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Hurt, tired and queasy: Specific variants in the ATPase domain of the *TRAP1* mitochondrial chaperone are associated with common, chronic "functional" symptomatology including pain, fatigue and gastrointestinal dysmotility

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ABSTRACT

Functional disorders are common conditions with a substantial impact on a patients' wellbeing, and can be diagnostically elusive. There are bidirectional associations between functional disorders and mitochondrial dysfunction. In this study, provided clinical information and the exon sequence of the *TRAP1* mitochondrial chaperone were retrospectively reviewed with a focus on the functional categories of chronic pain, fatigue and gastrointestinal dysmotility. Very-highly conserved *TRAP1* variants were identified in 73 of 930 unrelated patients. Functional symptomatology is strongly associated with specific variants in the ATPase binding pocket. In particular, the combined presence of all three functional categories is strongly associated with p.Ile253Val (OR 7.5, P = 0.0001) and with two other interacting variants (OR 18, P = 0.0005). Considering a 1–2% combined variant prevalence and high odds ratios, these variants may be an important factor in the etiology of functional symptomatology.

1. Background

Functional disorders such as chronic fatigue syndrome, migraine, cyclic vomiting syndrome, irritable bowel syndrome and fibromyalgia are common conditions, often with a substantial deleterious impact on patients' wellbeing, including disability. The term "functional" has varying usages among subspecialties and physicians, and is used herein in the manner common to gastroenterologists, to denote any condition without demonstrable gross "structural" etiology. This definition neither assumes a psychogenic or a non-psychogenic etiology, nor does it distinguish among these concepts. Symptoms of functional disorders commonly relate to neurological/autonomic dysfunction, and can be diagnostically elusive without an obvious physiological or anatomical cause. Physicians and other medical personnel often discount these patients, who generally present with multiple subjective symptoms that are not demonstrable by testing, as being complainers, mentally ill, or even causing their conditions (e.g. Munchausen, including by proxy). While the etiology of functional disorders is largely unknown, they generally run in families, suggesting genetic underpinnings. As these conditions are common in patients with mitochondrial disease, genes involved in energy metabolism are good candidates for the pathogenesis of functional disorders.

Ascertained by Next Generation (NextGen) sequencing, multiple cases with functional disorders were identified to have variants of interest in the *TRAP1* gene. This gene encodes tumor necrosis factor receptor-associated protein 1, a mitochondrial chaperone involved in antioxidant defense (Kadye et al., 2014; Matassa et al., 2012; Yoshida et al., 2013). Sequence variants in the C-terminal domain of this gene have recently been associated with congenital malformations and Leigh syndrome (Saisawat et al., 2014; Skinner et al., 2014). We show herein that specific variants encoding the ATPase domain of the TRAP1 protein are highly associated in a dominant fashion with multiple functional chronic symptomatology, including pain, fatigue and gastrointestinal (GI) dysmotility.

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2. Methods

Given the extreme genetic heterogeneity of mitochondrial disease, many clinicians are turning to Next Generation (NextGen) sequencing, such as Courtagen Life Sciences, Inc.'s nucSEEK® and mtSEEK® genetic panels, to aid in the diagnosis and treatment of patients with mitochondrial disease. nucSEEK® is a nuclear mitochondrial NextGen sequencing panel which includes the 1034 genes listed in MitoCarta (all genes encoding proteins with known mitochondrial location) (Pagliarini et al., 2008), as well as 161 additional genes, including all known peroxisomal genes, and many cytosolic "metabolic" genes. The genes included in the panel are available online at Courtagen's website (Courtagen life Sciences, Inc. nucSEEK™ Test, nd). Patients tested by this methodology represent the wide spectrum of mitochondrial disease presentation, with clinical manifestations varying from isolated pain syndromes to lethal Leigh disease. Considering the overlap between mitochondrial and functional disorders, the nucSEEK® referral population is enriched with patients suffering from functional symptomatology.

One of the first several patients sequenced by this methodology, a patient with multiple functional disorders, was identified to have the p.Ile253Val variant in the *TRAP1* gene. As per standard clinical genomics practice, candidate status was applied given genetic and biological plausibility, and suspicion of a genotype–phenotype association increased as additional cases were evaluated. The present study is a retrospective analysis of Courtagen's dataset, in patients with and without identified variants in the *TRAP1* gene, in 930 unrelated patients from a United States and Canadian population evaluated with the nucSEEK® gene panel.

As part of clinical diagnostics, saliva samples were obtained using commercially-available Oragene collection kits by DNA Genotek and sent by regular mail directly from the families to the laboratory. DNA was extracted from the samples using the SPRI-TE nucleic acid extractor and the SPRI-TE gDNA extraction kit according to manufacturer's protocols. Sequence-ready libraries for the MiSEQ DNA sequencer (Illumina) were prepared using the HaloPlex V2 library preparation kit (Agilent). Sensitivity and specificity for detection of known variants exceeded 99% and 99.99%, respectively, for the exonic sequence. Splice sites were evaluated as well. Variants suspected to be related to disease, including those in *TRAP1*, were confirmed by Sanger sequencing performed on the original sample.

For this study, a significant sequence variant was defined as one that is highly predicted to alter protein structure or function (e.g. start, stop, frameshift and clear splicing variants corresponding to ACMG categories 4 or 5), or very-highly conserved missense variants. The latter of which we defined as complete evolutionary conservation through all vertebrates (to spotted gar, a primitive fish; the lamprey sequence is absent from the database) per the UCSC Genome Browser. Poorly aligned sequences were discounted, and a single species with a different amino acid sequence was allowed to account for potential sequence error in the database. This was done with the understanding that very-highly conserved variants may or may not be relevant, yet lesser-conserved missense variants are not as likely to have biological relevance. Based on being relatively-common and per our prior clinical suspicion, p.Ile253Val was analyzed separately. In addition, the potential clinical relevance of sequence variants was determined based on the modeled protein's structure. The crystal structure of wild-type zebrafish TRAP1 protein was published (Lavery et al., 2014). Using this structure as the main template, the structure for human TRAP1 was modeled computationally (Zhou and Skolnick, 2011). All variants identified in our patients were mapped to the in silico model to predict pathogenesis, and those missense variants identified as likely pathogenic (hereafter termed as "possible missense disruptors" or "disruptors") were analyzed. Another experimental group consisted of patients with a nonsense mutation (stop codon) in the *TRAP1* gene. Control groups included published sequence databases representing the general population (1000Genomes, ESP, ExAC), and our patients in which no variants were identified in the *TRAP1* gene.

Limited clinical information was provided with the samples in order to facilitate clinical sequence interpretations, and only this pre-existing information was used for the present retrospective study. Individuals were excluded if no clinical information was available or the available information was inadequate. Adequate clinical information included an informative clinical summary, a comprehensive evaluation, or a completed checklist provided by a treating physician. The above-described limited data set, containing no personal identifiers, was used for the present study. Families were not contacted in regards to the current study.

Our primary, preexisting hypothesis, based upon our review of the available medical records in the first five cases, was that p.Ile253Val and other highly-conserved variants within the ATPase domain of the TRAP1 protein are associated with chronic pain, fatigue and GI dysmotility, which will be subsequently referred to in this paper as the "functional triad". Additionally, these are the only functional conditions common enough among our patient cohort to allow for statistical evaluation by the present methodology. A set of keywords was used to search the laboratory reports for these three functional disease categories. A manual search verified the inclusion, and identified strings such as "without migraine" or "family history of migraine" for exclusion. Pain was defined as the inclusion of the word "pain" or any of the common manifestations such as "headache", "migraine", "myalgia", etc. We defined fatigue to include exercise intolerance and muscle weakness, because we did not believe that the available clinical information reliably distinguished among these entities. We defined "GI dysmotility" to be the presence of any symptomatic diagnosis suggestive of abnormal gut transit speed including gastrointestinal reflux disease, gastroparesis, delayed gastric emptying, constipation, diarrhea, irritable bowel and the term "GI dysmotility". Since many of the initial patients had an autistic spectrum disorder, relevant search terms were queried in a secondary, exploratory analysis. No other clinical conditions were queried. All subjects are unrelated to our knowledge, and family members were excluded.

Statistics are given as odds ratio and 95% confidence interval (OR, 95% CI) with the calculated P value per http://www.medcalc.org/calc/odds_ratio.php. The amino acid change in either of two "salt bridge" variants abolishes their interaction, which we judged as crucial for protein function (see Discussion), and thus both variants were analyzed together.

Determination of the predominate racial, ethnic and geographic backgrounds of our patients was performed by principal component analysis using the smartpca program incorporated into the EIGENSOFT program along with variants that were part of the initial call set from the 1000 Genomes (1000 g) Project Phase3 analysis (ftp://ftp.1000genomes.ebi.ac.uk/vol1/ftp/release/20130502/) (Patterson and Price, 2006; Price et al., 2006). This release contains 79 million variants and is based on data from 2535 individuals comprising 26 different populations around the world. This set of 79 million variants is reduced to a set of at least 43,455 SNPs that overlaps our panel. Each of our patient samples is genotyped for these panel-overlap SNPs using the Genome Analysis Toolkit UnifiedGenotyper and the patient sample is put through the smartpca workflow along with genomes for the 1000 g samples. After smartpca completes, geometric distance scores are calculated between the patient sample and all 2535 1000 g individuals using the first 10 principal components (where n = the principal component, a = the patient sample, and b = the 1000 g

individual being interrogated):

$$\sqrt{\sum_{n=1}^{10}(a_n - b_n)^2}.$$

The distances are ranked by which are closest to the patient samples and the most commonly occurring ethnicity in the top ten is retained as the ethnicity that most closely resembles the patient sample.

3. Results

Among the 930 patients referred for sequencing in which adequate clinical information was provided, a very-highly conserved variant in the *TRAP1* gene was identified in 73 patients (7.8%), including 70 with missense variants in 23 different amino acid positions and 3 with nonsense variants (Table 1). Ten of the 23 very-highly conserved missense variants were predicted to be "disruptors" to protein function; there were no predicted disruptors among the lesser-conserved amino acids.

Functional disease categories were compared between patients with different types of variants in the *TRAP1* gene and in patients in which no *TRAP1* variants were identified (Table 2). In addition to the statistics listed in that table, very-highly conserved variants within the ATPase domain, compared to very-highly conserved variants outside the domain, are statistically associated with the following functional disease categories: pain (OR = 25, P = 0.0002), fatigue (OR = 6.1, P = 0.01), and the functional triad (the presence of all three categories in the same patient, OR = 18, P = 0.002). Disruptors within the ATPase domain, compared to disruptors outside the domain, are statistically associated with the following functional disease categories: pain (OR = 23, P = 0.01) and triad (OR = 15, P = 0.03).

Table 1
Very-highly conserved variants identified in the *TRAP1* gene.

Amino acid change	ATPase domain?	Number of patients	Clinical pain/fatigue/GI/triad[1,2]	Variant comments[2]
p.Ile113Val	Yes	1	1/1/0/0	
p.Arg114Trp	Yes	1	0/1/0/0	Disruptor
p.Arg128His	**Yes**	**3**	**2/2/2/2**	**Disruptor/salt bridge**
p.His144Asp	Yes	1	0/0/0/0	Disruptor
p.Glu192Lys	**Yes**	**4**[3]	**3/3/3/3**	**Disruptor/salt bridge**
p.Gln200Lys	Yes	1	1/0/1/0	Disruptor
p.Glu216Ter	Yes	1	1/1/1/1	Nonsense
p.Tyr229Ter	Yes	1	1/1/1/1	Nonsense
p.Ile253Val	**Yes**	**16**[3]	**11/10/9/8**	**"Common mutation"**
p.Glu267Lys	No	1[4]	0/0/0/0	
p.Arg271Gln	No	1	0/0/1/0	
p.Arg388Gln	No	2	0/0/1/0	
p.Tyr444Asn alone	No	5	0/2/1/0[5]	Disruptor
p.Tyr444Asn + p.Arg692His[6]	No	7[4]	1/2/2/0[5]	Disruptor
p.Arg449Trp	No	1	0/0/0/0	Disruptor
p.Glu457Lys	No	1	1/1/1/1	
p.Ala465Glu	No	1	1/1/1/1	Disruptor
p.Arg469His	No	8	0/1/1/0[5]	
p.Arg489His	No	1	0/0/0/0	
p.Arg495Gly	No	1	0/0/0/0	
p.Arg505Cys	No	1	0/0/0/0	Disruptor
p.Thr535Ser	No	5	0/1/2/0	
p.Arg602Gln	No	2	0/0/1/0	
p.Asp685Asn	No	4	0/2/0/0	
p.Pro686Ala	No	1	0/0/0/0	
p.Arg703Gln	No	1	0/0/0/0	
p.Arg703Ter	No	1	1/1/0/0	Nonsense

Bold text refers to the three highly-conserved variants that are statistically associated with functional disease.

[1] Number of patients with pain, fatigue, GI dysmotility and the triad of all three.

[2] Terms defined in the Methods section; salt bridges are covered in the Discussion.

[3] One case has both p.Glu192Lys and p.Ile253Val, phase unknown.

[4] One case has both p.Glu267Lys and (p.Tyr444Asn + p.Arg692His), phase unknown.

[5] The common, highly conserved C-terminal variants, Tyr444Asn (with or without Arg692His) and Arg469His, as well as the moderately-conserved polymorphism Arg692His, demonstrate no association with the three functional categories (data not shown).

[6] Phase was determined in five of the seven cases, all in cis.

Among all 930 unrelated patients tested by nucSEEK®, 95 have the functional triad per our records review. Among those 95 patients, 8 (8%) have p.Ile253Val, which is higher than that expected for this variant in the general population (0.37% per 1000Genomes, 0.62% per ESP, 0.61% per ExAC). As our referral and *TRAP1* populations are enriched in individuals with genetic results indicating that they are predominately of European heritage, and p.Ile253Val is more common in this population, statistical analysis used the prevalence values for individuals of European background (0.92% per 1000Genomes and 0.91% per ESP; OR 10, 95%CI 3.8–27, P < 0.0001). Of note, we did not use the figure of 0.76% in non-Finnish Europeans per ExAC. The general population prevalence (by ExAC) for the "salt bridge mutations" (see Discussion) is 0.14% for p.Glu192Lys and 0.32% for p.Arg128His. Collectively, the salt bridge mutations were identified far more often than by chance alone among patients with the functional triad versus that expected in the general population (OR 17, 95%CI 7.6–40, P < 0.0001).

Among the 16 patients with p.Ile253Val, five were referred for testing by the first author along with his physician note, and thus generally more comprehensive and consistent clinical information was available for the purposes of this retrospective study. All five have the triad of chronic pain, fatigue and GI dysmotility, which is increased relative to the other 11 p.Ile253Val patients (P = 0.04). Additional conditions that were listed as present in exactly 4 of the 5 patients included chronic dizziness and intermittent tachycardia/palpitations.

Additional potential relationships are possible based on our data, and while statistically significant by themselves, they were not part of our a priori hypotheses, and significance does not withstand a multiple testing correction. 1. The three nonsense *TRAP1* variants are significantly associated with the functional triad, and show a trend with two of the individual functional symptoms (Table 2). Of possible importance is that the two patients with triad have nonsense variants within the ATPase domain. 2. Very-highly conserved variants outside of the ATPase domain, including that subset defined as disruptors, demonstrate the opposite association with chronic pain syndromes, in that these conditions are less common than in our controls (patients without *TRAP1* variants). The association does not extend to fatigue or GI disease, and may be type I error, or alternatively suggests a possible protective effect of another domain of this protein that is specific for pain. 3. Excluding p.Ile253Val, the very-highly conserved missense variants in the ATPase domain are associated with autistic spectrum disorders (6/11, 55%) versus 4/45 (9%) with very-highly conserved missense variants outside the ATPase domain (OR 9.6, 95%CI 2.1–43, P = 0.003) and versus 198/808 (24%) of patients without *TRAP1* variants (OR 3.7, 95%CI 1.1–12, P = 0.03). However, autistic spectrum disorders do not appear to be associated with p.Ile253Val (3/16, 19%). In addition, none of the three patients with nonsense mutations has an autistic spectrum disorder per available records.

The racial and ethnic backgrounds by principal component analysis was quite similar between our 30 patients with a very-highly conserved *TRAP1* ATPase domain variant and 75 control patients, with substantial majorities of both groups placing them within the European cluster, indicating predominantly European heritage (25/30, 83%, *TRAP1* versus 64/75, 85%, controls). Control patients were randomly chosen from among our patients without such *TRAP1* variants. Among the patients of predominant European heritage, no obvious ethnic/geographic differences were noted, with the closest match being Toscani in Italy (8/30 *TRAP1* versus 23/75 controls), British in England and Scotland (6/30 and 12/75), Northern and Western European ancestry in Utah, USA (5/30 and 14/75), Iberian population in Spain (4/30 and 12/75), and Finnish in Finland (2/30 and 3/75). Patients with principal component analysis results

Table 2
Functional disease categories in patients with *TRAP1* variants.

TRAP1 variants	Pain syndrome[1]	Chronic fatigue[1]	Gastrointestinal dysmotility[1]	Triad[2] of pain, fatigue & GI	Total number of patients
p.Ile253Val "common mutation"	11 (69%) 5.7 (2.0–17) P = 0.001	10 (63%) 3.4 (1.2–9.4) P = 0.02	9 (56%) 3.4 (1.2–9.2) P = 0.02	8 (50%) 7.5 (2.8–20) P = 0.0001	16
Conserved in ATPase NOT I253V	7 (64%) 4.6 (1.3–16) P = 0.02	7 (64%) 3.6 (1.0 − 12) P = 0.04	6 (55%) *P = 0.06*	5 (45%) 6.2 (1.9–21) P = 0.003	11
All conserved in ATPase domain	17 (65%) 4.9 (2.2–11) P = 0.001	16 (62%) 3.3 (1.5–7.3) P = 0.004	14 (54%) 3.1 (1.4–6.8) P = 0.005	12[3] (46%) 6.4 (2.9–14) P < 0.0001	26[3]
Conserved elsewhere in protein	3 (7%) 0.19 (0.06-0.61) *P = 0.005* *In reverse*	10 (22%)	11 (24%)	2 (4%)	45
All disruptors[4]	6 (24%)	10 (40%)	7 (28%)	4 (16%)	25
Disruptors[4] in ATPase domain	6 (60%) 3.9 (1.1–14) P = 0.04	6 (60%) P = *0.09*	6 (60%) 4.0 (1.1–14) P = 0.03	5 (50%) 7.5 (2.1–26) P = 0.002	10
Disruptors[4] elsewhere in protein	1 (6.3%) P = *0.09* *In reverse*	5 (31%)	4 (25%)	1 (6.3%)	16
All non-disruptors[5]	19 (28%)	24 (35%)	15 (22%)	9 (13%)	69
Salt bridges[6] p.Arg128His, p.Glu192Lys	5 (71%) 6.5 (1.3–34) P = 0.03	5 (71%) P = *0.053*	5 (71%) 6.6 (1.3–34) P = 0.02	5 (71%) 18 (3.6–100) P = 0.0005	7
Nonsense[7]	3 (100%) *P = 0.06*	3 (100%) P = *0.08*	2 (67%)	2 (67%) 15 (1.3–170) P = 0.03	3
None	224 (28%)	266 (33%)	222 (27%)	95 (12%)	808

Statistics are given as: top row: number (percent) of patients with that phenotype; middle row: odds ratio (95% confidence interval); third row: significance compared to the bottom row (no *TRAP1* variants identified). P values not shown are >0.1. Statistics were performed by http://www.medcalc.org/calc/odds_ratio.php.
Italics refer to trends that are not statistically significant at the 5% level.
[1] The functional categories are defined in the Methods section.
[2] All three functional categories are present in the patient (per provided records).
[3] One patient with the triad has both Glu192Lys and Ile253Val.
[4] Missense variants predicted based on structure to affect protein function, as described in Methods, and excluding the relatively-common p.Ile253Val variant.
[5] Missense variants predicted based on structure NOT to affect protein function.
[6] The lid covering the ATP-binding pocket; see Discussion.
[7] Presence of a stop codon, which can be assumed to result in no protein.

outside of the European cluster included 2/30 and 0/75 in the South Asian cluster (both Punjabi), 1/30 and 3/75 in the East Asian cluster (3 Han, 1 Kinh), 0/30 and 1/75 in the African cluster (African American), and 2/30 and 7/75 outside of the clusters, whose ancestry most closely matched admixed Latin American populations (5 Puerto Rican and 4 Colombian).

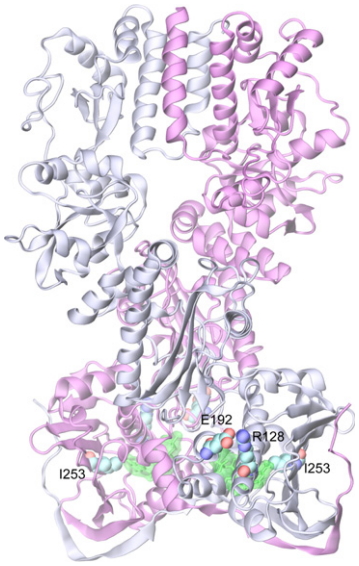
4. Discussion

Tumor necrosis factor receptor-associated protein 1 (TRAP1), also known as heat shock protein 75 (HSP75), is a member of the HSP90 family. The heat shock proteins (HSPs) were originally defined by their production in cells subjected to high-temperature exposure, but later the definition was extended to additional stressful conditions such as exposure to low temperature and ultraviolet radiation, as well as during tissue healing. Many of the HSPs are chaperones, which function by stabilizing new proteins to ensure correct folding or by helping to refold proteins that are damaged by cell stress. The *TRAP1* gene contains 19 exons and 701 amino acids, with an N-terminal mitochondria-targeting sequence spanning positions 1–59, an ATPase domain at 108 to 260, and a C-terminal chaperone domain at 292–701, with significant homology to other HSP peptides in its family. The HSP90 proteins, including TRAP1, are highly-conserved molecular chaperones that, in one of their functions inhibit cell death caused by reactive oxygen species (ROS) (Felts et al., 2000).

The ATPase domain of TRAP1 hydrolyses the energy-rich bond of ATP, which like in other HSP90 proteins (Marzec et al., 1823), is involved in processing proteins in an energy-requiring manner. In our data, three *TRAP1* variants were identified in at least two patients with the functional triad: p.Ile253Val, p.Glu192Lys and p.Arg128His (excluding the common polymorphism p.Arg692His). The hydrophobic side chain of the wild-type isoleucine residue 253 protrudes directly into the far end of the ATP binding pocket (Fig. 1). The mutant amino acid valine has a side chain one-carbon shorter, whereas it is predicted to make a slightly deeper ATP binding pocket. It is unclear if this variant exerts a clinical effect through alteration of the ATP binding site or through another mechanism. All of our cases result from the same single nucleotide transition (chromosome 16, position 3726094, T > C). It is unlikely that p.Ile253Val is a marker for a linked pathogenic variant as no common variants were found in or near the gene, although this cannot be totally excluded. Residue glutamate 192 is located at the tip of a loop known as the "lid" covering the ATP-binding pocket. The lid exhibits significant conformational change upon the activation of the protein. In our model, glutamate 192 forms a salt bridge with arginine 128 in one monomer, thus maintaining the active form of the protein. This salt bridge would be lost on mutation of 192 to lysine, or of 128 to histidine.

All 12 patients with the functional triad and highly-conserved variants in the ATPase domain of *TRAP1* had at least one of those three variants, p.Ile253Val, p.Glu192Lys or p.Arg128His (one patient has two of the variants). The "common mutation" p.Ile253Val alone, as well as the two "salt bridge mutations" collectively, are very-highly associated (P < 0.0005) with the functional triad of chronic pain, fatigue and GI dysmotility. The odds ratios are quite high with point estimates of 7.5 and 18 (Table 2). The data is just as striking when the proportion of patients with these variants in our study with the functional triad is compared to the proportion of patients with these variants in the general population, giving odds ratio point estimates of 10 and 17.

The major limitation of this study is the dearth of clinical information. In order to maintain the confidentiality of the subjects and the



TRAP1 functions as a homodimer. The two monomers are represented by cartoons in silver and purple, respectively. One ATP molecule is bound to each monomer, and are both shown in green. The three amino acids discussed above are labeled: I253 = isoleucine 253 (labeled in both monomers); E192 = glutamate 192 and R128 = arginine 128 (both labeled for one monomer only).

Fig. 1. Computer model of a TRAP1 protein dimer.

non-human-subject designation of the study, we were confined to a limited data set extracted from retrospective intakes and sequences. In most cases, the clinical information available to us was fairly limited, and the ascertainment of functional symptomatology is certainly incomplete. However, there is no reason to suggest that there is any systemic bias in assigning phenotype, which was done blind to genotype. Symptom ascertainment should be equally incomplete in all patients, and low sensitivity would create false negatives that would favor the null hypothesis. Indeed, when the five patients with p.Ile253Val referred by the first author (symptoms determined from the comprehensive clinic note) were compared to the other 11 patients (symptoms determined from any one of the means listed in the Methods section), it appears that our methodology underestimates patients with functional symptomatology, and the actual odds ratios may be higher than those presented herein.

Another disadvantage of the dearth of clinical information is that the extent of the phenotype associated with the three *TRAP1* variants cannot be presently elucidated. Based on the few patients for whom we have more detailed clinical information, it appears that the TRAP1-related phenotype does extend beyond the triad to include additional functional symptomatology such as chronic dizziness and episodic palpitations/tachycardia. Several of the patients are disabled by their disease, including persistent ongoing pain, severe fatigue meeting the Fukuda definition of chronic fatigue syndrome, intestinal failure on total parental nutrition and/or persistent dizziness that prevents walking at school. However, the *TRAP1* variants are compatible with a broad range of severity, as among the few cases in which full clinical evaluations were provided, relatives carrying the variant (determined by Sanger sequencing) demonstrate a wide range of symptomatology, from the lack of all functional symptoms to substantial disability resulting from the full triad. Additionally, there is not enough data to assess any cognitive effects of these variants, except to say that many patients appear to have normal to near-normal intelligence. It is presently unclear if the observed statistically-significant association of non-253 conserved ATPase variants in *TRAP1* with autistic spectrum disorders is real, although the presence of that spectrum in 5/7 patients with salt bridge mutations does suggest that further investigation is indicated.

Recently a single patient with a heterozygous p.Ile253Val variant in *TRAP1* was described as having multiple congenital anomalies consistent with VACTERL association (MIM #192350). However, this patient and the other four VACTERL patients described in that article all had one or two C-terminal variants in *TRAP1* (Anon, nd). In addition, one child with a homozygous C-terminal variant in *TRAP1* was reported to have the severe mitochondrial disorder of Leigh syndrome (Skinner et al., 2014). The structure of TRAP1 implies a very complicated dimerizing region in the C-terminal domain, and variants in this domain generally decrease ATPase activity while variants in the ATPase domain increase ATPase activity (Lavery et al., 2014). As a result, it is not surprising that patients with variants in the ATPase domain may exhibit a distinct phenotype from patients with C-terminal variants. Of particular interest, histidine 87 and glutamate 157 form another salt bridge within the ATPase domain, and mutating either of those amino acids to alanine in zebrafish Trap1 resulted in a tripling of ATPase activity over wild-type. This suggests the possibility that the three functional disease-associated ATPase variants that we report herein may predispose towards disease by futile ATP hydrolysis resulting in an energy-depleted state, with or without defective chaperoning of antioxidant proteins.

While primarily a mitochondrial matrix protein, *TRAP1* has also been identified in non-mitochondrial locations including pancreatic zymogen granules, insulin secretory granules, cardiac sarcomeres, and the nuclei of heart and pancreatic cells, suggesting broader functions (Cechetto and Gupta, 2000). The role of TRAP1 as a protector against oxidative stress was one of its earliest recognized functions. Different groups have observed that overexpressing *TRAP1* resulted in a reduction of oxidative damage and apoptosis in response to agents known to be toxic to mitochondria (deferoxamine and cisplatin, respectively), suggesting that TRAP1 plays a role in protecting mitochondria by reducing reactive oxygen species (ROS) levels (Im et al., 2007; Montesano Gesualdi et al., 2007). Inversely, one group observed that silencing *TRAP1* through RNA interference increases ROS accumulation (Hua et al., 2007).

Since the connection between oxidative stress and functional disease has been supported in several studies, it is not surprising that *TRAP1*-related disease can lead to functional symptomatology. Oxidative damage, as measured by lipid peroxidation, is associated with clinical symptoms in patients with fibromyalgia (Cordero et al., 2011). Headaches, in particular in these patients, appear to be related to oxidative stress (Cordero et al., 2012; Gupta et al., 2009a). Furthermore, antioxidants have been found to be valuable in treating functional disease, such as chronic fatigue (Logan and Wong, 2001; Gupta et al., 2009b), migraine (Chayasirisobhon, 2013) and gastrointestinal dysmotility (Yang et al., 2008). Early anecdotal clinical reports from our patient population suggest substantial improvement in symptoms, at least with regards to pain and fatigue, in those patients followed by the first author. Clinical studies are planned.

Defective chaperone function in the TRAP1 protein, due to mutations in the ATPase domain, might result in erroneous protein processing (e.g. mis-folding), and such a gain-of-function would likely result in the dominant inheritance (variant on one allele is related to disease despite a normal gene copy on the other allele) that is observed in our cases. Dominantly-inherited chaperonopathies have been described, including early-onset torsion (*DYT1*) (Valente et al., 1998; Ozelius et al., 1997) and myofibrillar myopathy (*CRYAB*) (Bhat, 1989; Vicart et al., 1998). Although numbers are small, the high degree of the functional triad among the three cases with nonsense variants in *TRAP1* (Table 2) suggest that the mechanism for p.Ile253Val-related functional symptomatology may instead be a loss-of-function/haploinsufficiency. Thus, the mechanism is unclear, and *in vitro* functional studies are planned.

The p.Ile253Val variant in the *TRAP1* gene is not rare as it is present in nearly one of 100 peoples from Europe. Collectively, p.Ile253Val, p.Glu192Lys or p.Arg128His is found in about 1–2% of peoples from Europe. Functional disease, including fatigue, pain and gut dysmotility, is relatively common, and therefore the presence of functional disease-predisposing variants being present in as many as 1–2% of some populations is not surprising. The presumed gross model herein is that these are common variants that predispose towards the development of some common conditions, in a multifactorial and polygenic manner.

5. Conclusion

Three very-highly conserved variants, p.Ile253Val, p.Glu192Lys and p.Arg128His, in the ATPase domain of the *TRAP1* gene are associated with a statistically-significant, several-fold increased, prevalence of common chronic functional conditions, including at least pain, fatigue and GI dysmotility. Considering the 1–2% combined prevalence of these variants in European-derived populations, and the odds ratios presented herein, these variants may be a factor in the pathophysiology of functional symptomatology experienced by substantial numbers of people. These variants may predispose towards the development of functional disease symptomatology via reduced antioxidant functions and/or futile ATP hydrolysis, which provides possible clues towards treatment. We propose the name of TRAP1-Related Disease (T1ReD), an acronym highlighting which is perhaps the most overall bothersome aspect of this disease. Additionally, our results demonstrate the importance of massively-parallel NextGen sequencing in large groups of patients coupled with modeling of the proteins' three dimensional structure to further the molecular understanding of complex disorders, such as functional disease.

Competing interests

Richard G. Boles, Julie M. Eggington, Holly A. Hornung, Kevin J. McKernan, Stephen McLaughlin, Kate M. Sheldon (past employee), Christine M. Stanley, Stacey A. Wong and Amir S. Zare are associated with Courtagen Life Sciences Inc. CLS offers large nextgen sequencing panels for clinical and research use, and some of the panels contain the *TRAP1* gene.

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Authors' contributions

Literature review RGB, HAH, KJM
Clinical interpretations RGB, HAH, SAW
Molecular interpretations RGB, CMS, JME, JS, KMS
Data analysis RGB, AEM, TBO, JME, ASZ, KMS
Statistical analysis RGB, JME
TRAP1 modeling RGB, MG, HZ, JS
Principal component analysis RGB, SM
Mechanism discussions RGB, JME, JS, KMS, KJM
Manuscript drafting RGB, HAH
Manuscript editing all

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MG and HZ are research scientists at the Center for the Study of Systems Biology and Georgia Institute of Technology.

AEM and TBO are students who are operating on this project as volunteers for Courtagen Life Science, Inc. Both had attended California Lutheran University, and AEM is now a student at the University of Nevada School of Medicine.

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