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# Sex-specific effects of serum sulfate level and *SLC13A1* nonsense variants on DHEA homeostasis

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ABSTRACT

*Context:* Sulfate is critical in the biotransformation of multiple compounds via sulfation. These compounds include neurotransmitters, proteoglycans, xenobiotics, and hormones such as dehydroepiandrosterone (DHEA). Sulfation reactions are thought to be rate-limited by endogenous sulfate concentrations. The gene, *SLC13A1*, encodes the sodium-sulfate cotransporter NaS1, responsible for sulfate (re)absorption in the intestines and kidneys. We previously reported two rare, non-linked, nonsense variants in *SLC13A1* (R12X and W48X) associated with hyposulfatemia ($P = 9 \times 10^{-20}$).
*Objective:* To examine the effect of serum sulfate concentration and sulfate-lowering genotype on DHEA homeostasis.
*Design:* Retrospective cohort study.
*Setting:* Academic research.
*Patients:* Participants of the Amish Pharmacogenomics of Anti-Platelet Intervention (PAPI) Study and the Amish Hereditary and Phenotype Intervention (HAPI) Study.
*Main outcome measures:* DHEA, DHEA-S, and DHEA-S/DHEA ratio.
*Results:* Increased serum sulfate was associated with decreased DHEA-S ($P = 0.03$) and DHEA-S/DHEA ratio ($P = 0.06$) in males but not females. Female *SLC13A1* nonsense variant carriers, who had lower serum sulfate ($P = 9 \times 10^{-13}$), exhibited 14% lower DHEA levels ($P = 0.01$) and 7% higher DHEA-S/DHEA ratios compared to female non-carriers ($P = 0.002$). Consistent with this finding, female *SLC13A1* nonsense variant carriers also had lower total testosterone levels compared to non-carrier females ($P = 0.03$).
*Conclusions:* Our results demonstrate an inverse relationship between serum sulfate, and DHEA-S and DHEA-S/DHEA ratio in men, while also suggesting that the sulfate-lowering variants, *SLC13A1* R12X and W48X, decrease DHEA and testosterone levels, and increase DHEA-S/DHEA ratio in women. While paradoxical, these results illustrate the complexity of the mechanisms involved in DHEA homeostasis and warrant additional studies to better understand sulfate's role in hormone physiology.


## 1. Introduction

Dehydroepiandrosterone (DHEA) and its inactive, sulfate ester, dehydroepiandrosterone sulfate (DHEA-S), are endogenous hormones secreted by the adrenal gland and serve as precursors for androgenic and estrogenic steroids [1,2]. DHEA can be converted into active androgens, or into DHEA-S via the enzyme DHEA sulfotransferase, also known as SULT2A1 [3]. Likewise, DHEA-S can be converted back into DHEA via the enzyme steroid sulfatase (STS) [4]. DHEA-S is the most abundant circulating steroid hormone in humans with levels of DHEA-S far exceeding that of DHEA [5].

The interconversion between DHEA and DHEA-S was previously assumed to occur continuously under the premise that inactive DHEA-S serves as a circulating storage pool for DHEA regeneration, and ultimately sex steroids [6]. However, with the exception of breast and prostate tissue [7,8], only low levels of *STS* expression and STS activity are present in adult human tissues [9], suggesting that regeneration of DHEA from DHEA-S is uncommon and that DHEA sulfation via SULT2A1 is the predominant reaction [6,10]. Moreover, genetic variants in *SULT2A1*, but not *STS*, have been shown to be associated with lower DHEA-S in women with and without polycystic ovary syndrome (PCOS) [4,11].

The sulfate donor 3′-phospho-adenosine-5′-phosphosulfate (PAPS) is required by all sulfotransferases including SULT2A1 [12,13]. PAPS is synthesized by one of two isoforms of PAPS synthase (PAPSS): PAPSS1 which is the major isoform in brain and skin, and PAPSS2 which

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predominates in the liver, cartilage and adrenal glands [14]. PAPS consists of inorganic sulfate and adenosine triphosphate (ATP) and is considered the "activated" form of sulfate [12]. In 2009, Noordam et al. reported inactivating *PAPSS2* mutations in a female who presented with premature pubarche that progressed to a PCOS phenotype, DHEA-S levels below the limit of detection, and testosterone levels approximately twice the upper limit of normal for her age and gender [3]. Recently, the same group reported a PAPSS2 deficiency in two brothers with compound heterozygous mutations in *PAPSS2* who presented with overt spondyloepimetaphyseal dysplasia (SEMD), low serum DHEA-S, but normal serum DHEA, androstenedione, and testosterone [6]. The concentration of PAPS equilibrates rapidly with that of serum sulfate, thus the rate of PAPS synthesis, and consequently the rate of sulfation, are thought to be dependent on endogenous sulfate concentrations [15,16]. Therefore, it is reasonable to presume that differences in serum sulfate concentration would impact levels of DHEA and DHEA-S, however this has never been examined.

We previously reported on two rare, non-linked, nonsense variants in *SLC13A1* (rs28364172, c.34C>T, p.R12X and rs138275989, c.144G>A, p.W48X) that are enriched in frequency in the Old Order Amish (Amish) population (1.2-fold (0.29% vs. 0.23%) and 3.7-fold (0.74% vs. 0.20%), respectively, compared to $ESP_{(EA)}$ allele frequencies) and associated with hyposulfatemia ($P = 9 \times 10^{-20}$) [17]. *SLC13A1* encodes the apical membrane, sodium-sulfate cotransporter NaS1, which is responsible for sulfate (re)absorption in the intestines and kidneys [18]. As a result, loss-of-function alleles in this gene cause in decreased serum sulfate levels [17, 20–22]. The enrichment of these *SLC13A1* nonsense variants in the Amish provides us the unique opportunity to dissect the role of sulfate and these sulfate-lowering variants in androgenic hormone homeostasis. With this is mind, we evaluated the effect of serum sulfate concentration and sulfate-lowering genotype on DHEA, DHEA-S, and DHEA-S/DHEA ratio.

## 2. Materials and methods

### 2.1. Study population

This report is based on the Old Order Amish community living in Lancaster County, PA, whom our research group has been studying since 1993. This community was founded by several hundreds of individuals who immigrated to Lancaster County, PA from central Europe during the early 18th century, with the present day Lancaster County Amish community comprised of their descendants [23]. Cultural and religious beliefs have maintained the Amish as distinct from the general population. Due to the availability of extensive genealogical records [24], virtually all present-day Amish can be linked into a single, 14-generation pedigree. To date, we have screened approximately 6500 Amish adults for a variety of risk factors related to cardiovascular disease [25], diabetes [26], and osteoporosis [27] as part of the Amish Complex Disease Research Program.

Subjects included in this report are members of the Amish community in Lancaster County, PA who were at least 18 years old and have previously participated in one or more Institutional Review Board-approved studies conducted at the University of Maryland Amish Research Center. Written informed consent was obtained from each participant.

In accordance to our human subject research protocols, we are unable to release individual-level data. Given that the Old Order Amish of Lancaster County, PA are a founder population with publicly available pedigree data, depositing individual-level data creates the potential for personal identification of our research participants.

### 2.2. Serum dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEA-S) measurements and calculations

DHEA and DHEA-S was measured in 203 participants of the Amish Pharmacogenomics of Anti-Platelet Intervention (PAPI) Study [28,29], for whom serum sulfate concentration, *SLC13A1* R12X and W48X genotypes, and fasting serum aliquots were available. This included 59 Amish carriers of *SLC13A1* R12X or W48X, as well as 144 participants who were randomly selected. DHEA and DHEA-S measurements were completed on frozen, fasting, serum aliquots stored at −80 °C. Serum DHEA and DHEA-S concentrations were measured by Johns Hopkins Bayview Medical Center's Advanced Biochemistry Laboratory (Baltimore, Maryland) via radioimmunoassay (RIA; Rocky Mountain Diagnostics, Inc., Colorado Springs, CO) and enzyme-linked immunosorbent assay (ELISA; ALPCO®, Salem, NH), respectively. The ratio of DHEA-S to DHEA (Ratio) was calculated by dividing DHEA-S by DHEA.

### 2.3. Genotyping

Genotyping of *SLC13A1* nonsense variants, R12X and W48X (rs28364172, c.34G>A, p.R12X and rs138275989, c.144C>T, p.W48X), was performed using TaqMan® SNP genotyping assays (Life Technologies, Foster City, California). For both single nucleotide variants (SNVs), the TaqMan genotype concordance was >99.8% in a subset of samples genotyped in duplicate.

### 2.4. Serum sulfate measurements

Sulfate was measured in participants of the Amish PAPI [28,29] Study and the Amish Heredity and Phenotype Intervention (HAPI) Heart [25] Study for whom fasting serum aliquots were available. Sulfate measurements were completed on frozen, fasting, serum aliquots stored at −80 °C. Sulfate concentration was determined by turbidimetry according to Dodgson & Price [30] using a Quantichrom™ Sulfate Assay Kit (BioAssay Systems, Hayward, CA). In order to improve accuracy, a quadratic least squares fit was used, instead of a linear fit, to generate the standard curve [31]. All standards and samples were measured in duplicate. For each sample, sulfate concentration was calculated as the mean of the duplicate measurements. Samples with an absolute difference > 20% were considered discordant duplicate measurements and were not included in the analysis.

### 2.5. Testosterone measurements and calculations

Total testosterone (TotalT), sex hormone binding globulin (SHBG), and free testosterone (FreeT) were previously measured in 177 female participants of the Amish PAPI Study [28,29] and the Amish HAPI Heart Study [25] for whom *SLC13A1* R12X and W48X genotypes were available. Measurements were completed on frozen, fasting, serum aliquots stored at −80 °C. Total testosterone was performed by radioimmunoassay by the University of Virginia Center for Research in Reproduction Ligand Assay Core (Charlottesville, VA). SHBG was measured by Immulite assay. Free testosterone was calculated according to the method of Vermeulen et al. [32]

### 2.6. Statistical analysis

Logarithm (base 10) transformations were used to normalize the distributions of DHEA, DHEA-S, Ratio, TotalT and FreeT (skewness > |1| in all cases), resulting in normal distributions for $DHEA_{Log}$, $DHEA\text{-}S_{Log}$, $Ratio_{Log}$, $TotalT_{Log}$, and $FreeT_{Log}$. Association analyses between genotypes and serum sulfate, and other phenotypic measures were conducted using a regression-based method that models variation of the trait of interest as a function of measured covariates, measured genotypes and a polygenic component that accounts for phenotypic correlation due to relatedness. This method was implemented using the Mixed Models Analysis for Pedigrees and Populations (MMAP) program [33]. For each association analysis performed, individuals with a missing covariate, genotype and/or trait of interest were excluded from the analysis. All analyses included age, age-squared, and gender as covariates. All analyses of hormone levels obtained in Amish PAPI Study [28,29] or

Amish HAPI Study [25] participants containing serum sulfate as a covariate were limited to subjects for whom serum sulfate was measured using a serum aliquot collected at the time of participation in that study.

## 3. Results

Amish research subjects selected for serum DHEA and DHEA-S measurements consisted of 92 males (45.3%) and 111 females (54.7%) with a mean age of 44.6 ± 15.2 years and a mean BMI of 26.8 ± 4.9 kg/m$^2$. Serum sulfate concentration (0.34 ± 0.11 mM) and *SLC13A1* R12X (MAF: 4.4%) and W48X (MAF: 10.1%) genotypes were available for all 203 subjects by design (Tables S1-S2). The average serum sulfate concentration among the 59 *SLC13A1* nonsense variant carriers in this cohort was 0.25 ± 0.07 mM (R12X: 0.23 ± 0.06 mM; W48X: 0.25 ± 0.07 mM), ranging from 0.06–0.43 mM. The average serum sulfate concentration among the 144 non-carriers in this cohort was 0.38 ± 0.10 mM, ranging from 0.10–0.55 mM. Serum sulfate concentration was significantly lower in *SLC13A1* nonsense variant carriers compared to non-carriers in all cohorts examined in this study (Table S3).

### 3.1. Serum sulfate concentration is inversely associated with DHEA-S/DHEA ratio in men

In the cohort of 203 subjects, no associations were observed between serum sulfate concentration, and $DHEA_{Log}$ ($P = 0.56$), DHEA-$S_{Log}$ ($P = 0.10$) or $Ratio_{Log}$ ($P = 0.46$) (Table 1). Stratifying by gender to examine the possibility of any gender-specific associations revealed a significant inverse relationship between serum sulfate concentration and $Ratio_{Log}$ in men ($\beta = -0.55$ μg/mL, $P = 0.02$) equivalent to a 3.6 unit decrease in DHEA-S/DHEA ratio per mM of sulfate (Table 1 and Fig. 1a). A trending inverse association was also noted between serum sulfate concentration and DHEA-$S_{Log}$ in men ($\beta = -0.43$ μg/mL, $P = 0.07$) (Table 1 and Fig. 1b). No associations were observed between serum sulfate concentration, and $DHEA_{Log}$ in males, nor $DHEA_{Log}$, DHEA-$S_{Log}$, or $Ratio_{Log}$ in females (Table 1 and Fig. 1).

### 3.2. SLC13A1 nonsense variants paradoxically increase DHEA-S/DHEA ratio and decrease DHEA in women

$Ratio_{Log}$ and $DHEA_{Log}$ were significantly associated with *SLC13A1* nonsense variant carrier status ($\beta = 0.13$, $P = 0.007$ and $\beta = -0.14$ ng/mL, $P = 0.01$ respectively) (Table 1). The unadjusted increase in $Ratio_{Log}$ observed in *SLC13A1* nonsense variant carriers is equivalent to a 1.3 unit, or a 6% increase in DHEA-S/DHEA ratio compared to non-carriers (Fig. 2a). The total variance in $Ratio_{Log}$ explained increased from 8% to 13% when *SLC13A1* nonsense variant carrier status was included in the model, suggesting that *SLC13A1* nonsense variant carrier status explains 5% of the total variance in $Ratio_{Log}$ in this cohort. The unadjusted decrease in $DHEA_{Log}$ observed in *SLC13A1* nonsense variant carriers is equivalent to a 1.5 ng/mL, or a 21% decrease in DHEA compared to non-carriers (Fig. 2c). The total variance in $DHEA_{Log}$ explained increased from 17% to 22% when *SLC13A1* nonsense variant carrier status was included in the model, suggesting that *SLC13A1* nonsense variant carrier status explains 5% of the total variance in $DHEA_{Log}$ in this cohort. No association was observed between *SLC13A1* nonsense variant carrier status and DHEA-$S_{Log}$ (Table 1).

Stratifying by gender revealed a significant association between *SLC13A1* nonsense variant carrier status and decreased $DHEA_{Log}$ in both males and females ($P_{Females} = 0.02$, $P_{Males} = 0.04$), while the association between *SLC13A1* nonsense variant carrier status and increased $Ratio_{Log}$ was only significant in females ($P_{Females} = 0.007$, $P_{Males} = 0.22$) (Table 1). Gender stratification did not reveal any association between *SLC13A1* nonsense variant carrier status and DHEA-$S_{Log}$ in either males or females (Table 1).

To determine if the aforementioned associations were driven directly by *SLC13A1* nonsense variant carrier status, or by the secondary decrease in serum sulfate resulting from these sulfate-lowering variants, we implemented a Mendelian randomization approach [34] through the use of a model that included both *SLC13A1* nonsense variant carrier status and serum sulfate concentration as covariates. Once again, no association was observed between serum sulfate concentration, and

**Table 1**
Associations between hormone levels and serum sulfate concentration, and *SLC13A1* nonsense variant carrier status. *P* represents significance from model including age, age-squared, and gender (for non-stratified analyses) as covariates, and accounting for phenotypic correlation due to relatedness; bold text indicates $P < 0.05$. Adjusted for age, age-squared, and gender (for non-stratified analyses). Abbreviations: SE, standard error; SD, standard deviation; SO4, sulfate; SNV, single nucleotide variant.

| Model | Trait | Cohort | n | # of nonsense alleles | Trait SD | $\beta_{SO4} \pm SE$ | $\beta_{SO4}/SD$ | $P_{SO4}$ | $\beta_{SNV} \pm SE$ | $\beta_{SNV}/SD$ | $P_{SNV}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| [Serum sulfate] | $DHEA_{Log}$ (ng/mL) | All | 203 | – | 0.33 | −0.12 ± 0.21 | −0.37 | 0.56 | – | – | – |
| | | Males | 92 | – | 0.33 | 0.17 ± 0.29 | 0.50 | 0.57 | – | – | – |
| | | Females | 111 | – | 0.33 | −0.01 ± 0.29 | −0.02 | 0.98 | – | – | – |
| | DHEA-$S_{Log}$ (μg/mL) | All | 203 | – | 0.31 | −0.29 ± 0.17 | −0.93 | 0.10 | – | – | – |
| | | Males | 92 | – | 0.27 | −0.43 ± 0.24 | −1.62 | 0.07 | – | – | – |
| | | Females | 111 | – | 0.32 | 0.10 ± 0.24 | 0.31 | 0.68 | – | – | – |
| | $Ratio_{Log}$ | All | 203 | – | 0.25 | −0.13 ± 0.17 | −0.50 | 0.46 | – | – | – |
| | | Males | 92 | – | 0.23 | −0.55 ± 0.23 | −2.35 | **0.016** | – | – | – |
| | | Females | 111 | – | 0.25 | 0.04 ± 0.23 | 0.15 | 0.87 | – | – | – |
| *SLC13A1* nonsense variant carrier status | $DHEA_{Log}$ (ng/mL) | All | 203 | 59 | 0.33 | – | – | – | −0.14 ± 0.06 | −0.43 | **0.011** |
| | | Males | 92 | 25 | 0.33 | – | – | – | −0.15 ± 0.07 | −0.44 | **0.036** |
| | | Females | 111 | 34 | 0.33 | – | – | – | −0.18 ± 0.08 | −0.55 | **0.022** |
| | DHEA-$S_{Log}$ (μg/mL) | All | 203 | 59 | 0.31 | – | – | – | 0.01 ± 0.05 | 0.03 | 0.87 |
| | | Males | 92 | 25 | 0.27 | – | – | – | 0.03 ± 0.06 | −0.10 | 0.67 |
| | | Females | 111 | 34 | 0.32 | – | – | – | −0.02 ± 0.07 | −0.07 | 0.73 |
| | $Ratio_{Log}$ | All | 203 | 59 | 0.25 | – | – | – | 0.13 ± 0.05 | 0.50 | **0.007** |
| | | Males | 92 | 25 | 0.23 | – | – | – | 0.08 ± 0.06 | 0.33 | 0.22 |
| | | Females | 111 | 34 | 0.25 | – | – | – | 0.17 ± 0.06 | 0.69 | **0.007** |
| [Serum sulfate] + *SLC13A1* nonsense variant carrier status | $DHEA_{Log}$ (ng/mL) | All | 203 | 59 | 0.33 | −0.34 ± 0.24 | −1.02 | 0.15 | −0.18 ± 0.06 | −0.53 | **0.006** |
| | | Males | 92 | 25 | 0.33 | 0.03 ± 0.32 | 0.08 | 0.93 | −0.14 ± 0.08 | −0.43 | 0.07 |
| | | Females | 111 | 34 | 0.33 | −0.40 ± 0.32 | −1.20 | 0.21 | −0.23 ± 0.09 | −0.70 | **0.010** |
| | DHEA-$S_{Log}$ (μg/mL) | All | 203 | 59 | 0.31 | −0.32 ± 0.19 | −1.04 | 0.10 | −0.03 ± 0.05 | −0.09 | 0.10 |
| | | Males | 92 | 25 | 0.27 | −0.56 ± 0.26 | −2.09 | **0.033** | −0.09 ± 0.07 | −0.33 | 0.21 |
| | | Females | 111 | 34 | 0.32 | 0.08 ± 0.28 | 0.24 | 0.78 | −0.01 ± 0.08 | −0.04 | 0.86 |
| | $Ratio_{Log}$ | All | 203 | 59 | 0.25 | 0.08 ± 0.19 | 0.33 | 0.66 | 0.14 ± 0.05 | 0.54 | **0.009** |
| | | Males | 92 | 25 | 0.23 | −0.50 ± 0.26 | −2.13 | 0.06 | 0.03 ± 0.06 | 0.11 | 0.68 |
| | | Females | 111 | 34 | 0.25 | 0.43 ± 0.25 | 1.73 | 0.09 | 0.23 ± 0.07 | 0.91 | **0.002** |
| | $TotalT_{Log}$ (ng/dL) | Females | 177 | 11 | 0.20 | −0.08 ± 0.22 | −0.41 | 0.72 | −0.14 ± 0.06 | −0.72 | **0.025** |
| | $FreeT_{Log}$ (ng/dL) | Females | 177 | 11 | 0.24 | 0.04 ± 0.28 | 0.18 | 0.88 | −0.12 ± 0.08 | −0.52 | 0.13 |

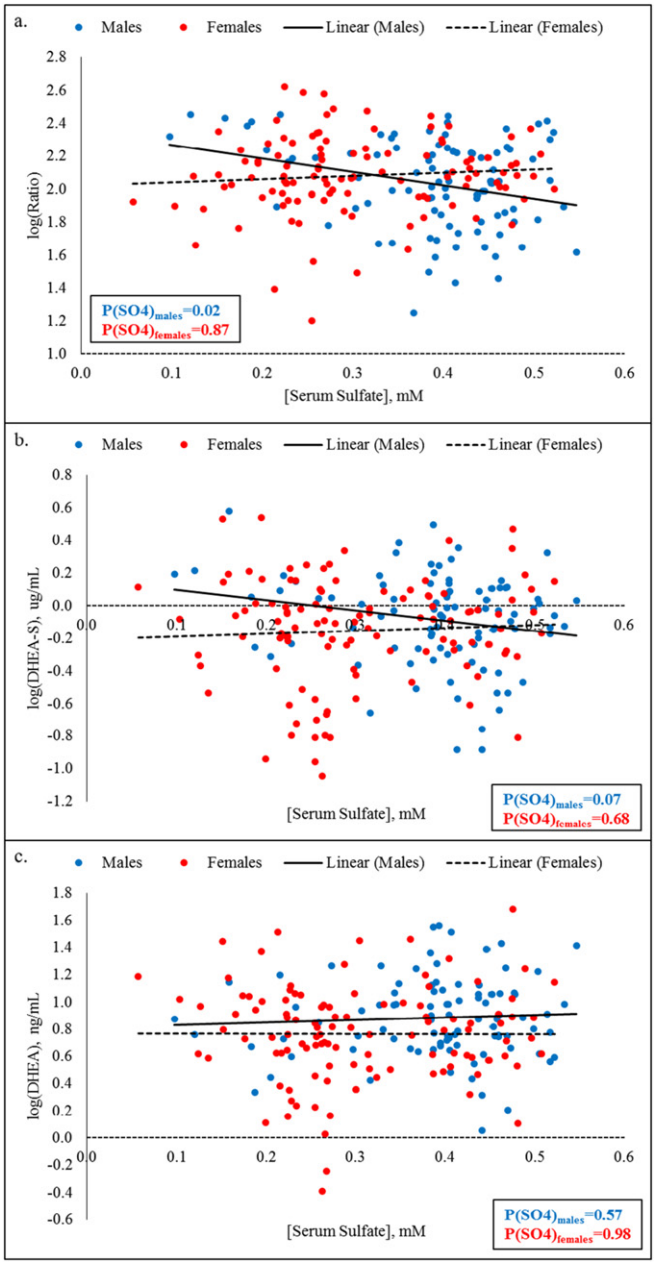


**Fig. 1.** Associations between DHEA hormone levels and serum sulfate concentration in men (n = 92) and women (n = 111). a) $Ratio_{Log}$, b) $DHEA\text{-}S_{Log}$, and c) $DHEA_{Log}$. *P* represents significance from model including age, age-squared, and gender as covariates, and accounting for phenotypic correlation due to relatedness.

$DHEA_{Log}$, $DHEA\text{-}S_{Log}$, nor $Ratio_{Log}$. However, this model strengthened the significance of the association between *SLC13A1* nonsense variant carrier status and $Ratio_{Log}$ ($\beta = 0.14$, $P = 0.009$), and $DHEA_{Log}$ ($\beta = -0.18$ ng/mL, $P = 0.006$), suggesting *SLC13A1* nonsense variant carrier status is the primary driver of these associations as opposed to the secondary decrease in serum sulfate resulting from these sulfate-

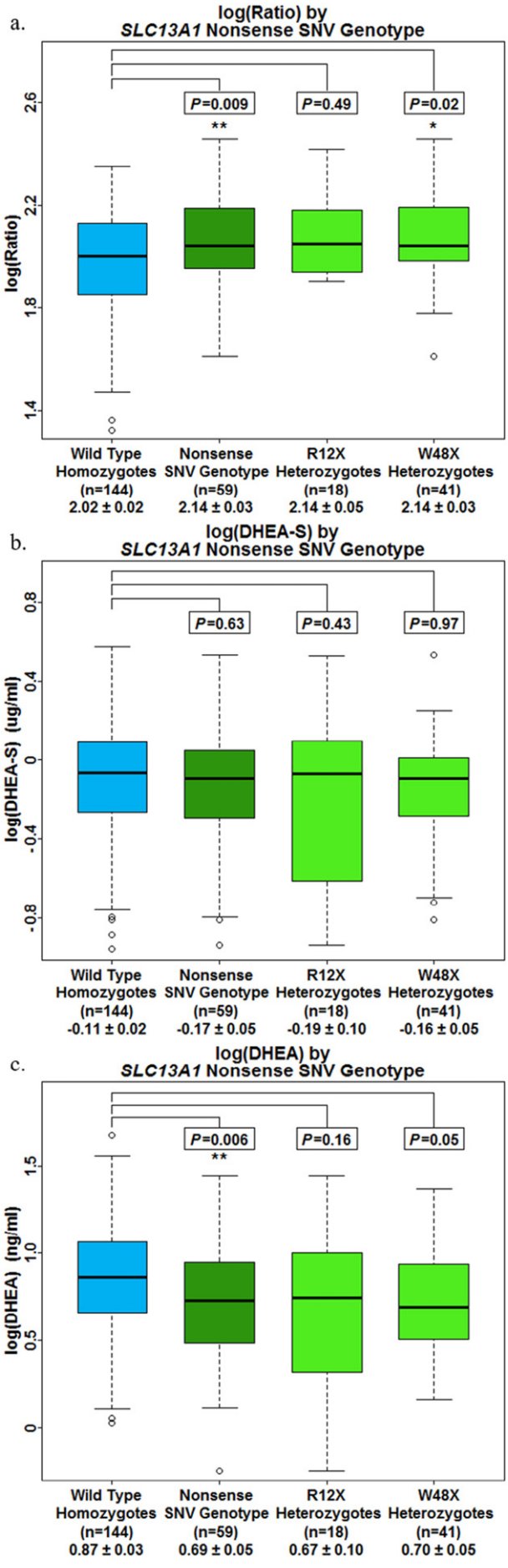


**Fig. 2.** DHEA hormone levels by *SLC13A1* nonsense single nucleotide variant (SNV) genotype in men (n = 92) and women (n = 111). a) $Ratio_{Log}$, b) $DHEA\text{-}S_{Log}$, and c) $DHEA_{Log}$. Box and whisker plots: box represents 2nd and 3rd quartiles (IQR, interquartile range); horizontal band represents median value; whiskers represent 1st and 4th quartiles; ends of whiskers represent minimum and maximum values, excluding outliers; open circles represents outliers (data point > 1.5*IQR below the 1st quartile or above the 3rd quartile). *P* represents significance from model including age, age-squared, gender, and serum sulfate concentration as covariates, and accounting for phenotypic correlation due to relatedness; * indicates $P < 0.05$, ** indicates $P < 0.01$.

lowering variants (Table 1 and Fig. 2). Interestingly, similar results from this model were observed among women but not men. In women, this model confirmed the lack of an association between serum sulfate concentration and $DHEA_{Log}$, DHEA-$S_{Log}$, nor $Ratio_{Log}$, while the significance of the association between *SLC13A1* nonsense variant carrier status and $Ratio_{Log}$ ($\beta = 0.23$, $P = 0.002$) and $DHEA_{Log}$ ($\beta = -0.23$ ng/mL, $P = 0.01$) was strengthened (Table 1 and Fig. 3). The unadjusted increase in $Ratio_{Log}$ observed in female *SLC13A1* nonsense variant carriers is equivalent to a 1.3 unit, or a 7% increase in DHEA-S/DHEA ratio compared to non-carriers (Fig. 3a). The unadjusted decrease in $DHEA_{Log}$ observed in *SLC13A1* nonsense variant carriers is equivalent to a 1.3 ng/mL, or a 14% decrease in DHEA compared to non-carriers (Fig. 3b). Conversely in men, no associations with either *SLC13A1* nonsense variant carrier status nor serum sulfate concentration gained significance with the exception of an inverse association between serum sulfate concentration and DHEA-$S_{Log}$ ($\beta = -0.56$ mg/mL, $P = 0.03$) (Table 1). Additionally, the aforementioned inverse association between serum sulfate concentration and $Ratio_{Log}$ in men was nearly-significant in this model ($\beta = -0.50$ mg/mL, $P = 0.06$) (Table 1).

### 3.3. SLC13A1 nonsense variants are associated with decreased testosterone levels in women

Serum sulfate concentration, *SLC13A1* nonsense variant genotype status, and testosterone measures were available in 177 women. Using the model that included both *SLC13A1* nonsense variant carrier status and serum sulfate concentration as covariates, an inverse association was observed between female *SLC13A1* nonsense variant carriers and $TotalT_{Log}$ ($\beta = -0.14$ ng/dL, $P = 0.03$) equivalent to a 1.4 ng/dL decrease in total testosterone compared to non-carriers (Table 1 and Fig. 4). Additionally, free testosterone was also lower in *SLC13A1* nonsense variant carriers; however, this association was not statistically significant ($P = 0.13$) (Table 1).

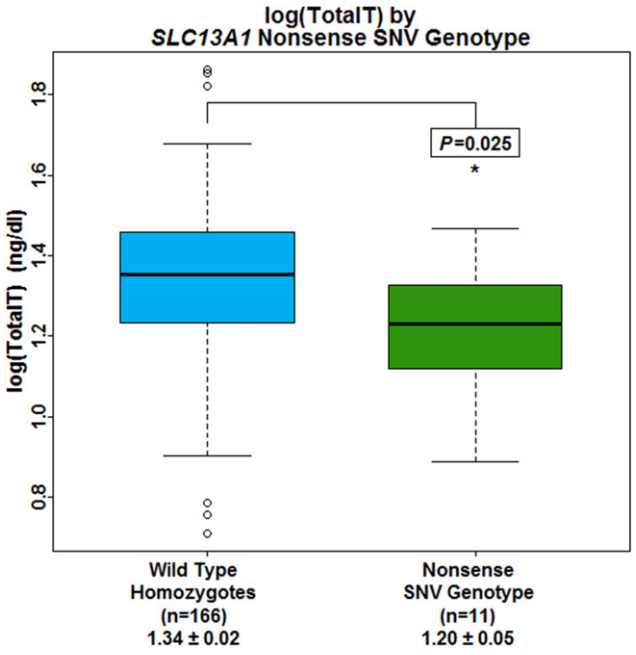


**Fig. 4.** Total testosterone levels by *SLC13A1* nonsense single nucleotide variant (SNV) genotype in women (n = 177). Box and whisker plots: box represents 2nd and 3rd quartiles (IQR, interquartile range); horizontal band represents median value; whiskers represent 1st and 4th quartiles; ends of whiskers represent minimum and maximum values, excluding outliers; open circles represents outliers (data point > 1.5*IQR below the 1st quartile or above the 3rd quartile). *P* represents significance from model including age, age-squared, gender, and serum sulfate concentration as covariates, and accounting for phenotypic correlation due to relatedness; * indicates $P < 0.05$.

## 4. Discussion

We conducted a study to examine the effect of serum sulfate concentration on DHEA and DHEA-S. Despite the likely relevance of serum sulfate levels on DHEA and DHEA-S, we are, to the best of our knowledge, the first to examine this association. This is likely due to the fact that serum sulfate is not routinely measured, in either clinical or research settings, and therefore association studies between serum sulfate concentration and clinical phenotypes have not previously been performed.

In contrast to our hypothesis that decreased serum sulfate would be associated with decreased DHEA-S and DHEA-S/DHEA ratio, we did not observe any association between serum sulfate concentration and these hormones in the cohort of 203 men and women. Similarly, we did not observe any association between serum sulfate concentration and

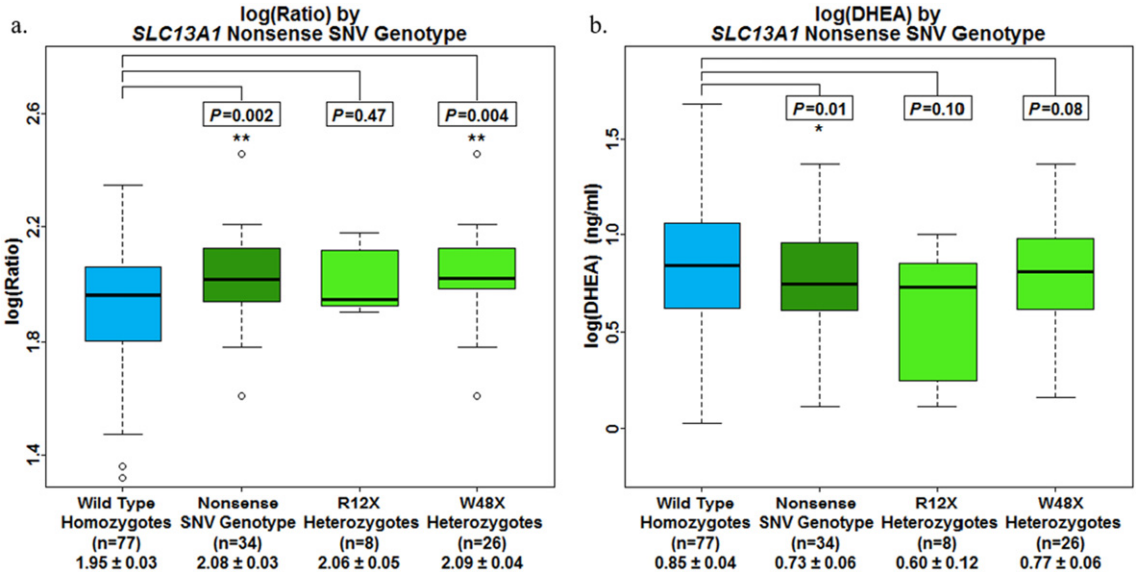


**Fig. 3.** DHEA hormone levels by *SLC13A1* nonsense single nucleotide variant (SNV) genotype in women (n = 111). a) $Ratio_{Log}$, b) $DHEA_{Log}$. Box and whisker plots: box represents 2nd and 3rd quartiles (IQR, interquartile range); horizontal band represents median value; whiskers represent 1st and 4th quartiles; ends of whiskers represent minimum and maximum values, excluding outliers; open circles represents outliers (data point > 1.5*IQR below the 1st quartile or above the 3rd quartile). *P* represents significance from model including age, age-squared, gender, and serum sulfate concentration as covariates, and accounting for phenotypic correlation due to relatedness; * indicates $P < 0.05$, ** indicates $P < 0.01$.

these hormones when analyzing women only. However, we found that in men, both DHEA-$S_{Log}$ and $Ratio_{Log}$ exhibit an inverse relationship with serum sulfate concentration that is significant or nearly significant in two different models. This in itself is an interesting finding, as it suggests that sulfation of DHEA is not limited by decreased substrate (sulfate) in men or women at the serum sulfate concentrations observed in this cohort (range: 0.06–0.55 mM; 0.30–0.50 mM in normal adults [18,37]) despite evidence that sulfation can be limited by decreased or absent levels of cofactor (PAPS) [3,6,35,36]. In fact, these results suggest that decreased serum sulfate encourages sulfation of DHEA in men, possibly through a gender-specific feedback mechanism that increase expression of *PAPSS2* in response to decreased serum sulfate, or through some other alternative mechanism. Future studies aimed at quantifying *PAPSS2* expression and/or PAPS activity in these samples would allow one to test the former explanation.

Contrary to our hypothesis that sulfate-lowering variants would be associated with decreased DHEA-S and DHEA-S/DHEA ratio, we instead observed a decrease in DHEA among carriers of the sulfate-lowering nonsense variants, *SLC13A1* R12X or W48X, compared to non-carriers. Interestingly, results from our Mendelian randomization analysis suggest *SLC13A1* nonsense variant carrier status is the primary driver of these associations as opposed to the secondary decrease in serum sulfate resulting from these sulfate-lowering variants. This suggests a role for these genetic variants in DHEA homeostasis that is independent of serum sulfate concentration, which could potentially explain our paradoxical findings. To examine the possibility of any gender-specific associations, both analyses were also performed after stratifying by gender. In both cases, the association between *SLC13A1* nonsense variant carrier status and decreased DHEA, as well as the association between *SLC13A1* nonsense variant carrier status and increased DHEA-S/DHEA ratio, remained significant in women but not in men, providing insight that these that these associations are primarily driven by female subjects. Given that testosterone is a downstream product of DHEA, we suspected that the lower DHEA levels observed in female *SLC13A1* nonsense variant carriers compared to non-carriers may also result in lower testosterone levels. Our results suggest that female *SLC13A1* nonsense variant carriers have lower total testosterone levels than non-carrier females, and may also exhibit lower free testosterone levels.

*SLC13A1* nonsense variants cause decreased serum sulfate, likely due to decreased sulfate (re)absorption in the intestines and kidneys [17]. However the mechanism by which these variants result in decreased serum DHEA is not as clear. It seems possible that while NaS1 functions to move sulfate into intestinal and kidney epithelial cells, NaS1 could function to move sulfate out of other types of cells if either NaS1 was expressed on the basolateral membrane or in the reverse orientation in such tissues. The cystic fibrosis transmembrane conductance regulator (CFTR) protein, for example, transports chloride out of epithelial cells in the lung, liver, pancreas, and digestive tracts, yet reabsorbs sodium chloride in the reabsorptive duct due the reverse orientation of the CFTR transporter in reabsorptive ducts [39,40]. This results in the paradoxical hypotonic mucus and hypertonic sweat seen in patients with cystic fibrosis, a genetic disease caused by mutations in *CFTR* [41]. Analogously, one could imagine a scenario where decreased sulfate transport, due to loss-of-function variants in *SLC13A1*, could cause reduced cellular export and increased intracellular concentrations of sulfate. If this were to occur in tissues involved in DHEA production, increased intracellular sulfate concentration would drive the equilibrium to a state of increased DHEA-S, decreased DHEA production, and increased DHEA-S/DHEA ratio. With DHEA-S levels far exceeding that of DHEA [5], smaller variations in DHEA levels may be more apparent and significant than changes in DHEA-S of equal absolute quantity. In such a scenario, sulfate supplementation may actually be contraindicated as it could possibly result in even lower levels of DHEA. Patients with homozygous and compound heterozygous loss-of-function mutations in *PAPSS2* have been reported, all presenting with clinical manifestations and phenotypes consistent with impaired sulfation [3,6,36,42]. In 1998, Faiyaz ul Haque et al. described a large, inbred Pakistani family with a form of autosomal recessive SEMD caused by a homozygous mutation in *PAPSS2* [42,43]. This phenotype is thought to result from impaired proteoglycan sulfation in growth-plate chondrocytes [3,42,44,45]. While the individuals of this particular family did not undergo endocrine investigations, additional individuals with bone dysplasia and PAPSS2 deficiency resulting from mutations in *PAPSS2* have been described, often revealing low DHEA-S but normal DHEA, androstenedione, and testosterone levels in subjects for whom serum androgens were measured [6,35,36]. However, due to a cause that remains unclear, a minority of patients with *PAPSS2* mutations present with signs of androgen excess and/or abnormal androgen metabolism [3,46]. We previously reported on R12X and W48X, two rare, non-linked, nonsense variants in *SLC13A1* that are enriched in the Amish (Table S2) and associated with a 27% decrease in serum sulfate [17]. Despite this enrichment, we have yet to identify an Amish individual homozygous or compound heterozygous for R12X and/or W48X. Furthermore, no individuals have been identified as homozygous for any of the loss-of-function variants listed for *SLC13A1* on The Exome Aggregation Consortium (ExAC) database [47], with the exception of one R12X homozygote. This suggests the possibility of an *SLC13A1* R12X genotype error and/or potential lethality for humans homozygous for loss-of-function variants in *SLC13A1*, as a 54% decrease in serum sulfate ($27\% \times 2$ assuming an additive effect) [17] may not be sufficient for necessary sulfation reactions to occur. Alternatively, if individuals with homozygous or compound heterozygous mutations in *SLC13A1* exist, they would not only be extremely rare, but might also be severely affected, preventing them from participating in studies such as our Amish Complex Disease Research Program and those included in the ExAC database.

In conclusion, while the findings in this study may be limited by its relatively small sample size, the clinical descriptions of individuals with hypomorphic or loss-of-function variants in *SULT2A1* [4,11] and *PAPSS2* [3,6,35,36,46,48] warranted a study aimed at examining the effect of serum sulfate concentration, as well as the effect of *SLC13A1* nonsense variants, on DHEA and DHEA-S. Our results suggest an inverse relationship between serum sulfate concentration and DHEA-S and DHEA-S/DHEA ratio in men. In addition, our findings show that the sulfate-lowering variants, *SLC13A1* R12X and W48X, decrease DHEA and testosterone levels, and increase DHEA-S/DHEA ratio in women. Clinicians and researchers should consider mutations in genes involved in sulfate biochemistry when treating patients with unknown causes of osteochondrodysplasia, premature pubarche, or PCOS. Lastly, through these investigations, what has become most clear is that the mechanisms involved in DHEA, DHEA-S, and testosterone homeostasis are complex and elaborate, and as such, are not entirely dependent on any one single component, such as serum sulfate concentration. Furthermore, these mechanisms inevitably vary between men and women. Despite these complexities, there is clear value in elucidating these mechanisms given the role of DHEA, DHEA-S, and testosterone in the brain [1,49,50] and their associations with autism spectrum disorder (ASD) [51,52]. Lastly, additional studies are warranted to better characterize the phenotypes of individuals with homozygous or compound heterozygous mutations in genes involved in sulfate biochemistry to better understand sulfate's role in human physiology, disease, and drug toxicity.

## Disclosure statement

CT, LA, AZ, PM, JM, and ES have nothing to disclose. AR, in addition to his part-time appointment at the University of Maryland School of Medicine, is Vice President and Co-Head of the Regeneron Genetics Center, LLC, a fully owned subsidiary of Regeneron Pharmaceuticals, Inc. The Regeneron Genetics Center focuses on early discovery research, applying human genomics to identify novel drug targets. LY is an employee stockholder of GlaxoSmithKline; however, her contribution to this

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ymgmr.2017.01.005.

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