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Hyperemesis gravidarum associated with *RYR2* genetic analysis of hyperemesis gravidarum reveals association with intracellular calcium release channel (RYR2)

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1 Hyperemesis Gravidarum associated with *RYR2*

2

3 **Genetic analysis of Hyperemesis Gravidarum reveals association with**
4 **intracellular calcium release channel (RYR2)**

5

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61 **Running Title:** RYR2 linked to Hyperemesis Gravidarum

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62 **ABSTRACT**

63 Hyperemesis Gravidarum (HG), severe nausea/vomiting in pregnancy (NVP), can
64 cause poor maternal/fetal outcomes. Genetic predisposition suggests the genetic
65 component is essential in discovering an etiology. We performed whole-exome
66 sequencing of 5 families followed by analysis of variants in 584 cases/431 controls.
67 Variants in *RYR2* segregated with disease in 2 families. The novel variant L3277R
68 was not found in any case/control. The rare variant, G1886S was more common in
69 cases (p=0.046) and extreme cases (p=0.023). Replication of G1886S using
70 Norwegian/Australian data was supportive. Common variants rs790899 and
71 rs1891246 were significantly associated with HG and weight loss. Copy-number
72 analysis revealed a deletion in a patient. *RYR2* encodes an intracellular calcium
73 release channel involved in vomiting, cyclic-vomiting syndrome, and is a thyroid
74 hormone target gene. Additionally, RYR2 is a downstream drug target of Inderal,
75 used to treat HG and CVS. Thus, herein we provide genetic evidence for a pathway
76 and therapy for HG.

77

78

79 **KEY WORDS:** Hyperemesis Gravidarum, Nausea, Vomiting, Pregnancy, RYR2

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81 **INTRODUCTION**

82 Nausea and vomiting of pregnancy is a common symptom affecting 70% of pregnant
83 women (Goodwin, 1998). Clinical intervention is necessary in the severest form,
84 Hyperemesis Gravidarum (HG), which affects up to 2% of pregnancies
85 (Christodoulou-Smith et al., 2011). HG leads to significant weight loss, dehydration,
86 electrolyte imbalance, and ketonuria (Fairweather, 1968; Goodwin et al., 1992;
87 Goodwin, 1998). Although maternal mortality is rare, 6 deaths due to HG have been
88 reported recently (MacGibbon et al., 2015), as well as morbidity including
89 Wernicke's encephalopathy (Chiossi et al., 2006), acute renal failure (Hill et al.,
90 2002), liver function abnormalities (Ahmed et al., 2013), splenic avulsion (Nguyen
91 et al., 1995), esophageal rupture (Woolford et al., 1993), pneumothorax (Schwarz et
92 al., 1994), and post-traumatic stress symptoms (Christodoulou-Smith, 2011). HG is
93 also associated with poor fetal/child outcomes including a 4-fold increased risk of
94 preterm birth and a 3-fold increased risk of neurodevelopmental delay in children
95 (Fejzo et al., 2013; Fejzo et al., 2015).

96 A variety of potential causative factors have been investigated, but the etiology
97 remains unknown. Evidence for a genetic predisposition is provided by classic twin
98 studies of Norwegian, Spanish, and Finnish cohorts (Colodro-Conde et al., 2016;
99 Corey et al., 1992). Family based studies provide evidence that female relatives of
100 patients with HG are more likely to be affected, with a 17-fold increased risk if a
101 sister has HG (Gadsby et al., 1993; Vellacott et al., 1998; Vikanes et al., 2010; Zhang
102 et al., 2011). Recently, mutations in the thyrotropin receptor gene have been linked
103 to hyperemesis gravidarum accompanied by gestational thyrotoxicosis. This

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104 suggests a genetic etiology has already been identified in, at minimum, a subgroup
105 of cases (Coulon et al., 2016). Thus, understanding the genetic component is
106 essential in discovering the causal pathway(s).
107 The objective of this study was to perform whole-exome sequencing on HG families
108 to identify rare variants conferring susceptibility to HG and to validate these
109 findings in a large cohort of affected and unaffected individuals from the United
110 States, followed by replication in cohorts from Australia and Norway.
111
112 **MATERIALS AND METHODS**
113
114 **POPULATIONS**
115 The size and minimum HG CASE and CONTROL criteria for the 3 populations (US,
116 Norway, Australia) used in this study are summarized in Figure 1A. The genetic
117 analysis methods used on each population are summarized in Figure 1B.
118
119 **US Population**
120 **Eligibility Criteria.** The source population for HG CASES in the US included
121 patients primarily recruited through advertising on the HER Foundation website
122 (www.helpher.org). The stringent study criteria were designed to exclude all cases
123 and controls that would increase phenotypic uncertainty. Briefly, the inclusion
124 criteria for affected individuals were a diagnosis of HG and treatment with
125 intravenous (IV) fluids or total parenteral nutrition/nasogastric feeding tube. Each
126 participant was asked to recruit a non-blood related acquaintance with at least 2

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127 pregnancies that went beyond 27 weeks. Controls were eligible if they experienced
128 normal or no nausea/vomiting in their pregnancy, no weight loss due to
129 nausea/vomiting and no medical attention in their pregnancy due to
130 nausea/vomiting. Participants were enrolled in the family study if an HG CASE had 2
131 or more additional family members with HG. Additional affected family members
132 were eligible if they reported severe NVP accompanied by > 5% weight loss, and
133 medication or hospitalization for HG. Control family members had the same
134 eligibility requirements as Controls.

135

136 **Description of US Families Analyzed by Whole-exome Sequencing and Sanger**
137 **sequencing.** The whole-exome sequencing study included 15 affected individuals
138 and 3 unaffected individuals. These 18 individuals came from 5 families (Figure
139 1A). Follow-up analysis to confirm segregation in Family 1 included additional
140 family members -3 unaffected and 1 affected individual from Family 1. Each family
141 submitted saliva samples for a minimum of three affected individuals. We chose to
142 analyze a total of 18 individuals: 3 affected individuals from each of 5 families with
143 HG in addition to 3 unaffected controls from 3 of the five families to further limit
144 potential causal variants by dismissing those variants identified in unaffected family
145 members. Pedigrees of two families of Caucasian/European descent analyzed in this
146 whole-exome sequencing study, and whose variants are described herein, are
147 shown in Figure 2. Family 1 is of mixed Finnish, Swedish, English, and German
148 descent. We collected DNA from 4 CASES (4 sisters) and 4 CONTROLS (2 sisters,
149 mother, and maternal aunt) from Family 1. This family consists of 9 sisters, 5

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150 affected and 4 unaffected, but only those siblings who participated are shown in
151 Figure 2A. Family 2 is of mixed Scottish, German, Swiss, English, and Italian descent
152 and we collected DNA from 3 affected sisters (Figure 2B).
153
154 **Description of US CASE/CONTROL Population Analyzed by Genotyping and**
155 **Copy-Number Analysis.** The follow-up CASE/CONTROL population from the United
156 States included 584 HG CASES and 431 unaffected CONTROLS that were all
157 genotyped. A subgroup analysis was performed comparing the most severe HG
158 CASES requiring total parenteral nutrition/nasogastric feeding tube to Controls who
159 reported no NVP in any pregnancy. The subgroup was also used for a copy-number
160 analysis. All participants gave informed consent. This study was approved by the
161 UCLA Institutional Review Board.
162
163 **Norwegian Population**
164 **Eligibility Criteria.** Eligibility was determined using data obtained from self-
165 reported questionnaires (Nilsen et al., 2009). HG CASES were eligible if they were
166 admitted to hospital for prolonged nausea and vomiting in pregnancy. Controls
167 were eligible if they were NOT admitted to hospital for prolonged nausea and
168 vomiting of pregnancy.
169
170 **Description of Norwegian Population Analyzed by GWAS and Correlation with**
171 **Weight Loss.** The samples in this HG study included 385 HG CASES and 2280
172 unaffected Controls. The samples included were singleton pregnancies of

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173 Norwegian ancestry. Summary statistics for *RYR2* were analyzed for independent
174 replication in the Norwegian Mother and Child Cohort Study (MoBa), a prospective
175 population-based pregnancy cohort conducted by the Norwegian Institute of Public
176 Health recruited from Norway during 1999-2008 (http://www.fhi.no/moba-en;
177 Magnus et al., 2016). In addition, data was collected for each participant on maternal
178 pre-pregnancy weight and weight change until week 18 of gestation and used as a
179 continuous variable in a regression analysis to study genetic associations with
180 weight loss in early pregnancy. Ethical approval for the MoBa study has been
181 approved by the Regional Committee for Medical Research Ethics and all women
182 provided informed written consent.
183
184 **Australian Population**
185 **Eligibility Criteria.** Eligibility was determined using data collected from health and
186 wellbeing surveys. Women reported their experience in their pregnancy with the
187 most severe NVP using a five-point questionnaire adapted from Zhang et al (2011).
188 HG CASES (1) were defined as NVP that disrupted daily routine accompanied by
189 weight loss and medication and/or IV fluids and/or feeding tube. A second less
190 stringent CASE criteria included HG CASES (1) in addition to HG CASES (2) that
191 reported NVP that disrupted their daily routine and medication treatment, but did
192 not lose weight. CONTROLS were defined as experiencing no NVP.
193 Women in between the extreme ends who reported MILD NVP (NVP for more than 7
194 days, but did not see a doctor or nurse/did not disrupt daily routine very much) and
195 MODERATE NVP (disrupted daily routine but it did not affect my weight and did not

196 need medication, were not included in the CASE: CONTROL study, but were included
197 in a continuous analysis of the GWAS data.
198
199 **Description of Australian Population GWAS Analyzed using CASE:CONTROL**
200 **and Continous Phenotype.** The Australian sample is composed of genotyped
201 women unselected for HG who are part of the Australian Endogene Study and the
202 QIMR Mothers of Twins Study, which are two of the cohorts participating in the NVP
203 Genetics Consortium (Colodro-Conde; 2016).
204 As part of health and wellbeing surveys, a total of 1440 women reported on NVP
205 severity. We conducted a CASE:CONTROL analysis using 946 women who were in
206 the extremes of the severity scale. Women reporting no NVP (n=677) were used as
207 controls and women reporting severe NVP (n=269) with disruption of their daily
208 routine and medication prescription, including those losing weight and put on a drip
209 or feeding tube, were used as CASES. We also conducted a more stringent
210 CASE/CONTROL analysis that excluded the 139 HG CASES with no weight loss, thus
211 limiting the study to 130 HG CASES and 677 CONTROLS. Finally, we analyzed NVP
212 as a continuous phenotype for all 1440 study participants, which included an
213 additional 163 CASES of MILD NVP and 331 CASES of MODERATE NVP.
214
215 **GENETIC METHODS**
216 **Genetic Methods for US Population**
217 **Whole-Exome Sequencing of Families.** Each study participant was asked to
218 submit a saliva sample for DNA analysis. A saliva collection kit (Oragene, Ottawa,

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219 Canada) was self-administered for submitting 2 milliliters of saliva. DNA was
220 extracted from 75% of the saliva sample according to the manufacturer's
221 instructions (Oragene, Ottawa Canada).
222 We sequenced the entire exomes (~50 Mb) of 15 affected individuals and 3
223 unaffected individuals from 5 HG families. Paired end reads 100 nucleotides (2 X
224 100 nucleotides) were generated on an Illumina HiSeq 2000. Each sample was
225 sequenced on 3 different lanes to avoid lane bias. Qseq files were converted into
226 Sanger-formatted FASTQ files and reads were mapped to the reference human
227 genome build hg19 using the Burrows Wheeler Alignment algorithm (BWA) (Li and
228 Durbin, 2009). Duplicated reads were marked by Picard. The Genome Analysis
229 Toolkit (GATK) was used for local realignment around indel sites followed by a base
230 quality recalibration (McKenna et al., 2010). For reliable SNP calling we used
231 genotype quality ≥ 10; read QUAL ≥ 30 and a minimum read depth of 4. The
232 combined total variants from all 18 individuals were filtered as shown in Figure 3.
233 Synonymous variants, which are unlikely to be causal, were discarded. The
234 identified variants were further filtered against variants present in the HapMap,
235 1000 Genomes Project and dbSNP132 databases, selecting for novel variants and
236 known variants with minor allele frequency <5% (McKenna et al., 2010;
237 International HapMap 3 Consortium et al., 2010). These variants were further
238 filtered by selecting variants predicted to affect protein function using PolyPhen and
239 SIFT (Ramensky et al., 2002, Ng and Henikoff, 2003). Variants were further filtered
240 by deleting variants present in the 3 unaffected family controls. All variants were
241 discarded that were not shared by all 3 whole-exome sequenced affected family

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242 members with each family. Finally, we identified a subgroup of genes involved in
243 more than one family, and screened these genes for a functional effect, which
244 included genes 1) functionally relevant to reproduction (ie hormones), 2) nausea
245 and vomiting (ie gastric tract, vomiting center of the brain), and 3) genes expressed
246 in relevant tissues (ie ovary, placenta, vomiting center of the brain).
247
248 **Sanger Sequencing to analyze segregation in Family 1.** Sanger Sequencing of the
249 novel variant in Family 1 (*RYR2* exon68:c.T9830G:p.Leu3277Arg) was performed to
250 confirm whole-exome sequencing results and to confirm or deny segregation with
251 the disease in the remaining family members who were not included in exome
252 sequencing (1 affected sister and 1 unaffected sister, the unaffected mother, and an
253 unaffected maternal aunt). PCR primer pairs GGAAGTCATACTGCCCATGC and
254 GGGGTACAATGTCTTCTTCCA were designed from genomic DNA to amplify and
255 sequence the variant. PCR amplification and sequencing were carried out using
256 standard methods.
257
258 **Protein prediction tools used to predict functional effect of L3277R.** The SIFT
259 protein prediction tool was used to determine that the novel SNP encoding L3277R
260 resulted in a damaging protein product, and the Provean Prediction tool was used to
261 determine that it was deleterious. (Choi et al., 2012; Ng and Henikoff, 2003).
262
263 **Genotyping.** Taqman primers were designed for both the novel variant L3277R in
264 Family 1 and the rare variant G1886S identified in Family 2, and used to screen

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265 individuals from ≥ 573 HG CASES and ≥ 426 controls using Applied Biosystems
266 PRISM 7900HT Sequence Detection System (TaqMan) for large-scale screening. The
267 call rate was > 96%.
268
269 **Statistical Analysis.** Statistical significance of association of genotype with HG was
270 determined by calculating the p-values using a 1-tailed Fisher's exact test
271 (http://graphpad.com/quickcalcs/contingency1/) and odds ratios were calculated
272 using the odds ratio calculator (https://www.medcalc.org/calc/odds_ratio.php). A
273 p-value <0.05 was considered statistically significant.
274
275 **Copy-number analysis of *RYR2*.** Quantitative real-time PCR analysis of *RYR2* was
276 performed in triplicate on 10 ng from 240 DNA samples (101 extreme CASES
277 requiring tube feeding and 139 extreme controls with no nausea/vomiting in ≥ 2
278 pregnancies) on 384-microwell optical plates using the predesigned Taqman Copy
279 Number Assay covering 90 base pairs within a likely pathogenic duplicated region
280 in autism (Soueid et al., 2016) (Assay ID: Hs00137466_cn FAM labeled, MGB probe,
281 Thermofisher Scientific, Waltham, MA) and the RNaseP Copy Number Reference
282 Assay (VIC labeled, TAMRA probe). Melt-curve analysis was applied and all results
283 were normalized to RNaseP levels and calculated using the $\Delta\Delta C_T$ method. One
284 sample with a deletion originally identified in the above triplicate assay along with 5
285 normal control samples, were assayed a second time in duplicate (re-diluted to 10
286 ng from the original DNA sample) to verify the deletion.
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288

289 **Genetic Methods for Norwegian Population.** Maternal genome-wide data were
290 obtained using Illumina HumanCoreExome genotyping BeadChip v1.1. Imputation
291 was performed with reference panel HapMap phase 3 build 36 using IMPUTE2
292 (Howie et al., 2009). Standard association analyses were performed in PLINK 1.7
293 (Purcell et al., 2007). Genotypes were analyzed with allelic and genotypic approach.
294 Regression analysis was performed using a *z-score transformed gestational weight*
295 *gain (GWG)* based on maternal pre-pregnancy weight and weight change until week
296 18 of gestation.

297

298 **Genetic Methods for Australian Population**. The samples were genotyped using
299 Illumina arrays and genotype imputation was completed using 1000 Genome Phase
300 3 version 5 as reference data. For validation of the rare imputed SNP G1886S, we
301 also conducted CASE/CONTROL analysis in a more stringent subset by removing the
302 participants who did not report weight loss from the CASES, thus limiting to a more
303 stringent CASE phenotype (n=130).

304

305 **RESULTS**

306

307 **Whole-exome sequencing identifies *RYR2* variants linked to HG in 2 of 5**
308 **families.** We sequenced the entire exomes (~50 Mb) of 15 affected individuals and
309 3 unaffected individuals from 5 families with HG. The mean coverage was 54 fold.
310 Reads were mapped to the human genome reference build UCSC hg19 using BWA

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311 (Li and Durbin, 2009). On average, 3223 single-nucleotide variants were detected in
312 each individual and a total of 58006 variants were detected in all 5 families
313 combined (Figure 3). The synonymous variants were subsequently discarded
314 resulting in 29856 variants. The identified variants were further filtered against
315 variants present in the HapMap, 1000 Genomes Project, and dbSNP132 databases,
316 resulting in 13509 novel variants and known variants with minor allele frequency
317 <5% (McKenna et al., 2010; International HapMap 3 Consortium et al., 2010). These
318 variants were further filtered by selecting variants predicted to affect protein
319 function using PolyPhen and SIFT (Ramensky et al., 2002; Ng and Henikoff, 2003).
320 Filtering for missense and stop gain or stop loss variants that were shared by any of
321 the 3 whole-exome sequenced unaffected family members resulted in 6481 variants.
322

323 As we did not find any single variant that was shared by all the affected members
324 across all of the families, we focused on variants within each family shared by all 3
325 whole-exome sequenced affected subjects. For example, 94 variants were shared by
326 all 3 affected individuals in Family 1, and 227 variants were shared by all 3 affected
327 individuals in Family 2. We searched for variants and/or genes that were shared by
328 more than one family. 27 genes were identified that carried rare variants in the
329 affected family members in more than one family. These variants were evaluated
330 based on a functional effect, which included variants located in genes functionally
331 relevant to reproduction (ie hormones), nausea and vomiting (ie gastric tract,
332 vomiting center of the brain), and genes expressed in relevant tissues (ie ovary,
333 placenta). This resulted in identification of the gene *RYR2* involved in 2 of 5 families

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334 as a strong candidate based on its functional potential: *RYR2* encodes an
335 intracellular calcium release channel that is part of a signaling pathway for emesis
336 expressed in the vomiting center of the brain (Giannini et al., 1995; Zhong et al.,
337 2014). It is the only gene identified with variants significantly linked to cyclic
338 vomiting syndrome, is a thyroid hormone target gene, and is differentially expressed
339 in cumulus cells of the pre-ovulatory follicle (Grøndahl et al., 2012; Jiang et al., 2000;
340 Li et al., 2015).
341 Synonymous and common variants in *RYR2* that were originally removed in the
342 filtering steps were re-investigated. None of the 6 variants identified segregated
343 with disease. In addition, variants in *RYR2* in the remaining families (Family 3,4,5)
344 were not identified in this study. Potentially causal variants in other genes in the
345 remaining families are currently under investigation.
346
347 **Genotyping the novel and rare *RYR2* variants in the US cohort provides**
348 **confirmation.** In the largest HG family (Family 1), the novel heterozygous variant in
349 the *RYR2* gene (*RYR2*:NM_001035:exon68:c.T9830G:p.Leu3277Arg) was confirmed
350 by Sanger Sequencing to be shared by four affected sisters and was not shared by
351 either of 2 unaffected sisters, the unaffected mother, nor the unaffected maternal
352 aunt (Figure 2A). The phenotype and genotype results suggest L3277R is of
353 paternal rather than maternal origin in this family. However, the DNA from the
354 father who is presumed to be a carrier, nor his sister who reportedly did not have
355 HG, was unfortunately not available. We do not have any additional information
356 about phenotype on the father's side (ie father's mother). Genotyping via Taqman

357 showed the *RYR2* variant to be unique in the sample to Family 1, as it was not
358 identified in 584 HG CASES and 431 unaffected CONTROLS (Table 1). The nucleotide
359 at the location of L3277R is 100% conserved across vertebrates and invertebrates.
360 The mutation changes a hydrophobic amino acid to an electrically charged amino
361 acid, and is predicted to be damaging and deleterious (SIFT Prediction Score=0;
362 Provean Prediction Score=-5.38).

363 In Family 2, the heterozygous variant G1886S
364 *RYR2*:NM_001035:exon37:c.G5656A:p.Gly1886Ser rs3766871 was shared by all 3
365 affected sisters (Figure 2B). Genotyping via Taqman identified the heterozygous
366 variant G1886S (Family 2) to be twice as common (p=.046) in CASES than
367 CONTROLS (in 38 out of 580 additional CASES and 17 out of 431 CONTROLS) and
368 four times more common (p=.023) when comparing the extreme ends of the clinical
369 spectrum, 9 out of 106 CASES requiring tube feeding compared to 3 out of 141
370 controls who reported no nausea/vomiting in pregnancy) (Table 1). The SNP
371 G1886S is already known to have a biological effect in the homozygous state.
372 Homozygous substitution of serine for glycine causes a significant increase in
373 cellular calcium oscillation activity compared to wild-type *RYR2* in HEK293 cells
374 (Koop et al., 2008). Interestingly, calcium oscillations are completely abolished by
375 homozygous substitution of a neighboring SNP in the double mutant
376 G1885E/G1886S. The estimated frequency of G1886S in the European_American
377 population (ESP6500) is 0.031 and the estimated frequency of G1885E is 0.023 (The
378 1000 Genomes Project Consortium, 2015). Therefore the estimated frequency of
379 carrying both mutations is very rare (<0.001), but may be selected for in the

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380 extreme control population where the phenotype (no NVP in at least 2 pregnancies)
381 is also rare. The SNP G1886S has also been associated with ventricular arrhythmias
382 and is an independent predictor of sudden cardiac death, while a neighboring SNP
383 rs790896 (G>A) was linked to a decreased risk of sudden cardiac death (Ran et al.,
384 2010). The frequency of the protective A allele rs790896 is predicted to be 0.415 in
385 the European population (The 1000 Genomes Project Consortium, 2015), so the
386 estimated frequency of carrying both G1886S and rs790896 is 0.013. In this study
387 we identified 3/141 extreme controls carrying G1886S and 2 cases of
388 G1886S/rs790896 are predicted in 141 extreme controls. Therefore, it will be
389 interesting to investigate if additional variants in controls carrying *RYR2* G1886S
390 (such as G1885E and rs790896) explain why this variant is also present in a subset
391 of controls with no NVP.
392
393 **Summary statistics were supportive but not statistically significant for variant**
394 **G1886S in *RYR2* in both a Norwegian and an Australian GWAS.** Genotype data
395 for G1886S were imputed in both Norwegian and Australian datasets. Although
396 statistical significance was not achieved probably due to the rarity of G1886S and
397 the small number of affected individuals, there is a supportive trend in both cohorts.
398 In the Norwegian cohort there is a 1.3-fold OR for this SNP (reference allele A), and
399 in the Australian cohort, after removal of the CASES with no weight loss to better
400 reflect the severe end of the clinical spectrum of NVP, there was a 1.2-fold OR for
401 G1886S (Table 1).
402

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403 **Common variants in *RYR2* (rs790899 and rs1891246) are significantly linked
404 to HG and are highly significant with respect to weight loss in early pregnancy.**
405 In addition to the rare variants, common *RYR2* SNPs (rs790899 and rs1891246)
406 were significantly linked to HG in both the Norwegian and Australian GWAS using
407 the CASE/CONTROL phenotypes (Table 1). No other common variants were
408 identified that reached statistical significance in both the Norwegian and Australian
409 datasets.
410 In the Norwegian dataset, adding the zscore for weight change until gestational
411 week 18 as a covariate increased the odds ratio and significance for the common
412 *RYR2* variants, and suggested a strong association with weight loss (p=2.12E-31 for
413 rs790899 and p=1.19E-31 for rs1891246, Table 2A). In the smaller Australian
414 dataset, using the continuous severity measure, neither rs790899 nor rs1891246
415 reached statistical significance.
416

417 **Copy-number analysis identifies a deletion in *RYR2* in an extreme HG CASE
418 requiring intravenous feeding (TPN).** We also performed copy-number analysis
419 to search for pathogenic duplications and/or deletions in *RYR2*. A deletion in exon
420 16 was identified in *RYR2* in DNA from one woman with HG requiring total
421 parenteral nutrition (TPN) among DNA isolated from 101 CASES requiring TPN for
422 severe HG (Table 1). The deletion was not observed in any of the remaining samples
423 including 139 extreme controls reporting no nausea and no vomiting in any of their
424 pregnancies.
425

426 **DISCUSSION**

427

428 This is the first whole-exome association study of HG and suggests *RYR2* may play a
429 role in the biology of HG. We have successfully identified two rare variants in *RYR2*
430 that are linked to HG using a whole-exome sequencing approach followed by
431 genotyping a large validation cohort from the US. Independent replication in GWAS
432 studies from Norway and Australia are suggestive of a role for *RYR2* and revealed 2
433 common variants very significantly associated with weight loss in early pregnancy.
434 Copy-number screening identified a deletion in *RYR2* in an extreme HG CASE
435 requiring intravenous feeding.

436 *RYR2* encodes an intracellular calcium release channel which localizes to the
437 intracellular Ca2+ stores (ER/SR), and that, in excitable cells, including cardiac
438 muscles and neurons, controls contraction and activity, respectively (Santulli and
439 Marks, 2015). *RYR2* is expressed in several other cell types in many tissues,
440 including the thyroid gland, ovaries, and pancreas (Craps et al., 2015; Grøndahl et
441 al., 2012; Santulli et al., 2015). An animal model of emesis shows that intracellular
442 Ca^{2+} release through ryanodine receptors in the brainstem initiate Ca2+-dependent
443 activation of CaMKIIa and ERK1/2, leading to emesis, which can be blocked by
444 dantrolene, an inhibitor of emesis (Zhong et al., 2014). Thus, we speculate that the
445 variants and deletions described here may result in abnormalities in the emesis-
446 signaling pathway via a hyper-functioning Ca^{2+} channel. Indeed, variants in *RYR2*,
447 including G1886S identified in this study (albeit in a heterozygous state), have been
448 shown to cause increased RYR2 channel activity in a homozygous state (Koop et al.,

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449 2008). Functional evidence for the other variants identified in this study remains to
450 be determined, but there are some clues. The novel variant L3277R is predicted to
451 be deleterious and map between a putative phosphorylation site (AA2947) and a
452 CALM interacting site (AA3581). And exon 16, the location of the deletion, contains,
453 an RIH Domain (CDD:250561) which may form a binding site for IP3. The common
454 variants identified in this study (rs790899 and rs1891246) both map in introns
455 toward the end of the gene in sites with no predicted regulatory significance (Kent
456 et al., 2002), and therefore, are not likely to have a phenotype on their own, but may
457 be linked to functional mutations/deletions not identified in this study.
458 The role *RYR2* variants play in HG etiology is unknown, but there are several
459 intriguing avenues to explore further. Firstly, *RYR2* encodes an intracellular calcium
460 release channel that is the only ryanodine receptor expressed in the vomiting center
461 of the brain (Giannini et al., 1995) and has been implicated in a signaling pathway
462 underlying emesis in an animal model (Zhong et al., 2014). Secondly, the thyroid
463 hormone has been shown to induce RYR2 overexpression (Jiang et al., 2000), while
464 the drug Inderal (Propranolol, used to treat hyperthyroidism) blocks RYR2
465 phosphorylation and lowers its expression (Yoshida et al., 1992). Hyperthyroidism
466 accompanies HG in as many as 60% of pregnancies (Goodwin et al., 1992) and
467 mutations in the thyrotropin receptor have been linked to HG (Coulon et al., 2016),
468 providing additional genetic evidence that this pathway may be causal in some
469 cases. However, because thyrotoxicosis is not normally associated with nausea and
470 vomiting, it is likely that another factor is involved, such as the additional
471 requirement of an emetic stimulus (ie pregnancy hormones). There could be a

472 viscious cycle of deterioration from NVP to HG as a result of hormone-induced
473 nausea/vomiting in conjunction with aberrant RYR2 Ca^{2+} signaling caused by
474 progressive thyroid dysfunction and/or mutant RYR2. Of note, two of 6 recent
475 maternal deaths secondary to HG were accompanied by severe
476 thyrotoxicosis/thyroid storm (Knight et al., 2014; MacGibbon et al., 2015). Thirdly,
477 in a NextGen sequencing study of Cyclic Vomiting Syndrome (CVS), *RYR2* was the
478 only gene among over 1,000 genes screened, with variants significantly linked to the
479 disease (Lee et al., 2015), and Inderal has been used to effectively treat 92% of
480 children with CVS (Haghighat et al., 2007). Likewise, in 1980 a patient presenting
481 with severe thyrotoxicosis and hyperemesis gravidarum reportedly responded
482 dramatically to Inderal treatment (Valentine et al., 1980). As her thyroid function
483 improved and the Inderal was discontinued, she again returned to the hospital with
484 severe vomiting. Upon restarting medication, her vomiting ceased and she
485 continued treatment until term. Our findings provide evidence for a biological
486 pathway, diagnostic marker, and potential targeted therapy for the etiology and
487 treatment of HG.

488 Lastly, limited evidence suggests *RYR2* may play a role in fertility. It is expressed
489 more than 50-fold in cumulus cells compared to mural granulosa cells of human pre-
490 ovulatory follicle, and its expression correlates with amphiregulin, a key mediator of
491 the effect of LH/hCG and a marker for oocyte competence (Grøndahl et al., 2012).
492 Ryanodine receptor variants are significantly associated with pig litter size (Omelka
493 et al., 2004), and women with HG produce an abnormally high number of mature
494 oocytes when undergoing follicle stimulation (Fejzo et al., 2010). A genetic link that

495 explains both the symptoms of HG and a potential increase in fertility, would
496 provide a rationale for why severe nausea in pregnancy has not been selected out in
497 nature despite its link to adverse outcomes.
498 The limitation of this study stems from the fact that full sequencing and copy
499 number analysis of *RYR2* in all cases and controls, is cost-prohibitive. We were only
500 able to study the 2 mutations involved in the 2 HG families in this study, not the
501 complete gene sequence, in the validation cohorts from the United States. Also,
502 while the US cohort used intravenous fluid treatment as its clinical criteria for HG,
503 and the Norwegian cohort used hospitalization, the Australian cohort used a less
504 severe phenotype, which may have led to a reduced effect for that dataset. The
505 small sizes for GWAS may also contribute to an underestimate of the effect for the
506 validation cohorts. Alternatively, the multiple analyses in different relatively small
507 cohorts can lead to over-interpretation of the results. Finally, the copy-number
508 analysis that identified a deletion only surveyed 90 bp of exon 16 in 240 individuals.
509 Mutation and copy-number analysis of the full *RYR2* gene in cases and controls is
510 now warranted to determine their frequencies in affected individuals and to
511 understand the role of RYR2 in HG pathogenesis.
512 In conclusion, this study uses an innovative approach to identifying the etiology of
513 HG. This disease has thus far eluded both scientists and clinicians, resulting in
514 largely ineffective treatments, significant maternal morbidity, and an increased risk
515 in adverse fetal outcome (Fejzo et al., 2013). Mutations in genes in the ryanodine
516 receptor-signaling pathway may account for a substantial amount of the attributable
517 risk of HG, although just how much must be deferred to a follow-up study as

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518 causality has not been definitively established. Additional studies are required, such
519 as functional analysis of the deleterious *RYR2* variant L3277R, complete deletion
520 analysis of *RYR2*, and a larger GWAS. However, this novel discovery may provide the
521 first step in understanding the etiology of HG. The identification of genes linking HG
522 to RYR2 provides an intriguing new avenue for diagnosis, research, and therapy.
523

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526

527 **DISCLOSURE OF INTERESTS**

528 The authors declare no competing financial interests.

529

530 **CONTRIBUTION TO AUTHORSHIP**

531 All authors fulfill authorship criteria as defined in the instructions for authors.

532

533 **DETAILS OF ETHICS APPROVAL**

534 This study was approved by the UCLA Institutional Review Board on 5/20/2011 as
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537

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759 **FIGURE 1. Summary of A) Populations and B) Methods used in this study.** The
760 size and minimum HG CASE and CONTROL criteria for the 3 populations (US,
761 Norway, Australia) used in this study are summarized in Figure 1A. The genetic
762 analysis methods used on each population are summarized in Figure 1B. *MILD
763 NVP was defined as participants who answered: 'had some NVP for more than 7
764 days, but did not see a doctor or nurse and did not disrupt daily routine very much'
765 **MODERATE NVP was defined as 'disrupted daily routine but did not affect weight
766 and did not need medication'.

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770 **FIGURE 2. Pedigrees and Genotypes of A) Family 1 and B) Family 2.** In Family 1,
771 A, B, C, and D all were affected with HG. Participant A reported pic line, medication
772 and weight loss to treat HG, B reported a 10 pound weight loss and medication to
773 treat symptoms until birth, C reported intravenous fluids (IV) and a 23 pound
774 weight loss, and D reported IV fluids, hospitalization, weight loss, and medication to
775 treat her HG. Among the unaffected family members, participant E reported 2
776 pregnancies with mild nausea, no weight loss, and no medication; F reported 5
777 pregnancies with no nausea and vomiting, no weight loss, and no medication in any
778 pregnancy, G reported 13 pregnancies with normal nausea and vomiting, no weight
779 loss, no medication, and H reported 3 pregnancies with normal nausea and
780 vomiting, no weight loss, and no medication. For Family 2, all 3 sisters were affected
781 and all 3 sisters required medication and IV fluids to treat their HG. Sisters A and C
782 were both hospitalized for HG. Their mother was also affected but did not
783 participate.
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785 **Figure 3. Whole-exome sequencing filtering steps identifies RYR2 variants.**

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788 **TABLE 1. *RYR2* variant table.**

RYR2 VARIANT	SOURCE	EXON/INTRON	METHOD	SCREENED	OR	P-value
L3277R*	FAMILY 1, USA	68:c.T9830G	Genotyping	584 HG, 431 C	NA	NA
G1886S	FAMILY 2, USA	37:c.G5656A	Genotyping	584 HG, 431 C	1.29	0.046
G1886S	FAMILY 2, USA2	37:c.G5656A	Genotyping	106 HG, 141 C	4.27	0.023
G1886S	NORWEGIAN	37:c.G5656A	GWAS	318 HG, 1823 C	1.32	0.661
G1886S	AUSTRALIAN	37:c.G5656A	GWAS	269 HG, 677 C	0.89	0.693
G1886S	AUSTRALIAN2	37:c.G5656A	GWAS	130 HG, 677 C	1.17	0.665
rs790899	NORWEGIAN	intron 95	GWAS	385 HG, 2280 C	1.19	0.033
rs790899	AUSTRALIAN	intron 95	GWAS	269 HG, 677 C	1.33	0.013
rs1891246	NORWEGIAN	intron 100	GWAS	385 HG, 2280 C	1.23	0.009
rs1891246	AUSTRALIAN	intron 100	GWAS	269 HG, 677 C	1.3	0.014
NOVEL DEL*	USA	16:237619976	Copy Number	101 HG, 139C	NA	NA

HG=Hyperemesis Gravidarum, C=Unaffected Control, TPN=Severe HG requiring tube feeding
L3277R*=novel deleterious SNP (c.9830T>G, p.Leu3277Arg)
***DEL=deletion in exon 16 of unknown size**

rs3766871 G1886S (NM_001035.2:c.5656G>A, NP_001026.2:p.Gly1886Ser)
rs790899 (NM_001035.2:c.13913+381G>A; XM_006711804.2:c.13943+381G>A)
rs1891246 (NM_001035.2:c.14434-490T>G; XM_005273224.1:c.14491-490T>G)
USA2 and AUSTRALIAN2 are datasets with more stringent criteria.
USA2 (HG=requiring iv feeding, C=no NVP)
AUSTRALIAN2 (HG=weight loss, C=no NVP)

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790 **TABLE 2. Adding zscore weight change until gestational week 18 as covariate**

791 **shows rs790899 and rs1891246 associated with weight.**

CHR	SNP	BP	A1	TEST	NMISS	OR	STAT	P-VALUE
1	rs790899	237957678	C	ADD	2499	1.267	2.649	0.00808
1	rs790899	237957678	C	COV1	2499	0.2447	-11.66	2.12E-31
1	rs1891246	237981846	G	ADD	2499	1.292	2.968	0.002993
1	rs1891246	237981846	G	COV1	2499	0.2424	-11.71	1.19E-31

rs790899 (NM_001035.2:c.13913+381G>A; XM_006711804.2:c.13943+381G>A)
rs1891246 (NM_001035.2:c.14434-490T>G; XM_005273224.1:c.14491-490T>G)

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FIGURE 1A. POPULATIONS SUMMARY

UNITED STATES

5 HG FAMILIES:
Primary family member: HG diagnosis requiring treatment with IV fluids or feeding tube, and
≥2 family members: severe NVP and >5% weight loss/medication or hospitalization for HG
Family 1: 4 HG, 4 C (3 HG, 1 C for whole-exome sequencing; all 8 used for Sanger-sequencing)
Family 2: 3 HG (3 HG for whole-exome sequencing)
Family 3: 3 HG (3 HG for whole-exome sequencing)
Family 4: 3 HG, 1 C (3 HG, 1 C for whole-exome sequencing)
Family 5: 3 HG, 1 C (3 HG, 1 C for whole-exome sequencing)

584 HG CASES: HG diagnosis requiring treatment with IV fluids or feeding tube
431 CONTROLS: 2 pregnancies with none/normal NVP, no weight loss, no treatment

NORWAY

385 HG CASES: hospitalized for NVP
2280 CONTROLS: not hospitalized for NVP

AUSTRALIA

130 HG CASES(1): disrupted daily routine/lost weight/medication or IV fluids or feeding tube
139 HG CASES(2): disrupted daily routine/medication/no weight loss
677 CONTROLS: no NVP

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FIGURE 1B. METHODS SUMMARY

UNITED STATES

WHOLE-EXOME SEQUENCING-15 HG CASES/3 CONTROLS FROM 5 FAMILIES
GENOTYPING IN 584 HG CASES/431 CONTROLS
GENOTYPING IN 106 HG CASES REQUIRING TPN/141 CONTROLS WITH NO NVP
COPY NUMBER ANALYSIS 101 HG CASES REQUIRING TPN/139 CONTROLS WITH NO NVP

NORWAY

GWAS ON 385 HG CASES/2280 CONTROLS, AND
ANALYSIS OF COVARIANCE WITH WEIGHT LOSS UNTIL 18 WEEKS GESTATION

AUSTRALIA

GWAS ON 269 HG CASES (1 AND 2)/677 CONTROLS
GWAS ON 130 HG CASES(1)/677 CONTROLS
ANALYSIS OF CONTINUOUS PHENOTYPE ADDED 163 MILD* AND 331 MODERATE** NVP CASES

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Figure 2A.

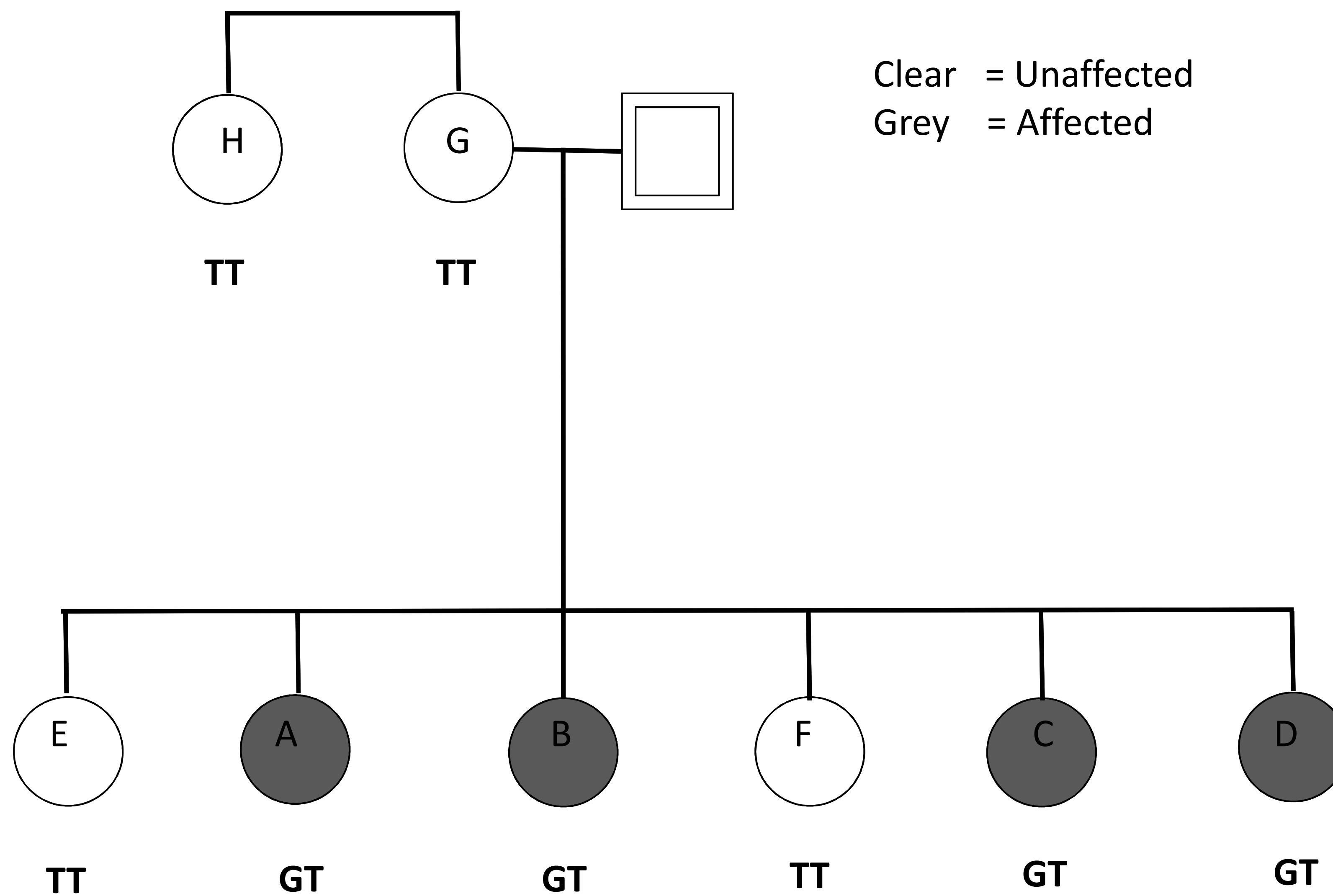

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Figure 2B.

Family 2

RYR2:NM_001035: rs3766871
exon37:c.G5656A:p.Gly1886Ser

Clear = Unaffected
Grey = Affected

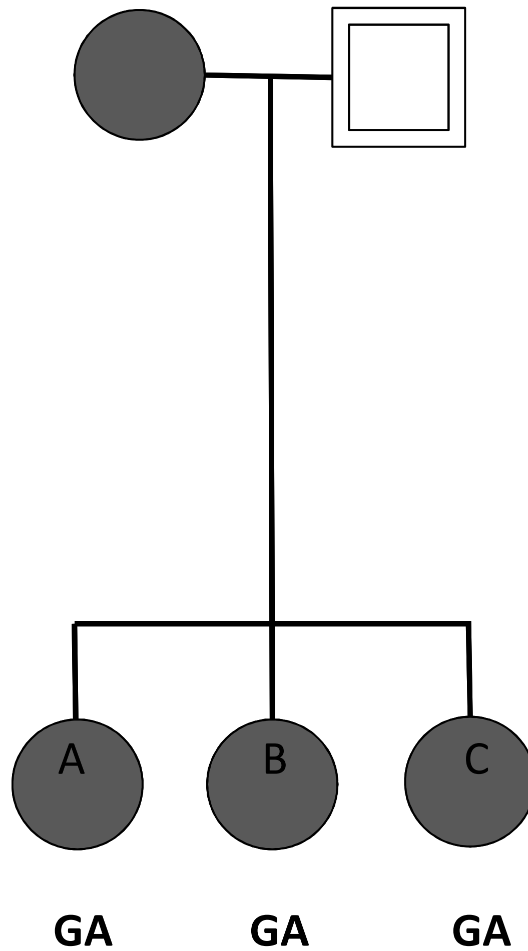
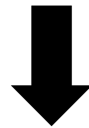
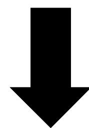
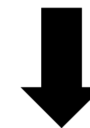


Figure 3. Whole-exome sequencing filtering steps

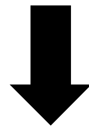
1. 58006 variants from 18 individuals in 5 families
2. 29856 after removal of synonymous variants
3. 13509 after removal of common variants
4. 9870 after removal of non-damaging variants
5. 6481 after removal of variants in unaffected

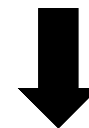
6A. 94 variants shared by Family 1

6B. 156 variants shared by Family 2

6C. 405 variants in remaining 3 Families



7. 27 Genes with variant(s) segregating in more than one family



RYR2

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HIGHLIGHTS

- **Whole-exome sequencing in Hyperemesis Gravidarum (HG) identifies link to RYR2**
- **Novel variant L3277R segregates with disease in large HG family**
- **US genotyping, and Norwegian and Australian GWAS support link to G1886S in RYR2**
- **RYR2 deletion identified in severe case treated with total parenteral nutrition**
- **Common variants rs790899 and rs1891246 significantly associated with weight loss**

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