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Interindividual Regulation of the BCRP/*ABCG2* Transporter in Term Human Placentas

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Number of text pages: 40

Number of tables: 10

Number of figures: 8

Number of references: 40

Number of words in Abstract: 250

Number of words in Introduction: 640

Number of words in Discussion: 1214

Abbreviations (alphabetical order):

5' UTR, 5' untranslated region; *ABCG2,* ATP-binding cassette subfamily G member 2; AF, allele frequency; ARE, antioxidant response element; AHR, aryl hydrocarbon receptor; BCRP, breast cancer resistance protein; CYP, cytochrome P450; DRE, dioxin response element; EGFR, epidermal growth factor receptor; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; ERE, estrogen response element; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HIF1α, hypoxia inducible factor 1 alpha; HRE, hypoxia response element; NRF2, nuclear factor erythroid 2-related factor 2; PPARγ, peroxisome proliferator-activated receptor gamma; PPRE, peroxisome proliferator response element; PBS/T, PBS with 0.5% Tween-20; PR, progesterone receptor; RPL13A, ribosomal protein L13A; SNP, single nucleotide polymorphism; SP1, SP1 transcription factor; SP3, SP3 transcription factor; TSS, transcriptional start site

Abstract

The breast cancer resistance protein (BCRP/*ABCG2*) is a maternally-facing efflux transporter that regulates the placental disposition of chemicals. Transcription factors and gene variants are important regulatory factors that influence transporter expression. Here, we sought to identify the genetic and transcriptional mechanisms underlying the interindividual expression of BCRP mRNA and protein across 137 term placentas from uncomplicated pregnancies. Placental expression of BCRP and regulatory transcription factor mRNAs was measured using multiplex branched DNA analysis. BCRP expression and *ABCG2* genotypes were determined using western blot and Fluidigm Biomark genetic analysis, respectively. Placentas were obtained from a racially and ethnically diverse population including Caucasian (33%), African American (14%), Asian (14%), Hispanic (15%) and mixed (16%) backgrounds as well as unknown origins (7%). Between placentas, BCRP mRNA and protein varied up to 47-fold and 14-fold, respectively. In particular, BCRP mRNA correlated significantly with known transcription factor mRNAs including NRF2 and AHR. Somewhat surprisingly, single nucleotide polymorphisms (SNPs) in the *ABCG2* non-coding regions were not associated with variation in placental BCRP mRNA or protein. Instead, the coding region polymorphism (C421A/Q141K) corresponded with 40-50% lower BCRP protein in 421C/A and 421A/A placentas compared to wild-types (421C/C). While BCRP protein and mRNA expression weakly correlated (r=0.25, p=0.040), this relationship was absent in individuals expressing the C421A variant allele. Study results contribute to our understanding of the interindividual regulation of BCRP expression in term placentas and may help to identify infants at risk for increased fetal exposure to chemicals due to low expression of this efflux protein.

Introduction

In the placenta, the breast cancer resistance protein (BCRP/*ABCG2*) efflux transporter plays an important role in limiting fetal exposure to chemicals including carcinogens, chemotherapy drugs, endogenous compounds, flavonoids, antibiotics, and antidiabetic drugs (Burger et al., 2004; Imai et al., 2004; Merino et al., 2005; Gedeon et al., 2006; van Herwaarden et al., 2006; Nakayama et al., 2011). BCRP is primarily localized to the apical membrane of syncytiotrophoblasts as well as fetal capillary endothelial cells. In syncytiotrophoblasts, BCRP actively transfers compounds out of the placenta and back to the maternal circulation (Maliepaard et al., 2001; Jonker et al., 2002; Szilagyi et al., 2017). Due to the critical role for BCRP in protecting the fetus from exposure to xenobiotics, it is important to identify regulators of constitutive BCRP expression in placenta including transcription factors and single nucleotide polymorphisms (SNPs).

BCRP/*ABCG2* expression can be regulated by various transcription factors, nuclear receptors, and steroid hormone receptors (Ee et al., 2004; Krishnamurthy et al., 2004; Tompkins et al., 2010; Basseville et al., 2014) (Figure 1). The promoter region of the *ABCG2* gene is well-characterized and contains response elements for transcriptional regulators including the aryl hydrocarbon receptor (AHR), (Tompkins et al., 2010), estrogen receptor alpha (ERα/ESR1) and beta (ERβ/ESR2), (Ee et al., 2004), hypoxia inducible factor alpha (HIF1α) (Krishnamurthy et al., 2004), nuclear factor erythroid 2-related factor 2 (NRF2) (Singh et al., 2010), peroxisome proliferator-activated receptor gamma (PPARγ) (Szatmari et al., 2006), progesterone receptor (PR) (Wang et al., 2008), SP1 transcription factor (SP1) (Bailey-Dell et al., 2001), and SP3 transcription factor (SP3) (Yang et al., 2013). Despite the identification of functional regulatory

elements, the relationship between these transcription factors and BCRP in term human placentas has largely been unexplored.

A number of SNPs have been identified in the *ABCG2* gene (chromosome 4q22, *ABCG2,* Figure 2) (Zamber et al., 2003; de Jong et al., 2004; Kobayashi et al., 2005; Poonkuzhali et al., 2008). The most well-characterized genetic variants are localized to the coding region of the *ABCG2* gene and result in amino acid changes in the subsequent BCRP protein (i.e., G34A→V12M and C421A→Q141K) (Zamber et al., 2003; de Jong et al., 2004; Kobayashi et al., 2005). The C421A variant is often observed in Asian (allele frequency, AF: 0.35) and Caucasian (AF: 0.10) populations (Zamber et al., 2003; Kobayashi et al., 2005). Importantly, the C421A variant has been associated with the altered pharmacokinetics and pharmacodynamics of drugs in patients that express one or two variant alleles, likely due to reduced BCRP function in the intestine, liver and/or kidneys. For example, lung cancer patients with one variant allele (421C/A) have a 3.7-fold greater risk of developing severe diarrhea, a toxic side effect of the BCRP substrate and chemotherapeutic drug gefitinib (Cusatis et al., 2006). Furthermore, healthy volunteers that were homozygous for the variant (421A/A) exhibited significantly greater area-under-the-curve values and maximal concentrations for the hypolipidemic drug rosuvastatin following a 20 mg oral dose as compared to those with no (421C/C) or one mutant allele (421C/A) (Keskitalo et al., 2009). Due to these data and additional reports, the International Transporter Consortium recommended that the C421A polymorphism be considered in clinical drug development (Giacomini et al., 2013).

Genetic variants that occur in the non-coding region of the *ABCG2* gene have begun to emerge as regulators of BCRP expression and are being evaluated for their clinical relevance. SNPs in the 5'-untranslated region and intronic regions of the *ABCG2* gene have been associated with variation in BCRP mRNA expression in intestinal, liver, and lymphoblast samples (Poonkuzhali et al., 2008). Importantly, the effect of non-coding variants on BCRP expression and function in the placenta warrants investigation. Therefore, the purpose of this study was to identify factors, including infant sex, ethnicity/race, transcription factor relationships and genetic variants, that contribute to the interindividual expression of BCRP mRNA and protein expression in term placentas from uncomplicated pregnancies.

Materials and Methods

Placenta Donor Selection

One hundred and thirty-seven placentas were obtained by written informed consent from women with uncomplicated pregnancies across two institutions (Robert Wood Johnson University Hospital, New Brunswick, NJ N=108 and the University of California, San Francisco, CA N=29). Participant inclusion criteria for this study were healthy women, ages 18-40, term gestation, scheduled Cesarean sections, and vaginal deliveries (University of California only). Exclusion criteria were pregnancy-induced medical conditions (i.e., pregnancy-induced hypertension, preeclampsia, gestational diabetes), chronic medical disorders (i.e., hypertension, diabetes, autoimmune diseases), maternal infection, clinical chorioamnionitis, medication use (except for prenatal vitamins), maternal alcohol or drug use, and known fetal chromosomal abnormalities. Placenta donor demographic information is listed in Table 1. Ethnicity and race were self-reported. Where possible, infant ethnicity and race were obtained from both maternal and paternal reporting. In the absence of paternal information, the ethnicity and race of the mother were used. This study was approved by the Institutional Review Boards of Robert Wood Johnson Medical School (Protocol #0220100258), Rutgers University (Protocols #E12-024 and #E14-325), and the University of California San Francisco (Protocol #10-00505).

Sample Collection

Placentas were collected within 10 min of delivery for processing within 1 h. Visible abnormalities and the location of the umbilical cord were assessed and only normal placentas with central or eccentric cord insertions were used. A small piece of umbilical cord close to the placenta was placed in a PAXgene Tissue Container (Qiagen, Germantown, MD) in the

PAXgene Tissue Fix for 4 h at 4°C, moved to PAXgene Tissue Stabilizer and stored at -80°C for *ABCG2* SNP genotyping. To sample placental tissue, the overlying membranes, maternal decidua, and chorionic plate were removed. From the maternal side of the placenta, two pieces of chorionic tissue were collected along the long axis approximately 1 cm distal to the cord insertion site as described (Memon et al., 2014). It was previously shown that BCRP expression does not change across the placental disc (Memon et al., 2014). The dimensions of each sample were approximately 2 cm x 1 cm x 0.5 cm. After rinsing three times with sterile PBS to remove maternal blood, each sample was further dissected into two sub-segments of equal size for RNA and protein analysis. Tissues were placed in PAXgene Tissue Containers and processed as described above. Samples were stored at -80°C until further analysis. For assessment of protein expression, samples were snap frozen in liquid nitrogen and stored at -80°C.

Single Nucleotide Polymorphism Genotyping

Umbilical cord samples were homogenized and total DNA was isolated with the PAXgene Tissue DNA Kit (Qiagen). A DropSense96 UV/Vis droplet reader was used to quantify total DNA and confirm the integrity of the DNA (Trinean, Gentbrugge, Belgium). The Fluidigm BioMark Genetic Analysis system was used to genotype 20 *ABCG2* gene SNPs (Figure 2) in the Bionomics Research and Technology Center at Rutgers University. Primer sequences used for *ABCG2* SNP analysis are provided in Supplemental Tables 1-4.

RNA Isolation and Multiplex Branched DNA Assay

PAXgene stabilized placental tissues were homogenized using a TissueLyser (2 min, 50 Hz; Qiagen) in TR1 buffer provided in the PAXgene Tissue RNA Kit (Qiagen) plus 1% β-

mercaptoethanol. Total RNA was isolated with the PAXgene Tissue RNA Kit (Qiagen) according to the manufacturer's instruction. Concentration of total RNA was determined using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and integrity was confirmed using a 2100 Bioanalyzer instrument (Agilent, Santa Clara, CA). A multiplex branched DNA signal amplification assay (QuantiGene Assay, Affymetrix, Santa Clara, CA) and a Bio-Plex MAGPIX multiplex reader (Bio-Rad, Hercules, CA) were used to quantify mRNA expression of BCRP, AHR, ERα, ERβ, HIF1α, NRF2, PPARγ, PR, SP1 and SP3. The number of target RNA molecules detected in each sample was proportional to the assay read out, median fluorescence intensity (MFI). Data were normalized to the geometric mean of the MFI for two reference genes that were previously determined to have low correlation with placental BCRP mRNA expression, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ribosomal protein L13A (RPL13A) (Memon et al., 2014). Probe information is provided in Supplemental Table 5.

Western Blot

Samples were processed for western blot analysis as previously described (Memon et al., 2014). Briefly, frozen placenta samples were homogenized in a sucrose (250 mM)-Tris base (10 mM) buffer (pH 7.5) with protease inhibitors (1%, Sigma Aldrich, St. Louis, MO). Following centrifugation (100,000 x *g*) for 1 h at 4°C, pellets containing crude membrane fractions were resuspended in sucrose-Tris base buffer with protease inhibitors. Protein concentrations were measured by a bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL). Western blot analysis was performed by adding 10 μg total protein to polyacrylamide 4-12% Bis-Tris gels (Life Technologies, Carlsbad, CA) that were resolved by electrophoresis. Gels were carefully trimmed as previously described (Kiyatkin and Aksamitiene, 2009) and the same size proteins

were transferred to polyvinylidene fluoride membranes in overnight transfer apparatuses onto the same membrane (Bio-Rad Criterion Blotter, Bio-Rad, Hercules, CA). This approach allowed protein samples from all placentas to be directly compared on the same transblotted membrane. This procedure was performed twice for each of the two sampling sites within the placentas. Membranes including all the samples were blocked in 5% non-fat dairy milk in PBS with 0.5% Tween-20 (PBS/T) for 1 h. Primary antibodies (BCRP, BXP-53, 1:5000, Enzo Life Scientific, Farmingdale, NY; Beta-ACTIN (β-ACTIN), Ab8227, 1:2000, Abcam, Cambridge, MA) were diluted in 2% non-fat milk in 0.5% PBS/T and incubated with the membranes overnight at 4°C. After washing with PBS/T, HRP-conjugated secondary antibodies were added to the blots for 1 h. Supersignal West Dura Extended Duration Substrate (Pierce Biotechnology) was used for chemiluminescent detection of proteins using a Fluorchem Imager (ProteinSimple, Sanata Clara, CA). Band densities were semiquantitated using the AlphaView Software (ProteinSimple). BCRP band density for each sample was normalized to its respective β–ACTIN (loading control) band density.

Statistical Analysis

Separate linear regression models were used to compare both relative BCRP mRNA expression and relative BCRP protein expression to race and ethnicity, infant sex, placenta weight, infant weight, transcription factor expression (BCRP mRNA expression only), and SNPs (using an additive model). For relative BCRP mRNA expression, these linear regression models also included a fixed effect of collection site to account for overall differences in expression between collection sites. Adjusted relative BCRP mRNA expression values were used for figures and were calculated from the linear regression model that included collection site. Because BCRP

protein expression values were calculated only at one site, no adjustment was needed. Effects are reported as the parameter estimate (b) from the regression model, i.e., the relative change in BCRP mRNA or protein expression associated with a 1 unit change in the predictor variable. The comparison between protein expression and BCRP mRNA expression used a Pearson correlation coefficient and only included samples from one site. To compare transcription factor expression to one another, a pairwise Pearson correlation analysis was employed. All statistical analyses were carried out using the R statistical package (Version 3.4.1). Statistical significance was set to $p<0.05$. P-values were adjusted using a Bonferroni correction to account for multiple comparisons.

Results

Interindividual Expression of BCRP in Term Human Placentas. From the lowest to the highest expressing placenta, BCRP mRNA and protein expression varied up to 47-fold (N=137) and 14-fold (N=70), respectively (Figure 3A and B). There was no association between infant ethnicity/race and either BCRP mRNA or protein (Figure 3A and 3B). Interestingly, BCRP protein expression demonstrated a weak, but significant, correlation with BCRP mRNA expression (r=0.25, *P*=0.040) (Figure 4). There were no significant differences in BCRP mRNA or protein between term placentas from female and male infants (Figure 5A and B). Additionally, neither infant weight nor placental weight correlated with BCRP mRNA expression (Supplemental Figure 1).

Correlation of Transcription Factor Expression with BCRP Expression. The mRNAs of 9 transcription factors known to regulate BCRP expression (AHR, ERα, ERβ, HIF1α, NRF2, PPARγ, PR, SP1, SP3) were quantified in human term placentas (N=137). Univariate regression analysis revealed that BCRP mRNA correlated most closely to NRF2 (b = 0.85; *P*<0.0001) and AHR (b = 0.29; *P*<0.0001) mRNAs (Table 2). Furthermore, the mRNA expression of multiple transcription factors correlated well with each other, notably NRF2 with SP1 (r=0.74; *P*<0.0001) and AHR with SP3 (r=0.73; *P*<0.0001) (Supplemental Table 6, Supplemental Figure 2).

Assessment of ABCG2 Genetic Variants as Contributors to Variation in Placental BCRP Expression. Twenty SNPs in the non-coding and coding regions of the *ABCG2* gene were assessed in 137 human term placentas (Figure 2). While allele frequencies varied by SNP, non-coding region SNPs occurred more frequently than those in the exonic regions (Table 3). Most of

the genetic variants located in the coding region of the *ABCG2* gene occurred at low frequencies (G41A, C369T, C376T; Table 3), such that statistical analysis in relation to BCRP mRNA or protein expression could not be performed.

Overall, individuals heterozygous for the A61562C variant in the 5' untranslated region (5'UTR) tended to exhibit reduced BCRP mRNA expression (Table 4), though this was not statistically significant. Interestingly, there were no individuals homozygous for the A61562C SNP. No other associations between SNPs and BCRP mRNA expression were observed.

In a representative population of samples from a single collection site (N=70), analysis of BCRP protein expression revealed a negative trend for two SNPs in non-coding regions, C72144T (5'UTR) and C93624A (Intron 1) (Table 5). The C421A SNP located in exon 5 occurred at 11% of the overall sample population but was most frequent in Asian and Hispanic populations occurring at 32% and 20%, respectively. While the C421A variant was not associated with altered BCRP mRNA expression (Table 4, Figure 6), at the protein level, the C421A genotype had a significant effect on BCRP protein expression. Importantly, individuals that were homozygous for the variant (421A/A) had 50% lower BCRP expression in placentas as compared to the wild-type genotype (421C/C) (Table 5, Figure 6). Of note, heterozygous individuals (421C/A) similarly exhibited 40% lower BCRP protein expression. Finally, BCRP mRNA and protein expression correlated in 421C/C individuals (r=0.29, *P*=0.032). This relationship was not observed in placentas expressing one or two variant alleles (421C/A r=0.13 and *P*=0.75, 421A/A r=0.16 and *P*=0.84) (Figure 6C).

Discussion

This study characterized interindividual differences in the mRNA and protein expression of the BCRP transporter in term placentas from ethnically and racially diverse pregnant women with uncomplicated pregnancies. There were no differences in BCRP mRNA or protein expression according to infant sex or ethnicity/race. Additionally, expression of BCRP mRNA weakly correlated with protein levels in a representative population of placentas (N=70). BCRP mRNA expression correlated to the greatest extent with the mRNA expression of two transcription factors, NRF2 and AHR. Overall, SNPs in the non-coding regions of the *ABCG2* gene were not associated with changes in BCRP mRNA or protein expression. However, there was up to a 50% reduction in BCRP protein in term placentas expressing a common SNP located in the 5th exon (C421A). Taken together, these data suggest that the C421A genetic variant has the greatest influence on placental BCRP protein expression.

We observed no significant differences in BCRP mRNA or protein expression based on infant sexes (Figure 5). In contrast, other laboratories have demonstrated that BCRP is more highly expressed in the intestines and livers of adult females compared males (Zamber et al., 2003; Prasad et al., 2013). Differences may be due to variation in the expression of BCRP regulatory factors across tissues or during different developmental ages.

Of the transcription factors assessed, AHR and NRF2 mRNAs exhibited the greatest associations with BCRP mRNA (Table 2). This is similar to the relationships reported between mouse placental Ahr and Bcrp mRNAs ($r=0.78$, $p<0.01$) compared to other screened transcription factor mRNAs (Hif1α, Erα, Erβ, Pr) (Wang et al., 2006). However, in human placenta cell culture, two

studies have reported conflicting findings regarding the regulation of BCRP by the AHR signaling pathway. The prototypical AHR activator 3-methylcholanthrene (24 h) significantly enhanced the mRNA expression of AHR target gene CYP1A1 without changing BCRP levels in human term placental trophoblasts (Stejskalova et al., 2011). By comparison, the AHR antagonist CH223191 prevented a maximal 10-fold up-regulation of BCRP mRNA by opioid receptor agonists methadone, buprenophrine, and norbuprenorphine in placenta choriocarcinoma cells (BeWo and JEG3) and primary human villous trophoblasts (Neradugomma et al., 2017). Based on these findings, additional mechanistic investigations are needed to demonstrate a causal relationship between AHR and BCRP expression in term placentas.

The relationship between the NRF2 transcription factor and BCRP has not been explored in the placenta. Typically, NRF2 localizes to antioxidant response elements (AREs) in the promoter region of genes involved in detoxification pathways including NAD(P)H quinone oxidoreductase 1 (NQO1). In rat brain capillaries, Bcrp protein and function were significantly increased following exposure to the prototypical NRF2 activator sulforaphane (Wang et al., 2014). Moreover, Singh et al. reported NRF2 directly interacts with an ARE in the promoter of the *ABCG2* gene and observed a down-regulation of BCRP mRNA in human lung cancer cells in response to the genetic knockdown of NRF2 with a targeted short hairpin RNA (Singh et al., 2010). In human choriocarcinoma JEG3 cells, NRF2 is expressed and upon treatment with acetaminophen (48 h) resulted in an approximate 40% increase in NQO1 gene expression, however BCRP mRNA expression was not assessed (Blazquez et al., 2014). A detailed investigation of the relationship between human placental NRF2 and BCRP may provide

mechanistic information regarding the fetoprotective pathways of the placenta during healthy conditions and in the presence of oxidative and cellular stress.

While BCRP mRNA expression correlated with AHR and NRF2 mRNA expression in term placentas, it is unclear whether this relationship is established early in pregnancy. There are conflicting data regarding the expression of BCRP across gestation as three independent research groups reported a reduction (mRNA and protein, (Meyer zu Schwabedissen et al., 2006), no change (protein and mRNA, (Mathias et al., 2005), and an increase (only protein, (Yeboah et al., 2006)) in BCRP expression with advancing gestational age. Future studies should assess the mRNA and protein expression of BCRP, AHR, and NRF2 of first, second, and third trimester placentas to better characterize the relationship between BCRP and the two transcription factors during placental development.

In a prior study, non-coding SNPs in the *ABCG2* gene correlated with high (A61562C, G94112A) or low (G46932C, C61785T, C78551T) BCRP mRNA expression in liver, intestine, and lymphoblasts (Poonkuzhali et al., 2008). The proposed underlying mechanisms for differential BCRP mRNA expression included changes in the binding of specific transcription factors (promoter region, 5'UTR) and/or disruption of gene splicing (introns) (Boccia et al., 1996; Wang and Sadee, 2015). Since the initial investigation by Poonkuzhali et al., three of the non-coding *ABCG2* SNPs have been linked to altered pharmacodynamics of BCRP substrates (Poonkuzhali et al., 2008). In the first intron, the C78551T and A92873G variants have been associated with the development of severe myelosuppression as a side effect of the anticancer drug irinotecan (Cha et al., 2009). Also in the first intronic region, the A92894C variant has been

associated with altered pharmacokinetic parameters in patients treated with the epilepsy drug, lamotrigine; significantly higher blood concentrations were observed in subjects heterozygous or homozygous for the A92894C variant (Zhou et al., 2015).

This is the first report investigating the association of placental BCRP expression with genetic variants in the non-coding region of the *ABCG2* gene. Only one SNP in the 5'UTR (A61562C) tended to be associated with lower BCRP expression. Despite prior associations between non-coding variants and BCRP expression in liver, intestine, and lymphoblasts (Poonkuzhali et al., 2008), there may be differences in gene regulation across tissues. Tissue-specific effects have been reported for intronic SNPs occurring in the *CYP3A4* gene. Human livers with the intronic variant CYP3A4*22 exhibited lower CYP3A4 mRNA expression, which was not observed in the intestines (Wang and Sadee, 2015). Segregation of mRNA expression data by ethnicity and genotype may uncover additional associations between *ABCG2* SNP and BCRP mRNA expression, however our sample size limited the ability to conduct this analysis. We also examined SNPs occurring in the coding region of the *ABCG2* gene. Individuals that were homozygous for the coding region variant (C421A) had 50% less BCRP protein than the wild-type controls, with no differences in BCRP mRNA. This is in agreement with a study performed in 2005, which examined BCRP expression in 100 placentas from healthy Japanese women (Kobayashi et al., 2005).

We observed a weak but statistically significant correlation between BCRP mRNA and protein (Figure 4) suggesting that, in some cases, mRNA levels may be a surrogate measure for understanding BCRP protein expression in term human placentas. However, the correlation

coefficient (r=0.25) revealed that variation in BCRP protein is not solely dependent on mRNA expression and that other regulatory mechanisms including microRNAs (Saito et al., 2013) and post-transcriptional modifications (Imai et al., 2005) may be important. Two other studies found no correlation between the BCRP gene and protein expression in other normal human tissues including intestine (N=42) and liver (N=65) (Zamber et al., 2003; Prasad et al., 2013), further emphasizing the importance of understanding tissue-specific differences in the regulation of BCRP expression.

In summary, these data demonstrate the interindividual expression of placental BCRP in term placentas from uncomplicated pregnancies and reveal associations of BCRP with genetic variants as well as transcription factors. These findings add to our understanding of the regulation of placental BCRP expression in normal term pregnancies and provide a foundation for identifying individuals that may be at risk for reduced BCRP expression and function.

Authorship Contributions

Participated in research design: Bircsak, Aleksunes

Conducted experiments: Bircsak, Moscovitz, Wen

Provided facilities and expertise in placenta collection: Archer, Yuen, Mohammed, Memon, Vetrano, Weinberger

Provided statistical expertise: Saba

Wrote or contributed to the writing of the manuscript: Bircsak, Aleksunes

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Footnotes

This work was supported by the National Institute of Environmental Health Sciences [Grants ES020522, ES005022, ES007148], a component of the National Institutes of Health. A portion of tissue samples were provided by the NIH Placental Bank in UCSF, funded by The Eunice Kennedy Shriver National Institute of Child Health and Human Development of the National Institutes of Health under award HD055764. Kristin Bircsak was supported by predoctoral fellowships from the American Foundation for Pharmaceutical Education and Pharmaceutical Research and Manufacturers of America. Jamie Moscovitz was supported by predoctoral fellowships from the American Foundation for Pharmaceutical Education and the Eunice Kennedy Shriver National Institute of Child Health and Development [Grant F31HD082965].

Figure Legends

Figure 1. Map of transcription factor and nuclear receptor response elements in the promoter region of the *ABCG2* gene. Location of response elements numbered with respect to the transcriptional start site (TSS, +1) and the first nucleotide of the gene (+1; AC084732).

Figure 2. Map of single nucleotide polymorphisms in the non-coding and coding regions of the *ABCG2* gene. Non-coding SNPs are numbered according to the 1st nucleotide of the gene (+1; AC084732) and coding SNPs are numbered according to the translational start site (TSS, +1) and exclude nucleotides in non-coding regions. When applicable, *ABCG2* SNPs that result in amino acid changes in the BCRP protein are denoted with an arrow (→).

Figure 3. Evaluation of placental BCRP expression by infant ethnicity/race. (A) Messenger RNA expression of BCRP was assessed in 137 term placentas using a multiplex branched DNA signal amplification assay. BCRP mRNA values were normalized to the geometric mean of two references genes, GAPDH and RPL13A. (B) Protein expression of BCRP was assessed in 70 term placentas by western blot analysis. β-ACTIN was used as a loading control. Data are presented as box and whisker plots using the Tukey method with medians marked by a horizontal line. The box contains 50% of the data while the length of the whiskers from the edge of the box is equal to the lesser of either 1.5 times the interquartile range or the distance to the minimum/maximum value. Dots represent individual expression values that fall more than 1.5 times the interquartile range beyond the 25th and 75th quartiles. The bars represent individual placental BCRP expression.

Figure 4. Correlation between BCRP mRNA and protein expression. Messenger RNA expression of BCRP was assessed in 70 term placentas using a multiplex branched DNA signal amplification assay. BCRP mRNA values were normalized to the geometric mean of two references genes, GAPDH and RPL13A. Protein expression of BCRP was assessed in the same term placentas by western blot analysis. β-ACTIN was used as a loading control. Correlation between BCRP mRNA and protein expression was determined using the Pearson linear method (r=0.25, *P*=0.040).

Figure 5. Evaluation of placental BCRP expression by infant sex. (A) Messenger RNA expression of BCRP was assessed in 137 term placentas using a multiplex branched DNA signal amplification assay. BCRP mRNA values were normalized to the geometric mean of two references genes, GAPDH and RPL13A. (B) Protein expression of BCRP was assessed in 70 term placentas by western blot analysis. β-ACTIN was used as a loading control. Data are presented as box and whisker plots using the Tukey method with medians marked by a horizontal line. The box contains 50% of the data while the length of the whiskers from the edge of the box is equal to the lesser of either 1.5 times the interquartile range or the distance to the minimum/maximum value. Dots represent individual expression values that fall more than 1.5 times the interquartile range beyond the 25^{th} and 75^{th} quartiles.

Figure 6. Association of the C421A coding region SNP with placental BCRP expression. (A) Messenger RNA expression of BCRP was assessed in 137 term placentas using a multiplex branched DNA signal amplification assay. BCRP mRNA values were normalized to the geometric mean of two references genes, GAPDH and RPL13A. (B) Protein expression of BCRP

was assessed in 70 term placentas by western blot analysis. β-ACTIN was used as a loading control. Data are presented as a representative western blot and according to C421A genotype using box and whisker plots using the Tukey method with medians marked by a horizontal line. The box contains 50% of the data while the length of the whiskers from the edge of the box is equal to the lesser of either 1.5 times the interquartile range or the distance to the minimum/maximum value. Dots represent individual expression values that fall more than 1.5 times the interquartile range beyond the 25^{th} and 75^{th} quartiles. Asterisks (*) represent statistically significant differences ($p<0.05$) compared with wild-type control (C/C) using a Kruskal-Wallis non-parametric test with a Dunn's multiple testing correction. (C) Association of BCRP mRNA and protein expression by genotype was assessed by Pearson correlation.

Tables

Table 1. Placenta donor demographic information (N=137).

Parameter	Clinical Characteristics of Pregnancies and Placentas Obtained
Gestational age (weeks)	38.8 ± 1.1[a]
Maternal age (years)	31.2 ± 4.9[a]
Placental weight (grams)[b]	660.0 ± 154.5[a]
Birth weight (grams)	3355.3 ± 426.3 [a]
Infant sex[c]	Female = 65, Male = 69

[a]Mean ± standard deviation
[b]Wet, untrimmed weight
[c]Sex was unknown for 3 infants

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Table 2. Univariate associations between individual transcription factors and BCRP mRNA expression.

	Parameter Estimate	Standard Error of Parameter Estimate	t-statistic	Unadjusted p-value	Adjusted p-value
NRF2	0.85	0.11	7.97	6.1e-13	5.5e-12
AHR	0.29	0.04	7.05	8.6e-11	7.7e-10
SP3	0.40	0.06	6.16	7.9e-09	7.1e-08
SP1	1.14	0.24	4.67	7.2e-06	6.5e-05
HIF1α	0.64	0.23	2.76	6.7e-03	6.0e-02
ESR2	36.57	19.86	1.84	6.8e-02	6.1e-01
ESR1	10.76	8.10	1.33	1.9e-01	1.0e+00
PR	10.86	8.19	1.33	1.9e-01	1.0e+00
PPARγ	1.20	0.94	1.27	2.1e-01	1.0e+00

Table 3. Allele frequencies of SNPs separated by ethnicity/race.

dbSNP	Gene Location	Gene Position[a]	Allele Frequency						
			African American	Asian	Caucasian	Hispanic	Mixed	Unknown	OVERALL
rs2127862	5'UTR	C46847T	0.47	0.21	0.45	0.28	0.3	0.28	0.35
rs2127861	5'UTR	G46932C	0.11	0	0.13	0.1	0.07	0.17	0.097
rs13135956	5'UTR	G59582A	0.53	0.13	0.20	0.15	0.21	0.06	0.22
rs141093520	5'UTR	A61562C	0.026	0.16	0.078	0.075	0.071	0	0.074
rs55930652	5'UTR	C61785T	0.11	0.13	0.17	0.13	0.18	0.22	0.15
rs2725226	5'UTR	C72144T	0.19	0.53	0.41	0.6	0.5	0.61	0.45
rs3114020	5'UTR	T73809C	0.32	0.53	0.41	0.63	0.5	0.61	0.47
rs2622604	Intron 1	C78551T	0.11	0.16	0.13	0.10	0.14	0.22	0.13
rs3109823	Intron1	A92873G	0.61	0.26	0.22	0.15	0.36	0.22	0.29
rs3114018	Intron 1	A92894C	0.24	0.55	0.47	0.70	0.57	0.78	0.52
rs2725250	Intron 1	T93490C	0.34	0.26	0.35	0.28	0.33	0.17	0.31
rs2622620	Intron 1	C93624A	0.24	0.53	0.38	0.58	0.45	0.78	0.44

rs2046134	Intron 1	G94112A	0.42	0.083	0.033	0.05	0.13	0	0.11
rs2231137	Exon 2	G34A*	0	0	0.011	0.13	0.09	0	0.036
rs186749266	Intron 2	A96567G	0.056	0.18	0.10	0.2	0.14	0.056	0.13
rs2231139	Exon 4	C369T*	0.11	0	0	0.025	0	0	0.019
rs72552713	Exon 4	C376T*	0	0	0.023	0	0.024	0	0.011
rs2231142	Exon 5	C421A*	0.026	0.32	0.011	0.2	0.048	0.28	0.11
rs2725267	Intron 14	T141300C	0.64	0.11	0.11	0.43	0.43	0	0.28
rs2231164	Intron 14	A141618G	0.63	0.37	0.13	0.6	0.45	0.22	0.36

[a] AC084732 (1st nucleotide of gene = 1)
[b] Allele frequency was calculated by dividing the number of variant alleles by the total number of alleles (# individuals x 2) in a population.
*Starting at translational start site (+1) and excluding nucleotides in non-coding regions

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Table 4. Univariate associations between SNPs and BCRP mRNA expression.

SNP[a,b]	Regression Parameter Estimate for Additive Effect on BCRP mRNA Expression	Standard Error of Parameter Estimate for Additive Effect on BCRP mRNA Expression	t-statistic for SNP Effect on BCRP mRNA Expression	p-value for SNP Effect on BCRP mRNA Expression	Bonferroni-adjusted p-value for SNP Effect on BCRP mRNA Expression
C46847T	-0.0082	0.0082	-1.00	0.318	1.000
G46932C	0.0014	0.0129	0.11	0.912	1.000
G59582A	0.0016	0.0094	0.17	0.864	1.000
A61562C	-0.0294	0.0155	-1.90	0.060	1.000
C61785T	0.0019	0.0104	0.18	0.857	1.000
C72144T	0.0040	0.0074	0.55	0.586	1.000
T73809C	0.0060	0.0074	0.82	0.415	1.000
C78551T	0.0066	0.0109	0.61	0.543	1.000
A92873G	0.0031	0.0079	0.39	0.695	1.000
A92894C	-0.0062	0.0074	-0.84	0.401	1.000
T93490C	0.0035	0.0081	0.43	0.665	1.000
C93624A	0.0091	0.0075	1.22	0.225	1.000
G94112A	-0.0147	0.0109	-1.36	0.177	1.000
A96567G	-0.0072	0.0127	-0.57	0.570	1.000
C421A*	0.0029	0.0109	0.26	0.794	1.000
T141300C	-0.0013	0.0085	-0.15	0.882	1.000
A141618G	-0.0009	0.0074	-0.12	0.903	1.000

[a]AC084732 (1st nucleotide of gene=1)
[b]SNPS G34A, C369T and C376T were removed from analysis due to small sample size
*Starting at translational start site (+1) and excluding nucleotides in the non-coding region

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Table 5. Univariate associations between SNPs and BCRP protein expression.

SNP[a,b]	Regression Parameter Estimate for Additive Effect on BCRP Protein Expression	Standard Error of Parameter Estimate for Additive Effect on BCRP Protein Expression	t-statistic for SNP Effect on BCRP Protein Expression	p-value for SNP Effect on BCRP Protein Expression	Bonferroni-adjusted p-value for SNP Effect on BCRP Protein Expression
C46847T	0.0247	0.0169	1.46	0.149	1.000
G46932C	-0.0023	0.0256	-0.09	0.928	1.000
G59582A	0.0213	0.0178	1.20	0.235	1.000
A61562C	0.0097	0.0326	0.30	0.767	1.000
C61785T	0.0120	0.0215	0.56	0.578	1.000
C72144T	-0.0279	0.0148	-1.88	0.065	1.000
T73809C	-0.0210	0.0147	-1.42	0.159	1.000
C78551T	0.0227	0.0227	1.220	0.227	1.000
A92873G	0.0142	0.0158	0.90	0.373	1.000
A92894C	0.0207	0.0151	0.137	0.175	1.000
T93490C	0.0102	0.0180	0.57	0.574	1.000
C93624A	-0.0294	0.0154	-1.91	0.060	1.000
G94112A	0.0104	0.225	0.46	0.646	1.000
A96567G	0.0008	0.0274	0.03	0.976	1.000
C421A*	-0.0666	0.0195	-3.42	0.001	0.018
T141300C	0.0097	0.0184	0.53	0.598	1.000
A141618G	-0.0227	0.0147	-1.54	0.127	1.000

[a]AC084732 (1st nucleotide of gene=1)
[b]SNPS G34A, C369T and C376T were removed from analysis due to small sample size
*Starting at translational start site (+1) and excluding nucleotides in the non-coding region

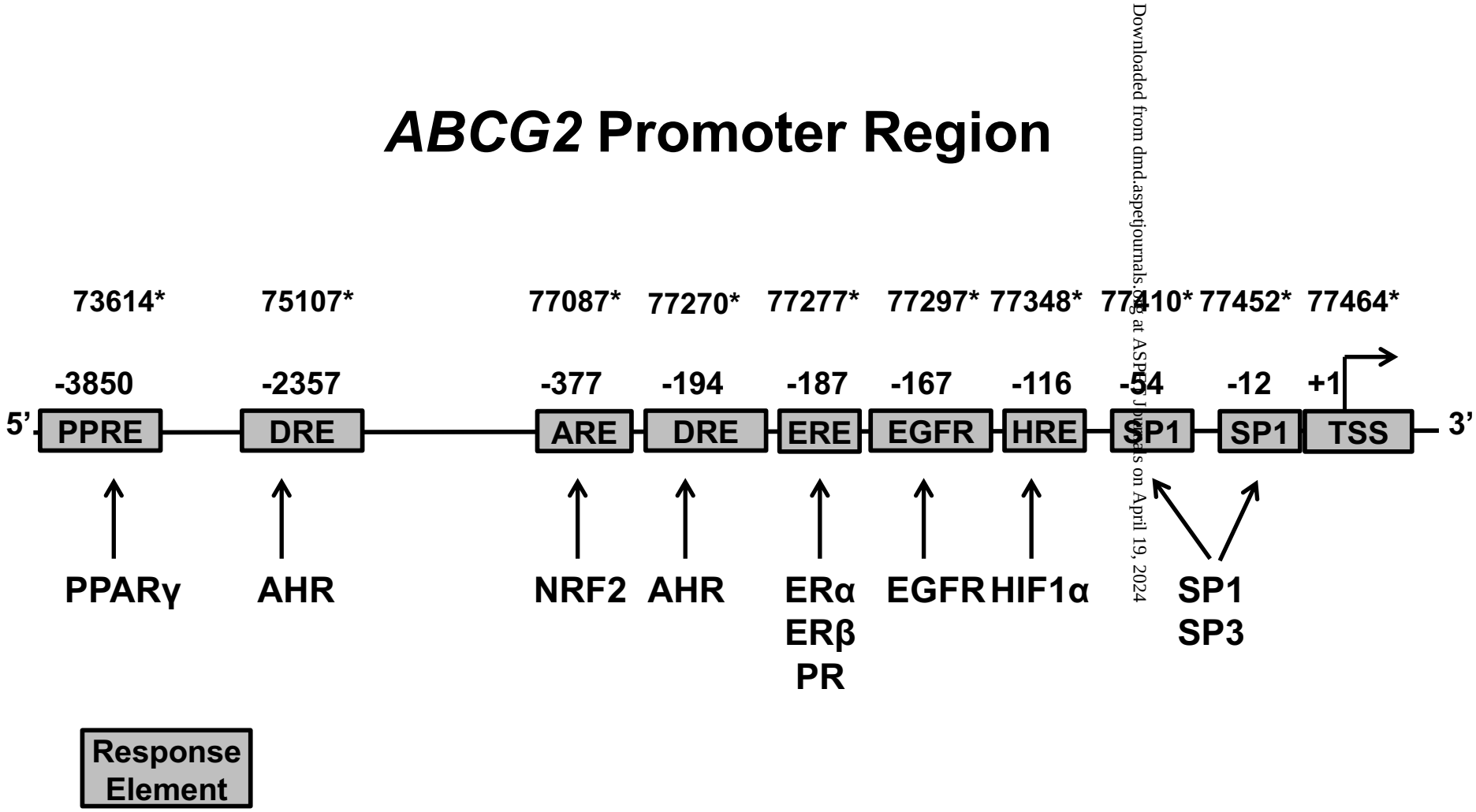
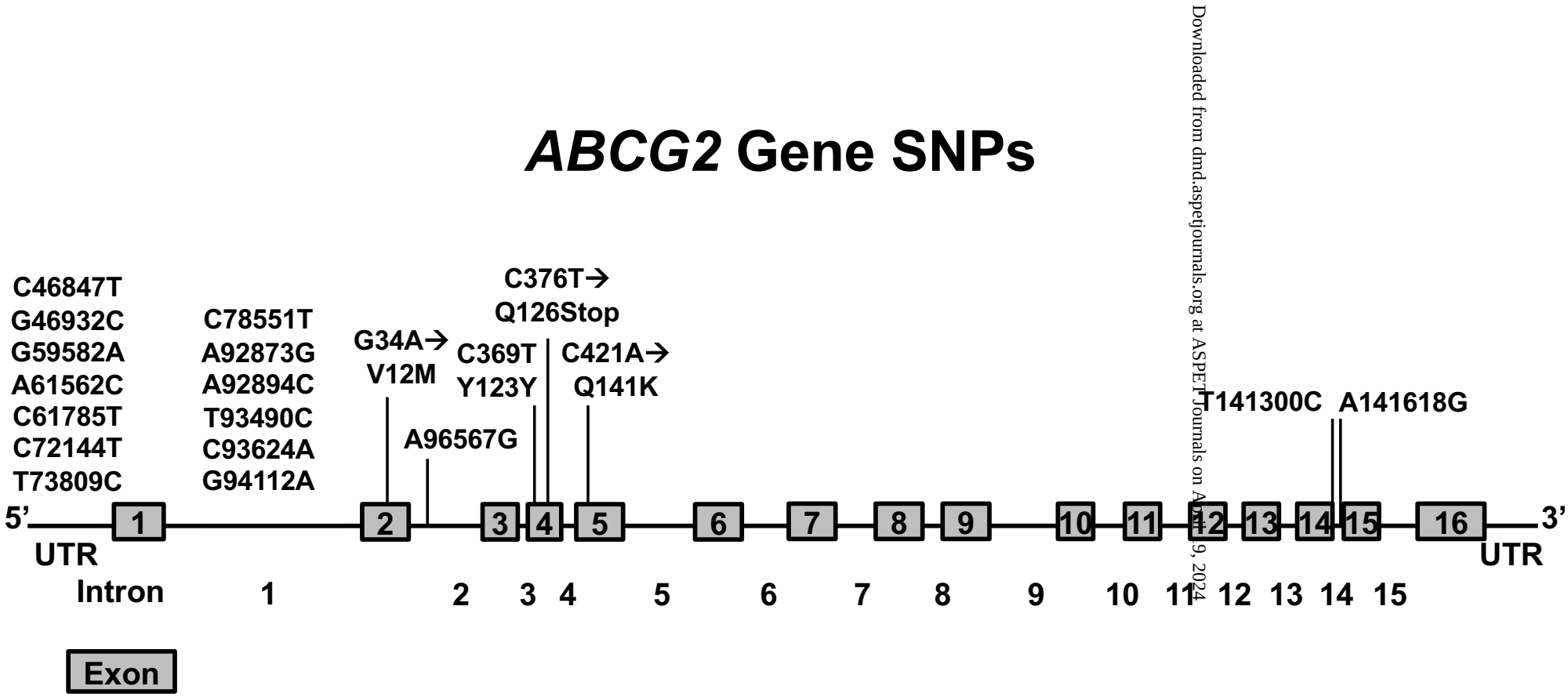


Figure 1



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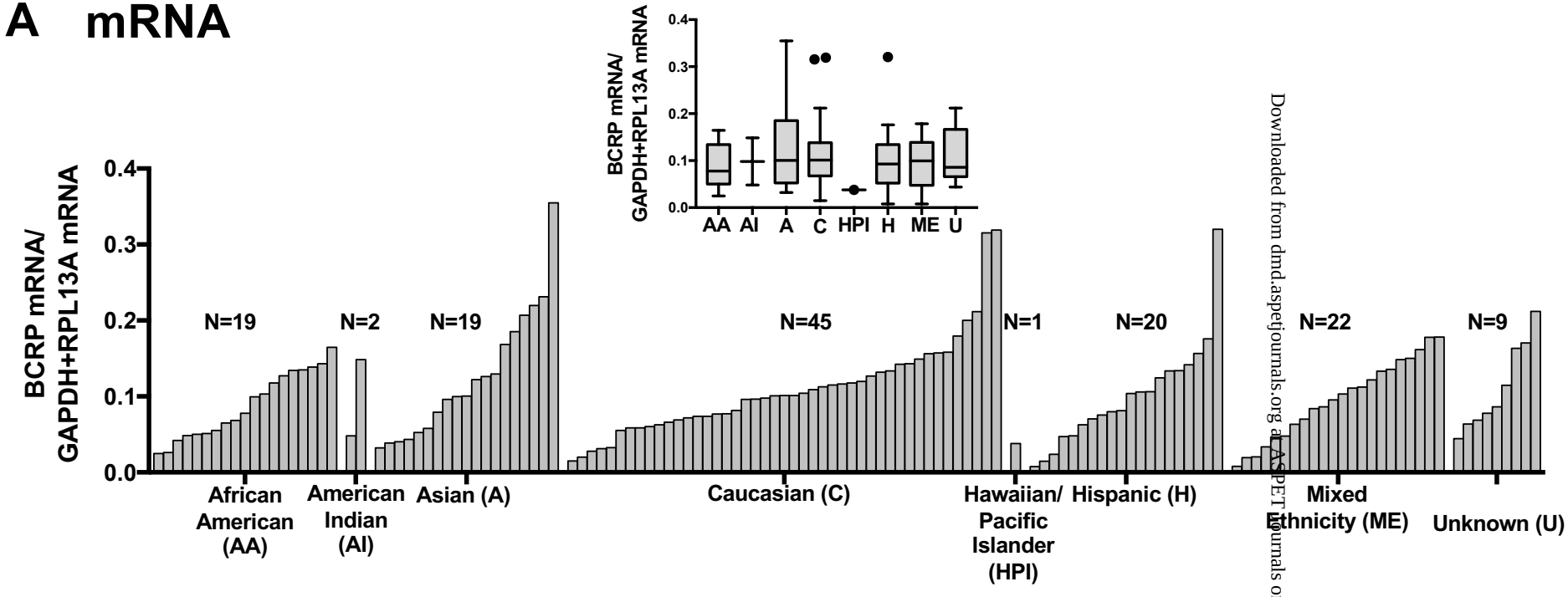

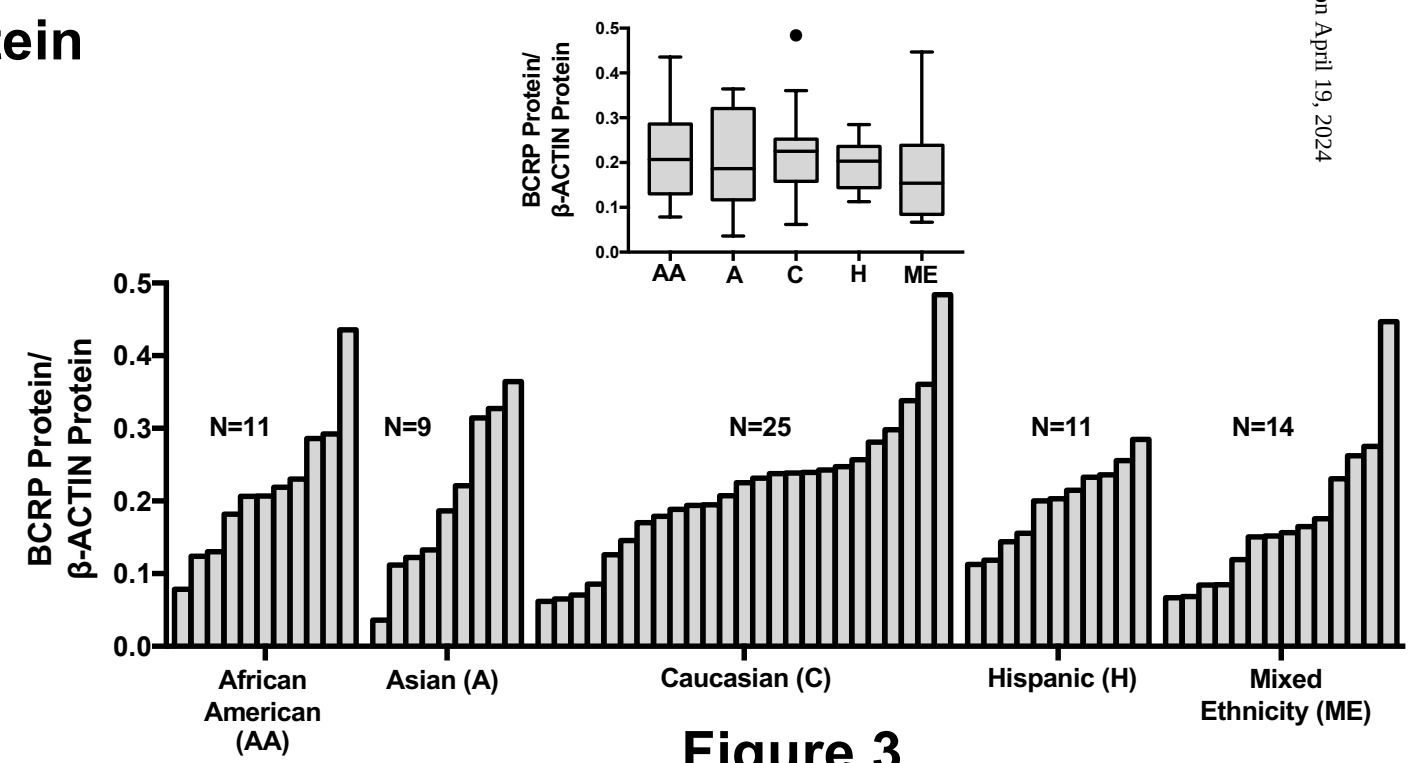

Figure 3

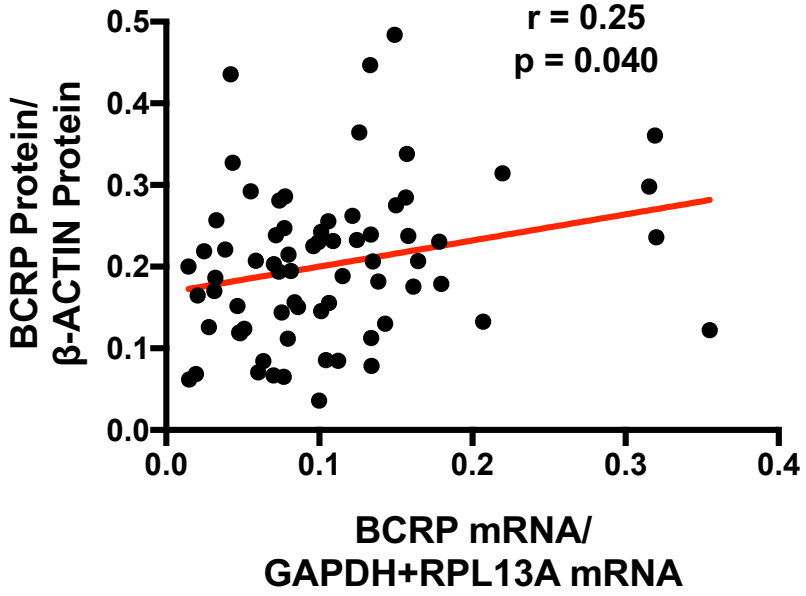

Figure 4

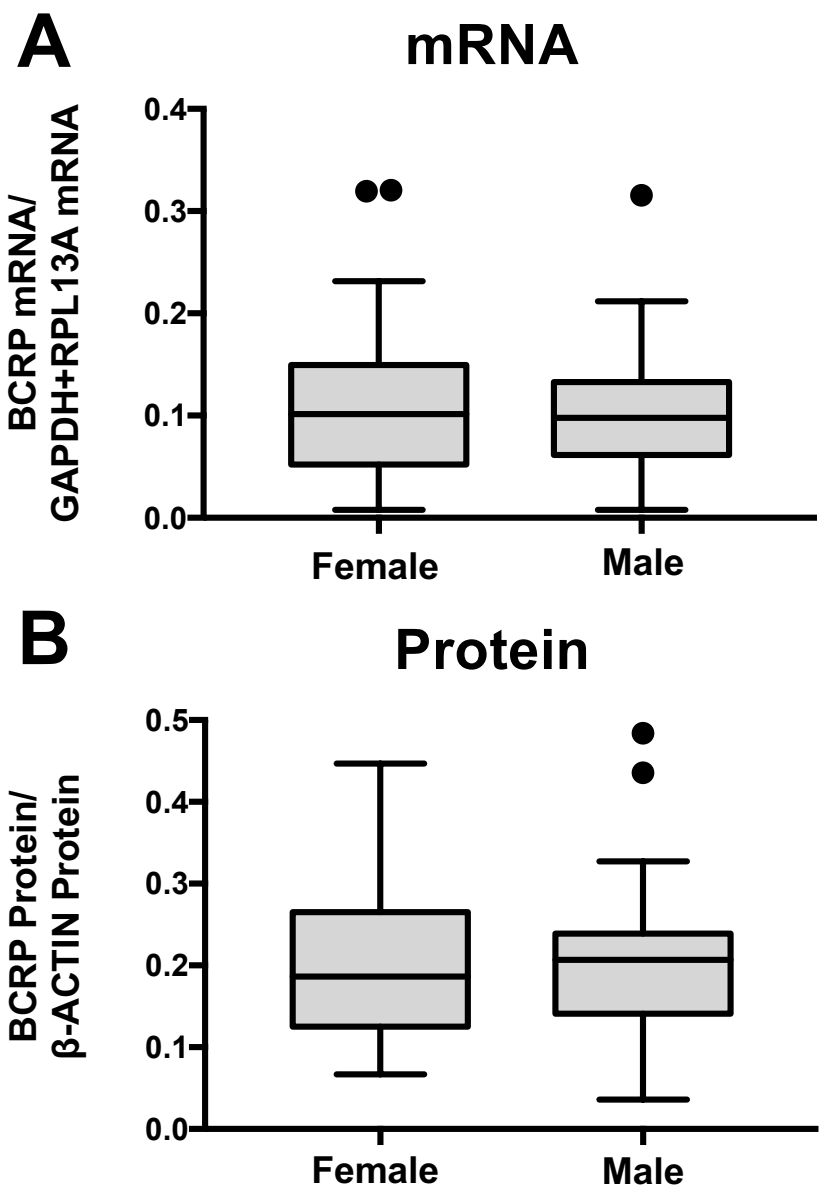

Figure 5

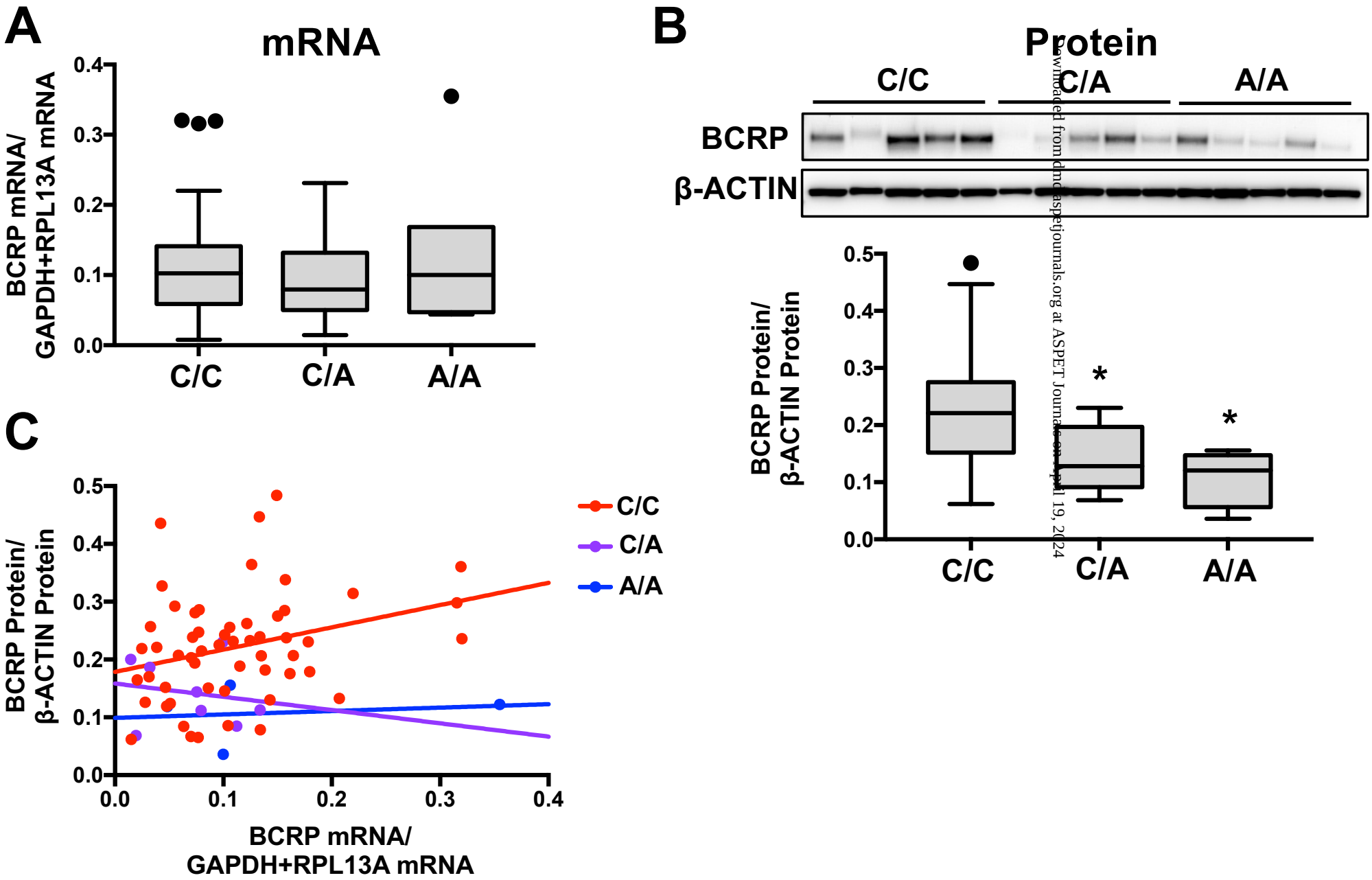

Figure 6