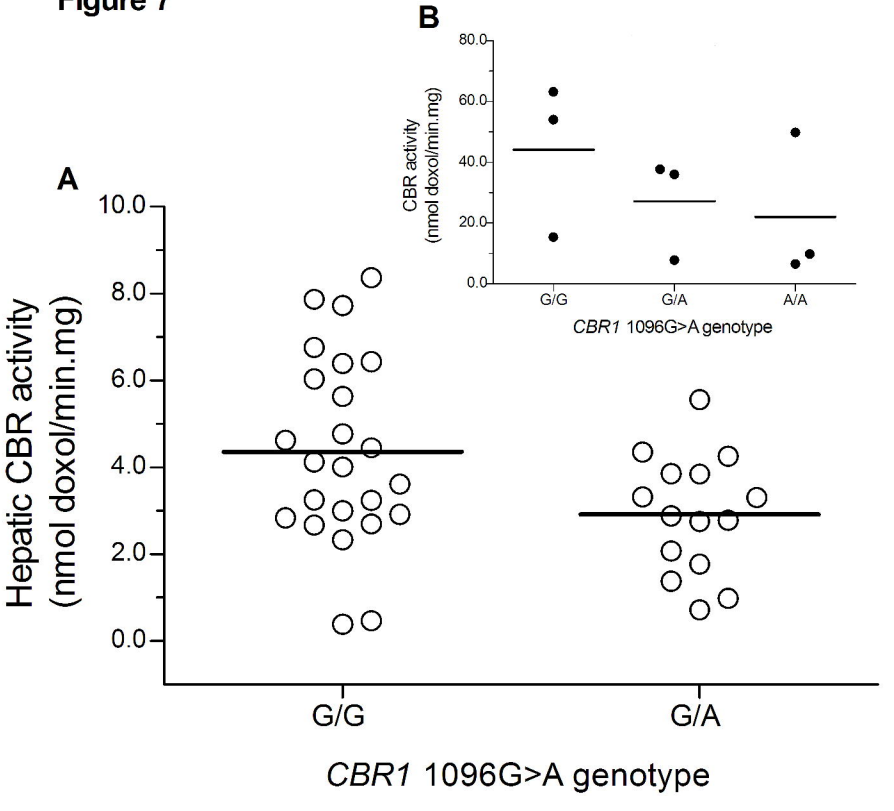

25.0
20.0
15.0
10.0
5.0
0.0
CBR1 protein
(CBR1 nmol/g cytosolic protein)
G/G
G/A
*CBR1* 1096G>A genotype

**Figure 7**


A
Hepatic CBR activity
(nmol doxol/min.mg)
10.0
8.0
6.0
4.0
2.0
0.0
G/G
G/A
CBR1 1096G>A genotype
B
CBR activity
(nmol doxol/min.mg)
80.0
60.0
40.0
20.0
0.0
G/G
G/A
A/A
CBR1 1096G>A genotype