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1 **Alternate steroid sulfation pathways targeted by LC-MS/MS analysis of disulfates.**
2 **Application to prenatal diagnosis of steroid synthesis disorders**

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15 **Short title**

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22
23

24 **ABSTRACT**

25 The steroid disulfates (*aka* bis-sulfates or bis(sulfates)) are a significant but minor
26 fraction of the urinary steroid metabolome that have not been widely studied
27 because major components are not hydrolyzed by the commercial sulfatases
28 commonly used in steroid metabolomics. In early studies, conjugate fractionation
29 followed by hydrolysis using acidified solvent (solvolysis) was used for the indirect
30 detection of this fraction by GC-MS. This paper describes the application of a specific
31 LC-MS/MS method for the direct identification of disulfates in urine, and their use as
32 markers for the prenatal diagnosis of disorders causing reduced estriol production:
33 STSD (Steroid Sulfatase Deficiency), SLOS (Smith-Lemli-Opitz Syndrome) and PORD
34 (P450 Oxido-Reductase Deficiency). Disulfates were detected by monitoring a
35 constant-ion-loss (CIL) from the molecular di-anion. While focused on disulfates, our
36 methodology included an analysis of intact steroid glucuronides and monosulfates
37 because steroidogenic disorder diagnosis usually requires an examination of the
38 complete steroid profile. In the disorders studied, a few individual steroids (as
39 disulfates) were found particularly informative: pregn-5-ene-3 β ,20S-diol, pregn-5-
40 ene-3 β ,21-diol (STSD, neonatal PORD) and 5 α -pregnane-3 β ,20S-diol (pregnancy
41 PORD). Authentic steroid disulfates were synthesized for use in this study as aid to
42 characterization. Tentative identification of 5 ξ -pregn-7-ene-3 ξ ,20S-diol and 5 ξ -
43 pregn-7-ene-3 ξ ,17,20S-triol disulfates was also obtained in samples from SLOS
44 affected pregnancies. Seven ratios between the detected metabolites were applied
45 to distinguish the three selected disorders from control samples. Our results show
46 the potential of the direct detection of steroid conjugates in the diagnosis of
47 pathologies related with steroid biosynthesis.

48

49

50 **INTRODUCTION**

51 From the earliest days of steroid metabolomics, the principal conjugated forms of
52 steroids (sulfates and glucuronides) have been hydrolyzed prior to analysis, and for
53 decades the instrument of choice for steroid separation and measurement has been
54 GC-MS (Shackleton and Marcos 2006). While this technique remains the gold-
55 standard for steroid profiling, LC-MS/MS has been increasingly adopted because of
56 the simplified sample preparation and speed of analysis, mainly provided by
57 absence of a derivatization step. This is in spite of the poor ionization for fully
58 reduced steroids by electrospray (ESI) (Pozo, et al. 2007). While an advance, this
59 methodology still retains the most time-consuming step of sample preparation, the
60 enzymatic or chemical hydrolysis of conjugates (Gomes, et al. 2009). Hydrolysis itself
61 can take several hours and requires a further solid phase extraction (SPE). Necessary
62 chemical derivatization for GC-MS can also take hours.

63 Intact steroid conjugates have been analyzed by mass spectrometry since the
64 introduction of particle beam ionization (e.g. Fast Atom Bombardment, FAB) in the
65 1980s (Shackleton and Straub 1982; Shackleton 1983). Their spectra have dominant
66 deprotonated molecules $[M-H]^-$ in negative ion mode allowing ease of mass
67 determination. Conjugate analysis was simplified with the introduction of
68 electrospray ionization (ESI) and incorporation of HPLC and MS/MS. Glucuronides
69 can be analyzed in both positive and negative ionization modes by monitoring
70 $[M+NH_4]^+$ and $[M-H]^-$ respectively (Fabregat, et al. 2013). In the case of
71 monosulfates, collision-induced-dissociation (CID) of the strong $[M-H]^-$ ions shows a
72 distinctive hydrogen sulfate (HSO_4^-) fragment at m/z 97 (Shackleton 1983; Galuska,
73 et al. 2013). Direct detection of steroid conjugates also circumvents the ionization
74 problems of reduced steroids (Pozo, et al. 2007) as phase II metabolites have readily
75 ionized functionality (i.e. a carboxylic acid in glucuronides and an acidic sulfate ester
76 in sulfates).

77 While mono-conjugates dominate the sulfate fraction of urinary steroids, it has been
78 known since the 1960s that disulfates (diS, also referred to as bis(sulfates) or bis-
79 sulfates to distinguish them from compounds containing the disulfate ($S_2O_7^{2-}$) unit)
80 are significant components of the metabolome (Pasqualini and Jayle 1962; Arcos
81 and Lieberman 1967; Shackleton, et al. 1968a; Shackleton, et al. 1968b; Jänne, et al.
82 1969). Early studies by GC-MS of separated conjugate fractions showed that, in
83 addition to the classic 3β -sulfated steroids, hydroxyls at positions 16β -, 17 -(α and β)
84 and 18 - in androgens and 20 - and 21 - in pregnanes were prone to sulfation (Jänne,
85 et al. 1969; Jänne and Vihko 1970; Laatikainen, et al, 1972; Meng and Sjövall 1997).

86 Since these original studies, disulfates have been a largely ignored component of the
87 metabolome that nevertheless had significant potential to expand the
88 understanding of steroid biosynthetic and metabolic pathways. Given this, we
89 sought to develop LC-MS/MS methodology to target this group. It was found that
90 constant-ion-loss (CIL) of hydrogen sulfate (HSO_4^-) fragment at m/z 97 from the
91 molecular di-anion $[M-2H]^{2-}$ was the most useful reaction to monitor (McLeod, et al.
92 2017).

93 The ease of steroid disulfate analysis led us to investigate their use in diagnosis of
94 steroid biosynthetic disorders. One particular area of interest to the authors has
95 been the pre-natal diagnosis of single-gene disorders of estriol (E3) synthesis by
96 urine analysis, of which we have studied three conditions by GC-MS, viz., Steroid
97 Sulfatase Deficiency (STSD), Smith-Lemli-Opitz Syndrome (SLOS, 7-dehydrosterol
98 reductase deficiency) and cytochrome P450 Oxido-Reductase Deficiency (PORD)
99 (Marcos et al. 2009; Shackleton, et al. 2004a; Shackleton et al. 2004b; Arlt et al.
100 2004; Reisch, et al. 2013; Shackleton et al. 2007). This communication offers our
101 preliminary observations of the disulfated steroids excreted in these disorders at
102 around mid-pregnancy. While focusing on disulfates, selected monosulfates and

103 glucuronides were also included; evaluating the complete steroid profile is crucial to
104 diagnosing aberrant steroid biosynthesis (Shackleton and Marcos 2006).

105

106 MATERIALS AND METHODS

107

108 *Reagents and chemicals*

109

110 Steroid starting materials were obtained from Steraloids (Newport, RI, USA).
111 Chemicals and solvents including sulfur trioxide pyridine complex (SO₃.py), *N,N*-
112 dimethylformamide (DMF) and ammonium formate (HPLC grade) were purchased
113 from Sigma–Aldrich (St Louis, MO, USA). Aqueous ammonia solution (25%), and
114 acetonitrile and formic acid (LC-MS grade) were from Merck (Darmstadt, Germany).
115 MilliQ water was obtained using a Milli-Q purification system (Millipore Ibérica,
116 Barcelona, Spain).

117

118 *Synthesis of reference steroid disulfates*

119

120 The qualitative synthesis of steroid disulfates as the ammonium salts was performed
121 as previously described (McLeod, et al. 2017) with small modifications. Briefly, 1 mg
122 of each steroid standard was directly dissolved in a freshly prepared solution of
123 SO₃.py complex (20 mg, 124 μmol, ~38 eq/steroid or ~19eq/hydroxyl group) in DMF
124 (100 μL) and incubated at room temperature for 72 hours. The success of synthesis
125 was confirmed by analysis of reaction using both LC-MS in scan mode and LC-MS/MS
126 for collision induced dissociation studies. The purification of synthesised disulfates
127 was performed using SPE as previously described (McLeod et al, 2017).

128 Steroid disulfate reference materials isolated as the corresponding ammonium salts
129 and used in this study included: 5α-pregnane-3β,20S-diol bis(sulfate), (3β5αPD-diS);
130 3β,21-dihydroxypregn-5-en-20-one bis(sulfate), (21-hydroxypregnenolone
131 bis(sulfate), 21OHPreg-diS); androst-5-ene-3β,17α-diol bis(sulfate), (5AD(17α)-diS);
132 androst-5-ene-3β,17β-diol bis(sulfate), (5AD(17β)-diS); 3β,16α-dihydroxyandrost-5-
133 en-17-one bis(sulfate), (16α-hydroxydehydroepiandrosterone bis(sulfate),

134 16 α OHDHEA-diS); 3 β ,16 β -dihydroxyandrost-5-en-17-one bis(sulfate) (16 β -
135 hydroxydehydroepiandrosterone bis(sulfate), 16 β OHDHEA-diS); pregn-5-ene-
136 3 β ,17 α ,20S-triol 3,20 bis(sulfate), (5PT-diS); pregn-5-ene-3 α ,20S-diol bis(sulfate),
137 (5PD-diS); 5 β -pregnane-3 β ,20S-diol bis(sulfate),; 5 β -pregnane-3 α ,20S-diol
138 bis(sulfate),; 5 α -pregnane-3 α ,20S-diol bis(sulfate),; 5 β -pregnane-3 β ,20R-diol
139 bis(sulfate), 5 α -pregnane-3 β ,20R-diol bis(sulfate),; 5 β -pregnane-3 α ,20R-diol
140 bis(sulfate),; In this manuscript the IUPAC terms for the 20-hydroxypregnane
141 diastereomers are used, S and R, in some publications often trivialized to α and β ,
142 respectively.

143 Two reference materials (3 β 5 α PD-diS and 21OHPreg-diS), were prepared on larger
144 scale and subjected to characterisation by spectroscopic methods. Experimental
145 details and characterization data for these new compounds, together with copies of
146 the ^1H NMR, ^{13}C NMR, and ESI LRMS spectra are available from the authors (MM).

147

148 ***Urine Samples***

149 One of our laboratories (Children's Hospital Oakland, Dr. Cedric Shackleton) has
150 been the recipient for urine samples from patients with suspected abnormal
151 steroidogenesis in an attempt to characterize the defects. The studies were
152 approved by the Children's Hospital Institutional Review Board (IBR#2010-038)).
153 Many of the samples used in this study were remnants of those sent to the
154 laboratory for investigation of low pregnancy estriol (generally defined as individuals
155 with serum unconjugated estriol < 0.3 MoM, multiples of median). Other samples
156 were from women who had had a previously affected SLOS child or other
157 symptomatic reasons for concern regarding steroidogenesis. The samples have
158 generally been collected between week 16 and 30 of gestation. They have been
159 stored frozen at -20 °C. Eleven STSD samples were analyzed, and six samples from
160 SLOS affected pregnancies. The neonatal PORD samples were collected at 7, 18 and

161 23 days. Urine samples from unaffected pregnancies were from a collection held by
162 IMIM (Institut Hospital del Mar d'Investigacions Mèdiques, Barcelona). Normal
163 neonatal urine specimens were from a control urine collection at the Institute of
164 Metabolism and Systems Research (IMSR), University of Birmingham UK.

165

166

167 ***Sample treatment***

168 Urine extraction was by C18 SPE. Generally, a 2 mL aliquot of urine was passed
169 through a pre-conditioned cartridge. After a washing step with 3 mL water, steroid
170 conjugate analytes were eluted using 2 mL of methanol. After evaporation of a 200
171 μL aliquot of the elution solvent, the extract was reconstituted in 100 μL of water
172 and 5 μL was injected into the UHPLC-MS/MS system. Stably labelled 17-S $\{^{18}\text{O}\}_3$ -5 α -
173 androstane-3 α ,17 β -diol 3,17-bis(sulfate) and 17-S $\{^{18}\text{O}\}_3$ -5 α -androstane-3 β ,17 β -diol
174 bis (sulfate) were used as internal standards. The labeled sulfate residue was
175 introduced to the steroidal diol mono-sulfate using labelled S $\{^{18}\text{O}\}_3$.py generated in
176 situ from labelled sulfuric acid (95% atom) and acetic anhydride in pyridine.
177 Experimental details and characterization data for these internal standards, together
178 with copies of the ^1H NMR, ^{13}C NMR, and ESI LRMS spectra are available from the
179 authors (MM)

180

181 ***UHPLC-MS/MS analysis***

182

183 *Disulfates*

184

185 The study was carried out using a triple quadrupole (XEVO TQ-S micro) mass
186 spectrometer equipped with an ESI source and interfaced to an Acquity UPLC system

187 for the chromatographic separation (all from Waters Associates, Milford, MA, USA).
188 Drying gas as well as nebulizing gas was nitrogen. The desolvation gas flow was set
189 to approximately 1200 L/h, and the cone gas flow was 50 L/h. A cone voltage of 30 V
190 and a capillary voltage of 0.4 kV were used in negative ionization mode. The
191 nitrogen desolvation temperature was set to 600 °C, and the source temperature
192 was 150 °C.

193 The UHPLC separation was performed using an Acquity UPLC CSH Phenyl-Hexyl
194 column (2.1 × 100 mm i.d., 1.7 µm) (Waters Associates), at a flow rate of 300
195 µL/min. Water and acetonitrile:water (9:1) both with formic acid (0.01% v/v) and
196 ammonium formate (25 mM) were selected as mobile phase solvents. A gradient
197 program was used; the percentage of organic solvent was linearly changed as
198 follows: 0 min, 15%; 0.5 min, 15%; 25 min, 30%; 26 min, 100%; 27 min, 100%; 28
199 min, 15%; 30 min, 15%. The total analysis time was 30 min.

200 For the constant ion loss (CIL) scan, dwell times of 6 ms and collision energies of 15
201 eV were selected for each ion transition. Due to the molecular masses of steroid
202 hormones and metabolites (250-400 Da), the precursor ions of disulfates ($[M-2H]^{2-}$)
203 were restricted to the range from m/z 199 to m/z 274. A Selected Reaction
204 Monitoring (SRM) approach containing 75 preselected transitions was used for the
205 simultaneous detection of steroid disulfates. Among them, the transition 228→359
206 corresponded to the internal standards used in the analysis.

207

208 *Monoconjugates*

209

210 While the focus has been on steroid disulfates we have acquired data on steroid
211 monosulfates and glucuronides previously reported as relevant for the studied
212 disorders. Based on previous studies (Gomez, et al. 2014) the product ions at m/z 97
213 and m/z 75 for sulfates and glucuronides respectively were chosen (Table 1).

214 Exceptions were estriol conjugates due to the influence of the aromatic ring. The

215 neutral loss of the conjugate (80 Da and 176 Da for sulfates and glucuronides
216 respectively were detected).

217

218 *Quantification*

219 For this study, accurate quantitative measurements have not been conducted for
220 two reasons: 1) lack of some authentic compounds prevented the determination of
221 relative responses of analyte transitions to internal standard transitions; 2) the urine
222 samples were random “spot” collections and not accurate 24 h collections. Instead,
223 we have determined “diagnostic-ratios” from raw mass spectrometric transition
224 responses. These ratios are of an analyte known to be *overproduced* to one known
225 to be *underproduced* in a particular disorder. Such ratios have long been used in GC-
226 MS analysis (Shackleton and Marcos,2006).

227

228

229 **RESULTS AND DISCUSSION**

230

231 ***Method development***

232 This communication applies recent LC-MS/MS studies on steroid disulfate analysis
233 using the constant-ion-loss (CIL) from the di-anionic precursor $[M-2H]^{2-}$ (McLeod, et
234 al. 2017). The method was developed for untargeted detection, and designed for
235 the analysis of a maximum number of natural disulfates. The use of this precursor
236 ion and the fact that the product ion has a higher m/z value is unusual for small
237 molecules. Determination of disulfates under these conditions gives clean
238 chromatograms and the main interferences observed in the chromatograms are due
239 to the relatively high natural abundance of the ^{34}S isotope (4.25%). The transition
240 coming from the m/z 97 loss from an unsaturated (Δ^4 , Δ^5 , etc.) $\{^{34}\text{S}\}_1$ -disulfate
241 isotope is completely indistinguishable from the one coming from an A-ring reduced
242 steroid disulfate.

243 To maximize isobaric steroid metabolite separation (e.g. pregnenediol-diS, the
244 pregnenediol-disulfates and the androstenediol-disulfates) in this study, a phenyl-
245 hexyl column with a relatively high amount of ammonium formate (25 mM) was
246 required to obtain sharp and well resolved chromatographic peaks. Column
247 temperature was critical for this purpose with 30 °C determined as optimum. Under
248 these conditions, a 25 min gradient from 15% to 30% of organic solvent provided
249 desired separation (Figure 1A).

250 Under optimized conditions the elution order of disulfates was
251 dihydroxyandrostanoines < dihydroxypregnanones < androstenediols <
252 pregnenediols. In a specific group, 17β hydroxysteroid disulfates eluted earlier than
253 their 17α -counterparts and 20S -hydroxysteroid disulfates eluted earlier than their
254 20R counterparts. Regarding A ring derivatives, Δ^5 steroid disulfates eluted before

255 the fully reduced metabolites, the elution order of the reduced steroids being $3\beta,5\beta$
256 $< 3\beta,5\alpha < 3\alpha,5\beta < 3\alpha,5\alpha$. The chromatographic conditions were also able to separate
257 the two estriol glucuronide isomers i.e. the 16-glucuronide and 3-glucuronide.
258 Unfortunately, sulfate and glucuronide conjugates of two useful steroids in PORD
259 diagnosis, androsterone and etiocholanolone, could not be separated under the
260 selected conditions even after increasing the gradient to 1 h (Figure 1B).

261

262

263 ***Application to prenatal detection of disorders affecting estriol synthesis***

264 We report preliminary studies to determine whether steroid disulfates in urine can
265 be useful markers in the prenatal detection of disorders affecting estriol synthesis;
266 until now only monoconjugates had been used. The background to this study being
267 that unconjugated serum E3 is frequently measured at mid-pregnancy as a marker
268 for Down's syndrome as part of a test called triple- or quad- marker screening
269 (Haddow, et al. 1994). If results are low the question remains as to the reason, and
270 our original research was directed to diagnosis of Smith-Lemli-Opitz Syndrome
271 (SLOS), the clinically most severe cause of low E3 (Shackleton, *et al.* 2007). These
272 studies led to investigation of other causes such as STSD and PORD.

273 Diagnostic ratios are frequently employed in steroid metabolomics and E3
274 frequently used as denominator. Dominant E3 conjugates are 3- and 16-
275 glucuronides (30% and 60%, respectively) with about 2.5% as monosulfate and
276 estriol-3-glucuronide-16-sulfate (6.5%) (Tikkanen, *et al.* 1973). We assessed E3
277 excretion from the measurement of glucuronide and monosulfate conjugates (Table
278 1).

279

280 *Steroid sulfatase deficiency (STSD) (OMIM , 308100, location, Xp22.31)*

281

282 This X-linked disorder prevents the release of steroid from steroid sulfates. A
283 summary of the biosynthetic pathway leading to estriol is shown in Figure 2,
284 illustrating that inactivity of the enzyme in placenta prevents 16α OHDHEA-S
285 conversion to E3. This fetal 16α OHDHEA-S, androst-5-ene- 3β , 16α , 17β -triol sulfate
286 (5AT-S) and other steroid sulfates pass through the placenta and mother to be
287 excreted in urine largely unchanged (Taylor and Shackleton, 1979).

288

289 STSD urine samples (N=11) and 11 controls were analyzed using the CIL scan method
290 for disulfates complemented with the acquisition of 16α OHDHEA-S and E3
291 monoconjugates (Table 1). Among the disulfates measured by the CIL method, we
292 found that the response ratio between six of them, namely 16α OHDHEA-diS,
293 5AD(17α)-diS, 5AD(17β)-diS, 5PT-diS, 21OHPreg-diS and 5PD-diS against E3
294 glucuronide (measured as sum of 3- and 16-glucuronides) was markedly increased in
295 STSD.

296

297 Representative chromatograms of a normal pregnancy urine and one with an STSD
298 affected fetus are shown in Figure 3. The ratio values for our normal and STSD data
299 sets are shown in Figure 4A and show all analytes clearly distinguish STSD from
300 normal. Additionally, we used the ratios to evaluate the relative efficacy of each
301 analyte in diagnosis. The best steroid discriminatory ratio would show greatest
302 difference between the lowest steroid sulfate/E3-G ratio value in STSD, and the
303 highest ratio found in controls (Figure 4B). Interestingly, the ratios that gave the
304 greatest differential were Δ^5 pregnenes; 5PD-diS, 5PT-diS and 21OHPreg-diS, not the
305 C_{19} steroid sulfates on the direct biosynthetic pathway to E3. Combining ratio data
306 can give an even greater separation for normal and affected; note the combined
307 data for 21OHPreg-diS and 5PD-diS in Figure 4C. Such pregnene metabolites should
308 be incorporated in MS based methodologies for detection of the disorder.

309

310 *P450 oxido-reductase deficiency (PORD) (POR OMIM 124015 location: 7q11.23)*

311

312 Several pregnant women carrying PORD fetuses have been studied by GC-MS
313 (Shackleton, et al. 2004; Reisch, et al. 2013), but for only two were samples available
314 for this study. Shackleton and co-workers (2004a) deduced that the dominant “feto-
315 placental” maternal urinary steroid in PORD pregnancies was $3\beta 5\alpha$ PD-diS. While this
316 steroid is also present in the disulfate fraction of normal pregnancy urine it is in
317 much greater amount in PORD affected pregnancies. From its dominance together
318 with reduced E3, it was concluded that this metabolite is a maternal excretory
319 product of fetal pregnenolone. An intermediate precursor would be fetal steroid
320 5PD-diS. Excess pregnenolone and its sulfate are the result of an apparent “block” in
321 17-hydroxylase/C17-20 lyase secondary to attenuated POR activity (Figure 5). This
322 block, together with suppressed 16α -hydroxylase (also due to PORD) causes
323 reduction of fetal 16α OHDHEA-S production leading to low maternal E3 production
324 and excretion. The precise sequence of reactions from fetal pregnenolone to
325 $3\beta 5\alpha$ PD-diS, and localities of the conversions (fetal adrenal, liver, placenta and
326 mother) is yet to be determined. The process is multi-step, probably including
327 placental 3β -desulfation and likely 3β -hydroxysteroid dehydrogenase/isomerase. It
328 has long been known that both $3\beta 5\alpha$ PD-diS and 5PD-diS are prominent disulfates in
329 umbilical cord blood (Laatikainen, et al. 1972) so are freely synthesized and
330 transported in the feto-placental unit. The synthetic sequence for pregnenolone
331 conversion to urinary metabolites in normal and PORD affected pregnancies and
332 neonate are shown in Figure 5. Evidence suggests the corresponding conversion of
333 pregnenolone sulfate to DHEA-S is not an available pathway (Neunzig, et al. 2014;
334 Sanchez-Guijo, et al. 2016; Rege, et al. 2017).

335 Besides the increased excretion of $3\beta 5\alpha$ PD-diS, we also observed an increase in the
336 transitions corresponding to 5PD-diS and $3\beta, 21$ -dihydroxy- 5α -pregnan-20-one
337 disulfate ($21\text{OHPreg}3\beta 5\alpha$ -diS), the latter in spite of a likely POR requirement by fetal
338 21-hydroxylase. However, it should be noted that this fetal enzyme differs from that

339 coded by CYP21A2 required in cortisol synthesis (Guerami et al., 1988, Corsan,
340 Macdonald and Casey., 1997).

341 In Figure 6 we illustrate the chromatographic profiles of the $3\beta 5\alpha$ PD-diS, 5PD-diS
342 and 21OHPreg $3\beta 5\alpha$ -diS and the E3 conjugates in a control (Figure 6A) and affected
343 pregnancy (Figure 6B). The dominance of the $3\beta 5\alpha$ PD-diS in the affected
344 pregnancies is striking. In GC-MS diagnosis of PORD prenatally the ratio of
345 $3\beta 5\alpha$ PD/E3 was used, i.e. the ratio of principal PORD fetal metabolite to E3, the
346 conventional feto/maternal metabolite. In Figure 6C are shown ratios for intact
347 conjugates in PORD and controls. For the denominator (E3) we summed the total of
348 both glucuronylated forms.

349

350 One of the GC-MS prenatal diagnostic ratios for PORD remains a challenge for LC-
351 MS/MS under conditions developed for this study. With fetal PORD there is
352 increased androsterone production as a result of the “alternative pathway” activity
353 (Arlt, et al. 2004) resulting in markedly increased androsterone/etiocholanolone
354 ratio (Shackleton et al 2004a). That ratio should theoretically be determined by
355 direct analysis of glucuronides and this separation has been already reported by C18
356 columns both in glucuronides (Pozo et al. 2008) and unconjugated (Marcos and
357 Pozo, 2016). Unfortunately, under current chromatographic conditions developed
358 for the disulfates such isobaric monoconjugates (sulfates or glucuronides) could not
359 be resolved.

360

361 Postnatal detection of PORD:

362

363 While this paper has focused on prenatal diagnosis of PORD by $3\beta 5\alpha$ PD-diS
364 measurement, Shackleton and co-workers (Shackleton et al. 2004b) have shown
365 that its precursor 5PD-diS is a key analyte in diagnosing the condition in the first
366 months of life suggested its inclusion here. In PORD neonatal samples this steroid is

367 dominant, excretory values exceeding the classical major metabolites such as
368 16α OHDHEA-S and 16α -hydroxypregnenolone sulfate whose biosynthesis by 16α -
369 hydroxylation is also POR dependent. In the first weeks of life the fetal zone of the
370 adrenal is still dominant, but diminishing, and is responsible for producing a large
371 amount of 3β -OH- Δ^5 steroids.

372 Figure 7 illustrates the separation of steroid disulfates in an affected PORD infant
373 and normal infant. We have included 16α OHDHEA-S as analyte to act as
374 denominator for a potential diagnostic ratio 5PD-diS/ 16α OHDHEA-S. This ratio is
375 shown for three affected infants and normal controls in Figure 7C, clearly defining
376 the condition. Interestingly, one of the first steroid disulfates to be identified in the
377 neonatal period were 5AD(17α and 17β)-diS (Shackleton, et al. 1968a, Laatikainen,
378 et al. 1972), and 16β OHDHEA-diS (Shackleton, et al. 1968b, Laatikainen, et al. 1972)
379 and these are clearly separated with this methodology (Figure 7).

380

381 Smith-Lemli-Opitz Syndrome. "7-dehydrosterol reductase" deficiency. (SLOS) (OMIM
382 602858 location: 11q13,4)

383

384 This condition is caused by deficiency in 7-dehydrosterol reductase and the notable
385 feature is a build-up of 7-and 8-dehydrocholesterol, which can be used to diagnose
386 the condition when measured in amniotic fluid (Kelley,1994). The affected fetus can
387 use these sterols as steroid precursors, resulting in the appearance in maternal urine
388 of dehydro (DH) versions of common natural steroids. For instance, 5β -pregn-7(and
389 8)-ene- $3\alpha,17\alpha,20S$ -triol, (7(8)-DHPT) and an estriol equivalent, principally 8-
390 dehydroestriol (8-DHE3) (Guo, et al. 2001, Shackleton, et al. 1999). The biosynthesis
391 of steroids in SLOS pregnancy is illustrated in Figure 8.

392 SLOS steroids are mainly excreted as glucuronides. Thus, distinct peaks
393 corresponding to different isomers of 8-DHE3-G were found in all SLOS samples
394 (Figure 9). On the other hand, the detection of 7(8)-DHPT-G provided more

395 difficulties due to endogenous interferences probably coming from other
396 pregnenetriols and DH-hydroxypregnenolones which would share the same
397 transition (Figure 9).

398

399 A systematic study has not been made of steroid sulfates in this condition and lack
400 of appropriate authentic compounds has meant that only *candidate*
401 chromatographic peaks were provisionally identified. Such peaks were chosen by
402 having the expected CIL transitions for steroids with additional unsaturation and to
403 be accepted as SLOS-specific “candidate” analytes these peaks had to be present in
404 all six confirmed SLOS pregnancies, and be absent from controls. Two main
405 metabolites were found. Peaks with the expected transitions for DHPT-diS and
406 DHPD-diS were observed in all SLOS samples. Additionally, other minor metabolites
407 such as DH-androstenediol-diS were also found. In Figure 9 the chromatograms of
408 the proposed steroid disulfate analytes with DH-pregnanetriol glucuronide and
409 DHE3-glucuronide are shown. Little information can be stated on stereochemistry of
410 candidate analytes; not only that of 3- and 5-positions but both Δ^7 and Δ^8 isomers
411 are likely present. The chromatograms illustrated were from one affected pregnancy
412 and one control. Similar chromatograms were produced for the other five affected
413 pregnancies and controls. Clearly this is the most challenging of the three conditions
414 for conjugate LC-MS/MS analysis although aberrant steroid conjugate peaks
415 definitive for SLOS were detected.

416

417 *Distinguishing the disorders: summary*

418

419 This study has focused on the mass spectrometric analysis of steroid disulfates, but
420 steroid monosulfates and glucuronides have been included where required to
421 determine ratios used for diagnosis. In order to evaluate the potential of the
422 approach based on the combined screening of glucuronides, monosulfates and
423 disulfates, we propose a panel of markers able to differentiate between the selected

424 disorders and control samples. We found that using the ratios $16\alpha\text{OHDHEA-S/E3-G}$,
425 $3\beta5\alpha\text{PD-diS/E3-G}$, $5\text{PD-diS}/16\alpha\text{OHDHEA-S}$, $21\text{OHPreg-diS}/\text{E3-G}$, 21OHPreg-
426 $\text{diS}/16\alpha\text{OHDHEA-S}$, $8\text{DHE3-G}/\text{E3-G}$ and $\text{DHPT-diS}/\text{E3-G}$ allowed for the successful
427 differentiation between the controls and the different disorders
428 Hopefully the study emphasizes the potential of LC-MS analysis of all conjugate
429 types in future development of steroid metabolomics.

430

431 ***General discussion***

432

433 The steroid disulfates are a minor fraction of the urinary steroid metabolome, but
434 may provide significant markers of aberrant steroid biosynthesis. As a family, intact
435 steroid disulfates have not been recently subject to detailed study due a lack of
436 suitable analytical methodology. In the past, studying this family always involved
437 time-consuming fractionation of free and conjugate families followed by solvolysis
438 and GC-MS analysis. Most of the available literature stems from the 60's and 70's
439 and it was shown early which secondary positions (assuming the primary sulfated
440 position is the 3-hydroxyl) could be sulfated. These were 17 (α - and β), 16β - and 18
441 in C_{19} steroids and 20S- and 21- in C_{21} steroids. During that early research period the
442 dominant biological materials chosen to study were associated with pregnancy. In
443 that respect our current studies have followed this lead and the major disulfate
444 components reported here were also noted in the early publications (Shackleton, et
445 al. 1968a; Shackleton, et al. 1968b; Jänne, et al. 1969; Jänne and Vihko 1970;
446 Laatikainen et al 1972; Meng and Sjövall 1997).

447

448 There is little definitive evidence as to which sulfotransferases are responsible for
449 the secondary sulfation (Mueller et al 2015), and how disulfates are transported
450 (Grosser et al 2017). Available sulfation evidence points solely to SULT2A1 which
451 appears to have an active site capable of encompassing a wide variety of steroid
452 substrates (both free and monosulfated) and conduct sulfation at either end of the

453 steroid molecule. Thus, it can sulfate free steroids or steroid monosulfates (Cook, et
454 al. 2009).

455 A question remains as to whether disulfation is purely a catabolic reaction or if such
456 steroids could be transportable reservoirs of active hormone precursors, as is likely
457 the case for DHEA and estrone sulfates. Guerami and co-workers (1988) have
458 proposed that 21OHPreg-diS is an 11-deoxycorticosterone (DOC) precursor during
459 pregnancy, particularly since circulating levels of this mineralocorticoid and its
460 sulfate are increased during gestation (Corsan, Macdonald and Casey 1997). It is
461 known that the placenta is capable of hydrolyzing 21-sulfates and the enzyme
462 responsible is the usual STS as 21-desulfation does not occur in STSD (Guerami, et al.
463 1988). Another possible reservoir for disulfates is 5AD(17 β)-diS, potentially a
464 testosterone or estradiol precursor. This steroid is also subject to STS action in
465 mammals. In contrast, it is believed that human sulfatases are inactive on 17 α - (C₁₉
466 steroids) or 20S--sulfates, a situation shared with the commercial snail and mollusk
467 enzymes used for hydrolysis in steroid analysis (Stevenson, et al. 2014).

468

469 In summary, we have provided analytical data on the steroid disulfates through their
470 measurement as intact molecules by LC-MS/MS, employing CIL scan monitoring. We
471 have attempted to use these additional members of the steroid metabolome to
472 distinguish fetal disorders of steroid synthesis. To the best of our knowledge, this is
473 the first time that direct analysis of steroid disulfates has proved its value for clinical
474 diagnosis.

475 The ultimate goal of these studies is the ability to quantify the whole urinary steroid
476 metabolome as unhydrolyzed conjugates, the monosulfates, disulfates, glucuronides
477 and mixed sulfate-glucuronide conjugates. Studies of the plasma steroid
478 metabolome should also be included. To achieve this goal will require the synthesis
479 of a multitude of authentic steroids including appropriate internal standards and an
480 improvement in chromatographic resolution.

481

482 Declaration of interest

483

484 There is no conflict of interest that could be perceived as prejudicing the impartiality
485 of the research reported

486

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500

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627
628
629
630

FIGURE LEGENDS

Figure 1

Chromatographic separation obtained for (A) seven synthesized isomers of pregnanediol disulfates and (B) androsterone and etiocholanolone glucuronide. Note that the method optimized for the separation of isomeric disulfate metabolites was not able to separate epimeric glucuronides.

Figure 2

Steroid synthesis in STSD pregnancy starting from fetal adrenal pregnenolone. Inactivity of STS prevents conversion of $16\alpha\text{OHDHEA-S}$ to estriol in placenta so the former (and its metabolites) is excreted as sulfates by mother. C_{21} steroid sulfates upstream from $16\alpha\text{OHDHEA-S}$ also pass the placenta without de-sulfation and are directly excreted in maternal urine.

Figure 3

Selected urinary Δ^5 steroid sulfate and estriol conjugate analysis in a control and STSD affected pregnancy. Note the markedly increased Δ^5 steroid mono and disulfates in STSD. Regarding the estriol conjugates it must be noted that glucuronides give lower MS transition responses than sulfates under the reported conditions. In reality, E3-S is a minor metabolite of estriol compared to the 3- and 16-glucuronides, although it appears contrary in the chromatograms.

Figure 4

Panel A: Ratios of steroid sulfates to E3 glucuronide (3+16) in STSD. The scale represents the ratios of raw peak areas of transitions, not the actual

amount quantified. All ratios show separation of controls from affected pregnancies. Panel B: a measure of the difference between the lowest STSD ratio and highest control ratio. The higher this value, the greater the separation between affected and normal. Two Δ^5 pregnene di-sulfates are the most efficacious diagnostic analytes. Panel C: combining data from 5PD-diS and 21OHPreg-diS increases discrimination between normal and STSD.

Figure 5

Steroid biosynthesis and metabolism in PORD and normal pregnancies and neonates. *Normal pregnancy*: fetal adrenal pregnenolone is converted to maternally excreted estriol conjugates (POR essential). *PORD pregnancy*: excess adrenal pregnenolone (due to PORD) is metabolized primarily to maternally excreted $3\beta5\alpha$ PD-diS. *Normal neonate*: Excretion product 16α OHDHEA-S and other sulfates. *PORD neonate*: major pregnenolone excretory product 5PD-diS.

Figure 6

Selected Reaction Monitoring (SRM) transition chromatograms of estriol conjugates and steroid disulfates in control (A) and PORD affected pregnancies (B). Note high excretion of $3\beta5\alpha$ PD-diS and 21OHPreg $3\beta5\alpha$ -diS. (C) Graph shows peak area ratios (analyte/E3-G) for 2 affected pregnancies and 11 controls.

Figure 7.

SRM chromatograms of control (A) and affected PORD babies (B). The key analyte is the pregnenolone metabolite 5PD-diS and its relative excess is determined by peak area ratio to 16α OHDHEA-S, normally a dominant metabolite in neonates. Discrimination obtained by the use of 5PD-

diS/16 α OHDHEA-S (C) and 21OHPreg-diS/16 α OHDHEA-S (D) between healthy and PORD babies.

Figure 8.

Deficiency of 7-dehydrosterol reductase (DHCR7, SLOS] causes 7-or 8-dehydro-cholesterol to be used as fetal precursor for downstream steroids which retain B- ring unsaturation. Dehydro-pregnanetriol (DHPT) and dehydroestriol (DHE3) glucuronides have been used classically for diagnosis but here are candidate disulfates tentatively identified, compounds not seen in controls.

Figure 9.

SRM chromatograms of candidate analytes in SLOS pregnancy. (A) control pregnancy and (B) affected pregnancy. Transition chromatograms for known diagnostic steroid glucuronides and candidate disulfates useful for diagnosis. While authentic steroids are not available, these steroids, with appropriate transitions were only present in affected pregnancies. Steroid A/B ring stereochemistry including Δ 7/ Δ 8 unsaturation is as yet unknown. In the control chromatograms E3 conjugates are shown but all SLOS candidate disulfates and glucuronides are absent.

Table 1. SRM parameters of selected steroids

Analyte	Disorder	MW	Retention time (min)	Precursor ion (m/z)	Product ion (m/z)	Collision energy (eV)
<i>Monoconjugates</i>						
E3-3G	all	464	2.3	463	287	30
E3-16G	all	464	9.3	463	287	30
E3-3S	all	368	7.3	367	287	35
16OHDHEA-S	STSD/PORD*	384	16.0	383	97	40
DHE3-G	SLOS	462	8.6/9.1	461	285	30
DHPT-G	SLOS	510	21.3	509	75	30
<i>Disulfates</i>						
5AD(17 α)-diS	STSD	450	15.5	224	351	15
5AD(17 β)-diS	STSD	450	13.4	224	351	15
16 α OHDHEA-diS	STSD	464	10.0	231	365	15
16 β OHDHEA-diS	STSD	464	8.2	231	365	15
5PT-diS	STSD	494	10.5	246	395	15
21OHPreg-diS	STSD/PORD	492	18.2	245	393	15
5PD-diS	STSD/PORD*	478	16.7	238	379	15
3 β 5 α PD-diS	PORD	480	17.6	239	381	15
DH5AD-diS	SLOS	448	11.4	223	349	15
DHPT-diS	SLOS	494	12.6	246	395	15
DHPD-diS	SLOS	478	15.6	238	379	15

* PORD neonatal

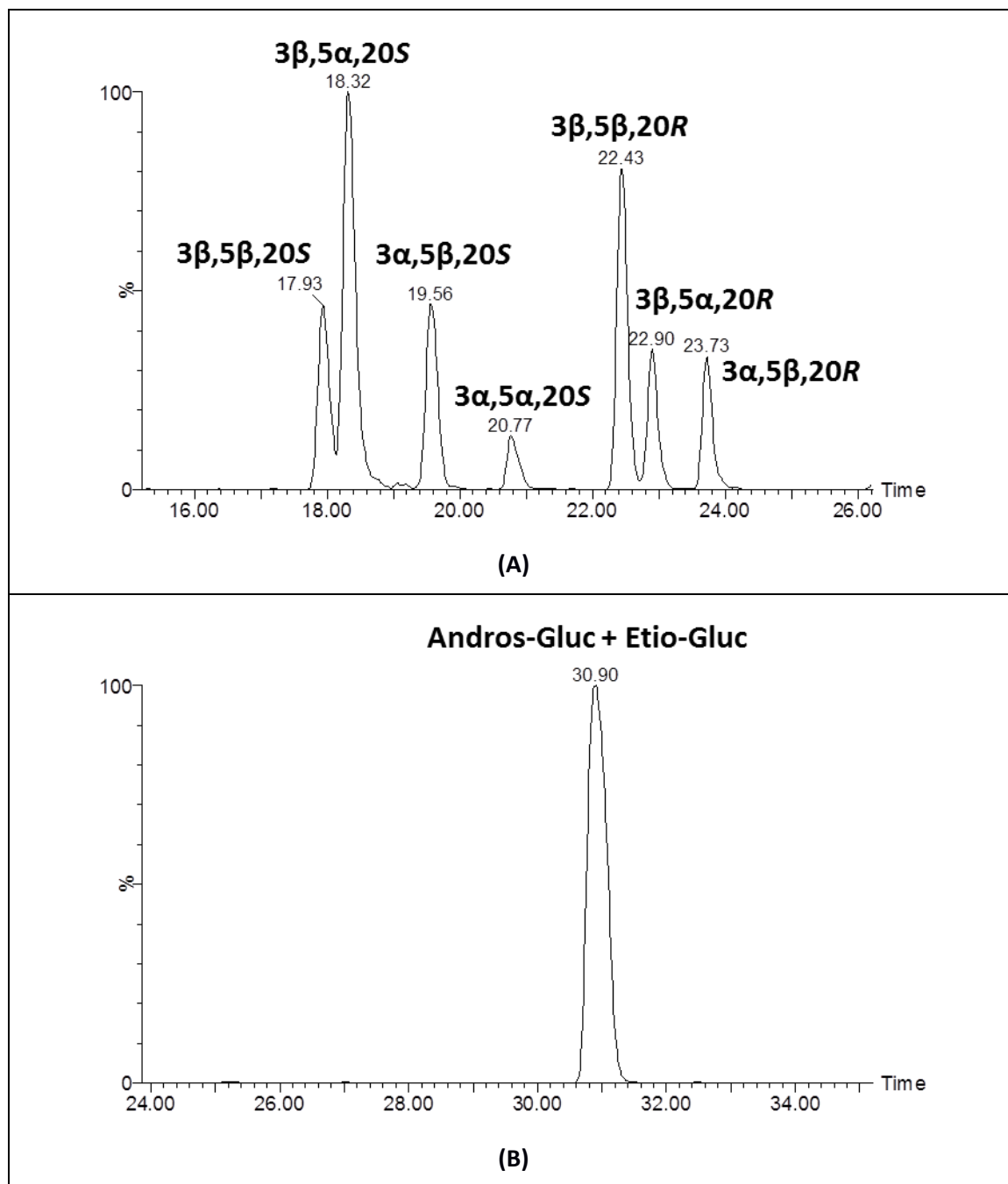


Figure 1

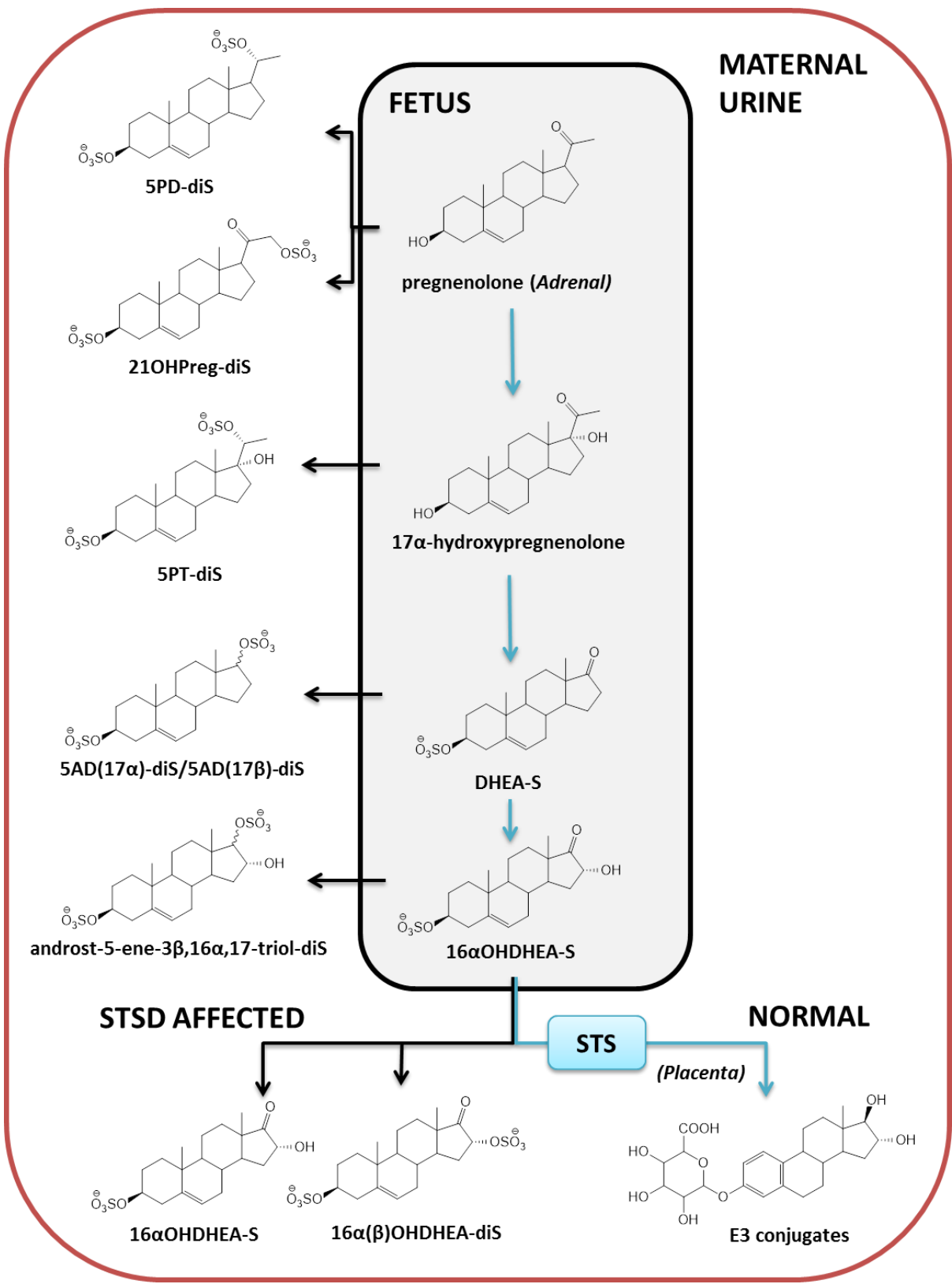


Figure 2

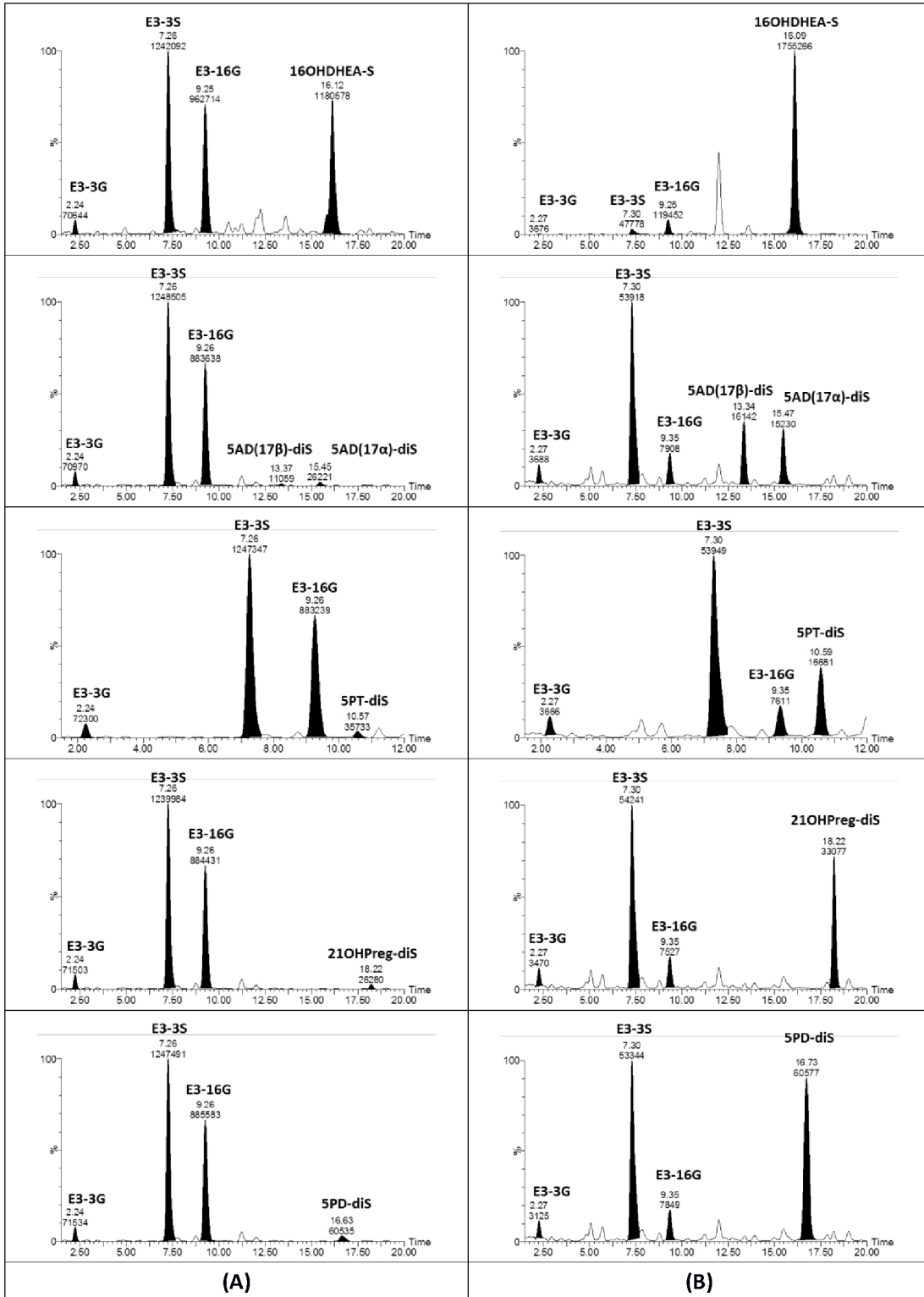


Figure 3

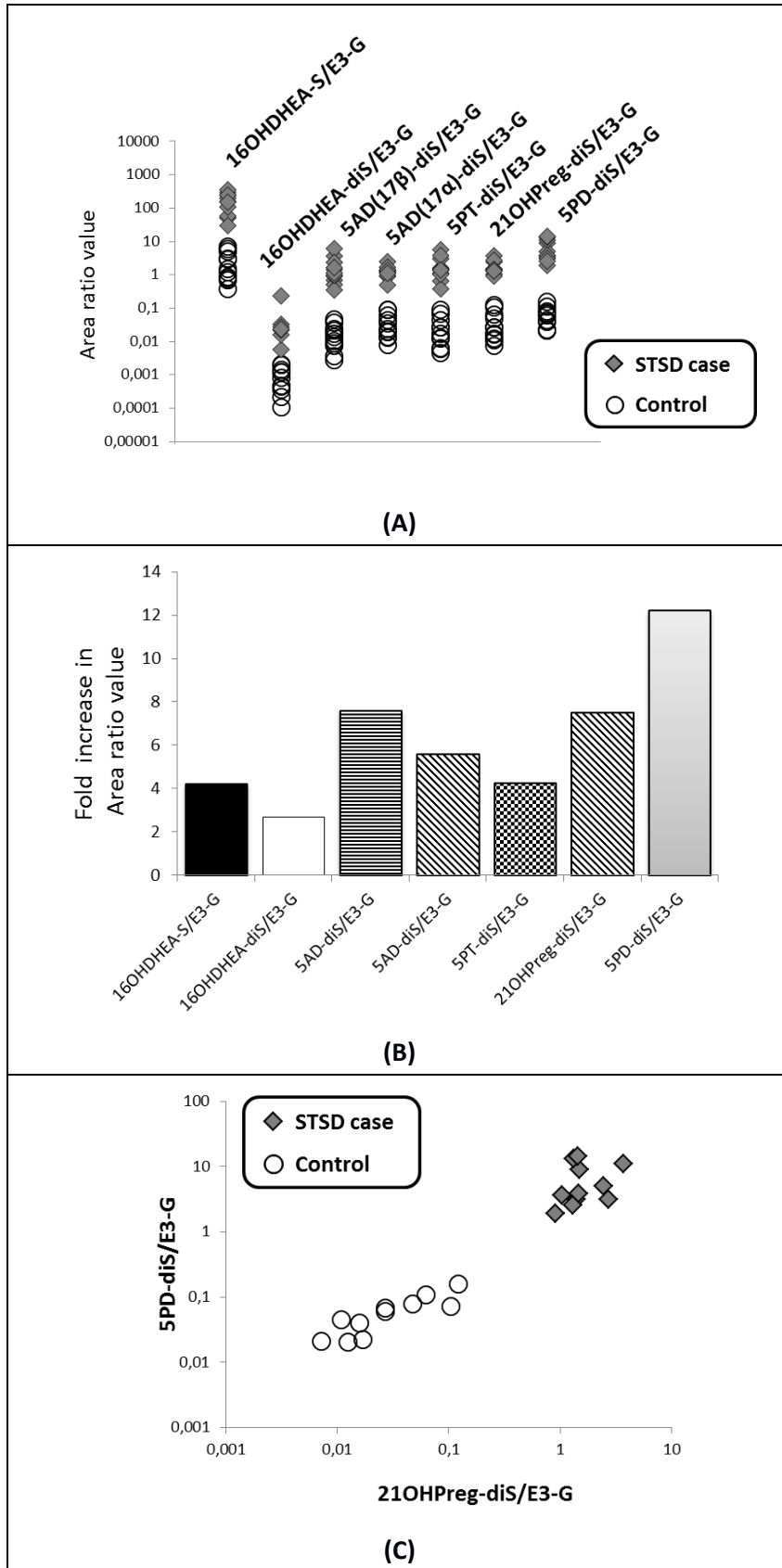


Figure 4

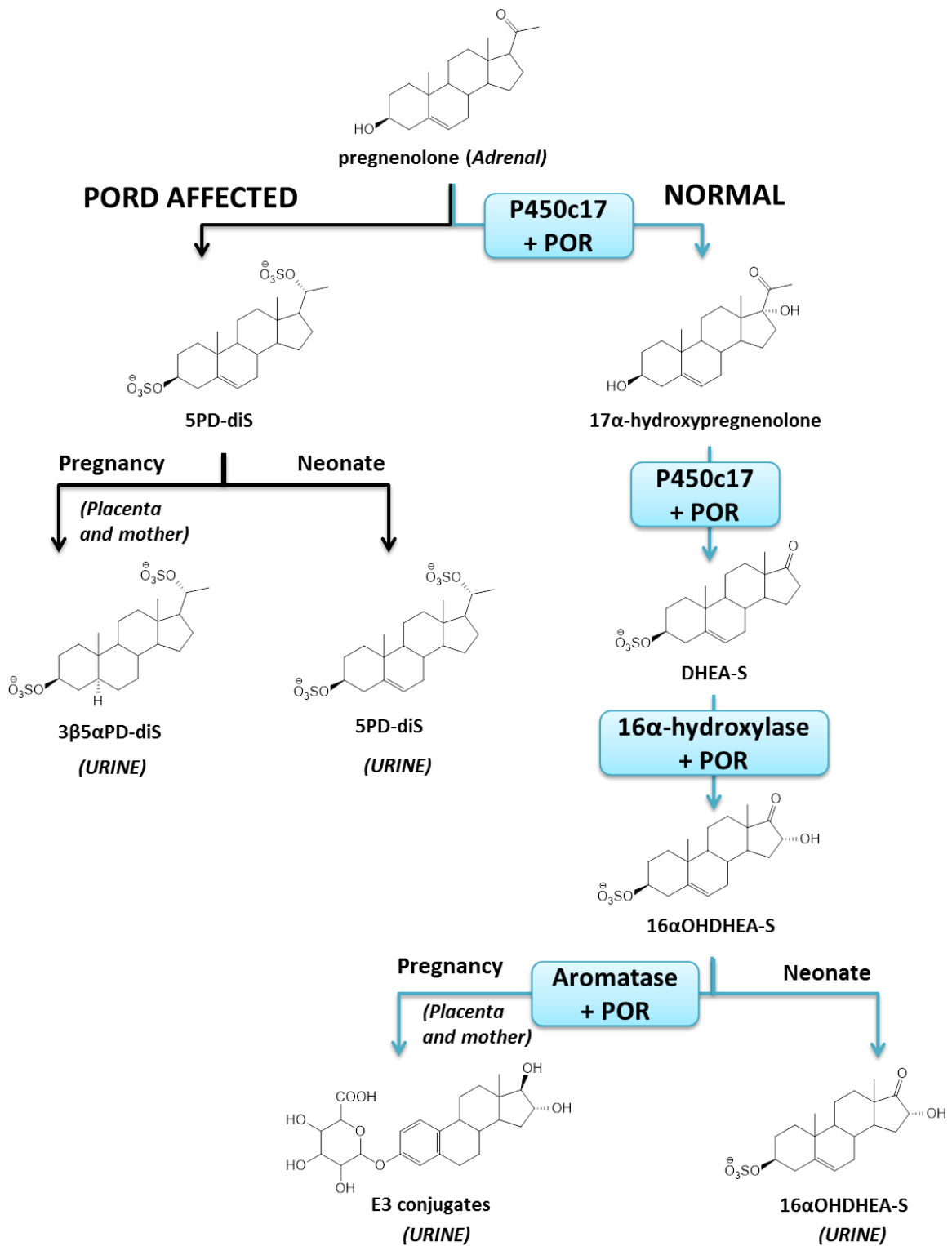


Figure 5

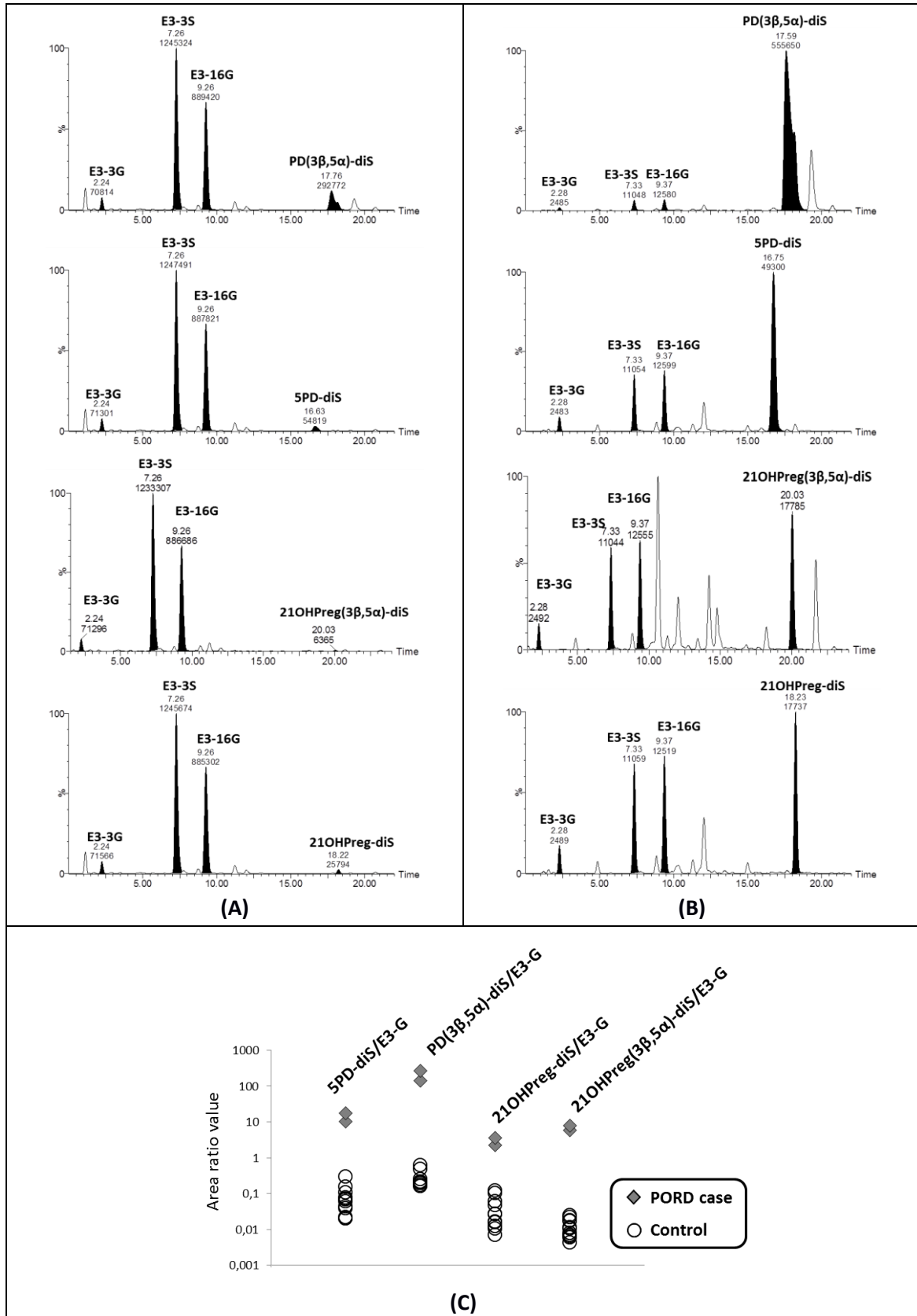


Figure 6

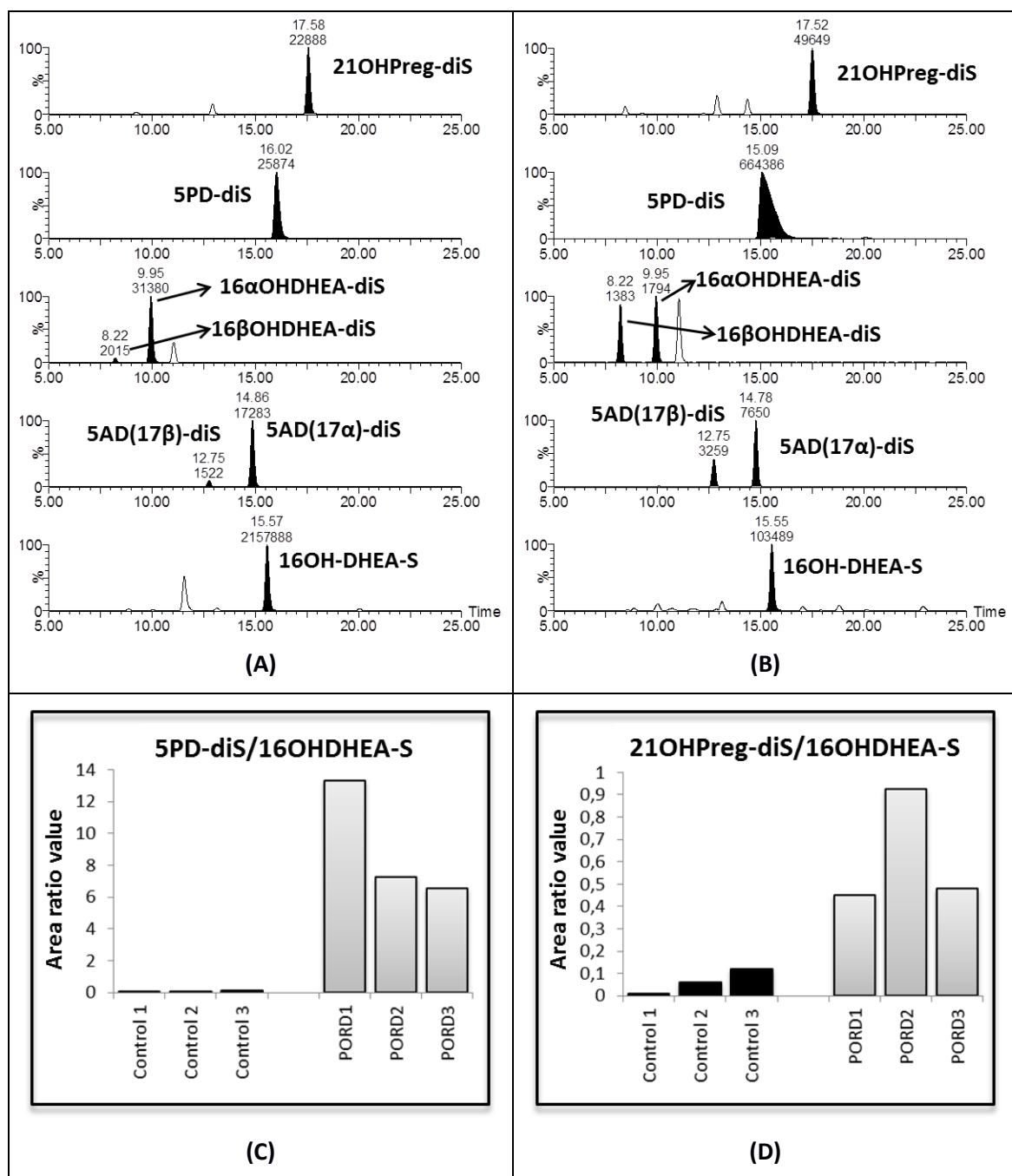


Figure 7

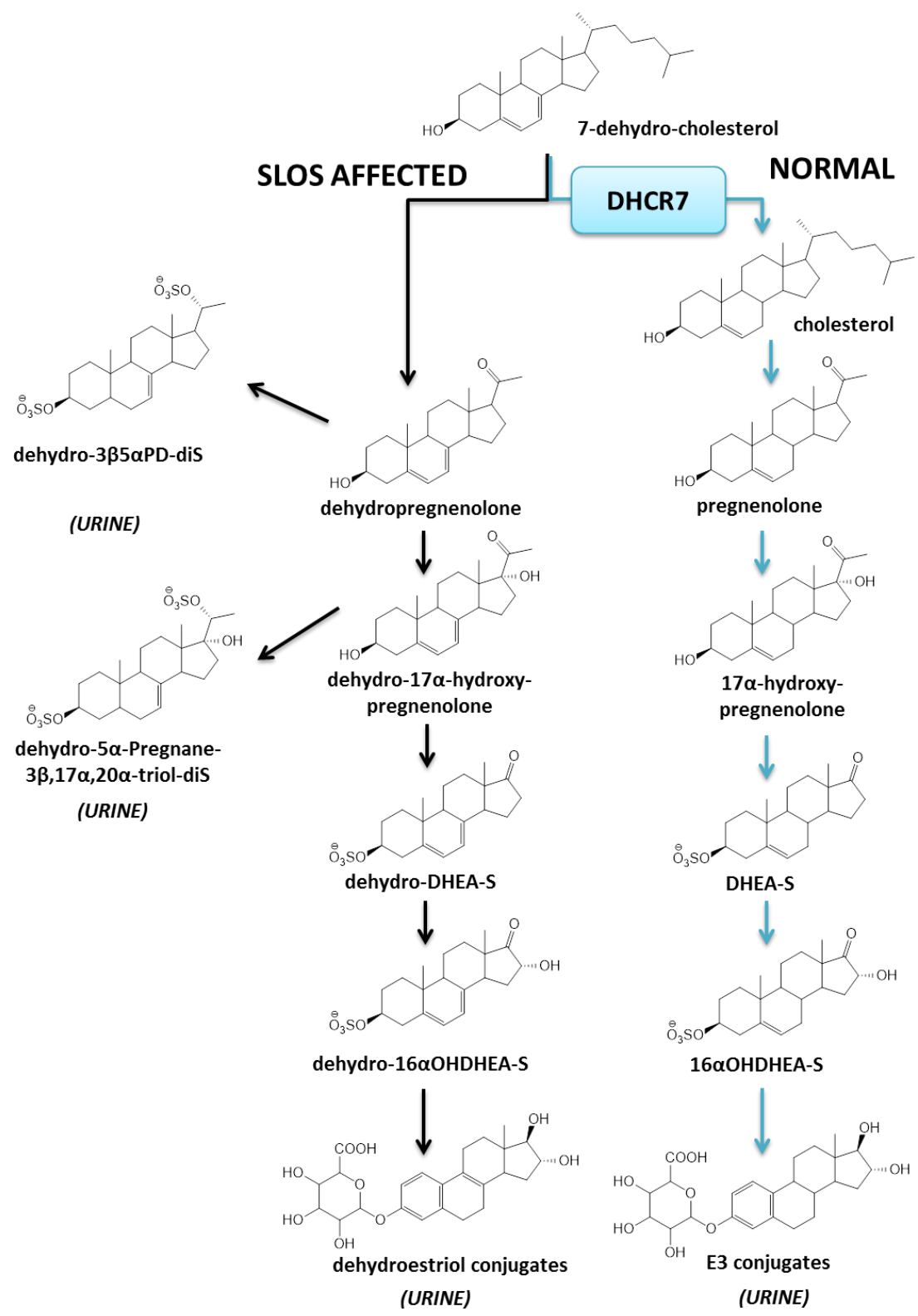


Figure 8

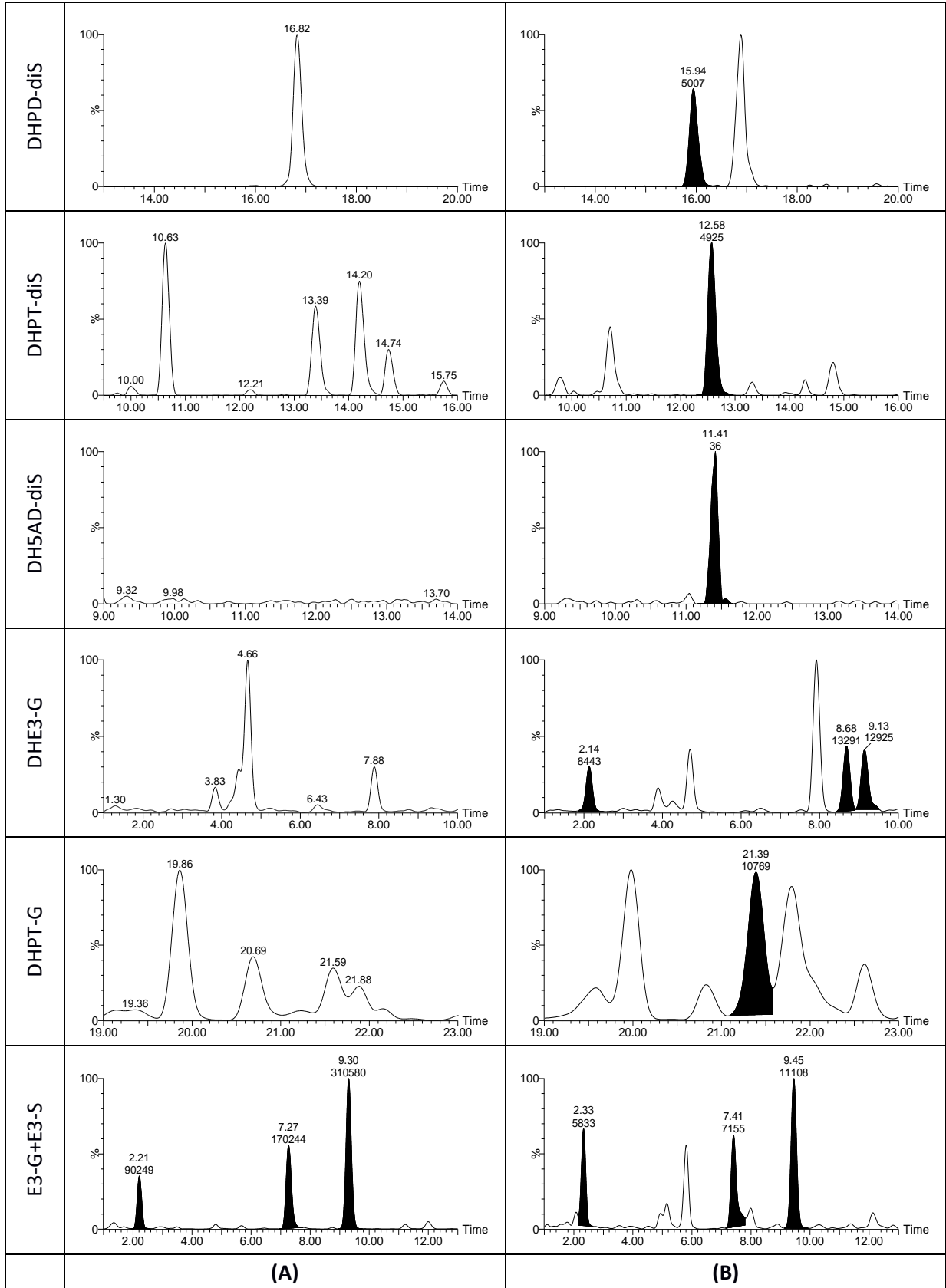


Figure 9.