IMMUNE FUNCTION IN ATP6V0A2-RELATED CUTIS LAXA

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ABSTRACT

Cutis laxa (CL) is a genetic disorder of connective tissue. CL has known effects on multiple body systems, but its relationship to immune function has not been characterized. Recently, the laboratory of Jordan Orange, MD, PhD identified a patient with immune deficiency disease who also had a mutation in a known CL-causing gene, *ATP6V0A2*. This observation prompted us to look for evidence of immune dysfunction among individuals with *ATP6V0A2*-related CL. We administered an infection history survey to study participants with *ATP6V0A2*-related CL, their unaffected relatives, and individuals with *ELN*-related CL. We calculated a novel age weighted infection score for each participant and compared the three groups. We found that individuals with *ATP6V0A2*-related CL had significantly higher infection scores than unaffected family members ($p=0.0004$) or individuals with *ELN*-related cutis laxa ($p=0.0016$). These data suggest that *ATP6V0A2* mutations have a deleterious effect on immune function. Our findings have public health significance because they further characterize the natural history of *ATP6V0A2*-related CL and thus enhance our ability to assess this condition in the population. The infection history questionnaire has the potential to be used in guiding genetic testing and in guiding medical management, support the public health function of policy development. In addition, research in CL is relevant to aging and to conditions that affect larger populations, such as asthma, chronic obstructive pulmonary disease, and heart disease.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

I would like to thank my advisor Zsolt Urban, PhD, my assistant program director Andrea Durst, MS, DrPH, LCGC, and my committee member Louise D'Cruz, PhD. I would also like to thank my program director Robin Grubs, MS, PhD, LCGC, my class of 2017 family, my colleagues Emily Spoth and Jennifer Peck the Magnificent, Elizabeth Lawrence and the rest of the Urban lab. I could not have done this without the support of my husband David Fraser, my daughter Milly Fraser, the Huen-Hillers, the Finkels, Anu Hoey, Jennifer Furin, Chelsea Dawn, my parents and siblings, and all the friends who make my life better. You know who you are.

1.0 INTRODUCTION

Cutis laxa (CL) is a family of connective tissue disorders (Berk, Bentley, Bayliss, Lind, & Urban, 2012) affecting approximately 300 families worldwide. The most prevalent feature in CL is loose, wrinkled skin, giving an aged appearance to individuals affected by this condition. There is variable systemic involvement, although pulmonary and cardiovascular complications are common. The nervous system, musculoskeletal system, and genitourinary system can also be affected. The constellation of symptoms seen in a particular individual is largely dependent on the underlying genetic cause of the condition. CL is usually diagnosed by a combination of clinical exam, abnormal structure and reduced number of elastic fibers by skin biopsy, and genetic testing (Berk et al., 2012).

Approximately one third of CL cases in our study group have a known genetic cause, and the others are of unknown etiology. Mutations in a dozen genes have been associated with CL and the resulting clinical phenotypes have been characterized. These monogenic forms of CL can be present from birth or, less commonly, can manifest in childhood or early adulthood. An acquired form of cutis laxa is also known (Lewis, Bercovitch, Dill, & Robinson-Bostom, 2004) , for which no genetic cause or susceptibility factor has been identified with the exception of one case (Hu, Reymond, Pinel, Zabot, & Urban, 2006).

While CL is known to be a multi-system disorder, investigators have not examined potential effects of CL on the immune system. We began to study immune function in

ATP6V0A2-related CL in collaboration with the laboratory of Jordan Orange, MD, PhD, after a case report identified an individual with CL and Epstein-Barr-associated smooth muscle tumors. These rare tumors are seen in conditions associated with immune deficiency, such as AIDS (Deyrup et al., 2006). The individual's presentation included CL, aortic root dilation, fetal alcohol syndrome, intellectual disability and developmental delay. Immunological investigations determined that the patient had a natural killer (NK) cell deficiency and that this abnormality was the mechanism for the patient's immune deficiency (Shaw et al., 2012). Exome sequencing later revealed an *ATP6V0A2* mutation (Orange, personal communication).

We hypothesized that other individuals with *ATP6V0A2*-related CL would have reduced immune function. To test this idea, we collected and analyzed data on infection history from individuals enrolled in our CL study, which characterizes genotype-phenotype relationships in individuals with cutis laxa and their unaffected first-degree relatives. A subset of individuals enrolled in the CL study traveled to Pittsburgh to participate in a research clinic, at which we collected blood and biopsy samples. Participants also underwent echocardiograms, DXA bone density scans, pulmonary function testing, chest CT scans, facial imaging, and skin and vascular elasticity measurements. They also had a genetic evaluation and a meeting with Zsolt Urban, PhD, the principal investigator of the study. Participants also completed a battery of questionnaires, among them an infection history questionnaire. We calculated a novel infection score from the questionnaire data, and tested the hypothesis that individuals with *ATP6V0A*2-related CL would have a higher infection score than unaffected relatives or individuals with other types of CL. Our findings support this hypothesis, and add to our understanding of the clinical phenotype in *ATP6V0A2*-related CL.

2.0 LITERATURE REVIEW

2.1 ELASTIC FIBER FORMATION

Elastic fiber abnormalities are present in all types of CL, and it is necessary to understand elastic fiber composition in order to understand the molecular basis of CL. Elastic fibers are part of the extracellular matrix (ECM), a complex and dynamic biological scaffolding that not only provides support for cells, but also regulates many biological processes through storage of growth factors and cytokines, interactions with cell surface receptors, alterations in cytoskeletal structures in response to environmental changes, and initiation of signaling cascades (Baldwin, Simpson, Steer, Cain, & Kielty, 2013). Other components of the ECM are proteoglycans, elastin, collagens, and a large number of glycoproteins including fibronectin and laminin. Components of the ECM are made inside cells and secreted into the extracellular space, where they are assembled into fibers, sheets, or three-dimensional networks. Modifications to these components can occur at both pre-secretion and post-secretion stages (Carson, 2004).

An elastic fiber is made up of two components, the amorphous core and the microfibrils. The amorphous core, composed primarily of elastin, accounts for the bulk of the elastic fiber. Elastin is a polymeric protein that is formed by crosslinking of tropoelastin monomers. The crosslinking is accomplished through the action of lysyl oxidase, which is a copper-dependent enzyme. The second component of the elastic fiber, the microfibril, contains fibrillin and

glycoproteins. Microfibrils provide the structure upon which elastin is deposited. Other components of the ECM, such as fibulins, deposit elastin at the point where the microfibrils meet the amorphous core (Alberts et al., 2002b).

2.2 REVIEW OF GENETIC FORMS OF CUTIS LAXA

2.2.1 *ELN*-related cutis laxa

Elastin (*ELN*)-related CL is also known as autosomal dominant CL (ADCL). Until recently, *ELN*-related CL was the only known form of CL inherited in an autosomal dominant manner. However, autosomal dominant forms of *ALDH18A1*-related CL have also been reported recently ((Bhola et al., 2017; Fischer-Zirnsak et al., 2015; Nozaki et al., 2016). *ELN*-related CL is the mildest form of CL, and most affected individuals live a normal lifespan. Symptoms of *ELN*-related CL can be limited to loose skin, but systemic manifestations are possible. These include emphysema, bronchiectasis, aortic dilation and aortic aneurysm (Szabo et al., 2006; Urban, Gao, Pope, & Davis, 2005). The musculoskeletal, genitourinary and GI systems can be affected, with resulting hernias, genital prolapse, and diverticula. Individuals with *ELN*-related CL have characteristic facial features, which include large ears and a hooked nose with a long philtrum (Bert Callewaert et al., 2011; Hadj-Rabia et al., 2013).

The *ELN* gene codes for tropoelastin, a precursor monomer that is crosslinked to form the elastin polymer. *ELN*-related CL is an autosomal dominant condition, but roughly 30% of cases are not inherited but are instead caused by a *de novo* mutation (Berk et al., 2012). The majority of mutations causing *ELN*-related CL are frameshift mutations near the C-terminus of the gene

that result in an elongated version of tropoelastin. The abnormal tropoelastin monomers exhibit reduced binding to fibrillin scaffolding but are prone to self-aggregation. This prevents the formation of normal elastin polymers and results in decreased elasticity in skin and other tissues (Bert Callewaert et al., 2011; Zhang et al., 1999). *ELN* mutations are also associated with increased TGF-beta signaling, upregulation of unfolded protein response, and increased apoptosis, which are hypothesized to contribute to emphysema in *ELN*-related CL (Hu et al., 2010; Urban, Gao, Michael Pope, & Davis, 2005).

2.2.2 *FBLN5*-related cutis laxa

Fibulin-5 (*FBLN5*)-related CL is also known as autosomal recessive CL type 1A (ARCL1A). It is characterized by congenital onset and severe systemic involvement. Pulmonary artery stenosis and severe developmental emphysema make this condition life-limiting (Van Maldergem & Loeys, 2014). Inguinal hernias, genitourinary diverticula, and supravalvular aortic stenosis have also been reported (Loeys et al., 2002). *FBLN5* contributes to the formation of connective tissue by depositing tropoelastin onto fibrillin-1 microfibril scaffolding (Papke & Yanagisawa, 2014). It binds fibrillin-1 (FBN1) (Freeman et al., 2005) and lysyl oxidase-like-1 (LOXL1), an enzyme involved in the crosslinking of elastic fibers (Liu et al., 2004). It also binds latent transforming growth factor beta binding protein 2 (LTBP2) (Hirani, Hanssen, & Gibson, 2007), and latent transforming growth factor beta binding protein 4 (*LTBP4*) (Noda et al., 2013), two other ECM proteins involved in the assembly of elastic fibers.

2.2.3 *FBLN4*-related cutis laxa

Fibulin 4 (*FBLN4*)-related CL is also known as epidermal growth factor domain containing fibulin-like extracellular matrix protein 2 (*EFEMP2*)-related CL or autosomal recessive CL type 1B (ARCL1B). Like *FBLN5*-related CL, it is also characterized by congenital onset and systemic involvement. Developmental pulmonary emphysema, arterial tortuosity, aneurysms, and diaphragmatic hernia are common features. Physical features such as retrognathia, arachnodactyly, and bone fragility have also been described (Loeys, De Paepe, & Urban, 2015). The severity of this condition is variable and ranges from lethal cardiovascular and pulmonary features to benign arterial tortuosity without skin or lung manifestations (Renard et al., 2010). From a molecular standpoint, *FBLN4* interacts with fibrillin-1 microfibrils. *FBLN4* also participates in elastic fiber formation by binding to lysyl oxidase, which mediates the crosslinking of elastic fiber components (Papke & Yanagisawa, 2014). Loss of function mutations in *FBLN4* lead to impaired secretion of elastic fiber components, dysfunctional *FBLN4* protein associations and abnormal elastic fiber production (Sasaki et al., 2016).

2.2.4 *LTBP4*-related cutis laxa

LTBP4-related CL is also known as autosomal recessive cutis laxa type 1C (ARCL1C) or Urban-Rifkin-Davis Syndrome (URDS). It is a congenital condition characterized by a severe clinical phenotype, with a childhood mortality rate of 76% (Callewaert & Urban, 2016). Pulmonary emphysema is the primary cause of death. In addition to respiratory symptoms, pulmonary artery stenosis, inguinal hernias, diverticula, diaphragmatic hernia, congenital heart disease, and physical features such as sagging cheeks and sparse hair have been described

(Urban et al., 2009). *LTBP4*, or latent transforming growth factor beta binding protein 4, participates in elastogenesis by interacting with both the fibrillin-1 scaffolding of the extracellular matrix and with tropoelastin-bound *FBLN5* (Noda et al., 2013). *LTBP4* is also a regulator of signaling by TGF-beta, an essential growth factor controlling the production of the ECM (Su et al., 2015).

2.2.5 *PYCR1*-related cutis laxa

Pyrroline-5-carboxylate reductase-1 (*PYCR1*)-related CL is also known as ARCL2B. Due to significant clinical overlap with aldehyde dehydrogenase-18 family member A1 (*ALDH18A1*)-related CL, it has also been grouped with ARCL3A. It is characterized by triangular facies and a progeroid appearance. Poor growth, joint laxity, developmental delay and intellectual disability are also commonly reported (Dimopoulou et al., 2013; Reversade et al., 2009). Other features with predictive value but variable frequency in this form of CL include corneal clouding, agenesis of the corpus callosum, osteopenia, and athetoid movements. Mutations in exons at the beginning of the gene correlate with a milder phenotype than mutations in exons in the middle or end of the gene (Dimopoulou et al., 2013).

PYCR1 is a mitochondrial enzyme which reduces pyrroline-5 carboxylate to proline while converting NAD(P)H into NADP+. Proline is an amino acid abundant in elastin and collagen. Fibroblast cells containing *PYCR1* mutations exhibit increased rates of apoptosis with oxidative stress (Reversade et al., 2009).

2.2.6 *ALDH18A1*-related cutis laxa

ALDH18A1-related CL is also called DeBarsy syndrome or autosomal recessive CL type 3. This condition shares significant phenotypic overlap with the proton-transporting adenosine triphosphatase V0 subunit A2 (*ATP6V0A2*)-related CL, and some individuals with a clinical diagnosis of DeBarsy syndrome have been identified as having mutations in *ATP6V0A2*. *ALDH18A1* is most often an autosomal recessive condition, but recent reports describe several *de novo* cases associated with monoallelic mutations (Bhola et al., 2017; Fischer-Zirnsak et al., 2015; Nozaki et al., 2016).

Features commonly reported include lax skin that is prominent on hands and feet, developmental delay, microcephaly, inguinal hernia, translucent skin, congenital hip dislocation, joint laxity, hypotonia, brisk reflexes, cataracts, distal contractures, and failure to thrive. (Bicknell et al., 2008; Skidmore et al., 2011; Wolthuis et al., 2014; Zampatti et al., 2012).

The *ALDH18A1* gene codes for the mitochondrial enzyme delta 1-pyrroine-5-carboxylate synthase (P5CS). P5CS is essential in the proline synthesis pathway, as well as in the synthesis of arginine in the gut. P5CS deficiency leads to proline deficiency, which prevents collagen synthesis. Proline is also required for the production of particular neuromoduators and supportive polypeptides for neuronal function (Pérez-Arellano, Carmona-Alvarez, Martínez, Rodríguez-Díaz, & Cervera, 2010).

2.2.7 *ATP7A*-related cutis laxa

Copper-transporting adenosine triphosphatase alpha (*ATP7A*)-related CL is also called occipital horn syndrome (OHS) or X-linked CL (XLCL), due to the gene's location on the X

chromosome. It was also previously known as Ehlers-Danlos type 9. Low serum copper is a diagnostic marker for this form of CL (Kaler et al., 1994). Mutations in *ATP7A* cause three disorders (in order of increasing severity); X-linked distal motor neuropathy, X-linked CL, and Menkes syndrome.

Menkes syndrome is a neurodegenerative disorder with an onset in infancy, characterized by hypotonia, neurodevelopmental regression, seizures, arterial tortuosity, failure to thrive, and early mortality. Individuals with Menkes syndrome also have sparse, brittle hair with a twisted, kinky texture (pili torti) (Menkes, Alter, Steigelder, Weakley, & Sung, 1962).

OHS is a milder form of Menkes syndrome (Das et al., 1995; Tümer & Horn, 1997). In addition to loose skin, the pathognomonic feature is the presence of the so-called occipital horns, bilateral calcifications that form along the occipital ridge of the skull at the site of neck muscle attachments. Other features that have been reported include bladder diverticula and diarrhea (Tsukahara, Imaizumi, Kawai, & Kajii, 1994). Neurological manifestations include muscle weakness, dysautonomia, and mild intellectual disability (Wakai et al., 1993).

ATP7A-related distal motor neuropathy is the least severe of the phenotypes associated with *ATP7A* mutations. Its manifestations are limited to progressive weakness in distal limbs (Kennerson et al., 2010).

ATP7A plays a role in the extracellular matrix and elastic fiber assembly through its role as a copper transporter. Copper is essential for the activity of lysyl oxidase and lysyl oxidase-like enzymes, necessary for crosslinking of monomers in elastin and collagen assembly (Alberts et al., 2002b). Severity in *ATP7A*-related disorders appears to be correlated with levels of functional ATP7A protein produced, such that mutations resulting in production of some functional protein will cause milder forms of the disease (Møller, 2015). For example, splice

site mutations that allow stable transcripts to be produced through exon skipping have been associated with OHS (Kaler et al., 1994).

2.2.8 Geroderma Osteodysplasticum

Geroderma osteodysplasticum (GO) is a form of CL caused by mutations in the *GORAB* gene (this gene is also known as SCY1-like 1 binding protein 1 (*SCYL1BP1*)). The *GORAB* gene codes for a Golgi-associated protein that interacts with the G-protein Rab6. GORAB, through its interactions with Rab6, participates in vesicular transport processes of the secretory pathway (Hennies et al., 2008). The main distinguishing characteristics of GO are a progeroid appearance, drooping cheeks, maxillary hypoplasia and reduced bone density resulting in fractures of vertebrae and long bones (Kariminejad et al., 2017; Rajab et al., 2008).

2.2.9 MACS syndrome

Macrocephaly, alopecia, cutis laxa, and scoliosis (MACS) syndrome is caused by mutations in Ras and Rab interactor 2 (RIN2), a Golgi protein that interacts with the G protein Rab5. Rab5 participates in vesicular trafficking, and it is hypothesized that mutations in *RIN2* result in defects in vesicular transport of extracellular matrix components (Basel-Vanagaite et al., 2009; Kimura, Sakisaka, Baba, Yamada, & Takai, 2006; Syx et al., 2010). In addition to the distinguishing features of macrocephaly, alopecia, cutis laxa and scoliosis, individuals with MACS syndrome generally have significant developmental delay and intellectual disability, as well as coarse facial features (Syx et al., 2010).

2.2.10 *ATP6V0A2*-related cutis laxa

ATP6V0A2-related CL is also known as autosomal recessive CL type 2A or Debre-type CL. As mentioned, there is significant phenotypic overlap between *ATP6V0A2*, *ALDH18A1*, and *PYCR1*-related CL subtypes, as well as with GO. In addition, *ATP6V0A2*-related CL has a variable phenotype. The milder forms have been clinically classified as wrinkly skin syndrome. The more severe forms fall under the clinical diagnosis of Debre type CL. Generally, *ATP6V0A2*-related CL is characterized by wrinkled skin that becomes less wrinkled with age, and distinct craniofacial features including oxycephaly, delayed closure of anterior fontanels, downslanting palpebral fissures, a long philtrum, anteverted nares, and microcephaly (Van Maldergem et al., 2008). Other features include joint laxity, congenital hip dislocation, hernias, hearing loss and ocular abnormalities, especially strabismus and myopia (Morava, Guillard, Lefeber, & Wevers, 2009). Neurological involvement is common and can include seizures, developmental delay, intellectual disability, and abnormalities of cortical development such as cobblestone brain malformation (Fischer et al., 2012; Hucthagowder et al., 2009). Pulmonary complications are uncommon. One recent study found that out of 211 subjects who presented with cutis laxa and no pulmonary deficits, 24% of them had *ATP6V0A2* mutations (Fischer et al., 2012).

ATP6V0A2-related CL is also a congenital disorder of glycosylation (CDG). Morava et al. used transferrin and apolipoprotein C-III isoelectric focusing to identify N- and O-linked glycan biosynthesis defects in a cohort of CL patients fitting the phenotypic profile of *ATP6V0A2*-related CL (Morava et al., 2005). Although *ATP6V0A2*-related CL is currently the only CDG recognized as a cutis laxa subtype, other cases of CDGs with skin laxity have been reported. These cases were associated with mutations in two Golgi-related genes for the

conserved oligomeric Golgi complex subunit COG7, and MAN1B1, a protein that targets misfolded proteins for degradation. (Rymen et al., 2013; Wu et al., 2004).

ATP6V0A2 also contributes to proper Golgi function. The *ATP6V0A2* gene encodes a component of a vacuolar ATPase (V-ATPase), the A2 subunit of the membrane-associated V0 domain. The V-ATPase is an ATP-driven proton pump, and it functions to regulate the acidity of membrane-bound organelles such as the Golgi, lysosomes, endosomes, and vesicles (Hurtado-Lorenzo et al., 2006). Acidification is essential for both secretory and endocytic cellular functions (Forgac, 2007). In *ATP6V0A2*-related CL, loss-of-function mutations are associated with abnormal protein trafficking that results in abnormal elastin production. Fibroblasts still produce tropoelastin monomers, but there is reduced secretion of tropoelastin from the vesicles. As a result, *ATP6V0A2* knockdown fibroblasts secrete low levels of elastin with abnormal aggregation of tropoelastin monomers in the Golgi apparatus (Hucthagowder et al., 2009; Kornak et al., 2008).

2.2.11 Recently identified *ATP6V*-related cutis laxa subtypes

Van Damme and colleagues recently described seven individuals with cutis laxa caused by mutations in *ATP6V1E1* or *ATP6V1A*, subunits of the ATP hydrolytic domain of the vacuolar ATPase. The phenotype in these individuals overlapped with that previously described for *ATP6V0A2* mutations, including craniofacial features and musculoskeletal features such as joint laxity and congenital hip dysplasia. Abnormal fat deposition, differences in skin features, and cardiovascular and pulmonary complications distinguished these forms of CL from *ATP6V0A2*-related CL. Like *ATP6V0A2* mutations, *ATP6V1* mutations cause abnormal Golgi trafficking. They also caused deficits in lysosomal function and were associated with impaired N- and O-

type glycosylation. Homology models predicted that the regions of the protein disrupted by the mutation were essential for the formation of stable interactions between subunits of the ATPase (Van Damme et al., 2017)

2.3 ATP6V0A2 AND IMMUNE FUNCTION

2.3.1 Possible roles for ATP6V0A2 in immune function

At least three potentially intersecting pathways exist by which ATP6V0A2 function could impact the immune system. First, endocytosis and destruction of invasive microbes and viruses depend on acidification of vesicles by ATPases. Second, the acidification of intracellular and extracellular environments influences immune regulatory processes. Finally, ATPase regulation of Golgi pH is important in the process of glycosylation, which is an essential mechanism for immune function.

2.3.2 Vacuolar ATPases: structure, expression pattern, function

The vacuolar ATPase is a molecular motor consisting of 13 subunits and two main domains, the V1 ATP-hydrolytic domain and the V0 membrane-associated domain. The V1 domain generates protons through the conversion of ATP to ADP. The V0 domain contains multiple transmembrane helices that form a rotating ring. The ring moves protons across the membrane. (Nishi & Forgac, 2000). One of four possible "A" subunits is used in the V0 domain. The A2 subunit is expressed throughout the body in Golgi and endosomes, especially

macrophages, while A1, A3, and A4 have tissue-specific expression patterns involving cell surface interactions (Guillard et al., 2009; Toyomura et al., 2003; Uhlen et al., 2015). The A2 isoform was previously thought to be exclusively vacuolar, but certain membrane-associated roles have since been recognized (Kulshrestha et al., 2015; Ntrivalas, Gilman-Sachs, Kwak-Kim, & Beaman, 2007; Ota et al., 2013).

The primary role of the vacuolar ATPase is in acidification of membrane-bound cellular compartments that are essential for secretory and endocytic cellular processes. Membrane-bound organelles include the endoplasmic reticulum, the Golgi, secretory vacuoles, endosomes, and lysosomes. The function of these organelles depends on the pH of their respective lumens which get progressively more acidic in the order listed above (Mellman, Fuchs, & Helenius, 1986). In the lysosome, acidity permits activation of hydrolases that initiate degradation processes. In receptor-mediated endocytosis, a change in pH stimulates conformational changes that allow ligand-receptor dissociation and receptor recycling (Mellman, 1992). ATPases can also affect the acidity of extracellular environments, and so can also be found at the plasma membrane of certain cells such as osteoclasts, renal intercalated cells, and macrophages. The ability of ATPases to regulate extracellular pH is also a reason that they are involved in tumorigenesis (Alzamora et al., 2013; Jaiswal et al., 2012; Kulshrestha et al., 2015; Nishi & Forgac, 2002; Ota et al., 2013; Toyomura et al., 2003)

2.3.3 Glycoimmunology: potential roles for ATP6V0A2

Glycosylation is essential to cellular function. Most proteins that are expressed on the surface of a cell or secreted are glycosylated, and disorders resulting in complete loss of glycans are incompatible with life. (Freeze, Chong, Bamshad, & Ng, 2014).

Glycosylation plays an essential role in both the innate and adaptive immune systems (Lyons, Milner, & Rosenzweig, 2015). This relationship was first brought to light by studies showing changes in glycosylation patterns associated with arthritis (Parekh et al., 1985) and studies demonstrating that the presence of different levels of N-glycosylation on a critical fragment of immunoglobulin G (IgG) determine whether the IgG will produce a pro-inflammatory or anti-inflammatory response (Kaneko, Nimmerjahn, & Ravetch, 2006; Schwab & Nimmerjahn, 2014).

Because glycans are a highly variable group of molecules, they provide versatility in immune signaling and antigen recognition processes. Many categories of immune cell receptors recognize glycans. These include C-type lectins, which function to recognize and internalize pathogen-associated molecular patterns (PAMPS), siglecs, which recognize sialic acid-containing glycans and which participate in immunological signaling, and galectins, which regulate signaling through cell surface interactions (Monticelli, Ferro, Jaeken, Dos Reis Ferreira, & Videira, 2016).

Selectins are a category of immune cell receptors whose function depends upon glycan recognition. Selectins are involved in facilitating the transfer of leukocytes from blood to infected tissue (extravasation). Extravasation of leukocytes is a multi-stage process involving capture and rolling along the endothelial wall, activation, and adhesion at the appropriate site for exit into tissue. Patterns of glycosylation on leukocytic selectin ligands determine the affinity of a particular selectin ligand for its complementary selectin on the endothelial wall of the vessel. High affinity will "capture" the selectin ligand (Ley, Laudanna, Cybulsky, & Nourshargh, 2007).

Other categories of immune receptors that recognize glycans include the toll-like receptors and T cell receptors. T cell receptors recognize even minor changes in glycosylation

patterns on cell surfaces, and can use them to create novel antigens for recognition by CD4+ and CD8+ T cells as well as by antibodies (Lyons et al., 2015). Nucleotide oligomerization domain-containing proteins 1 and 2 (NOD1 and NOD2) recognize peptidoglycans associated with pathogenic microorganisms (Philpott & Girardin, 2010). Natural killer cells recognize glycolipids presented by CD1 cells (Zajonc & Girardi, 2015).

There are extensive roles for glycosylation in immune function. For example, Notch signaling pathways are involved in multiple processes, including the production of T and B lymphocytes, and also the development of the CD4 immune subset. The particular ligands that bind to Notch, and therefore, the processes that are initiated, are determined by the identity of gylcans that are bound to Notch through the action of Fringe glycosyltransferases (Moloney et al., 2000). As another example of the diverse roles for glycosylation in the immune system, studies of human milk have demonstrated that sialic acid-containing oligosaccharides found on milk proteins provide a first layer of defense when pathogens attack mucous membranes in the neonate (Newburg, 1999).

At a systems level, a role for ATP6V0A2 in glycosylation-dependent immune function is suggested by the observation that patients with *ATP6V0A2*-related CL have abnormal N- and/or O-glycosylation patterns. The same studies have shown defective trafficking of tropoelastin in *ATP6V0A2*-related CL (Fischer et al., 2012; Hucthagowder et al., 2009; Kornak et al., 2008). This trafficking defect was localized to abnormal Golgi structures in *ATP6V0A2* mutant cells (Fischer et al., 2012; Hucthagowder et al., 2009). In addition to a potential role in Golgi formation, maintenance of the appropriate acidity in the Golgi is essential for appropriate protein sorting and glycosylation (Klionsky, Nelson, & Nelson, 1992; Rivinoja, Hassinen, Kokkonen, Kauppila, & Kellokumpu, 2009).

Production of abnormal elastin fibers due to dysfunctional protein trafficking may provide an adequate explanation for the skin manifestations in the *ATP6V0A2*-related CL phenotype. However, glycosylation functions regulated by ATPases that are involved in the formation and maintenance of the extracellular matrix may also play a role in the phenotype. Hinek and colleagues demonstrated that removal of sialic acid caps from glycoproteins in the extracellular matrix causes an increase in elastin deposition, and that a deficiency in the desialylating enzyme is linked to abnormal elastin deposition (Hinek, Pshezhetsky, von Itzstein, & Starcher, 2006).

Additional glycosylation-dependent mechanisms may also be involved in the CL phenotype. Hucthagowder et al. proposed reduced sialylation as a potential mechanism for the neurological sequelae of *ATP6V0A2*-related CL (Hucthagowder et al., 2009). Xia and collagues described failure of brain vascular development in mice lacking O-type glycans due to abnormal interactions between ECM and endothelial cells (Xia et al., 2004). Since many ECM proteins are glycosylated, Wopereis and colleagues suggested that N- and O- linked glycosylation deficits in ATP6V0A2 may underly the neurological aspects of the cutis laxa phenotype (Wopereis et al., 2005). It is also reasonable to suggest that glycosylation abnormalities could contribute to immune deficiencies.

2.3.4 ATP6V0A2 regulation of immune response

Several lines of experimental evidence suggest that ATP6V0A2 contributes to the regulation of immune responses. First, ATP6V0A2 may have a direct role in the prevention of infection. It is expressed in the neutrophil, where it is involved in exocytosis of granules containing antimicrobial molecules. In experimental conditions stimulating an immune response

pathway in neutrophils, ATP6V0A2-containing granules were trafficked to the cell surface (Gilman-Sachs et al., 2015).

Other roles for ATP6V0A2 in immune function are complex. In some contexts, expression is inversely correlated with immune response, and in other contexts, expression is positively correlated with immune function.

Reduced *ATP6V0A2* expression can negatively impact the immune system. A recent report from Udono and colleagues described a protective response known as the senescence-associated secretory phenotype, triggered by reduced *ATP6V0A2* expression. Cell senescence is a generally adaptive phenomenon that arrests the cell cycle in at-risk cells. The senescence-associated secretory phenotype is a deleterious version of senescence, and it is triggered in response to cellular cues indicating that a cell's DNA has been damaged. Damaged DNA places a cell at risk for malignant transformation. The senescence-associated secretory phenotype transforms senescent fibroblasts into pro-inflammatory cells and causes changes in the cell microenvironment. These changes promote tissue repair in the short term, but have damaging consequences in the long term (Udono et al., 2015).

Other studies indicate that increased ATP6V0A2 expression is associated with disease susceptibility. Lee and colleagues provided evidence that ATP6V0A2 is involved in down regulation of the immune response. They cloned cDNA of *ATP6V0A2* (alternative name J6B7), and found that it produced a protein that suppressed lymphocyte reactions in vitro (Lee, Ghoshal, & Beaman, 1990).

The A2 subunit of the V-ATPase is upregulated in mulitiple types of tumors. Immunohistochemical analysis of patient glioma tissue revealed upregulation of ATP6V0A2 ((Di Cristofori et al., 2015). In another study, ATP6V0A2 was overexpressed at the surface of

ovarian tumor cells along with a stabilizing protein known to be localized at the leading edge of cancer cells (Kulshrestha et al., 2015). Parmathy and colleagues demonstrated that ATP6V0A2 associates with invasion assembly related proteins, and may promote migration of triple negative breast cancer cells. Conversely, knockdown of *ATP6V0A2* resulted in reduced tumor cell viability, increased cancer cell apoptosis and reduced cancer cell migration. Altered interactions in the Notch signaling pathway may mediate these responses (Pamarthy et al., 2015).

A2NTD is the N-terminal portion of the ATP6V0A2 protein and has been referred to as the RTF soluble fragment. In monocytes, the a2NTD of monocytic ATP6V0A*2* can be cleaved from the protein. When the a2NTD is released, it behaves like a cytokine signal, and causes the monocyte to transform into a tumor associated macrophage, which promotes tumor growth and regulates inflammation in cancer (Boomer et al., 2001) Cancer-related inflammation prevents tumor cells from being targeted by the immune system (Kwong, Gilman-Sachs, & Beaman, 2011). A2NTD induces immune cells to produce interleukin-1beta, which regulates inflammatory conditions (Ntrivalas, Derks, et al., 2007). A2NTD also modulates production of and signaling functions of immune cytokines (Boomer et al., 2001; DuChateau, Lee, Westerman, & Beaman, 1999; Ntrivalas, Gilman-Sachs, et al., 2007). As a result, researchers are examining the potential of V-ATPase inhibitors as cancer therapeutic agents (Izumi et al., 2003).

The acidic extracellular environment generated by membrane ATPases promotes tumor growth through activation of proteases that dismantle the extracellular matrix (Bergamaschi et al., 2008). In addition, the increased acidity in tumor cells allow them to escape immune mediated apoptosis (Ishisaki, Hashimoto, Amagasa, & Nishihara, 1999). Conversely, inhibition of ATPases induces apoptosis in tumor cells (Aiko et al., 2002; De Milito et al., 2007).

In mammalian pregnancy, maternal immune responses must be adjusted to permit tolerance of the fetus. ATP6V0A2 participates in the regulation and coordination of pregnancy-related immune responses. Successful implantation during pregnancy depends on signaling between "host" (mother) and "allograft" (fetus) (Aplin & Kimber, 2004; Jaiswal et al., 2013). During bovine embryonic implantation, the expression of A, B, and C subunits of the vacuolar ATPase is increased (Skinner, MacLaren, & Wildeman, 1999). Levine and colleagues later characterized the role of ATP6V0A2 in implantation. Implantation requires an inflammatory response and subsequent vascularization. The authors showed that the inflammatory response was triggered through a multi-step process, in which ATP levels in the cell resulted in the release of the pro-inflammatory cytokine IL-1beta and the promotion of the inflammatory response (Levine, Derks, & Beaman, 2005).

ATP6V0A2 is expressed in sperm and contributes to the acidity of seminal fluid. Fertile men have higher sperm ATP6V0A2 expression levels than infertile men, and motile sperm have higher ATP6V0A2 expression levels than immotile sperm (Ota et al., 2013). Jaiswal and colleagues reported that capacitated sperm released cleaved a2NTD in the uterus, causing increased expression of pro-inflammatory immune molecules (Jaiswal et al., 2012). Studies in HIV-infected individuals demonstrated increased ATP6V0A2 (alternative name RTF) expression that was positively correlated with the degree of immune deficiency (DuChateau et al., 1999). Boomer and colleagues demonstrated involvement of ATP6V0A2 (alternative name regeneration and tolerance factor RTF) in protecting cells from immune attack. They hypothesized that ATP6V0A2 established immunological tolerance through clonal anergy, the inactivation of self-reactive lymphocytes (Boomer et al., 2001).

Because functional evidence exists for both supportive and deleterious roles for ATP6V0A2 in immune function, it is likely that an *ATP6V0A2* mutation can have different effects on different cellular processes. This could occur through the stimulation of different signaling pathways (Alberts et al., 2002a). Regulatory mechanisms within the same system can also produce a paradoxically identical effect when the same gene is upregulated or downregulated (Feng, 2012; Wang et al., 2014). Wang et al described a protein SRPK1 that is needed for recruitment of a phosphatase that downregulates consitutitive activation of the oncogenic protein Akt. They demonstrated that overexpression of SRPK1 caused squelching of the phosphatase. On the other hand, reduced expression of SRPK1 prevented recruitment of the phosphatase. Changes in either direction of expression caused an increase in oncogenic activity (Wang et al., 2014). It is possible that the immune-suppressive upregulation of ATP6V0A2 reflects a similar phenomenon. Further functional immunological studies may elucidate the mechanisms of action for ATP6V0A2.(Lyons et al., 2015)

3.0 MANUSCRIPT: INCREASED INCIDENCE OF INFECTIONS IN *ATP6V0A2*-RELATED CUTIS LAXA

3.1 BACKGROUND

Cutis laxa (CL) is a family of connective tissue disorders affecting approximately 300 families worldwide. The most prevalent feature in CL is loose, wrinkled, skin, conferring an aged appearance to individuals affected by this condition (Berk et al., 2012) . There is variable systemic involvement, and the constellation of symptoms seen in a particular individual is largely dependent on the underlying genetic cause of the condition. Over twelve CL-causing genes have been identified, and one of these is *ATP6V0A2* (Kornak et al., 2008). This gene codes for the A2 subunit of the vacuolar-ATPase's V0 domain. Vacuolar ATPases are proton pumps that are responsible for the acidification of membrane-bound organelles such as the ER, Golgi, endosomes, lysosomes, and vesicles.

ATP6V0A2-related CL is caused by homozygous or compound heterozygous mutations in the *ATP6V0A2* gene. It has been associated with generalized skin laxity, characteristic craniofacial features including delayed fontanel closure, downslanting palpebral fissures and a broad nasal bridge, musculoskeletal features including joint laxity, congenital hip dislocation and hernias, and neurological involvement including developmental delay, seizures, and brain malformations (Fischer et al., 2012).

The mutations that cause *ATP6V0A2*-related CL disrupt the process of elastic fiber production (Hucthagowder et al., 2009). Normally, tropoelastin monomers are trafficked from the Golgi to the cell surface and are secreted into the extracellular space. There, tropoelasin monomers aggregate through a pH and temperature-driven process called coacervation, and are crosslinked by the oxidation of lysyl residues to form the elastin polymer. Through the actions of fibulins, the elastin is deposited onto microfibrils and is incorporated into the extracellular matrix (Baldwin et al., 2013).

In *ATP6V0A2*-related CL, elastic fiber formation is dysfunctional due to abnormalities in the Golgi trafficking process. Experiments in fibroblasts containing patient-derived mutations in the *ATP6V0A2* gene demonstrated multiple abnormalities in the elastin production pathway. These included fragmented Golgi, swollen Golgi fragments holding large amounts of tropoelastin monomers, increased formation of abnormal tropoelastin aggregates, reduced formation of elastin polymers, and increased apoptosis rates (Fischer et al., 2012; Hucthagowder et al., 2009). Abnormal Golgi, abnormal aggregation of tropoelastin, and dysfunction in the secretory process can all be linked to dysregulation of pH.

ATP6V0A2-related CL is also recognized as a congenital disorder of glycosylation (CDG), (Morava et al., 2005). A subset of CDGs have significant immune involvement, and susceptibility to infection may also be increased for individuals with *ATP6V0A2*-related CL. Glycosylation defects can have a negative impact on immunity because many aspects of immune function are glycosylation-dependent. Glycans are a highly variable group of molecules that provide versatility in immune signaling and antigen recognition processes, and many categories of immune cell receptors recognize glycans (Monticelli et al., 2016).

Shaw and colleagues recently identified an individual with Epstein-Barr-associated smooth muscle tumors, which are seen in conditions of immune deficiency such as AIDS (Deyrup et al., 2006). This individual's presentation also included CL, aortic root dilation, presumed fetal alcohol syndrome, intellectual disability and developmental delay. Immunological investigations determined that a lack of natural killer cells (NK) was the underlying mechanism for the patient's immune deficiency (Shaw et al., 2012). Exome sequencing revealed an *ATP6V0A2* mutation (Orange, personal communication).

This observation suggests that the *ATP6V0A2*-related CL phenotype may include immune deficits. However, because immunological deficits have also been reported as a consequence of fetal alcohol syndrome (Johnson, Knight, Marmer, & Steele, 1981), further investigation is warranted. Immunological function in CL has not been studied, but it would be of clinical importance to characterize this aspect of CL involvement. To investigate possible immunological consequences of *ATP6V0A2* mutations, we administered an infection history questionnaire to individuals with *ATP6V0A2*-related CL, their unaffected first-degree relatives, and, as a control, individuals with *ELN*-related CL.

Using the infection history questionnaire data, we calculated an age-weighted infection score for each participant. We compared the scores for each group and found that individuals with *ATP6V0A2*-related CL had higher infection scores than individuals in either control group, suggesting that there is an as-yet-unrecognized immune component to the *ATP6V0A2*-related CL phenotype.

3.2 SUBJECTS AND METHODS

3.2.1 Study Participants

All study participants gave their informed consent for participation in a study examining genotype-phenotype relationships in individuals with CL and in first degree relatives of individuals with CL (CL study). All procedures were approved by the Institutional Review Board of the University of Pittsburgh's Office for the Protection of Human Subjects in Research (Appendix A).

Participants in the CL study were volunteers who were recruited through their physicians, or volunteers who contacted the study after finding our study information online (www.cutislaxa.pitt.edu). The Urban lab is the only US research group that studies CL, and because of this, physicians and patients contact the study with requests to enroll. After initial contact, the participants were screened by telephone to determine if they met study criteria (Appendix D). Individuals met study criteria if they exhibited multiple skin features such as lax or prematurely aged skin, reported other systemic features such as arterial tortuosity, or had a confirmed genetic diagnosis of CL through testing by an outside lab. The first-degree relatives of these individuals were also eligible to participate in the study. As part of our study procedures, we screened participants' DNA for mutations in known CL causing genes using Sanger sequencing. For participants with a genetic diagnosis identified by an outside lab, we confirmed the diagnosis through sequencing. We obtained demographic and clinical information for each participant through a CL clinical questionnaire that was filled out by a physician (Appendix C).

A subset of study participants volunteered to attend a CL research clinic held at the University of Pittsburgh. We contacted individuals with ATP6V0A2-related CL and invited

them to the clinic. The remainder of the clinic participants contacted the study with a request to attend. Testing included a physical, a genetic exam, a blood draw, a skin biopsy, pulmonary function testing, echocardiogram, chest CT scans, facial imaging, and skin and vascular elasticity measurements. The clinic also included a meeting with the PI and a patient support group and information session.

3.2.2 Methods

We compiled data from four participants with *ATP6V0A2*-related CL. These individuals had homozygous or compound heterozygous mutations in the *ATP6V0A2* gene. We also collected data from four unaffected relatives, each with a single mutation in the *ATP6V0A2* gene. As another comparison, we collected infection history data from two participants with elastin (*ELN)*-related CL. *ELN*-related CL is an autosomal dominant condition caused by a mutation in the *ELN* gene. It is not known to be associated with immune deficiency. Table 1 contains mutation information for each participant.

Table 1. Mutation data

			Mutation 1		Mutation 2	
ID	**GENE**	**REFERENCE SEQUENCE**	**cDNA**	**AMINO ACID CHANGE**	**cDNA**	**AMINO ACID CHANGE**
7093	ATP6V0A2	NM_012463	c.397_398insCATGCTGA	p.R133fs*3	c.2466_2470delGGTAG	p.W822*
7226	ATP6V0A2	NM_012463	c.1351C>T	p.R451W	c.2040C>A	p.C680*
7264	ATP6V0A2	NM_012463	c.1351C>T	p.R451W	-	-
7263	ATP6V0A2	NM_012463	c.2040C>A	p.C680X	-	-
7346	ATP6V0A2	NM_012463.3	c.78dupC	p.S27Qfs*28	-	-
7363	ATP6V0A2	NM_012463.3	c.78dupC	p.S27Qfs*28	c.78dupC	p.S27Qfs*28
7364	ATP6V0A2	NM_012463.3	c.600delC	p.I201Sfs*20	-	-
7365	ATP6V0A2	NM_012463.3	c.600delC	p.I201Sfs*20	c.2176-5C>G	intronic
7342	ELN	NM_001278939.1	c.1418G>A	p.G473D	-	-
7197	ELN	NM_001278939.1	c.5C>A	p.A2E	-	-

Shaded rows indicate individuals with cutis laxa

Clinical characteristics of the participants were collected as a part of their participation in CL research clinics organized at the University of Pittsburgh. As part of the CL research clinic, each participant underwent a clinical genetics assessment including a physical exam. The study team also reviewed participants' relevant medical records. Results of the examination and details of the family history were entered in a cutis laxa clinical questionnaire (Appendix C). Clinical photographs were taken to document relevant anatomical features. Additional tests performed during the clinic included lung function testing, echocardiography and 3-dimensional facial imaging.

We administered an infection history questionnaire to clinic participants (Appendix B). This questionnaire was modified from a questionnaire used by the laboratory of Jordan Orange. The modifications were designed to make it easier for patients to complete the questionnaire without the help of a clinician. The questionnaire asked whether participants had ever experienced a particular infection type. If the infection was a bacterial or fungal infection, or other infection that could be experienced more than once, we asked the participants to provide a frequency estimate (yearly or lifetime) for that type of infection. Infection types included viral infections such as varicella, measles, and stomach flu, bacterial infections such as strep throat and otitis media, and fungal infections such as yeast infections and ringworm. For infection types having the potential for recurrence, we asked participants to provide a lifetime or annual frequency. To minimize errors caused by self reporting, if a participant indicated that he or she had experienced a particular infection, study staff contacted that participant by phone to follow up with questions such as, "Was this infection confirmed by testing?"

To summarize each participant's data across infection types, we calculated a total infection score weighted by participant age. We devised this novel method in order to be able to quantify the infection rate among our participants. Quantification permitted us to capture subtle differences between study groups. The infection score incorporated two factors; the number of different types infections that a person experienced, and the frequency of infections. First, we assigned a score for each infection type. If a participant experienced an infection type between one and three times, we used a score of one for that infection type. If a participant experienced an infection type more than three times, we used a score of two. We then summed scores across all infection types to create a total infection score for each participant. Table 2 shows total number of infection types as well as the total infection score for each participant.

Table 2. Infection history data

	Participant ID									
Infection Type	**7363**	**7365**	**7264**	**7263**	**7346**	**7093**	**7226**	**7364**	**7197**	**7342**
Oral herpes										
CMV, EBV, mononucleosis					+					+
Varicella	+	+	+	+		+				+
HPV				+		+				
Roseola, human herpes virus, other DNA virus										
Flu, cold, stomach flu	+	+			++	+	+	+	+	
Flu vaccine reaction					+				+	
Mycobacteria, listeria, atypical intracellular bacteria										
Strep throat	+	+	+	++	++	+				
Urinary tract infection	+			++	++	+				
Conjunctivitis		+		+	+	+				+
Otitis media	++	+	+		++	+	++	+		
Pneumonia*	+				++	+	++	+		
Staph aureus skin infection			+	+						
Bronchitis	+		++	++	++	++			+	
Bacterial vaginosis						+				
Ringworm	+	+			+					++
Yeast infection				+	+	++			+	
Toenail/fingernail infections						++				++
Total number of infection types	8	6	5	8	11	12	3	3	4	5
Total infection score	9	6	6	11	17	15	5	3	4	7
+ = 1 to 3 times, ++ = >3 times, * ++ = >1 time										

We modified the scoring criteria for infection types in the following cases: If a participant gave a numerical range for incidences of an infection type, we used the lowest reported number. If a participant reported experiencing an infection "a few" times, we used a score of one. If a participant reported an infection as "ongoing, we used a score of one. If a

participant reported experiencing an infection "many" times, we used a score of two. If a participant reported experiencing pneumonia two or more times, we used a score of two for the pneumonia infection type.

We weighted each total infection score by participant age. To do this, we divided each participant's total infection score by years of life. We chose this approach because two participants with *ATP6V0A2* were under 10 years old, and our study group lacked age matched comparisons in the unaffected heterozygote or *ELN* groups. We weighted infection scores by age to account for the fact that younger participants would naturally be exposed to fewer infectious agents than older participants. The unweighted total infection scores of the child participants tended to be lower than adult scores in our study sample (Figure 1).

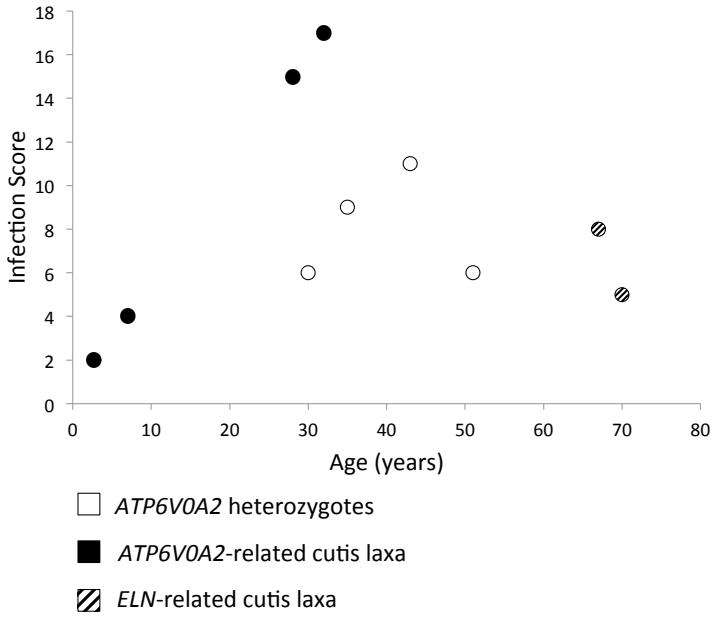

Figure 1. Age vs. total infection score (unweighted)

We calculated group means for the *ATP6V0A2* group, the unaffected heterozygous relative group, and the *ELN* group. We compared the group means with t-tests. For each comparison, a variance ratio test confirmed that the samples did not have unequal variance. We also performed power calculations for our t-tests. All statistics were calculated using Stata software. To address concerns about the small size of this sample and the need for age-weighting to include child participants, we performed additional statistical analyses on a data set that excluded child participants.

3.3 RESULTS

Three of the four participants with *ATP6V0A2*-related CL had compound heterozygous mutations, and one had a homozygous mutation (Table 1). A total of seven different mutations were observed; two nonsense, one missense, three frameshift, and one splice site mutation, consistent with prior reports (Kornak et al. 2008, Hucthagowder et al. 2009). Two of these mutations from a single participant (c.397_398insCATGCTGA, c.2466_2470delGGTAG) have been described previously (Hucthagowder et al., 2009). One additional mutation had been previously described in a patient who was not part of this study (c.600delC) (Fischer-Zirnsak et al., 2015). Out of the six mutations not involving a splice site, three were located in the long initial cytoplasmic region of the protein. The other three mutations were located within or near transmembrane domain regions spread across the protein (Figure 2) (The UniProt Consortium, 2017). These observations are consistent with a previous report, in which approximately one

third of identified mutations were located in the initial cytoplasmic domain, and most of the remaining mutations were in or near transmembrane domains (Hucthagowder et al., 2009). Transmembrane domains appear to be essential for APT6V0A2 function (Hucthagowder et al., 2009; Kornak et al., 2008).

The software prediction algorithm MutationTaster predicted all but one variant to be pathogenic. The splice site mutation (c.2176-5C>G) was predicted to be a polymorphism, but with the potential to affect splicing. The identification of the variant in a patient suggests that deleterious splicing abnormalities do occur. Another variant, c.78dupC, had previously been reported as pathogenic in the ClinVar database. MutationTaster reported that the single missense mutation (c.1351C>T, p.R451W) was conserved or partly conserved in all species having a homologue to *ATP6V0A2*. PolyPhen2 and SIFT also predicted the R451W amino acid substitution to be disease causing.

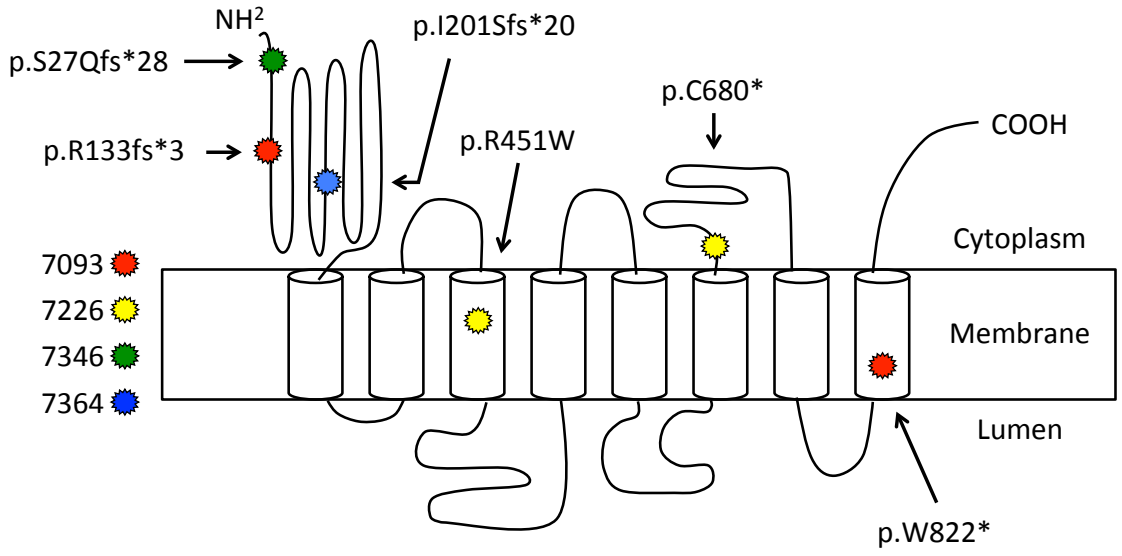

Figure 2. Location of mutations on ATP6V0A2 protein (adapted from Hucthagowder et al. 2008)

Three parents and one sibling were included in the study as controls (Figure 3, Table 2). All four were unaffected heterozygous mutation carriers. As cutis laxa controls, two individuals with heterozygous elastin mutations were included. Both of these participants had missense mutations (Table 1).

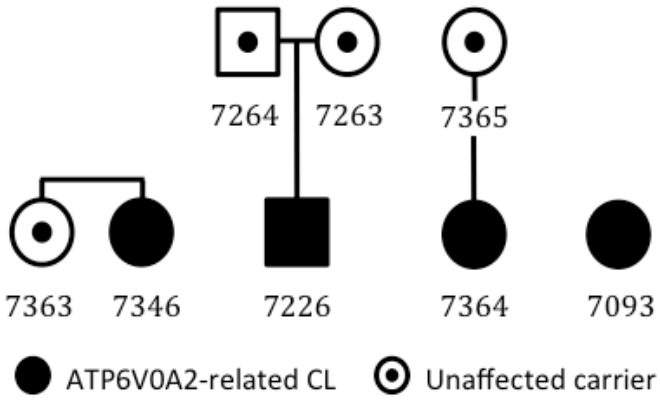

Figure 3. Familial relationships between individuals with *ATP6V0A2*-related CL and unaffected relatives

The *ATP6V0A2*-related CL group was composed of two adult females, one seven-year old boy, and one two-year old female (Table 3). All participants had dysmorphic features. Among the participants with *ATP6V0A2* mutations, at least ¾ exhibited a history of congenital

skin laxity, patent anterior fontanel, blue sclera, developmental delay, joint laxity, or abnormal pulmonary function testing. At least 2/4 reported a history of prematurity, low birth weight, microcephaly, eye alignment abnormalities, hearing loss, seizures, or hernia (Table 3). These findings are consistent with previous observations with the exception of pulmonary deficits which have not been reported as a characteristic feature of ATP6V0A2-related CL (Fischer et al., 2012).

Table 3. Clinical characteristics of individuals with cutis laxa

ID	7093	7346	7364	7226	7342	7197
***ATP6V0A2* mutation**	+	+	+	+	-	-
***ELN* mutation**	-	-	-	-	+	+
Age at exam	28	32	2	7	67	70
Gender	F	F	F	M	M	F
Prematurity	-	-	+	+	?	?
Low birth weight	-	-	+	+	?	-
Congenital skin findings	+	+	+	+	-	-
Patent anterior fontanel	+	+	-	+	-	-
Dysmorphic features	+	+	+	+	+	+
Microcephaly	-	-	+	+	-	-
Myopia	-	-	-	+	+	+
Eye alignment abnormalities	-	-	+	+	-	-
Blue sclera	+	-	+	+	-	-
Hearing loss	-	+	-	+	+	-
Hoarse voice	+	-	-	-	-	+
Developmental delay	+	+	+	+	-	-
Seizures	+	-	-	+	-	-
Hip dysplasia	-	-	+	-	-	-
Joint laxity	+	+	+	?	-	-
Hernia	-	-	+	+	-	+
Aortic aneurysm	-	-	+	-	+	-
Small left ventricle	-	+	-	-	-	+
Abnormal pulmonary function	+	+	n/a	+	+	+
Asthma/emphysema	+	+	-	+	-	+

The infection questionnaire results (Table 2) revealed severe cold/flu episodes, strep throat, urinary tract infections (UTIs), conjunctivitis, otitis media (OM), pneumonia, bronchitis, and yeast infection in the two adult participants with *ATP6V0A2*-related CL. The two juvenile patients with *ATP6V0A2*-related CL reported severe cold/flu episodes, OM, and pneumonia. The adult unaffected relatives collectively reported varicella, HPV, severe cold/flu episodes, strep throat, UTIs, conjunctivitis, OM, pneumonia, staph infection of the skin, bronchitis, ringworm, and yeast infections. The adults with *ELN*-related CL reported cytomegalovirus, varicella, severe cold/flu episodes, flu vaccine reaction, conjunctivitis, bronchitis, ringworm, yeast infection, and toenail/fingernail infections.

The adult participants with *ATP6V0A2*-related CL had the highest unweighted total infection scores (average number of infection types = 16) (Figure 1, Table 2). They also reported the largest variety of infection types (average number of infection types = 11.5) (Table 2). The child participants with *ATP6V0A2*-related CL reported the smallest variety of infection types (average number of infection types = 3, Table 2), and their unweighted total infection scores were among the lowest (average total infection score = 4, Table 2). But when total infection scores were weighted by age, the child participants with *ATP6V0A2*-related CL had higher scores than the unaffected heterozygous relatives and the participants with *ELN*-related CL (Figure 4).

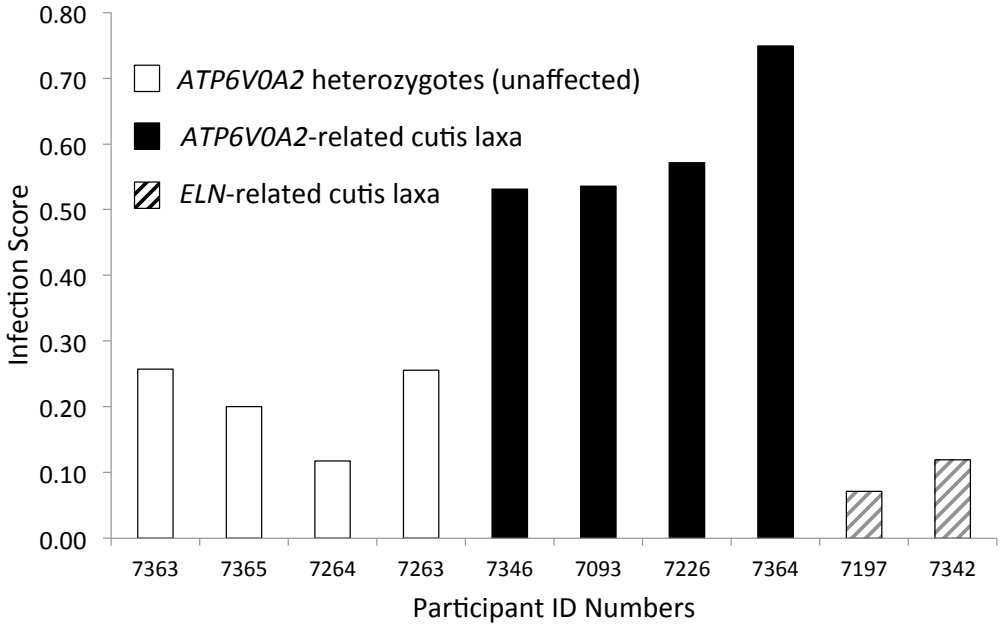

Figure 4. Age-weighted total infection score for each participant

Total age-weighted infection scores (Figure 4) were the highest in *ATP6V0A2*-related CL participants, and ranged from 0.53 to 0.75. The heterozygous relatives' scores ranged from 0.12 to 0.26, and the *ELN*-related cutis laxa scores ranged from 0.07 to 0.12.

The mean age-weighted infection score (Figure 5) for participants with *ATP6V0A2*-related cutis laxa (ATP6 aff) was significantly higher than the mean score for unaffected relatives with heterozygous *ATP6V0A2* mutations (ATP6 het) (Pr(ATP6 aff > ATP6 het) = 0.0107) (Fig 5). In addition, the age-weighted mean infection score of participants with *ATP6V0A2*-related cutis laxa was significantly higher than that of participants with *ELN*-related cutis laxa (*ELN* aff) (Pr(ATP6 aff > *ELN* aff) = 0.0108) (Figure 5, Table 4).

We also examined data calculated using an age-adjustment by decades of life. We hoped this approach would mitigate any over-weighting of the young participants' infection scores that occurred as a result of age-weighting by year. The age adjustment by decades of life comparisons, while still showing significant differences between groups, did not have as much statistical power as the age adjustment by years of life (Table 5).

We also analyzed the data without including the child participants, thus eliminating the need for the age-weighting procedures. Despite the smaller sample size, we still found that individuals with *ATP6V0A2*-related CL had higher total infection scores than unaffected heterozygous relatives (p=0.0072) or individuals with *ELN*-related CL (p=0.0171) (Figure 6). Calculated power for both comparisons exceeded 0.80 (Table 5).

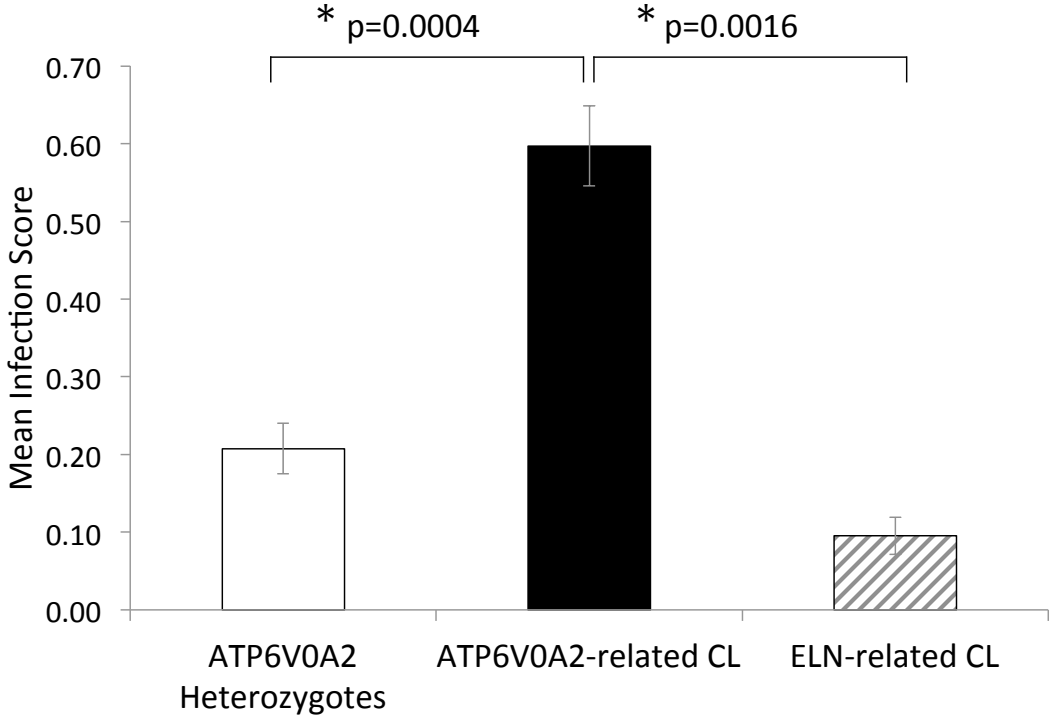

Figure 5. Between-group comparison of age-weighted mean infection scores

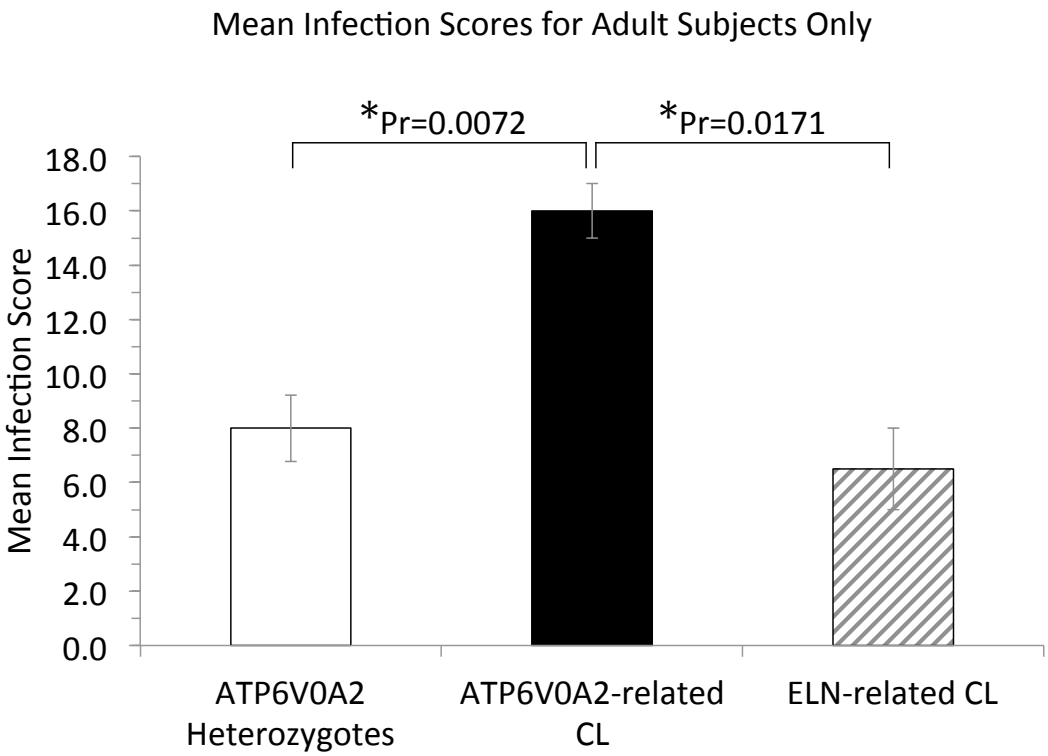

Figure 6. Between group comparisons of mean infection scores (adults only)

Table 4. Statistical test results (all subjects)

	ATP vs heterozygotes			ATP vs ELN		
	Infection score	**Decades adjusted**	**Years adjusted**	**Infection score**	**Decades A0.djusted**	**Years adjusted**
Equality of variance test p value	.0954	.1714	.4784	0.4037	0.2765	.4727
T test p value	.3599	.0247	.0004	0.3153	0.0275	.0016
power	Not calculated	.6	.99	0.1504	0.9174	1

Table 5. Statistical test results (adults only)

	ATP vs heterozygotes	ATP vs ELN
	Infection score	Infection score
Equality of variance test p value	.7916	0.7487
T test p value	.0072	.0171
power	.9886	.8960

3.4 DISCUSSION

In this study, we characterized infection history among individuals with *ATP6V0A2*-related CL. We also compared infection variety and infection rate to individuals with heterozygous mutations in *ATP6V0A2* and to individuals with another type of cutis laxa, *ELN*-related CL. We hypothesized that the *ATP6V0A2* group would have a higher rate of infections than the comparison groups of unaffected heterozygous relatives and individuals with *ELN*-related CL.

Our study group included four individuals with *ATP6V0A2* mutations, all of whom had experienced multiple categories of infections. Adult participants with *ATP6V0A2*-related CL reported a wider variety of infection types and more frequent infection rates than their heterozygous unaffected relatives. Between-group comparisons of the mean age-adjusted infection scores demonstrated that individuals with *ATP6V0A2*-related CL experienced more infections per year of life than individuals with a single *ATP6V0A2* mutation or individuals with

ELN-related CL. This suggests a previously unrecognized deficit in immune function for individuals with *ATP6V0A2*-related CL.

ATP6V0A2-related CL is a multi-systemic disorder, with features that frequently affect the skin, the musculoskeletal, pulmonary, digestive, and genitourinary systems (Fischer et al., 2012). Some features in all of these systems could be explained sufficiently by structural abnormalities in connective tissue caused by abnormalities in the Golgi and abnormalities in trafficking elastic fiber proteins (Hucthagowder et al., 2009). However, some features of *ATP6V0A2*-related CL, such as neurological abnormalities, are likely to have a complex pathogenesis incorporating abnormalities in extracellular matrix, glycosylation, and cellular trafficking processes (Van Maldergem et al., 2008) (Guillard et al., 2009). The immune deficiencies suggested by our data are also likely to have a complex pathogenesis.

Both glycosylation and cellular trafficking processes have the potential to impact multiple body systems, and could be involved in features of *ATP6V0A2*-related CL. Glycosylation can have effects on development of brain, bones, and organ systems through its regulation of signaling pathways (Harper, Yuan, Tan, Visan, & Guidos, 2003; Ishikawa et al., 2005). There are also many potential roles for glycosylation in immune function. Many receptors in the immune system recognize glycosylated molecules. These include C-type lectins, which function to recognize and internalize pathogen-associated molecular patterns (PAMPS), siglecs, which recognize sialic acid-containing glycans and which participate in immunological signaling, and galectins, which regulate signaling through cell surface interactions (Monticelli et al., 2016).

Symptoms of *ATP6V0A2*-related CL could also be caused by abnormalities in vacuolar function and cellular trafficking processes. ATPases regulate pH of notochord vacuoles, which

participate in body axis elongation in early stages of vertebrate embryonic development (Ellis, Bagwell, & Bagnat, 2013). Trafficking is also an essential component of normal neuronal growth, differentiation and development (Chapleau, Larimore, Theibert, & Pozzo-Miller, 2009).

To characterize the nature of the immune deficiency in our patient population, our collaborators performed immunological analyses on peripheral blood mononuclear cells collected from the individuals in our study group. Their experiments showed that natural killer (NK) cells were negatively impacted by mutations in *ATP6V0A2*. Natural killer cells are part of the innate immune system, and are best known for their role in eliminating viral pathogens and tumor cells (Orange, 2013). The Orange group found that peripheral blood samples of individuals with *ATP6V0A2*-related CL contained a smaller number of NK cells. The NK cells that were present expressed markers that were associated with inhibition and immaturity, and they expressed lower levels of markers associated with maturity and effector functions. The cytotoxicity of the NK cells was also diminished. Together, these data suggest that NK cell development and maturation may be impacted by the loss of ATP6V0A2 (Watkin et al., 2017).

Reduced NK cell cytotoxicity has been reported in multiple conditions including myelodysplastic syndromes (Hejazi et al., 2015), the immune disorder Hermansky-Pudlak syndrome subset 2 (Fontana et al., 2006), familial haemophagocytic lymphohisiocytosis subset 3 (Marcenaro et al., 2006), cancer (Hodge et al., 2014), and endometriosis, which is also hypothesized to have an immune component (Oosterlynck, Cornillie, Waer, Vandeputte, & Koninckx, 1991). NK cell reduction has been reported in patients with rheumatoid arthritis, scleroderma, insulin-dependent diabetes, and psoriasis (Koreck et al., 2002; Sumida et al., 1995; Wilson et al., 1998; Yanagihara, Shiozawa, Takai, Kyogoku, & Shiozawa, 1999). A recent study described a mutation in *MCM4* associated with systemic manifestations such as adrenal

insufficiency, growth deficiency, and NK cell deficiency. The mutation caused a failure in the promotion of DNA replication and lack of maturation in NK cells (Gineau et al., 2012). An interaction between ATP6V0A2 (or its downstream functions) and the *MCM4* system, or a similar system yet to be identified, could affect the NK phenotype (Orange, 2012). Future work may elucidate the role of ATP6V0A2 in NK cell development and maturation as well as the mechanisms leading to dysfunction in these processes.

This report has several limitations that should be addressed in future studies. First, it will be necessary to confirm and expand our analysis with a larger sample size. Although the statistical power of our analyses were sufficient to give us confidence in the results, our results must be interpreted with caution due to the small numbers of subjects. A larger sample may also allow us to assess susceptibility in CL to less prevalent and later onset consequences of NK cell deficiency, such as cancer.

Second, it would be desirable to have age-matched controls for our child participants with *ATP6V0A2*-related cutis laxa. We found that our youngest participants had few types of infections, resulting in lower unweighted infection scores. When data was adjusted for age, child participants with *ATP6V0A2*-related CL had higher infection scores than unaffected relatives. This approach has the disadvantage of potentially overweighting the immune scores for younger participants. However, the unweighted scores of the adult *ATP6V0A2*-related CL participants were significantly higher than the scores of the adult *ELN*-related CL participants, who were also older. This indicates that the relationship between mutation status and infection score was unchanged by weighting.

Third, it would be preferable to obtain additional data from the *ELN*-related CL control comparison group. One reason for this is that neither member of the *ELN* group had the

"classical" form of the CL-causing *ELN* mutation. This classical mutation is a frameshift at the end of the gene, which results in translation of an elongated elastin precursor that cannot form normal elastin polymers (Callewaert et al., 2011). The participants in this category were older than any of the other participants, which is another potential age-related confounding factor. However, it is relevant to note that the older age of the *ELN*-related CL group does not appear to have translated into higher infection scores for these individuals, as might be expected if our infection scores were simply a result of having more years in which to be exposed to pathogens.

Other future research directions would be to examine genotype-phenotype relationships between particular mutations and immune function. To date, weak genotype-phenotype relationships have been identified *ATP6V0A2*-related CL with missense mutations resulting in slightly less severe disease (Hucthagowder et al., 2009). Although our study sample was small, it is of note that the adult heterozygous participant with the highest unweighted infection score was the mother of the child with the higher unweighted infection score (Table 2, Figures 1, 3). It is possible that particular mutations have a higher impact on immune function, and that among unaffected heterozygous relatives, a single copy of a particular mutation may be more detrimental to immune function. Studies focused on the cellular consequences of particular mutations may shed light on this question.

Van Damme and colleagues recently described seven individuals with cutis laxa caused by mutations in *ATP6V1E1* or *ATP6V1A*, subunits of the ATP hydrolytic domain of the vacuolar ATPase. The phenotype in these individuals overlapped with that previously described for *ATP6V0A2* mutations. Homology models predicted that the regions of the protein disrupted by the mutation were essential for the formation of stable interactions between subunits of the ATPase (Van Damme et al., 2017). Interestingly, other proteins that are involved in assembly of

subunits, such as ATP6AP1, have been implicated in immune deficiency (Jansen et al., 2016). The use of homology models may be similarly useful in elucidating the pathophysiology of *ATP6V0A2*-related CL.

We would also like to expand our analysis to individuals with other forms of CL, to see if there are any other forms that have an unrecognized susceptibility to infections. It is also possible that some of the genetically undiagnosed cases of CL have an immune component. If true, infection history review could provide clues to diagnosis. Infection history assessment also has the potential to provide guidance when planning genetic testing for individuals in whom an autosomal recessive form of CL is suspected.

Understanding the increased susceptibility to infection in *ATP6V0A2*-related CL has the potential to change treatment for individuals with this rare condition. It may be important for these individuals to receive regular immunizations against pathogens with available vaccines that are optional in the general population, such as the flu, *Pneumococcus*, and HPV vaccines. Prophylactic antibiotic therapy under certain conditions, and/or aggressive treatment of infections antibiotics, antiviral and antifungal agents may be advised. Individuals may also choose to seek medical care for conditions that might not prompt a physician visit in a person without *ATP6V0A2*-related CL.

3.5 CONCLUSIONS

In our study sample, individuals with *ATP6V0A2*-related CL experienced a larger variety of infections and higher rates of these infections when compared to their heterozygous unaffected relatives and individuals with *ELN*-related CL.

Although interpretation is limited by our small sample size, this finding suggests an increased susceptibility to infections, a feature that has not previously been reported in *ATP6V0A2*-related CL. This finding may ultimately improve our understanding of the *ATP6V0A2*-related CL phenotype and provide important information about the natural history of CL. Our results also have potential impact for medical management of this rare condition, including increased surveillance and medical intervention as appropriate.

4.0 RESEARCH SIGNIFICANCE TO GENETIC COUNSELING AND PUBLIC HEALTH

Rare diseases are an important area of focus for public health. Individually, they are rare, but taken together, they affect between twenty-five and thirty million Americans (National Institutes of Health, n.d.). The principle of justice requires that even though a disease is rare, affected individuals are equally entitled to treatment as individuals with more common conditions. The principle of beneficence also requires that we do not abandon individuals who are suffering (Gericke, Riesberg, & Busse, 2005). These principles make it an ethical imperative to develop treatments for rare conditions. Understanding the features and natural history of a disease is necessary for the core public health function of assessment. This information also provides a foundation for research on treatments (Field & Boat, 2010).

Rare diseases often have relevance to more prevalent disorders (Gericke et al., 2005). CL, in particular, has features in common with pulmonary diseases such as asthma and COPD. Cutis laxa also has relevance for congenital heart disease and adult onset cardiovascular disease. *ATP6V0A2*-related cutis laxa, as a congenital disorder of glycosylation, is part of a larger family of disorders. There are over 100 known CDGs and more are being identified all the time (Lyons et al., 2015). Therefore, new knowledge about cutis laxa and the mechanisms that cause its features has the potential to impact a large population.

CL is a progeroid disorder, and as such has relevance to aging. While CL has not yet been studied in this context, evidence from other progeroid conditions suggests that there is some similarity at a cellular level between progeria and normal aging (Dreesen & Stewart, 2011). Needless to say, aging affects one hundred percent of the population. As the average age of US population continues to rise, this issue will continue to increase in relevance (He, Goodkind, & Kowal, 2016). The aged immune system is less robust than the immune system of an adult (Maue et al., 2009), and identification of the mechanism causing immune deficiency in *ATP6V0A2*-related CL may lead to greater understanding of immune function in old age.

CL is a multisystem disease, and our findings here suggest that the immune system may also be impacted in at least one form of CL. This serves as an example of the need for a multidisciplinary approach in the management of rare diseases. The approach used to manage care for CL applies to other connective tissue conditions, such as Marfan syndrome (von Kodolitsch et al., 2016) and Loeys-Dietz syndrome (MacCarrick et al., 2014). In addition, our immune questionnaire provides an example of a non-invasive clinical instrument that has the potential to guide genetic testing. It could be used for patients with a complex presentation and a differential diagnosis that includes a CDG or a CL subtype. Standardized assessment and management approaches support the public health function of policy development, and evaluation tools such as the infection history questionnaire support the public health function of assessment.

Our CL clinic also contributes to the public health functions of assessment and policy development by generating data about genotype-phenotype relationships that can lead to improvements in diagnosis and management guidelines. Characterization of the features of CL allows for both improved assessment and improved management. This study demonstrated the

potential of the newly developed immune history questionnaire to characterize immune involvement in CL and to identify individuals who may benefit from increased interventions to prevent or limit infections. Another recent study that emerged from the CL clinic demonstrated that a non-invasive skin elasticity measurement could differentiate individuals with CL from individuals without CL (Kozel et al., 2014). Since the diagnosis of CL is subjective unless a genetic cause can be identified, this technique has the potential to dramatically improve our ability to diagnose CL.

CL and other rare conditions have special relevance in the practice of genetic counseling. Among families and individuals affected by rare diseases, the search for a diagnosis is commonly referred to as "the diagnostic odyssey". This term conveys the fatigue and frustration expressed by parents and affected individuals during a time of uncertainty (Carmichael, Tsipis, Windmueller, Mandel, & Estrella, 2015). Rare diseases are frequently misdiagnosed, prolonging the odyssey (Anderson, Elliott, & Zurynski, 2013; Carmichael et al., 2015). In addition to the stress of uncertainty, families affected by rare diseases often have financial concerns, concerns about competence, and social isolation (Pelentsov, Fielder, Laws, & Esterman, 2016).

A diagnosis is beneficial because it can provide a prognosis, promote the formation of coping strategies, and facilitate connections to medical and social support. Lenhard and colleagues demonstrated that parents of children with a diagnosis of Down syndrome scored higher on measures of psycho-emotional status than parents of children with a nonspecific diagnosis of intellectual disability (Lenhard, Breitenbach, Ebert, Schindelhauer-Deutscher, & Henn, 2005).

In the case of rare disease, the diagnosis does not always provide these sources of relief for individuals and their families. There are few experienced care providers for rare conditions,

and social support is less likely to be available locally. In these cases families would benefit from additional resources and psychosocial support (Anderson et al., 2013; Dogba et al., 2016; Doyle, 2015; Rosell et al., 2016; Takeuchi, Muraoka, Yamada, Nishio, & Hozumi, 2016). A genetic counselor is uniquely positioned to bridge this gap by researching information about the condition, information about clinical research and online support groups, as well as immediate psychosocial supports for individuals and families (Helm, 2015).

The CL research clinic is the only one of its type in the world. At present, it is the only US event at which individuals and families affected by CL can meet one another. In addition, the clinicians and technicians who participate in the CL research clinic have more experience with CL than any other group in the world. The CL clinic is more than a data collection event. It is also an opportunity for a population affected by rare disease to come together to enhance their own health and psychosocial well-being while adding to scientific knowledge about the condition.

It is important to have a complete picture of the range of possible manifestations in CL, so that parents and patients are empowered with as much information as possible (Aymé, Kole, & Groft, 2008). This study provides information about previously unknown immunological features in *ATP6V0A2*-related CL, and thus has the potential to improve the experiences of families and individuals coping with a diagnosis, and to improve medical management for individuals affected by this condition.

APPENDIX A: IRB APPROVAL LETTER

University of Pittsburgh
Institutional Review Board

3500 Fifth Avenue
Pittsburgh, PA 15213
(412) 383-1480
(412) 383-1508 (fax)
http://www.irb.pitt.edu

Memorandum

To: Zsolt Urban, PHD
From: Margaret Hsieh, MD, Vice Chair
Date: 1/26/2017
IRB#: REN17010069 / PRO10020125
Subject: Genetics of Extracellular Matrix in Health and Disease

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The Renewal for the above referenced research study was reviewed and approved by the Institutional Review Board, Committee G, which met on 1/18/2017.

Please note the following information:

The risk level designation is Greater Than Minimal.

Approval Date: 1/18/2017
Expiration Date: 1/17/2018

Please note that it is the investigator's responsibility to report to the IRB any unanticipated problems involving risks to subjects or others [see 45 CFR 46.103(b)(5) and 21 CFR 56.108(b)]. Refer to the IRB Policy and Procedure Manual regarding the reporting requirements for unanticipated problems which include, but are not limited to, adverse events. If you have any questions about this process, please contact the Adverse Events Coordinator at 412-383-1480.

The protocol and consent forms, along with a brief progress report must be resubmitted at least **one month** prior to the renewal date noted above as required by FWA00006790 (University of Pittsburgh), FWA00006735 (University of Pittsburgh Medical Center), FWA00000600 (Children's Hospital of Pittsburgh), FWA00003567 (Magee-Womens Health Corporation), FWA00003338 (University of Pittsburgh Medical Center Cancer Institute).

Please be advised that your research study may be audited periodically by the University of Pittsburgh Research Conduct and Compliance Office.

APPENDIX B: INFECTION HISTORY QUESTIONNAIRE

Infection History Questionnaire

We are asking all study participants to complete this questionnaire, which will help us to investigate a possible link between cutis laxa and immune disorders. **Your responses are CONFIDENTIAL.** Clinic staff may contact you to obtain additional information about any suspected or diagnosed infections you have had. If you have any questions about the questionnaire items, please contact Michelle Morrow by email (cutislax@pitt.edu) or by phone (412) 383-7369.

1) Have you had (diagnosed or suspected) **oral or genital herpes**? Symptoms of oral herpes include blisters or sores around the mouth, symptoms of genital herpes include blisters or sores on or around the genitals or rectum.

   _____Not sure (go to question #2)
   _____No (go to question # 2)
   _____Yes (if yes, clinic staff will contact you with additional questions)

   FOR CLINIC STAFF: please answer questions below

   A) Has the infection been proven?
   _____No
   _____Yes

   B) Was the infection proven by culture, PCR or fluorescent assay?
   _____No
   _____Yes
   If yes, which? _____

   C) Has the infection resulted in hospitalization?
   _____No
   _____Yes
   If yes, how many times?_____

   D) Was the infection associated with any complications?
   _____No
   _____Yes
   If yes, what?_____

   E) Did the infection require treatment?
   _____No
   _____Yes
   If yes, what treatment?_____

2) Have you had (diagnosed or suspected) **cytomegalovirus (CMV), Epstein Barr virus (EBV), or mononucleosis**? These viruses may cause no symptoms, but they can also produce symptoms similar to mononucleosis, including fatigue, malaise, sore throat, fever, swollen glands, swollen tonsils, headache, skin rash, or swollen spleen.

   _____Not sure (go to question #3)
   _____No (go to question #3)
   _____ Yes (if yes, clinic staff will contact you with additional questions)

FOR CLINIC STAFF: (please answer questions below)

A) Has the infection been proven?

_____No

_____Yes

B) Was the infection proven by culture, PCR or fluorescent assay?

_____No

_____Yes

If yes, which? _____

C) Has the infection resulted in hospitalization?

_____No

_____Yes

If yes, how many times?_____

D) Was the infection associated with any complications?

_____No

_____Yes

If yes, what?_____

E) Did the infection require treatment?

_____No

_____Yes

If yes, what treatment?_____

3) Have you had (diagnosed or suspected) **chicken pox (varicella virus, varicella zoster virus)** or **complications from the chicken pox vaccine**? Symptoms of chicken pox include an itchy rash with raised bumps and blisters, as well as fever, headache, and malaise.

_____Not sure (go to question #4)

_____No (go to question #4)

_____ Yes (if yes, clinic staff will contact you with additional questions)

FOR CLINIC STAFF: please answer questions below

A) Has the infection been proven?

_____No

_____Yes

B) Was the infection proven by culture, PCR or fluorescent assay?

_____No

_____Yes

If yes, which? _____

C) Has the infection resulted in hospitalization?

_____No

_____Yes

If yes, how many times?_____

D) Was the infection associated with any complications?

_____No

_____Yes

If yes, what?_____

E) Did the infection require treatment?

_____No

_____*Yes*

*If yes, what treatment?*_____

F) If you experienced complications from the chicken pox vaccine, please describe below.

4) Have you had a (diagnosed or suspected) infection with **human papilloma virus (HPV)**? HPV causes **warts** on the hands, feet, face, neck, or genitals.

_____Not sure (go to question #5)
_____No (go to question #5)
_____ Yes (if yes, clinic staff will contact you with additional questions)

FOR CLINIC STAFF: please answer questions below

A) Has the infection been proven?

_____*No*
_____*Yes*

B) Was the infection proven by culture, PCR or fluorescent assay?

_____*No*
_____*Yes*

If yes, which? _____

C) Has the infection resulted in hospitalization?

_____*No*
_____*Yes*

*If yes, how many times?*_____

D) Was the infection associated with any complications?

_____*No*
_____*Yes*

*If yes, what?*_____

E) Did the infection require treatment?

_____*No*
_____*Yes*

*If yes, what treatment?*_____

5) Have you had a (diagnosed or suspected) infection with **roseola**, **human herpes virus (HHV)** or other **DNA virus** not referred to previously? Symptoms of roseola can include a sudden high fever followed by a rash that starts on the trunk and spreads to the face and limbs.

_____Not sure (go to next question)
_____No (go to next question)
_____ Yes (if yes, clinic staff will contact you with additional questions)

FOR CLINIC STAFF: please answer questions below

A) Has the infection been proven?

_____*No*
_____*Yes*

B) Was the infection proven by culture, PCR or fluorescent assay?

_____*No*
_____*Yes*

If yes, which? _____

C) Has the infection resulted in hospitalization?

_____No

_____Yes

If yes, how many times?_____

D) Was the infection associated with any complications?

_____No

_____Yes

If yes, what?_____

E) Did the infection require treatment?

_____No

_____Yes

If yes, what treatment?_____

6) Have you had multiple cases or severe cases of **influenza, the common cold, stomach flu**, or **other RNA viral infections**?

_____Not sure (go to question #7)
_____No (go to question #7)
_____ Yes (if yes, clinic staff will contact you with additional questions)

FOR CLINIC STAFF: please answer questions below

A) How many times have these illnesses occurred?_____

B) How many times have these illnesses occurred in the past year?_____

C) Has the infection been proven?

_____No

_____Yes

F) Was the infection proven by culture, PCR or fluorescent assay?

_____No

_____Yes

If yes, which? _____

G) Has the infection resulted in hospitalization?

_____No

_____Yes

If yes, how many times?_____

H) Was the infection associated with any complications?

_____No

_____Yes

If yes, what?_____

I) Did the infection require specific treatment?

_____No

_____Yes

If yes, what treatment?_____

7) Have you had **adverse reactions to a flu vaccine**?

_____Not sure (go to question #8)
_____No (go to question #8)

_____Yes (please answer the following question)
A) was the vaccine an injection or a nasal spray?_____

8) Have you had a documented or suspected infection with **mycobacteria, listeria**, **or atypical intracellular bacteria?**

_____Not sure (go to question #9)
_____No (go to question #9)
_____Yes (if yes, clinic staff will contact you with additional questions)

FOR CLINIC STAFF: please answer questions below

A) Has the infection been proven?
_____No
_____Yes

B) Was the infection proven by culture, PCR or fluorescent assay?
_____No
_____Yes
If yes, which? _____

C) Has the infection resulted in hospitalization?
_____No
_____Yes
If yes, how many times?_____

D) Was the infection associated with any complications?
_____No
_____Yes
If yes, what?_____

E) Did the infection require treatment?
_____No
_____Yes
If yes, what treatment?_____

9) Have you had any of the following **bacterial infections**? If yes, how many times?

A) Strep throat
_____No
_____Yes
If yes, how many times?_____

B) Urinary tract infections (UTI
_____No
_____Yes
If yes, how many times?_____

C) Conjunctivitis (pink eye)
_____No
_____Yes
If yes, how many times?_____

D) Otitis media (ear infection)
_____No
_____Yes
If yes, how many times?_____

E) Pneumonia
_____No
_____Yes
If yes, how many times?_____

F) Staph infection of the skin
_____No
_____Yes
If yes, how many times?_____

G) Bronchitis or chronic cough
_____No
_____Yes
If yes, how many times?_____

H) Bacterial vaginal infections
_____No
_____Yes
If yes, how many times?_____

10) Have you had any of the following **fungal infections**? If yes, how many times?

A) Ringworm, athlete's foot, or jock itch
_____No
_____Yes
If yes, how many times?_____

B) Yeast infections (oral thrush, vaginal yeast infection, yeast infection of the skin)
_____No
_____Yes
If yes, how many times?_____

C) Toenail or fingernail infections
_____No
_____Yes
If yes, how many times?_____

D) Other (list)_____
_____No
_____Yes
If yes, how many times?_____

APPENDIX C: CUTIS LAXA CLINICAL QUESTIONNAIRE

For Study Use Only:
Family ID: _____
Participant ID: _____

CUTIS LAXA

Patient/Physician questionnaire

Date of Exam: _____

Physician Name: _____ Physician Specialty: _____

(Please complete contact info on last page of questionnaire for both the participant and the physician.)

RACE:

American Indian or Alaskan [ ]

Asian [ ]

Black or African American [ ]

Native Hawaiian or Other Pacific Islander: [ ]

Other race (please specify): _____

Two or More Races (please specify): _____

White: [ ]

ETHNICITY:

Hispanic or Latino [ ]

Other ethnicity (please specify): _____

Definitions:
American Indian or Alaska Native: A person having origins in any of the original peoples of North, Central, or South America, and who maintains tribal affiliation or community attachment.
Asian: A person having origins in any of the original peoples of the Far East, Southeast Asia, or the Indian subcontinent including, for example, Cambodia, China, India, Japan, Korea, Malaysia, Pakistan, the Philippine Islands, Thailand, and Vietnam.
Black or African American: A person having origins in any of the black racial groups of Africa. Terms such as "Haitian" or "Negro" can be used in addition to "Black or African American."
Native Hawaiian or Other Pacific Islander: A person having origins in any of the original peoples of Hawaii, Guam, Samoa, or other Pacific Islands.
White: A person having origins in any of the original peoples of Europe, the Middle East, or North Africa.
Hispanic or Latino: A person of Cuban, Mexican, Puerto Rican, South or Central American, or other Spanish culture or origin, regardless of race. The term, "Spanish origin," can be used in addition to "Hispanic or Latino".

PAST MEDICAL HISTORY

Birth Data:

Date of birth (mo/day/year): ___/___/_____ Male: [ ] Female: [ ]

Gestational age at birth: _____ weeks

Birth weight: _____ Length: _____ Head circumference: _____

Amniotic fluid: Normal: [ ] Oligohydramnios: [ ] Polyhydramnios: [ ]

Fetal movements: Normal: [ ] Reduced: [ ] Increased: [ ]

Fetal ultrasound: Normal: [ ] Abnormal: [ ] (see table) Not Performed: [ ]

Ultrasound Abnormalities:	**Gestational Age:**

Death Data:

If deceased, date of death (mo/day/year): ____/____/____

Cause of death: _____

_____

_____

_____

_____

Previous Surgical History:

History of abdominal vascular surgery: Yes [ ] No [ ] ? [ ]

History of any facial surgeries: Yes [ ] No [ ] ? [ ]

If yes, type(s) of surgeries:

Cosmetic surgeries:

Face lift: Yes [ ] No [ ] Don't Know [ ] Date(s):_____

Other: _____ Date(s):_____

_____ Date(s):_____

Previous Hospitalizations:

History of any trauma that affected the facial structure: Yes [ ] No [ ] Don't Know [ ]

If yes, document here:

Medications:

Allergies: Yes [ ] No [ ] Don't Know [ ]

Allergens:

Skin affected by allergies: Yes [ ] No [ ] Don't Know [ ]

3 GENERATION PEDIGREE

Patient:_____
Historian:_____
Completed By:_____ Date:_____
Updated By:_____ Date:_____

Paternal Ethnicity:_____
Maternal Ethnicity:_____
Consanguinity:_____

Family Review of Systems (Please note findings on pedigree):

LD/DD/MR	Yes	No	Gastrointestinal Problems	Yes	No	Diverticulae	Yes	No
Respiratory Problems	Yes	No	Genitourinary Problems	Yes	No	Hernias	Yes	No
Cardiovascular Problems	Yes	No	Skeletal Problems	Yes	No	Growth Delay	Yes	No
Skin: At least 2-3 of the following (i) Loose, lax; (ii) Redundant; (iii) Inelastic; (iv) Premature aging of the skin; (v) Skin wrinkling							Yes	No

CLINICAL DESCRIPTION

Age at examination: _____

Age when symptoms began: _____

SKIN:

Lax:	Yes [ ]	No [ ]	? [ ]	
Redundant:	Yes [ ]	No [ ]	? [ ]	
Inelastic:	Yes [ ]	No [ ]	? [ ]	
Loose facial skin:	Yes [ ]	No [ ]	? [ ]	
Premature aging of skin:	Yes [ ]	No [ ]	? [ ]	
Skin wrinkling:	Yes [ ]	No [ ]	? [ ]	
Affected skin:	Generalized [ ]		Localized [ ]	
If localized:	Symmetric [ ]		Asymmetric [ ]	Flexural [ ] Focal [ ]
Transparent skin:	Yes [ ]	No [ ]	? [ ]	
Inflammatory skin disease:	Yes [ ]	No [ ]	? [ ]	
Alopecia:	Yes [ ]	No [ ]	? [ ]	Location: _____

Other skin findings:

CRANIOFACIAL:

Facial drooping:	Yes [ ]	No [ ]	? [ ]	
Wizened appearance:	Yes [ ]	No [ ]	? [ ]	
Large fontanels:	Yes [ ]	No [ ]	? [ ]	
Delayed closure of fontanels:	Yes [ ]	No [ ]	? [ ]	
Oxycephaly:	Yes [ ]	No [ ]	? [ ]	
Macrocephaly:	Yes [ ]	No [ ]	? [ ]	OFC: _____
Microcephaly:	Yes [ ]	No [ ]	? [ ]	OFC %ile: _____
Frontal bossing:	Yes [ ]	No [ ]	? [ ]	
Reversed-V eyebrows:	Yes [ ]	No [ ]	? [ ]	
Downslanting palpebral fissures:	Yes [ ]	No [ ]	? [ ]	
Hooked nose:	Yes [ ]	No [ ]	? [ ]	
Long philtrum:	Yes [ ]	No [ ]	? [ ]	Length: _____ (cm)
Retrognathia/Micrognathia:	Yes [ ]	No [ ]	? [ ]	(If Yes, circle which type)
Occipital horns:	Yes [ ]	No [ ]	? [ ]	

Other craniofacial findings:

ORAL:

Vocal cord laxity:	Yes [ ]	No [ ]	? [ ]	
Hoarse voice:	Yes [ ]	No [ ]	? [ ]	
Dental caries:	Yes [ ]	No [ ]	? [ ]	Details: _____
Cleft palate	Yes [ ]	No [ ]	? [ ]	Details/type: _____
Bifid uvula	Yes [ ]	No [ ]	? [ ]	

Other oral findings:

EYES/EARS:

Retinovascular tortuosity:	Yes [ ]	No [ ]	? [ ]	
Corneal opacities:	Yes [ ]	No [ ]	? [ ]	
Corneal arcus:	Yes [ ]	No [ ]	? [ ]	
Cataract:	Yes [ ]	No [ ]	? [ ]	
Myopia:	Yes [ ]	No [ ]	? [ ]	
Blindness:	Yes [ ]	No [ ]	? [ ]	
Hearing Loss:	Yes [ ]	No [ ]	? [ ]	dB loss (left):_____
Deafness:	Yes [ ]	No [ ]	? [ ]	dB loss (right):_____

Other eye findings:

NEUROBEHAVIORAL:

Deep tendon reflexes:	Present [ ]	Absent [ ]	Not Checked [ ]	
Hypotonia:	Yes [ ]	No [ ]	? [ ]	
Cong. bilateral athetosis:	Yes [ ]	No [ ]	? [ ]	
Seizures:	Yes [ ]	No [ ]	? [ ]	Frequency: _____
Autism:	Yes [ ]	No [ ]	? [ ]	Details: _____
Mental retardation:	Yes [ ]	No [ ]	? [ ]	IQ Test:_____ Score:_____

Education history:

Social history:

Other neurobehavioral findings:

RESPIRATORY:

Bronchiectasis:	Yes [ ]	No [ ]	? [ ]
Hypoplastic lungs (newborn):	Yes [ ]	No [ ]	? [ ]
Emphysema:	Yes [ ]	No [ ]	? [ ]
Tachypnea:	Yes [ ]	No [ ]	? [ ]

Pneumonia: Yes [ ] No [ ] ? [ ] Frequency: _____

Asthma: Yes [ ] No [ ] ? [ ] Type: _____

Chest CT findings and date: _____

Pulmonary function test (PFT) findings and date: _____

Current smoker: Yes [ ] No [ ]

Cigarette packs per day: _____

Years smoked: _____

Other respiratory findings:

CARDIOVASCULAR:

Cor pulmonale: Yes [ ] No [ ] ? [ ]

Right ventricular hypertrophy: Yes [ ] No [ ] ? [ ]

Infundibular stenosis: Yes [ ] No [ ] ? [ ]

Pulmonary valve stenosis: Yes [ ] No [ ] ? [ ]

Pulmonary artery stenosis: Yes [ ] No [ ] ? [ ]

Peripheral pulmonary stenosis: Yes [ ] No [ ] ? [ ]

Supravalvular aortic stenosis: Yes [ ] No [ ] ? [ ]

Valvular aortic stenosis: Yes [ ] No [ ] ? [ ]

Heart Murmur: Yes [ ] No [ ] ? [ ]

Murmur (6 scale):_____

Aortic dilatation: Yes [ ] No [ ] ? [ ]

Level:_____ Diameter:_____

Arterial tortuosity: Yes [ ] No [ ] ? [ ]

Which arteries:_____

Arterial aneurysms: Yes [ ] No [ ] ? [ ]

Which arteries:_____

Raynaud's phenomenon: Yes [ ] No [ ] ? [ ]

Venous varicosity: Yes [ ] No [ ] ? [ ]

Echocardiogram: Yes [ ] No [ ] ? [ ]

(Echo) findings and date:_____

Edema? Yes [ ] No [ ] ? [ ] Details: _____

Other cardiovascular findings:

GASTROINTESTINAL:

Diverticulae: Esophageal: Yes [ ] No [ ] ? [ ]
Intestinal: Yes [ ] No [ ] ? [ ]
Rectal: Yes [ ] No [ ] ? [ ]
Other:_____

Other gastrointestinal findings:

GENITOURINARY:

Bladder diverticulae: Yes [ ] No [ ] ? [ ]
Obstructive uropathy: Yes [ ] No [ ] ? [ ]
Bladder neck obstruction: Yes [ ] No [ ] ? [ ]
Other genitourinary findings:

MUSCULOSKELETAL:

Hernias: Inguinal: Yes [ ] No [ ] ? [ ]
Diaphragmatic: Yes [ ] No [ ] ? [ ]
Hiatal: Yes [ ] No [ ] ? [ ]
Umbilical: Yes [ ] No [ ] ? [ ]
Genital/Bladder/Rectal Prolapse Yes [ ] No [ ] ? [ ] Details: _____
Hip dislocation: Yes [ ] No [ ] ? [ ]
Thumbs and/or toes dislocated: Yes [ ] No [ ] ? [ ]
Other dislocations/subluxations: Yes [ ] No [ ] ? [ ] Details: _____

_____

Joint pain: Yes [ ] No [ ] ? [ ]
Joint laxity: Yes [ ] No [ ] ? [ ]
Fractures: Yes [ ] No [ ] ? [ ] #/Site(s): _____
Osteopenia/Osteoporosis: Yes [ ] No [ ] ? [ ] Hip? [ ] Spine? [ ] Total? [ ]
Pectus carinatum: Yes [ ] No [ ] ? [ ]
Pectus excavatum: Yes [ ] No [ ] ? [ ]
Scoliosis: Yes [ ] No [ ] ? [ ] Degree: _____
Flat feet: Yes [ ] No [ ] ? [ ]
Short broad clavicles: Yes [ ] No [ ] ? [ ]
Fused carpal bones: Yes [ ] No [ ] ? [ ]
Other musculoskeletal findings:

GROWTH:

Growth deficiency/delay:

Prenatal: Yes [ ] No [ ] ? [ ]

Postnatal Yes [ ] No [ ] ? [ ]

Dwarfism Yes [ ] No [ ] ? [ ]

Physical data:

Age	Weight	Height	Head Circumference

OTHER ABNORMALITIES NOT LISTED ABOVE:

_____

_____

_____

LABORATORY:

Abnormal elastic fibers:

Histology: Yes [ ] No [ ] ? [ ]

EM: Yes [ ] No [ ] ? [ ]

Glycosylation Testing: Yes [ ] No [ ] ? [ ] Result: _____

Genetic testing: Yes [ ] No [ ] ? [ ] Details/Result: _____

_____

Serum ceruloplasmin:_____

Serum copper:_____

ADDITIONAL INFORMATION:

Clinical photographs available: Yes [ ] No [ ]

DNA available: Yes [ ] No [ ]

Cultured cells available: Yes [ ] No [ ]

Pathology slides available: Yes [ ] No [ ]

Frozen tissue available: Yes [ ] No [ ]

Imaging Studies: Yes [ ] No [ ] Details: _____

Patient available for re-examination: Yes [ ] No [ ]

CUTIS LAXA

Contact Information

PARTICIPANT:

Family ID: _____ (to be filled in by study coordinator)

Participant ID: _____ (to be filled in by study coordinator)

Participant's Name:_____

Parent's or Guardian's Name (if participant is minor):_____

Address:_____

_____

_____

Home Phone: _____ Cell Phone: _____

Fax:_____

EMAIL:_____

REFERRING PHYSICIAN:

Physician's Name:_____

Physician Address: _____

_____

_____

Phone: _____

Fax:_____

EMAIL:_____

APPENDIX D: CUTIS LAXA STUDY SCREENING SCRIPT

Participant ID _____

Family ID _____

Phone Screening Script for
Genetics of Extracellular Matrix in Health and Disease

Telephone Screen Results:

___ Agreed and Eligible ___ Refused ___ Not eligible

_____
Printed Name of Participant

_____ _____
Printed Name of Person Screening Date and Time of Screen

Notes: _____
_____
_____
_____

Script ----------

"Hello. My name is _____ and I am researcher with the University of Pittsburgh Department of Human Genetics. May I please speak with _____?"

{If the potential participant is not available}
"OK, thank you. I'll call back at another time. Is there a better day or time to reach them?"

{If yes} Document here: _____
"Great. I'll call back then. Thank you. Goodbye."
{If no} *"OK. Thank you. Goodbye."*

{If the potential participant is available on the phone}
"Hello. Again, my name is _____ and I am a researcher at the University of Pittsburgh. I work on studies of cutis laxa. You expressed interest in participating in our study about looking at the genetic causes of cutis laxa.

May I talk to you today about our study that is looking at cutis laxa causing genes?"

{If no}: *"OK. Thank you very much for your time. Have a nice day."*

{If yes}: ***"OK. In this study, we are looking at several genes that have been shown to cause cutis laxa. We are interested in using clinical and laboratory information to find disease-causing changes in known genes and to identify new cutis laxa genes. We also will study the effects of the gene changes and how they cause cutis laxa.***

Are you interested in hearing more about the study?"

{If no}: *"OK. Thank you very much for your time. Have a nice day."*

{If yes}: *"Great! But before enrolling people in the study, we need to make sure that you are eligible. What I would like to do now is ask you a series of questions about your or your family member's health and medical history, specifically pertaining to the skin. All information that I receive from you today by phone will be strictly confidential and stored under lock and key in our files to be reviewed only by our research staff. The purpose of these questions is to determine if you are eligible for our study, and if you are found not to be eligible the information will be destroyed. Your participation in this interview is voluntary; you do not have to answer these questions, and can choose to stop answering these questions at any time.*

Do I have your permission to ask you these questions now?"

No ___ Yes ___

_____ _____
Printed Name of Person Obtaining Consent Role in Research Study

_____ _____
Signature of Person Obtaining Consent Date and Time of Consent

____ All questions were answered to the participant's satisfaction:

Document here:
_____
_____
_____
_____

____ Participant had no questions.

{If no}: *"OK. Thank you very much for your time. Have a nice day."*

{If yes}: *"Great! If at any time you have questions, please stop me and ask your question.*

1) "*Have you or a family member ever been diagnosed with cutis laxa, has cutis laxa been suspected in you or a family member, or have you or a family member had genetic testing that identified a change in a cutis laxa related gene?"*

___ (A) Yes, cutis laxa has been diagnosed in participant

*"Who diagnosed you with cutis laxa?"*_____

*"How old were you when diagnosed?"*_____

___ (B) Yes, cutis laxa has been suspected in participant

*"Who suspected that you have cutis laxa?"*_____

*"How old were you when cutis laxa was suspected?"*_____

___ (C) Yes, cutis has been diagnosed in a participant's family member

*"How are you related to your family member who has cutis laxa?"*_____

___ (D) Yes, cutis laxa has been suspected in family member

"How are you related to your family member who is suspected to have cutis laxa?"
_____

___(E) Yes, genetic testing has identified a variant (change) in a cutis laxa related gene in participant

*"What gene contained the change?"*_____

*"What type of genetic testing found the change?"*_____

___(F) Yes, genetic testing has identified a variant (change) in a cutis laxa related gene in participant's family member

*"What gene contained the change?"*_____

*"What type of genetic testing found the change?"*_____

*"How are you related to your family member with the cutis laxa related genetic change?"*_____

___ (G) No. Continue with question #2.

2) *"I am now going to ask you a few questions about [your/your family member's] skin:*

[Do you/ does your family] have:

(A) *Loose, lax skin*	___ Yes	___ No
(B) *Skin in redundant folds*	___ Yes	___ No
(C) *Inelastic (doughy) skin*	___ Yes	___ No

(D) *Premature aging of the skin* ___ Yes ___ No

(E) *Excessive premature wrinkling* ___ Yes ___ No

Additional notes about features:

_____

_____

_____

___ Participant or participant's *first degree* family member <u>has 3 or more</u> of the above features. {If yes}: Subject is eligible to participate. Skip question #3 below.

___ Participant or participant's family member <u>does not have 3 or more</u> of the above features. {If no}: Continue with additional screening question #3 below to determine eligibility.

NOTE: A check mark next to A, B, C, D, E, or F in Question 1 counts as one feature.

3) *"I am now going to ask you a few additional questions about [your/your family member's] health:*

[Do you/ does your family] have:

(A) A*rterial or venous tortuosity?* ___ Yes ___ No

(B) A*ortic dilation or aneurysm?* ___ Yes ___ No

(C) *Vascular or valve stenosis?* ___ Yes ___ No

(D) *Emphysema or chronic lung disease?* ___ Yes ___ No

(E) *Hernia or diverticulae?* ___ Yes ___ No

(F) *Spinal disease such as herniated disc or dural ectasia?* ___ Yes ___ No

Additional notes about features:

_____

_____

_____

___ Participant or participant's *first degree* family member has 1 of the above features and possible cutis laxa. If yes, subject is eligible to participate.

___ Participant or participant's family member does not have any of the above features. **If this option is checked, stop screening – subject is ineligible.** Tell the subject. *"Ok. Well I am*

(D) *Premature aging of the skin* ___ Yes ___ No

(E) *Excessive premature wrinkling* ___ Yes ___ No

Additional notes about features:

_____

_____

_____

___ Participant or participant's *first degree* family member <u>has 3 or more</u> of the above features. {If yes}: Subject is eligible to participate. Skip question #3 below.

___ Participant or participant's family member <u>does not have 3 or more</u> of the above features. {If no}: Continue with additional screening question #3 below to determine eligibility.

NOTE: A check mark next to A, B, C, D, E, or F in Question 1 counts as one feature.

3) *"I am now going to ask you a few additional questions about [your/your family member's] health:*

[Do you/ does your family] have:

(A) A*rterial or venous tortuosity?* ___ Yes ___ No

(B) A*ortic dilation or aneurysm?* ___ Yes ___ No

(C) *Vascular or valve stenosis?* ___ Yes ___ No

(D) *Emphysema or chronic lung disease?* ___ Yes ___ No

(E) *Hernia or diverticulae?* ___ Yes ___ No

(F) *Spinal disease such as herniated disc or dural ectasia?* ___ Yes ___ No

Additional notes about features:

_____

_____

_____

___ Participant or participant's *first degree* family member has 1 of the above features and possible cutis laxa. If yes, subject is eligible to participate.

___ Participant or participant's family member does not have any of the above features. **If this option is checked, stop screening – subject is ineligible.** Tell the subject. *"Ok. Well I am*

sorry to tell you, but you are not eligible for our study. We do appreciate your time today on the phone. Thank you. Goodbye."

For subjects who have passed the screening questions: *"Based on the questions I have asked, you <u>are</u> eligible for the study.*

"The next step is to provide you with all the relevant information you will need to make an informed decision about participating in the study. Do you have the packet you received [in the mail / from your physician] called 'Consent to Act as a Participant in a Research Study'?"

{If no}: "OK. What we can do is send you another packet, and you can contact us once you have received the packet."

{If yes}: "This will take about 15 minutes to go through, do you have enough time right now?"

{If yes}: Move onto "Informed Consent Script for Genetics of Extracellular Matrix in Health and Disease"

{If no}: "When will be a better time to contact you to go through this packet?"

Contact date and time: _____

"*Before going, I would like to ensure that we have your correct contact information:*

Name: _____

Contact Address: _____

_____

_____

_____

Contact Telephone: _____

Best time to call: _____

Contact Email: _____

Thank you very much for your time. Have a nice day."

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