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New Insights into Chronic Itch: A New Mouse Model Investigation of the Consequences of *COL6A5*-p.Glu2272* Gene Variant

Discipline: Neurosciences

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Résumé en Anglais

Itching (pruritus) is an unpleasant sensation that triggers the need to scratch. Chronic itch is defined as lasting more than six weeks. Chronic pruritus is classified into four main categories according to clinical etiology: (1) dermatological or pruritoceptive, originating in the skin with often skin inflammation, (2) systemic, caused by metabolic, hematological, or immune diseases, infections, cancers, or induced by drugs, (3) neurological or neuropathic depending on the absence or presence of lesions in the nervous system, (4) psychogenic or psychiatric. In some cases, the cause of pruritus is unknown and will be classified as "other" or "of unknown origin" (Misery et al., 2014; Nowak & Yeung, 2017). Neuropathic pruritus is caused by nervous system damage due to nerve compression, neurodegeneration, and other nervous system pathologies, infections or tumors. Neuropathic pruritus is often associated with other neurological symptoms, such as pain or sensory disorders. One cause of neuropathic pruritus is small fiber neuropathy or SFN (Misery et al., 2014).

In 2017, Martinelli-Boneschi and colleagues identified a mutation in the collagen VI α5 (*COL6A5*) gene variant, p.Glu2272**,* in families associated with chronic neuropathic itch through exome sequencing. These patients who carry *COL6A5*-p.Glu2272*** mutation have similar features to neuropathic itch with unknown origin. The *COL6A5* gene in these patients who carry *COL6A5*-p.Glu2272*** mutation contains a stop codon instead of the glutamic acid codon at position 2272 of the protein, which contains 2615 residues in total (Martinelli-Boneschi et al., 2017). The *COL6A5*-p.Glu2272*** variant was found in five patients affected by a chronic itch in two families and a sporadic patient with Typ1 painless diabetic neuropathy. In vitro analysis of complementary DNA from fibroblasts from some patients showed functional haploinsufficiency. Expression of COL6A5 was reduced in the skin of tested patients with the *COL6A5*-p.Glu2272*** variant. In family 1, 2 of 3 patients had small fiber neuropathy characterized by a reduction in intraepidermal nerve fiber density (IENF). In family 2, both patients had small fiber neuropathy. The sporadic patient with diabetic neuropathy had normal IENF density. The *COL6A5*-p.Glu2272*** mutation was identified as the common mutation in 6 out of 8 patients from three families.

To understand the role of this mutation and its involvement in chronic pruritus, the corresponding mouse model was generated, a line of mice (named as *Col6a5*^{E2302*}mutant mice) carrying this mutation in the collagen VI α5 gene . This *Col6a5*^{E2302*}mutant mouse model was generated by using the CRISPR-CAS9 system. The mutation was successfully transmitted to the offspring of the first mutant mice obtained, established the mutant line. We determined the expression of the p.Glu2272* mutant gene in the dorsal root ganglia (DRG) of mutant and wild-type (wt) control mice. These DRGs contain the cell bodies of sensory neurons. The mutant gene was expressed at a comparable level to the wt gene in DRGs. Nevertheless, mutant gene expression was found significantly decreased compared to the wt gene in skin. Scratching behavior of mutant mice in a given time of 30 min was performed. Mutant mice showed an increased spontaneous scratching as compared to control mice. Mutant mice were also analyzed for the anxiety and depressive consequences of chronic pruritus using behavioral tests. Mutant mice showed an increased anxiety-like behavior in the elevated plus maze (EPM) test.

In conclusion, the *COL6A5*-p.Glu2272* mutation increased the scratching behavior in mice, confirming that this mutation was the potentially cause of chronic itch in patients carrying the mutation. Mutant mice also showed alterations in certain emotion-related behaviors as a consequence of this mutation. Thereby, further investigation was required to explore the effects of the mutation on neuro-immune interactions at the level of the skin or sensory neurons. In addition, the neural circuits underlying itch are becoming increasingly well understood (Dong & Dong, 2018), and studying the impact of the mutation on these neural circuits, such as its effects on the activation of central nervous system regions involved in itch control (Schmelz, 2021), is a promising avenue for next research.

Résumé en Français

Le prurit, ou la démangeaison (“itch” en anglais), est une sensation désagréable qui provoque le besoin de se gratter. Le prurit est défini comme chronique lorsqu'il dure plus de 6 semaines. Les prurits chroniques sont classés en quatre catégories principales suivant les profils cliniques et leur étiologie : (1) dermatologiques ou pruritoceptifs, dont l'origine se situe dans la peau avec souvent une inflammation de la peau, (2) systémiques, causés par des maladies métaboliques, hématologiques ou immunitaires, infections, cancers ou induits par des médicaments, (3) neurologiques ou neuropathiques suivant l'absence ou en la présence de lésions du système nerveux, (4) psychogéniques ou psychiatriques. Dans certains cas la cause du prurit n'est pas connue et il sera classé en ‘autres’ ou ‘d'origine indéterminée’ (Misery et al., 2014; Nowak & Yeung, 2017). Les prurits neuropathiques sont provoqués par des lésions du système nerveux dues à des compressions de nerfs, une neurodégénérescence, des tumeurs du système nerveux et d'autres pathologies ou infections du système nerveux. Les prurits neuropathiques sont souvent associés à d'autres symptômes neurologiques tels que des douleurs ou des troubles de la sensibilité. L'une des causes du prurit neuropathique est la neuropathie à petites fibres ou SFN pour “Small Fiber Neuropathy”en anglais (Misery et al., 2014).

En 2017, Martinelli-Boneschi et collègues ont identifié par séquençage des exomes de patients présentant un prurit chronique d'origine inconnue, un variant du collagène 6A5, le variant *COL6A5*-p.Glu2272**.* Le gène *COL6A5* de ces patients p.Glu2272*** contient un codon stop au lieu du codon de l'Acide Glutamique en position 2272 de la protéine, qui content 2615 résidus en tout (Martinelli-Boneschi et al., 2017). Le variant *COL6A5*-p.Glu2272*** a été trouvé chez 5 patients affectés par le prurit chronique dans 2 familles et chez une patiente sporadique avec neuropathie diabétique modérée et du prurit chronique sans douleur. L'analyse *in vitro* de l'ADN complémentaire des fibroblastes de certains patients a montré une haplo-insuffisance fonctionnelle. L'expression du *COL6A5* était réduite dans la peau des patients avec le variant p.Glu2272*** testés. Dans la famille 1, 2 des 3 patients présentaient une neuropathie à petites fibres caractérisée par une réduction de la densité des fibres nerveuses intraépidermales (IENF). Dans la famille 2, les 2 patientes présentaient une neuropathie à petites fibres. La patiente sporadique avec de la neuropathie diabétique avait

une densité d'IENF normale. La mutation *COL6A5*-p.Glu2272* a été identifiée comme la mutation commune chez ces 6 patients.

Afin de comprendre le rôle de cette mutation et son implication dans le prurit chronique, nous avons généré le modèle murin correspondant, une lignée de souris (*Col6a5*^{E2302*}mutant mice) portant cette mutation dans le gène du collagène VIα5. Ces souris mutantes ont été générées par le système Crispr-Cas9. La mutation a bien été transmise à la descendance des première souris mutantes obtenues, ce qui a permis d'établir la lignée mutante. Nous avons déterminé l'expression du gène mutant p.Glu2272* dans les ganglions dorsaux des souris mutantes et contrôles sauvages (wt). Ces ganglions dorsaux contiennent les corps cellulaires des neurones sensoriels. Le gène mutant était exprimé à un niveau comparable au gène wt. Mais une diminution significative de l'expression gène mutant a été observée dans la peau. Nous avons ensuite évalué le comportement de 'grattage' des souris mutantes sur une durée de 30 min. Les animaux mutants présentaient une augmentation de la durée de grattage par rapport aux animaux contrôles. Les animaux mutants ont aussi été analysés pour les conséquences anxio-dépressives du prurit chronique à l'aide de tests comportementaux. Les animaux mutants présentaient une augmentation du comportement anxieux dans le test de la croix surélevée (EPM, « elevated plus maze » en anglais).

En conclusion, nous avons montré que la mutation *COL6A5*-p.Glu2272* induit bien une augmentation du comportement de grattage chez la souris, confirmant que cette mutation est la cause du prurit chronique chez les patients portant la mutation. Les souris mutantes présentaient également des altérations de certains comportements liés aux émotions. En perspective, il conviendrait d'étudier plus avant les conséquences de cette mutation. En effet, les circuits nerveux sous-tendant le prurit sont de mieux en mieux connus (Dong & Dong, 2018). Par exemple, les effets de la mutation sur les interactions neuro-immunes au niveau de la peau ou des neurones sensoriels (Ruppenstein et al., 2021), ou sur l'activation des régions du système nerveux central impliquées dans le contrôle du prurit (Schmelz, 2021), pourraient être explorés dans le futur.

Abbreviations

- AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
- B6N WT: C57BL/6N commercial wild type mice
- bp: base pairs
- Cas9: CRISPR associated gene 9
- CMHs: Mechano-heat-sensitive C-fibers
- CNS: Central Nervous System
- COL6A5: Collagen6a5
- CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats
- crRNA: CRISPR RNA
- DRG: Dorsal Root Ganglion
- CT : Computed tomography
- dsDNA: double stranded DNA
- DSB: Double Strand Break
- EPM: Elevated plus maze
- GABA: γ-aminobutyric acid
- GPCR: G protein-couple receptor
- gR: gRNA: guide RNA
- GRP: Glutamate, gastrin-releasing peptide
- IENFD: Intraepidermal Nerve Fiber Density
- IL: Interleukin
- KI: Knock-in
- LDT: Light Dark test
- LMT: Live mouse tracking
- MRI : Magnetic resonance imaging
- nt: nucleotide
- OD: Optical density
- OFT: Open Field Test
- PAM: Protospacer Adjacent Motif
- PCR: Polymerase Chain Reaction
- Pgp9.5: Protein gene product 9.5
- PM: Point Mutation
- PNS: Peripheral Nervous System
- pre-crRNA: precursor CRISPR RNA
- PVC: Polyvinyl chloride
- RM-ANOVA: Repeated measures analysis of variance
- RT-ddPCR: Real Time droplet digital PCR
- S: Stranger
- SFN: Small Fiber Neuropathy
- SNPs: Single nucleotide polymorphisms
- ssgRNA: single-stranded guide RNA
- ssODN: single-stranded Oligo-DeoxyNucleotide
- TEWL: Transepidermal water loss
- TLR: Toll-like receptors
- tracrRNA: *trans*-activating
- TSLP: Thymic stromal lymphopoietin
- TTS: Trigeminal trophic syndrome

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Chapter 1: Introduction

1.1 General introduction

Itch, also known as pruritus, is “an unpleasant cutaneous sensation which provokes the desire to scratch” and is a common experience in daily life. Most of us experience itch, an unpleasant sensation associated with perceived disruption to the skin, multiple times a day, and a quick scratch near the itchy area usually abolishes it. This everyday itching and scratching are probably beneficial; itching can alert us to potentially harmful stimuli, such as insects, on the skin, and scratching is a movement that can remove them. Scratching can also have a valuable social function, such as self-scratching used by primates (including humans) when experiencing stress (Whitehouse et al., 2017).

Much of our everyday itching and scratching is potentially harmless and beneficial. Unfortunately, itching and scratching can also represent debilitating problems and reduce the quality of life. Considering the different types in which itch manifests, one cannot regard it as a singular uniform entity. The International Forum for the Study of Itch (IFSI) has internationally accepted and defined four main categories (and a total of six categories) for the clinical classification of itch based on its origin, as outlined (Twycross, 2003). These categories have been defined according to etiology (Weisshaar et al., 2019).

Dermatological (or pruritoceptive) itch: it is generated in the skin, usually by inflammation or other visible pathological processes involving the skin. It is the most common type of itch confronted by dermatologists. Predominantly pruritoceptive itch occurs due to scabies, urticaria and insect bite.

Systemic itch: it does not originate in the skin but could result from systemic abnormality. These abnormalities that can cause systemic itch include renal insufficiency, cholestasis, Hodgkin's lymphoma, polycythemia vera, solid tumors, and many others (Hashimoto & Yosipovitch, 2019; Kremer et al., 2020).

Neurological-neuropathic itch: it can be generalized as diseases or disorders of the central or peripheral nervous system”, e.g., nerve damage, nerve compression, and nerve irritation. It can be further divided into two sub-categories: a) neurogenic itch and b) neuropathic itch (Rajagopalan et al., 2017).

Neurogenic itch; is derived from the central nervous system, in which itch is induced and transmitted through mediators and receptors without nerve damage. In other words, it is produced in the central nervous system by circulating pruritogens. This type of itch could be due to abnormal processing of afferent sensory signaling from periphery nerves or excitation of central processing (Song et al., 2018).

Neuropathic itch; occurs due to damage to peripheral afferent sensory nerves or in the central nervous system. Neuropathic itch is accompanied by symptoms of paresthesia (a burning or prickling sensation), hyperesthesia (increased sensitivity to noxious stimuli), or hypoesthesia (partial or total loss of sensation in a part of one's body) (Pereira et al., 2021).

Somatoform (Psychogenic itch): a disorder caused by psychological abnormalities of psychiatric origin. Indeed, depression, obsessive-compulsive disorder, anxiety, somatoform disorders, mania, psychosis, and substance abuse have been associated with itch (Kahremany et al., 2020). In addition, a parasitic phobia is a common disorder characterized by psychogenic pruritus (Song et al., 2018). Besides these four main categories, the itch can be presented as "mixed origin" if it involves multiple etiology. All remaining forms of itch are classified as "others" when no etiology has been identified (Weisshaar et al., 2019).

Also, the itch can occur as an acute or chronic condition. Acute itch occurs in the absence of disease. Various stimuli, ranging from mosquito bites to poison ivy, can cause an acute itch that tends to resolve itself quickly. While chronic itch is a symptom of many diseases, it can last for months or years.

In general, initially, repetitive or vigorous scratching can cause skin damage, which leads to inflammation of the skin and increases itch. Later, neural processing of itching and scratching can exacerbate the problem. In addition, scratching becomes perceived as pleasurable due to the involvement of brain reward circuits which increases the desire to scratch more and more. As a result, when patients experience neuropathic itch, it can be challenging to break the itch-scratch cycle, which may worsen their condition over an extended period of time.

Itch being complex can arise through endogenous factors such as endogenous chemical mediators that drive neuronal activity. For example, that can originate from the complex interaction between keratinocytes, inflammatory cells, nerve endings, upregulated immune

cascades, epidermal barrier dysfunction, and genetics etc. In addition, it may arise through exogenous factors or environmental stimuli such as microbiota, allergens, irritants, and dryness (H. S. Kim & Yosipovitch, 2020).

From the treatment perspective, an acute itch is simpler than a chronic one and easier to treat and manage. On the other hand, chronic itch is more complex due to the involvement of several mediators, receptors, channels, and many unknown factors. This complexity makes treating and managing chronic itch more challenging and demanding (Fowler & Yosipovitch, 2019).

Besides, chronic itch is more problematic when there is no visible lesion on the skin. Therefore, only skin examination remains insufficient to assess as a chronic itch. Therefore, it is vital to identify itch at the cutaneous level and through the wide range of other known causes. As the causes of itch are multiple, having a standard method or recommendation for itch treatment is challenging. The first step for effective treatment and management of itch is to identify the precise cause of the itch and determine the type of itch in a particular patient (Leslie, 2016).

The first treatments for itch are topical therapies. They include coolants, inhibitors, and local anesthetics, depending on the type of itch. In the second option, with the advancement of research, many proteases, peptides, receptors, and their functions are known, and they can also be used as therapeutic targets. For treatment, several itch mediators and sensitized neurons (in PNS or CNS) could be targeted (Raap et al., 2011). The most common and well-known itch treatment is antihistamines, which can aid patients with comfortable sleep and help to break the itch-scratch cycle. It could also be treated differently using mu-opioid antagonists or kappa opioid agonists (Mores et al., 2019) and other possible treatments such as phototherapy and antidepressants.

Moreover, it is essential to know and assess the severity of the patient's itch and recommend the most effective treatment. Medical professionals consider several factors, including the patient's medical history, age, use of other itch-provoking medications, and the intensity of the itch. Questionnaires related to itch may also be used to gather additional information (Weisshaar et al., 2019).

Also, to find new treatments and management of itch, it is essential to consider several other factors directly and indirectly associated with itch, including microbiota, skin barrier, and skin collagen etc.

In healthy skin, various microbiota (bacteria, fungi, and viruses) cover the skin's exterior and are considered an integral part of the skin barrier. Some of these microbiota produce antimicrobial compounds, which block the growth of competitors. In dysbiosis, Bacterial enzymes, such as proteases, can impact lipases and break down the skin surface, leading to a disruption of the skin barrier. This disruption can further provoke an immune reaction and cause itching.

Not only externally but internally, skin integrity can also be compromised through collagen fragmentation (H. S. Kim & Yosipovitch, 2020). This fragmentation can be due to an exogenous factor, such as UV light, or endogenous factors, such as genetic mutation (Potekaev et al., 2021).

Collagens are a large family of triple helical proteins and have functions of cell-matrix interactions along with tissue structure and function. The collagen superfamily comprises 28 members in vertebrates with given Roman counting (I-XXVIII), a collective term used as collagen for the whole family of glycoproteins. They are the most common and abundant structural protein in the animal kingdom. Even simple multicellular organisms express prototype genes of collagens. In vertebrates, collagens are the primary components of connective tissues, comprising approximately one-fourth of the total protein in the human body and three-fourths of the dry weight of human skin. Collagen family members were discovered on the bases of their blueprint sequence [Gly-X-Y]n (Sorushanova et al., 2019). However, tissue distribution and function of many types of collagens are still unknown. Also, in the collagen family, all types are not equally distributed in different tissue. Collagen I, II, and III have a significant share in the family. It comprises 80-90% of total body collagen (Shoulders & Raines, 2009; Sorushanova et al., 2019).

Collagens regulate a diverse yet crucial set of functions within the skin. For example, basement membrane collagens, such as collagen IV, VII, and XVII, are essential for the structural integrity of the basement membrane zone and anchorage of the epidermis to the

dermis. In the dermis, collagen V, VI and XII mediate the assembly of larger macromolecules and influence tissue mechanics (Figure 1.1).

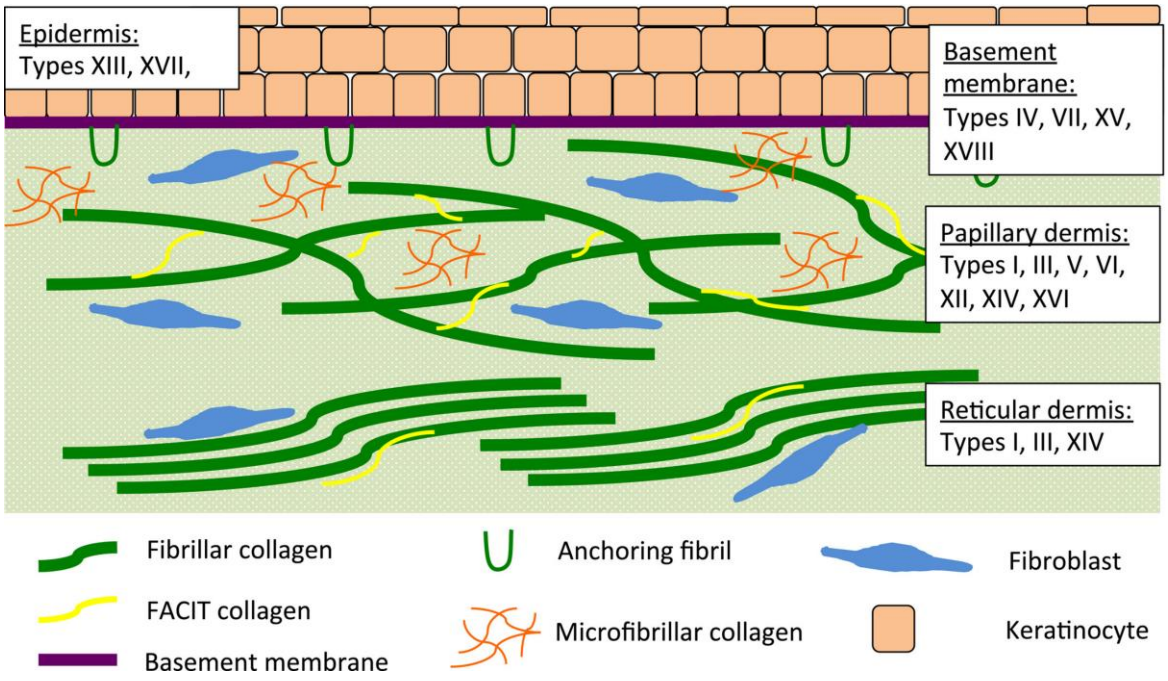

Figure 1.1 Schematic representation of the skin shows each collagen preferentially localized to the epidermis and basement membrane, showing fibrillar collagens within the papillary and reticular dermis. FACIT (fibril-associated collagens with interrupted triple helices). The figure was taken from (Theocharidis & Connelly, 2019).

These collagens performed their function according to their structure in the skin. Collagens assemble into fibrils (Triple helical collagen molecules assemble to form fibrils) and fibers (Fibrils bundle together to form fibers), thus categorized as fibril-forming collagens. Others, such as collagen IV, VI, VIII and X, are non-fibrillar and are organized into different types of networks. However, the role of other FACIT (fibril-associated collagens with interrupted triple helices) molecules in the skin is not yet well understood (Pfisterer et al., 2021; Theocharidis & Connelly, 2019).

In 2017, a study by Martinelli-Boneschi and colleagues identified that a mutation in collagen VI was associated with chronic neuropathic itch (Martinelli-Boneschi et al., 2017). Collagen VI is a beaded filament structure expressed in many connective tissues. The main monomeric unit is a heterotrimer comprising α1(VI), α2(VI) and α3(VI). Later, three more α3(VI)-like chains were identified: α4(VI), α5(VI) and α6(VI). These chains are believed to substitute the α3(VI) chain and form triple helices with the α1(VI) and α2(VI). However, the overall

distribution of the newly discovered chains is limited to a few select locations within the skin, skeletal muscle and reproductive organs (Gara et al., 2011; Sabatelli et al., 2011). Collagen VI is expressed throughout the papillary, reticular, and hypodermis in neonatal and adult skin. It is also found around nerve fibers and blood vessels, and the levels are enriched just below the basement membrane at the dermal-epidermal junction (Gara et al., 2011).

Martinelli-Boneschi and colleagues found, by whole exome sequencing, that two families and a diabetic patient carried the nonsense c.6814G>T (p.Glu2272* or can be noted as E2272*) variant. Further segregation analysis was made for the p.Glu2272* mutation in COL6A5, revealing its association with itch. Moreover, heterozygous mutation p.Glu2272* in COL6A5 carrying patients' fibroblasts were used to confirm the null alleles test. RT-PCR analysis showed that patients carrying *COL6A5*-p.Glu2272* mutation nonsense variant have functional haploinsufficiency due to RNA decay. Also, patients carrying mutation p.Glu2272* in COL6A5 had low COL6A5 protein expression in the skin. From this evidence, mutation p.Glu2272* in COL6A5 association with idiopathic itch was concluded and later inferred as neuropathic itch (Martinelli-Boneschi et al., 2017). Of note, this same mutation has also been found in rare patients with Chiari malformation type 1 (Urbizu et al., 2021).

Therefore, with the increasing research and importance of mutation p.Glu2272* in COL6A5 was necessary to investigate the consequences of this mutation in a mouse model corresponding to the human.

My Ph.D. project was on generating a mouse model for this mutation and characterizing this model, mainly for itch phenotype. Therefore, in this project, I focused on establishing and characterizing the first mutant mouse model *Col6a5*^{em1} (E2302*), also called named as *Col6a5*^{E2302*}, corresponding to the human *COL6A5*-p.Glu2272* mutation using CRISPR/Cas9 technology to understand the genotype-phenotype association and assess potential consequences of COL6A5 mutations in relation to chronic itch.

Briefly, this thesis is divided into chapters as follows: Chapter 1: Introduction; it explains the detailed background of itch, its types, several factors causing itch, the mechanism of itch, collagen, collagen VI, mutation p.Glu2272* in COL6A5, its association with chronic neuropathic itch and some potential treatment and briefly about thesis project etc. Next, this

thesis has a result section as Chapter 2: Results. It contains two parts 1) manuscript in preparation and 2) supplementary methods and results; this part has supplementary methods and results achieved in this project. To avoid redundancy in creating a new chapter for the materials and methods, it is noted that most of the materials and methods used in this thesis have already been incorporated into the "manuscript preparation" under the subsection ‘material and method’. The remaining supplementary methods have been included in the "supplementary methods and results" section under the supplementary methods subsection.

Further, this thesis contains a detailed general discussion under Chapter 3: General Discussion. To conclude and give future direction of this project, this thesis has a chapter containing a conclusion, prospectives, and concluding remarks as Chapter 4: Conclusion and perspectives. Lastly, in the end, the thesis contains Chapter 5: References section that gives references alphabetically. Additionally, the table of content on page 1 of this thesis, with the bold letter heading of each part clearly divides and describes each section for the reader’s convenience.

1.2 Itch

1.2.1 Definition of Itch and its historical background

Itch is a sensation that causes the desire to scratch (Weisshaar et al., 2019). Organismal survival needs systems to sense, warn and defend against noxious environmental changes at the skin level. For this, a wide range of sensations must be felt and analyzed, which will engage specific reactions. For example, human beings can feel pain against noxious stimuli; thus, pain constitutes an alarm system for potential damage to our skin or other body parts. Nausea and vomiting sensations warn us about pathogens or something toxic that have entered our gastrointestinal system (Canziani et al., 2018). Likewise, the itch sensation warns us of harmful stimuli that we should remove from our skin and induces skin scratching. Insensitivity to these sensations can be life-threatening (Sanders, Fast, et al., 2019).

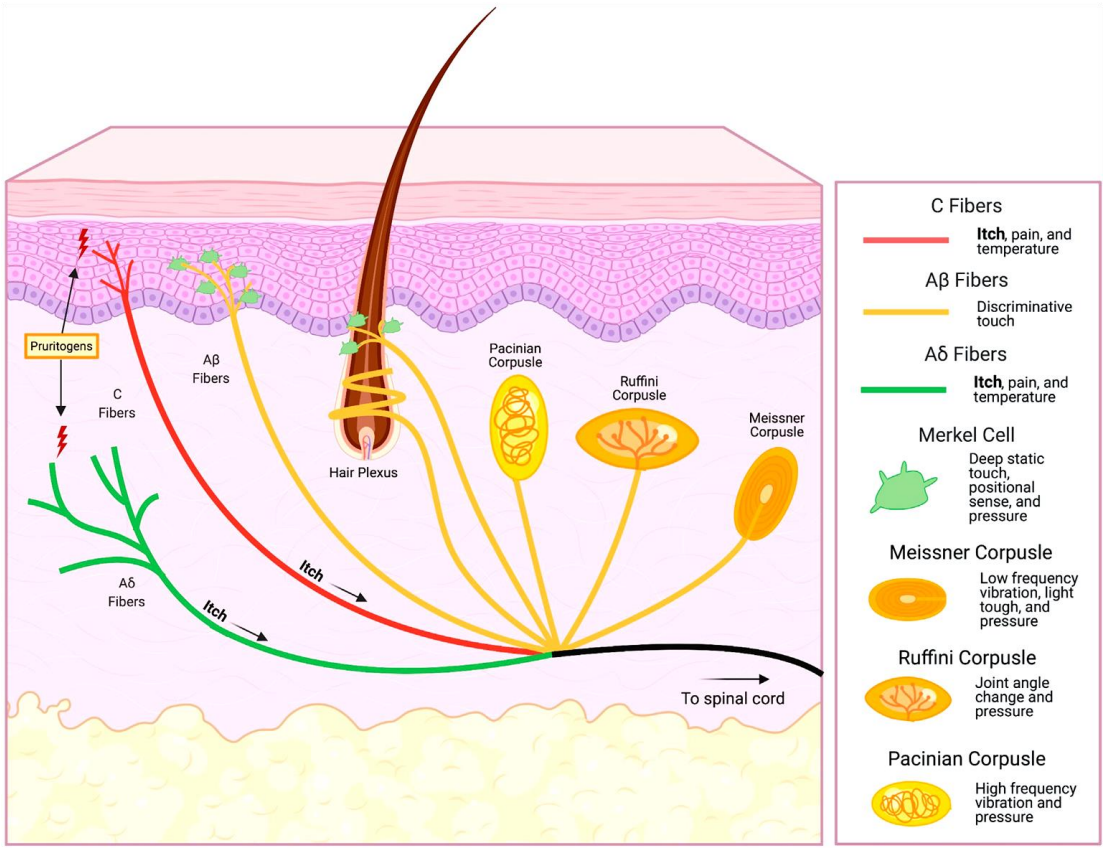

Figure 1.2 Nerve fibers innervating the skin. Itch is transmitted primarily via Aδ and C fibers. Above figure has been obtained from source (Sutaria et al., 2022)

However, the itch sensation has long been a mystery and is not as simple as it seems. If we ignore an itch, it may get worse, irritating, and disturbing. Moreover, the itch was poorly understood in the past due to scarce research on it. Nevertheless, the itch was already considered to be transmitted by the same pathway as pain (Figure 1.2).

Both itch and pruritus terms are used interchangeably, but these terms have different origins. The term “Itch” was used in Greek with the related word ‘Knêsmos’ for the sense of pleasure and irritation. While the term “pruritus” was used in Latin with a similar word to ‘prurigo’ for itchiness, irritation, and sexual excitement. From this, it is clear that either ‘Knêsmos’ or ‘prurigo’ and associated words were used in the past for both meanings of scratching, scrapping, and pleasure sensation (Weisshaar et al., 2009).

The itch was described in ancient literature; however, the terminology was generalized and unclear. In the 10th and 11th centuries, the itch was mainly related to the skin and often was thought to arise due to salty water in the skin (Weisshaar et al., 2015). To treat itch, there

were several traditional remedies available at that time. German physician Samuel Hafenreffer first introduced the itch in 1660 as “an unpleasant sensation that provokes the desire to scratch”. However, there was no clear definition of pruritus or itch to differentiate it from scabies- a skin infestation caused by a mite known as the ‘Sarcoptes scabiei’ and other skin diseases. In the middle of the 19th century, new classes of dermatoses were introduced, and several diseases were also classified in the field of dermatology. At that time, pruritus was seen as an illness, and the itch was no longer a symptom. Then the question was how itch could be defined as skin disease when skin is normal but itchy. So, it made itch unfit in a category of other skin diseases. For that, Max Joseph added in 1898 another definition of itch as ‘‘the appearance of the skin is fully normal and without pathological findings, but there is hyperesthesia accompanied by intense itching”. Again, a question arose: is itch associated with cutaneous symptoms? We can deduce the answer from the attention-seeking argument by Joseph Jadassohn (1863-1936), who elaborated that when itching is the only cutaneous symptom, ‘‘we can merely state that a patient suffers from itching, while we are unable to discover the nature and the cause of this condition.” Further, to not consider itch just as a symptom associated with another disease, Erhard Riecke defined itch as ‘‘a sensory neurosis of the skin’’ and emphasized itch as a disease in its own right. In the early 20th century, "itch" was defined as "itchy skin with no known cause”. However, with advancements in scientific explanations and knowledge about the pain research and nerve-ending pathways, our understanding of itch has improved (Weisshaar et al., 2015).

Bickford found in 1939 that itch was transmitted through C nerve fibers(Weisshaar et al., 2015). Later in 1948, Bishop described itch transmission from sensory fibers to the cerebral cortex and thalamus, from the periphery to the central nervous system (BISHOP, 1948). In the mid-20th century, Graham's study changed the scenario by showing two distinguishable transmissions of itch sensation through C and Aδ sensory fibers. Finally, in the 1990s, a new era of itch research evolved due to critical discoveries, including performing psychophysics on itch (Simone et al., 1991), identifying a subset of nerves specific to itch (Schmelz et al., 1997), and finding the beneficial effect of gabapentin for the treatment of pruritus (Bueller et al., 1999).

With the progress of science and the itch field evolution over several decades, still, a need to find unknown causes of itch, the association of itch with other diseases, for example, diabetes, and to investigate new therapeutic targets for the itch. Moreover, still, we need to answer some fundamental questions, e.g., why do we scratch an itch? Why scratching an itch feels so good?

1.2.2 Better classification of itch

There is an internationally accepted classification of itch. Originally, Twycross et al. (Twycross, 2003) proposed a neuropathophysiologically-based classification following the origin of itch. This classification of the itch is now divided into pruritoceptive (generated in the skin), neurogenic, neuropathic, and psychogenic. In addition, the International Forum for the Study of Itch (IFSI) recently defined a classification of chronic pruritus according to its etiology. The categories are “dermatological (or pruritoceptive)”, “systemic”, “neurological-neuropathic”, “somatoform”, “mixed origin” and “others” (Weisshaar et al., 2019) (Table **1.1,** Figure 1.3).

To deal pruritus effectively, a two-step approach can be adopted to make the classification of underlying etiology practical and useful.

At 1st step: a comprehensive history and physical examination are important before resorting to laboratory and radiological tests. Patients can be classified into three groups based on clinical observations of skin condition.

Group I patients includes inflammatory, infectious, autoimmune, and other skin diseases that manifest with itch and specific skin changes.

Group II comprises patients with pruritus originating from systemic, neurological, or psychogenic /psychiatric conditions, without primary skin lesions.

Group III involves chronic pruritus leading to mechanical reactions like scratching, resulting in secondary scratch lesions. These patients typically have severe chronic scratch lesions and may have a systemic or skin disease origin. They often experience pruritus for an extended period and may not recall the initial skin changes.

At 2nd step: further investigations such as histological, laboratory, and radiological tests are performed to identify the underlying causes of pruritus. Based on differential diagnosis, an etiological classification is proposed. Category I) includes dermatological diseases, category II) encompasses systemic diseases, category III) involves neurological conditions, and category IV comprises psychogenic diseases. In some cases, multiple underlying diseases may contribute to itch and are classified as "mixed" (category V), while patients without any identified underlying disease are categorized as "others" (category VI). When diagnostic tests fail to determine the underlying origin, the term "pruritus of undetermined origin" is used(Weisshaar et al., 2019) (Table **1.1,** Figure 1.3).

In the succeeding stages, detail about these categories (I, II, III , IV) is presented below.

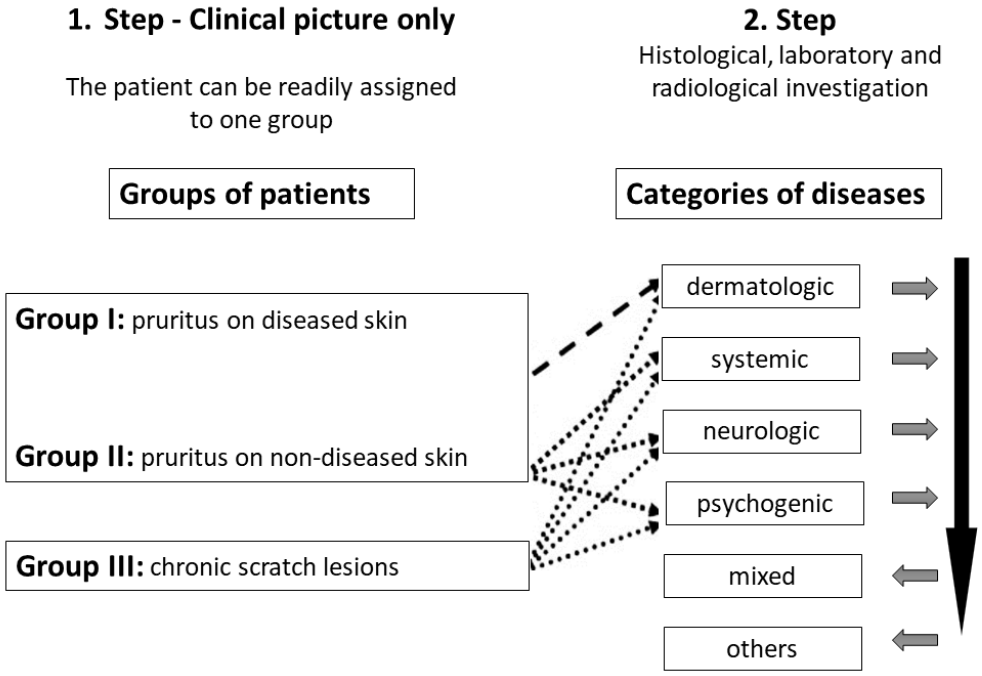

Figure 1.3 Clinical classification in the management of chronic pruritus patients. As a first step, patients are grouped according to their clinical picture and history. Although group I and II may already suggest a category, the classification of the patient is performed in a second step based on histological, laboratory, and radiological investigation. If no category fits or several diseases are found (small arrows), the patients are classified into “mixed” or “others” (arrow on the right). Above figure has been obtained from source (Ständer et al., 2007).

Table 1.1 Classification of pruritus according to its origin obtained from (Ständer et al., 2007)

Category	Diseases
I. Dermatological	Arising from "diseases of the skin", such as psoriasis, atopic dermatitis, dry skin, scabies and urticaria
II. Systemic	Arising from "diseases of organs" other than the skin, such as liver (e.g., primary biliary cirrhosis), kidney (e.g. chronic renal failure), blood (e.g. Hodgkin's disease), and certain multifactorial (e.g. metabolic) states or drugs
III. Neurological	Arising from "diseases or disorders of the central or peripheral nervous system", e.g., nerve damage, nerve compression, nerve irritation
IV. Psychogenic/ Psychosomatic	Somatoform pruritus with co-morbidity of "psychiatric and psychosomatic diseases"
V. Mixed	Overlapping and coexistence of several diseases
VI. Other	Undetermined origin

1.2.2.1 Dermatological itch

Pruritoceptive (or dermal) itch is the most common type of itch confronted by dermatologists. Its origin is in layers of skin to produce the sensation of itch. Most clinical cases of pruritus belong to pruritoceptive itch, that is why most of the pruritus research that took place in the past fell in this category. Common causes of pruritoceptive itch may have exogenous (external) and /or endogenous (internal) factors. Pruritoceptive itch can be induced and modulated by several exogenous factors, including chemical, mechanical, and environmental factors. It can be induced or modulated endogenously, such as pruritogen produced by keratinocytes cells and immune cells. It can be transmitted through C fibers. Examples of pruritoceptive itch include itch due to scabies, urticaria, and insect bite (Yosipovitch et al., 2003). The pathways of itch in the skin for itch sensation is illustrated in Figure 1.4 below.

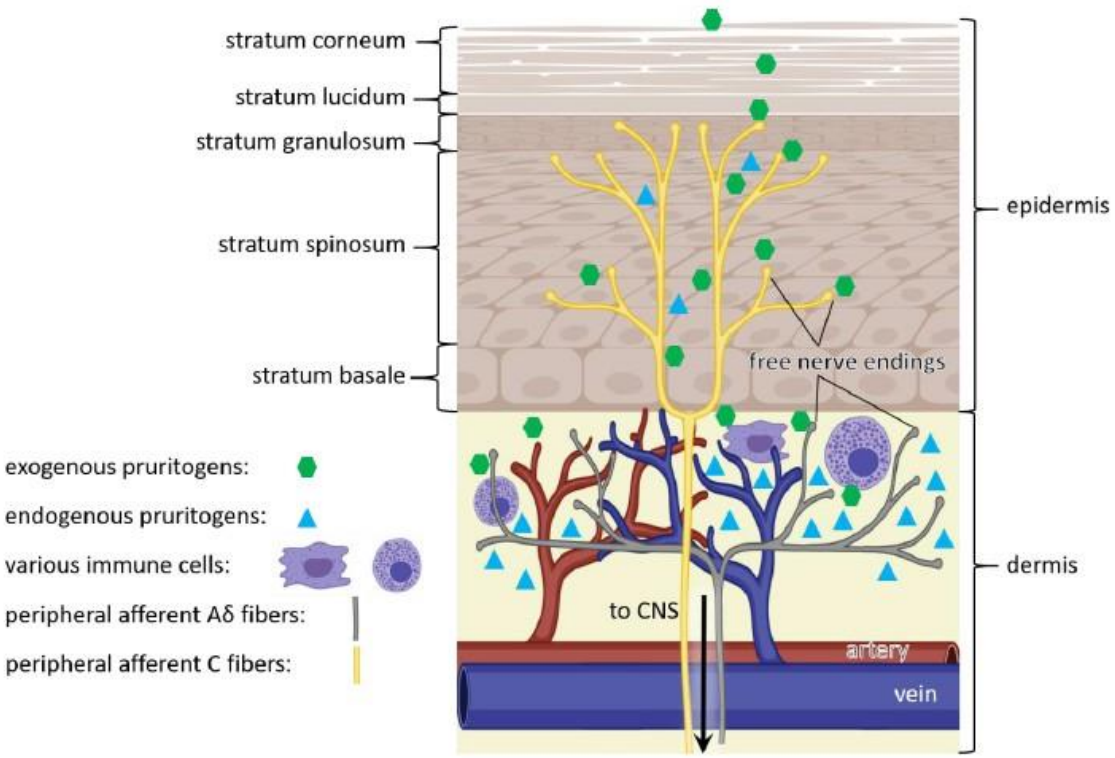

Figure 1.4 Itch pathway illustration. Itch sensation is caused by external (green) and internal (cyan) pruritogens that bind to itch receptors in free nerve endings of C fibers within the epidermis (yellow) and Aδ fibers within the dermis (grey). Besides, internal pruritogens (cyan) can be produced by epidermal keratinocytes and dermal immune cells (purple). The triggered signal is transmitted through peripheral afferent nerve fibers (yellow and grey) of the peripheral nervous system to the central nervous system (CNS), eventually resulting in an itch sensation. Above figure has been obtained from source (Kahremany et al., 2020).

Histamine was the first described mediator of itch and remained the most commonly acknowledged mediator of pruritoceptive itch. The majority of skin histamine arises from the degranulation of dermal mast cells. Histamine can excite sensory fibers (itch selective C-fibers) (Schmelz et al., 1997). The histamine receptors on C-fibers belong to the G protein-coupled receptors (GPCR) family. Out of four subtypes of histamine receptors H1-H4, histamine induces itch by activating H1 and H3 or H4 receptors in C-fibers (Kahremany et al., 2020). In this way, antihistaminic drugs remain a successful treatment against histamine-induced itch. Since then, many other skin mediators and itch receptors have been discovered.

1.2.2.2 Systemic itch

Systemic itch refers to “diseases of organs” with an origin different from the skin, such as liver (e.g., primary biliary cirrhosis), kidney (e.g., chronic renal failure), blood (e.g., Hodgkin’s disease), and certain multifactorial states (e.g., metabolic) or drugs. Several causes of systemic itch include endocrine and metabolic diseases, infections, hematologic and lymphoproliferative disorders, solid tumors of the internal organs, pregnancy, and drug-induced pruritus.

Endocrine and metabolic diseases caused by systemic itch can be commonly seen in an individual with chronic renal failure or kidney diseases, liver diseases with or without cholestasis, hyperthyroidism, and malabsorption. Infectious diseases cause can occur in an individual having HIV-infection, helminthosis, and parasitosis. Haematological and lymphoproliferative diseases cause can lead an individual to the following diseases: iron deficiency, polycythaemia vera, hodgkin’s disease, non-hodgkin’s lymphoma, and plasmocytoma, resulting in systemic itch.

Additionally, disorders of the thyroid gland such as hypothyroidism and hyperthyroidism, as well as solid tumors of the cervix, prostate, or colon and drugs such as opioids, angiotensin-converting enzyme (ACE)-inhibitors, amiodarone, hydrochlorothiazide, estrogens, simvastatin, hydroxyethyl starch, and allopurinol can induce itchiness (Hashimoto & Yosipovitch, 2019; Kremer et al., 2020).

1.2.2.3 Neurologic itch

The neurologic itch can be generalized as a disease or disorder of “the central or peripheral nervous system”, e.g., nerve damage, compression, and irritation. Therefore, it can be further divided into two subcategories 1) neurogenic itch and 2) neuropathic itch (Rajagopalan et al., 2017).

1) Neurogenic itch: it is derived from the central nervous system, in which itch is induced and transmitted through mediators and receptors without nerve damage. This type of itch could be due to abnormal processing of afferent sensory signaling from periphery nerves or excitation of central processing (Song et al., 2018). Neurogenic itch is associated with different

disorders including cholestasis, chronic renal failure, hematologic, lymphoproliferative conditions, and malignancies. There are difficulties in assessing and proposing the exact treatment of neurogenic itch associated with the disorders mentioned above, such as cholestasis, which refers to liver disease. In normal circumstances, the liver produces bile fluid that helps fat digestion. However, in the case of cholestasis, bile fluid is blocked. It could be due to primary sclerosing cholangitis, gallstones, carcinoma or viral hepatitis etc. The neurogenic itch can be induced through increased bile levels and opioid peptides acting on the μ-opioid receptor in the central nervous system. To assess the possible treatment, it was shown that antihistamines are ineffective for neurogenic itch as they are effective in the case of pruritoceptive itch, as mentioned earlier. Therefore, assessing the potential treatment of neurogenic itch has difficulties. So far, one strategy is to reduce cytochrome P450 in the liver to reduce liver pruritogens. A second strategy is to treat with mu-opioid receptor antagonists (Kremer et al., 2020). Still, the study is ongoing to find more itch-specific targets. Contrary to cholestasis, chronic renal failure refers to the gradual loss of kidney function over a period of time, and treatment remains more challenging and ambiguous. Interestingly, neurogenic itch associated with chronic renal failure rarely appears in children and does not appear in acute renal failure patients. The pathogenic basis of itch in chronic renal failure is not clear yet, and several pathomechanisms have been suggested. They include elevated levels of opioid peptides, mast cells, and plasma histamine. However, antihistamines treatment was found generally ineffective, as in the case of cholestasis. The effects of opioid antagonists to improve itch have raised some controversy along with opioid level association to itch. Also, itching, for instance, was caused by opioid peptides acting on the μ-opioid receptor (Berger & Steinhoff, 2011; Pauli-Magnus et al., 2000). The opioid hypothesis for treating neurogenic itch originated as the administration of opioids in epidural anesthesia induced frequent itch, which suggests that endogenous opioids may contribute to neurogenic itch. On this basis, the administration of opioid antagonists has been proposed as a treatment for neurogenic itch. Also, kappa opioid agonists such as nalfurafine and difelikefalin have shown clinical promise for treating various chronic pruritic conditions. Such treatments could also benefit neuropathic itch associated with peripheral nerve injury. Research is ongoing to find more specific and selective sets of neurons and pruritogens associated with the neurogenic itch in the central nervous system (Kahremany et al., 2020).

2) Neuropathic itch occurs due to damage to peripheral afferent sensory nerves or the central nervous system. According to the Münster Competence Center of Chronic Pruritus, 8% of chronic itch patient has a neuropathic origin (Stumpf & Ständer, 2013). It could be acute and in mostly found as chronic. Neuropathic itch is accompanied by symptoms of paresthesia (a burning or prickling sensation), hyperesthesia (increased sensitivity to noxious stimuli), or hypoesthesia (partial or total loss of sensation in a part of our body). In neuropathic pain, many patients have common symptoms of allodynia in which they are extremely sensitive to touch to induce pain. Similarly, many cases of neuropathic itch are due to the sensitization of nerve fibers, patients experience alloknesis (extremely sensitive to touch to induce itch) (Steinhoff et al., 2018). Then what characteristics make it unique and different from other types of itch? There are two main characteristics 1) it is related to other sensory signs in dermatomal distribution 2) it occurs together with other neurological signs or neural damage (motor and autonomic). However, neuropathic itch mechanisms are not well understood yet. One proposed reason is that damage to C fibers which transmit itch and pain, can cause neuropathic itch. Second, an excessive firing of neurons can happen. Third, the destruction or damage of inhibitory neurons in the spinal tract. That can cause changes in the somatosensory pathway, leading to chronic neuropathic itch (Steinhoff et al., 2018).

At the peripheral level, several conditions or diseases are associated with neuropathic itch, categorized as peripheral neuropathic itch. These include small fiber neuropathy, scars and burn radiculopathies, postherpetic neuralgia, trigeminal trophic syndrome, brachioradial pruritus, cheiralgia paresthetica etc. For example, small fiber neuropathy (SFN) cases can occur due to injury in unmyelinated C and lightly myelinated Aδ fibers. They will result in localized or generalized itch and pain. Patients commonly have itch, pain, burning, tingling, etc. These symptoms worsen during night time. The most common causes are diabetes mellitus, pre-diabetes, and impaired glucose tolerance. Other causes or conditions are infections, drug-induced (alcohol), autoimmune (post-viral, hepatitis C) diseases, genetic diseases (hereditary sensory-autonomic neuropathy, familial amyloid polyneuropathy), and other diseases (nutritional, cancer). An example of peripheral neuropathic itch is a trigeminal syndrome, in which a trigeminal nerve or root lesion cause a facial itch. The cranial nerve linked to specific itch syndrome is the trigeminal (V) nerve. Damage to the trigeminal nerve can cause neuropathic pain, but it also causes itch syndrome. Common causes of it are

generally known as 1) shingle-a viral infection that causes a painful rash, and 2) compression of the intracranial portion of the trigeminal nerve. Medicines such as gabapentin, pregabalin, and carbamazepine are commonly used to treat this type of itch. Another similar syndrome is a trigeminal trophic syndrome (TTS). It results from trigeminal nerve damage and is less commonly known as neuropathic itch syndrome. In associated symptoms are burning, numbness, dense sensory loss, and chronic itch. In 75% of cases, itch arises after surgical ablation of the Gasserian ganglion, while in other cases, it may be caused by rare infections such as Herpes simplex and leprosy. TTS initial assessment for treatment depends on its diagnosis as, in most cases, it is misdiagnosed as dermatitis artefacta or psychogenic pruritus (Sadeghi et al., 2004).

At the central level, damage or lesion is associated with central neuropathic itch, which is less common than peripheral neuropathic itch. It includes strokes, cysts, vascular malformations, abscesses (Summers & Macdonald, 1988) cerebral tumors, and Creutzfeldt-Jakob disease, etc. Several brain diseases are associated with itch, while itch associated with the spinal origin is uncommon. Still, the itch of spinal origin was reported in multiple sclerosis, spinal pilo-cytic astrocytoma, and spinal cavernomas. In all three diseases, itch symptoms appear before pain. One cause of neuropathic itch can be Creutzfeldt-Jakob disease, a common prion protein disease due to conformational changes in prion protein that becomes pathological and, after aggregating in the central nervous system, can damage the inhibitory gating mechanism of itch (Cohen et al., 2011). Another example of the cause of the central neuropathic itch is multiple sclerosis. It comprises paroxysmal pattern of itch, which can awake patients from sleep, and may cause pain, and 5% of patients have reported itch. Itch related to multiple sclerosis can be treated through carbamazepine (Freiha et al., 2020).

Due to difficulties distinguishing and diagnosing neuropathic itch, it is essential to know the detailed medical history of patients to exclude other types of itch. To assess neuropathic itch, in addition to medical history, it generally evaluated with the following important clinical features of neuropathic itch e.g., appearance of itch on non-lesioned skin, dysesthesias-abnormal sensation related to painful burning, prickling, or aching feeling, alleviation of itch with cold packs or water or ice, occurring of itch in attacks, and becomes worsen with warmth. Further, to estimate neuropathic itch, a physical examination of the skin is generally

performed by examining the entire skin, secondary lesions, and alloknesis. Moreover, a standardized questionnaire to determine the four factors in the pain field, known as 'douleur neuropathique 4 (DN4)', has been adapted to discriminate neuropathic pain. Similarly, such a score tool has been adapted in the field of itch. Five independent factors are chosen to differentiate neuropathic itch from non-neuropathic itch, known as 'Neuropathic Pruritus 5 (NP5)'. These independent factors include 1) twinges, 2) absence of burning, 3) worsening of the itch with activity, 4) no worsening with stress, and 5) relief of itch with cold temperature. The presence of 2 out of 5 factors gives the specificity to decimate neuropathic itch from the non-neuropathic itch 77%. After the non-invasive assessment, further invasive techniques are used primarily to assess small fiber neuropathy (Huguen et al., 2019). Intraepidermal nerve fiber density (IENFD) is a common standard. A skin sample is obtained through a punch biopsy of non-lesioned pruritus skin. It is recommended not to choose a lesioned part of the skin to avoid false results. Then the biopsy sample is stained with the specific axonal marker protein gene product 9.5 (Pgp9.5). Nerve fibers are counted based on crossing from the dermis layer to the epidermis. Skin biopsies from non-affected subjects are used as a control to compare the difference in IENFD. Further laboratory tests can be performed to assess the causes of neuropathic itch or to rule out non-neuropathic parameters (Lauria et al., 2010).

1.2.2.4 Psychogenic itch

The fourth type of itch is the psychogenic itch, a disorder caused by psychological abnormalities of psychiatric origin. Indeed, depression, obsessive-compulsive disorder, anxiety, somatoform disorders, mania, psychosis, and substance abuse have been associated with itch (Garibyan et al., 2013). In a previous study, 32% of patients in dermatology clinics had the psychogenic itch, but its incidence is unknown in the general population (Mazeh et al., 2008). It can often comprise a psychiatric condition such as parasitosis hallucinations, depression, or compulsive scratching syndromes. It can occur with some known psychological anomalies or be due to association with any other type of itch. In some reports, abnormalities such as depression, anxiety, obsessive-compulsive disorder, and mental stress-related disorders are associated with itch. A previous study suggested depression could be the primary clinical state of psychogenic itch (Mazeh et al., 2008). Secondary skin changes associated with psychogenic itch are body parts more accessible to hands to scratch. The face

is the most common body area to be accessed along with other areas, including arms, legs, abdomen, and shoulder. Due to scratches, patients can keep scratching, scoping, or even inserting some object into the skin and get relief from the itch. It can cause scars on the skin, bleeding, or ulceration. Often itch is so intense that patients who know what they are doing still feel less control, so they keep scratching. On the contrary, it is also possible that psychogenic itch could be without any skin signs (Buteau & Reichenberg, 2018).

1.2.3 Difference between acute and chronic itch

The itch can be clinically categorized into two categories: acute and chronic. Like pain, the **acute itch** is a physiological sensation and helps to protect us by inducing scratching that can remove toxic substances and disease-carrier insects from the skin. Acute itch occurs without disease and signals through primary somatosensory neurons. Activation of somatosensory neurons could be due to exogenous (e.g., mosquito bit or dust) or endogenous pruritogens (mast cell activation through endogenous histamine in the skin). Moreover, the itch is considered acute when it lasts less than six weeks (Yosipovitch et al., 2018).

Different pruritogens can trigger the acute itch, for example, compound C48/80, which activates mast cell degranulation. Similarly, following a mosquito bite, keratinocytes in the skin epidermis are damaged, and these keratinocytes, as well as local immune cells, detect this damage. This can induce the degranulation of mast cells, which can release different mediators including histamine, cytokines, proteases, and serotonin, in response. To clear the damage and avoid potential pathogens, histamine and other mediators help to trigger local vasodilation and bring circulating immune cells and leukocytes toward the damaged areas. Sensory nerves then detect these changes at their peripheral endings, resulting in an itch sensation that usually normalizes in very few days. The histaminergic pathway is the best understood, and much research has been performed on itch associated with histamine (Yosipovitch et al., 2018). Mast cells and basophils mainly secrete histamine. The histaminergic pathway Figure 1.5 can be seen below.

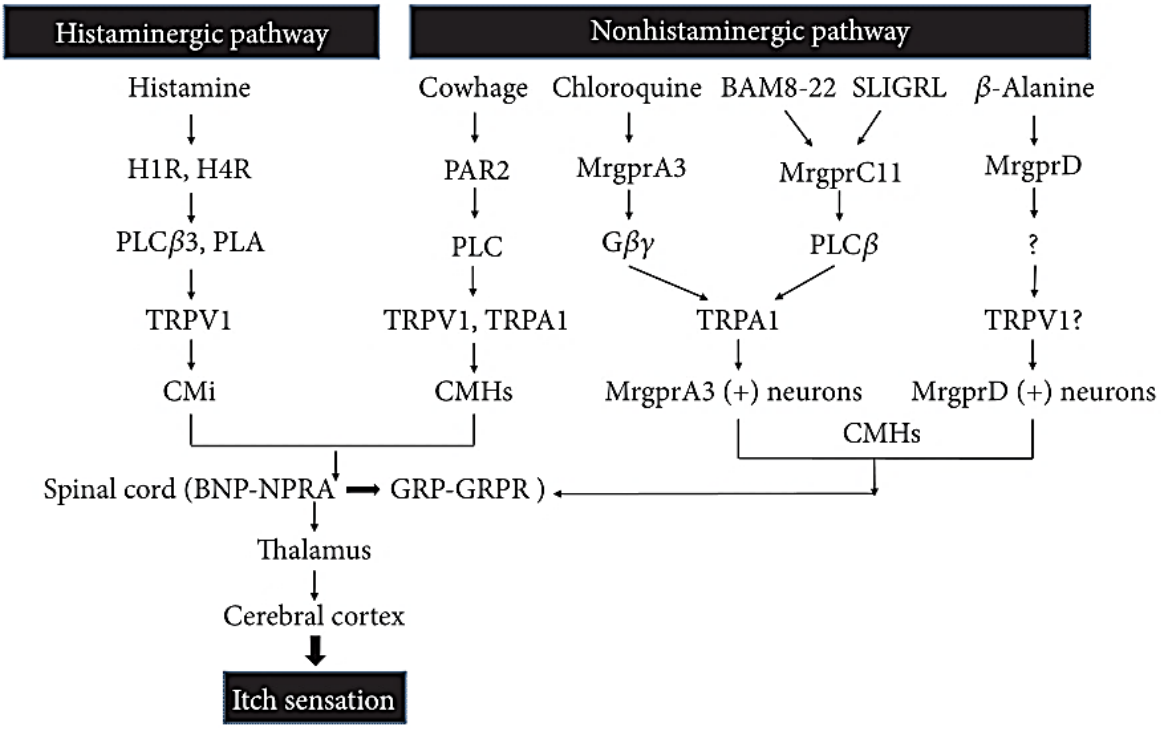

Figure 1.5 Schematic illustration of pruritic signaling pathways. According to different responses, signal pathways of itching are shown as histamine-dependent (histaminergic) signaling pathways and histamine-independent (non-histaminergic) signaling pathways. In the histaminergic pathway, histamine promotes PLCβ3 and PLC activation by binding to their specific receptors, particularly the H1 receptor and H4 receptor. These further induce the activation of downstream target TRPV1. Then, itch signals are transferred to the central nervous system via Cmi, which finally leads to an itchy sensation. Conversely, many pruritogens exist in the nonhistaminergic pathway, such as cowhage, CQ, BAM8-22, SLIGRL, and β-Alanine. For example, cowhage initially stimulates PAR2, which in turn sensitizes PLC. Then the downstream targets, including TRPV1 and TRPA1, are activated. Ultimately, itch signals are transferred to the central nervous system via CMHs, producing an itch sensation. At the same time, Mrgprs are linked and activated by CQ, SLIGRL, BAM8-22, and β-Alanine, further coupled to Gβγ or PLC or other; then they promote TRPA1/ TRPV1 activation, and Mrgpr-positive neurons detect itch signals; via afferent fibers (CMHs), these signals are sent to the spinal cord and are regulated byGRP-GRPR and BNP-NPRA systems; finally, itching sensation is present. PLCβ3, phospholipase Cβ3; TRPV1, transient receptor potential cation channel V1; TRPA1, transient receptor potential cation channel A1; Cmi, mechanically insensitive C-fibers; PAR2, protease-activated receptor; CMHs, mechanically sensitive C-type fibers; BAM8-22, bovine adrenal medulla 8-22 peptide; Mrgprs, Mas-related G protein-coupled receptors; GRP, gastrin-releasing peptide; GRPR, gastrin-releasing peptide receptor. Above figure has been obtained from Song et al. (2018) (Song et al., 2018).

Following these receptor-ligand bindings and signal transductions, specific C fibers (Cmi: mechanically insensitive C-fibers) transmit these signals to the dorsal horn of the spinal cord

shown in (Green) **Figure 1.6.** Further, the signal from the spinal cord is transmitted to the thalamus and the cerebral cortex to produce an itchy sensation (J. S. Lee et al., 2016).

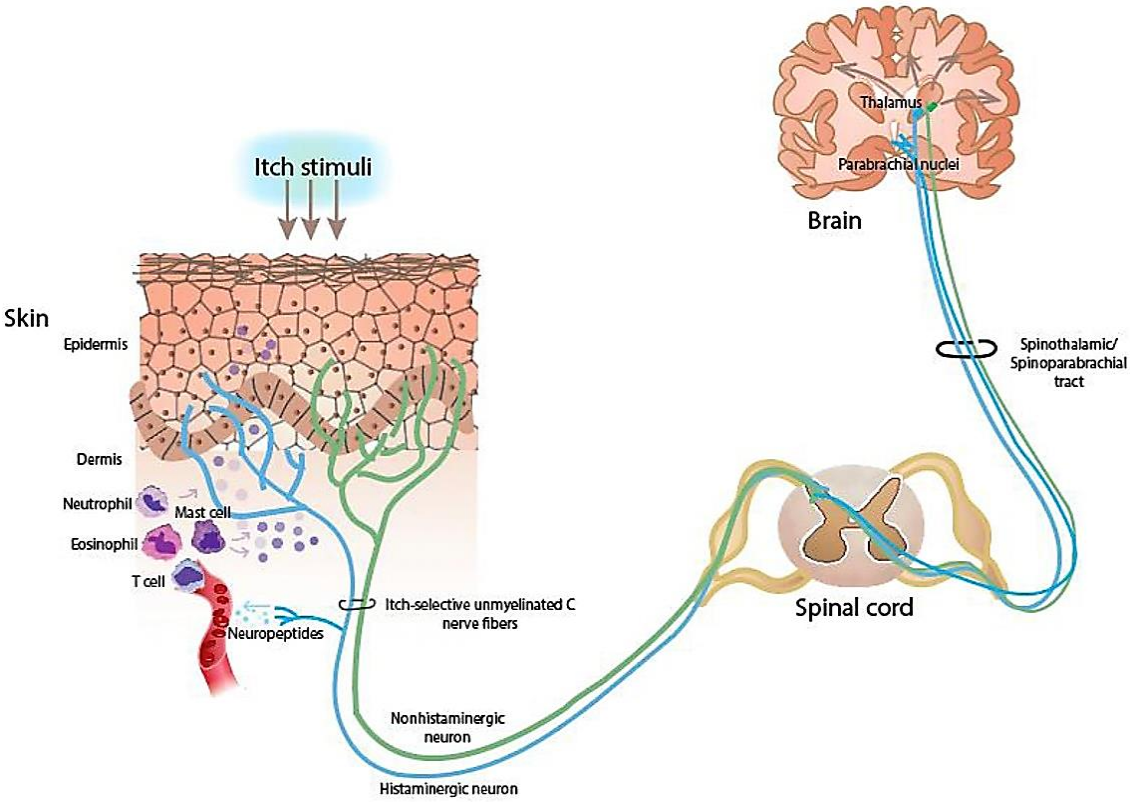

Figure 1.6 Schematic diagram shows two different pathways of itch transmission. Itch stimuli are detected through nerve endings in the epidermis and dermis. Selective C fibers transmit itch depending on the nature of stimuli toward the spinal cord. From the spinal cord, the signal is transmitted to the brain. Above figure has been obtained from source (Fowler & Yosipovitch, 2019).

In contrast, chronic itch is a pathological condition of persistent itch that lasts longer and does not serve a protective function. Itch is considered a chronic itch when it lasts more than six weeks. Chronic itch dramatically impairs quality of life. Chronic itch is associated with pathological conditions such as atopic dermatitis, psoriasis, bacterial infection, kidney disease, and cutaneous T-cell lymphoma etc. Due to the disease's severity and persistency, itch sensation alleviation is a severe issue in patients.

Moreover, some critical factors, such as skin barrier impairment and neuropathic changes, can assist in chronic itch. This barrier is crucial for exogenous object entry and also controls endogenous objects. Disruption of the epidermal barrier can cause pruritogen to enter the

skin and induce itch or cause water evaporation and trans-epidermal water losses, further promoting itch by inducing dry skin. However, the chronic itch has different pathways from the acute itch, and antihistamines are ineffective against several forms of chronic itch. Chronic itch is mediated through a non-histaminergic pathway (Yosipovitch et al., 2018). Non-histaminergic pathways can be evoked by exogenous or endogenous pruritogens (Figure 1.5).

These pruritogens include such as proteases, amines, cytokines, and chemokines. In contrast to the histaminergic pathway, one of the critical differences of the non-histaminergic pathway is its transmission through C fibers. C fibers mediating non-histaminergic pathways are CMHs (mechano-heat-sensitive C-fibers) (shown in (Blue) **Figure 1.6**) with their nerve endings in the epidermis. CMHs transmit signals from the epidermis to the central nervous system (Johanek et al., 2008a).

1.2.4 Mechanisms of itch

The itch mechanism is not fully revealed due to several types of itch and mediators involved in it. To understand how itch signal is transmitted and propagated from the periphery to the central nervous system, mechanisms are categorized into two major parts peripheral and central. In short, primary afferent nerve fibers convey itch signals from the periphery to the spinal cord or brainstem areas through sensory neurons with cell bodies in the dorsal root ganglia and the trigeminal ganglia, respectively. The spinal cord's second-order neurons collect this information and transmit it to the brain (**Figure 1.7**). Brain process this information and interpret it as an itch and send back information through the motor neuron to scratch (Sutaria et al., 2022).

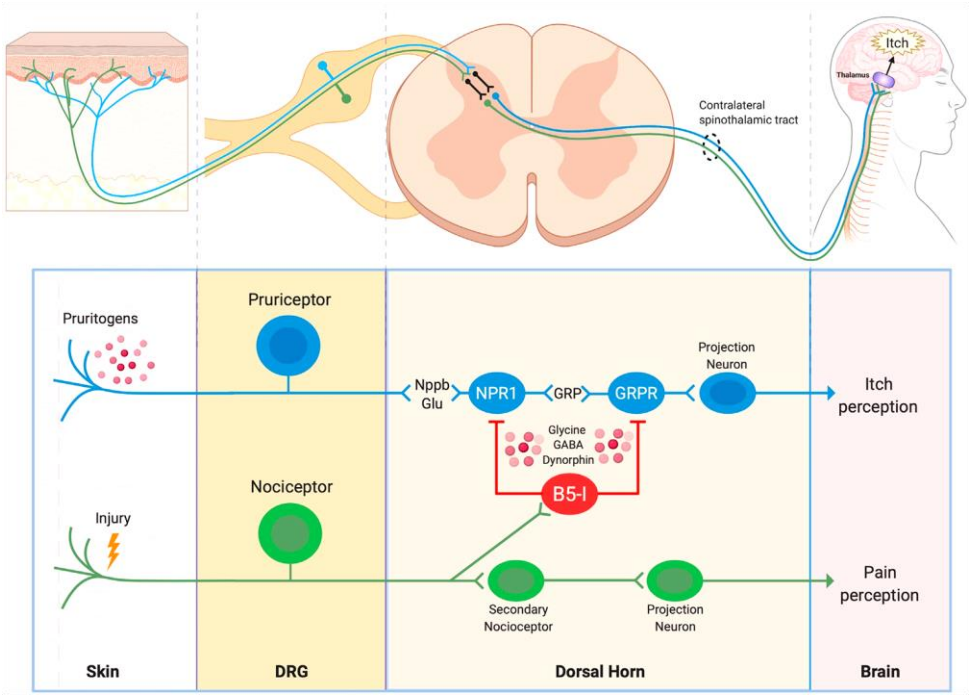

Figure 1.7 Itch pathways in the spinal cord (Sutaria et al., 2022)**.** DRG, Dorsal root ganglion; Nppb, natriuretic peptide B; Glu, glutamate; NPR1, natriuretic peptide receptor 1 positive neurons; GRP, gastrin-releasing peptide; GRPR, gastrin-releasing peptide receptor positive neurons; GABA, gamma-amino butyric acid; B5-I, helix-loop-helix family member B5 (bhlhb5) positive inhibitory interneurons.

1.2.4.1 Peripheral mechanisms

Proper skin functioning and communication with the external environment are crucial for the body survival. Skin detects exogenous stimuli and helps to avoid any damage. For that, peripheral mechanisms are vital in transmitting signals from an exogenous environment to the brain for interpretation. In the pain field, nerve fibers that sense noxious stimuli and transmit pain signals are called nociceptors. Similarly, nerve fibers that sense pruritogen stimuli are named pruritoceptors. In the epidermis, free nerve endings are sensors for examining the environment and communicating with skin cells and other mediators. These sensory nerve fibers are characterized according to their velocity, diameter, and other features. These two nerve fibers are known in this processing, i.e., A-fibers and C-fibers. A-fibers are myelinated and have fast conduction velocity, while C-fibers are unmyelinated fibers with slow conduction velocity. The A-fibers are further divided into Aδ (thinly myelinated) and Aβ-fibers (fully myelinated). The C-fibers are unmyelinated, so they perform

conduction with slow velocity and have smaller diameters than myelinated A-fibers. These C-fibers are involved in itch sensation and transmission (Ikoma et al., 2006). C-fibers can be divided further into two populations of C-fibers: CMH and CMi, as mentioned earlier. Interestingly, CMi fibers respond to histamine and release neuropeptides as substance P and calcitonin gene-related peptides. Both of these neuropeptides help in vasodilation and activation of the mast cell. On the other hand, CMH fibers show no response to histamine but to cowhage. Most itch sensations are transmitted by CMH fibers, as mentioned above in the section on chronic itch.

Several mediators, receptors, and channels are involved in itch transmission at the peripheral level. The receptors for pruritogens belong to mainly three classes, 1) G protein-coupled receptors (GPCR), 2) Toll-like receptors (TLR), and 3) cytokine receptors. Itch-related mediators and their cognate receptors are listed in **Table 1.2**

Table 1.2 Pruritogens and receptors in the periphery, adapted from (Dong & Dong, 2018).

Molecular Mediator	Main Cellular Source	Neuronal Receptor	Ion Channel	DRG Neuron Subtypes	Cause of Itch
Histamine	mast cells	H1R, H4R	TRPV1, TRPV4	NP2, NP3	insect bites, dermatitis
Serotonin (5-HT)	mast cells, keratinocytes	HTR7, HTR2	TRPA1, TRPV1, TRPV4	NP3	atopic dermatitis
Proteases	mast cells, plants	PAR2, MrgprC11	TRPA1, TRPV1	NP2	cowhage, dermatitis
TSLP	keratinocytes	TSLP receptor (IL-7Rα + TSLPR)	TRPA1		atopic dermatitis
IL-31	Th2 T helper cells	IL-31 receptor (IL-31Rα+ OSMR)	TRPA1, TRPV1	NP3	atopic dermatitis, T cell lymphoma
IL-33	keratinocytes	IL-33 receptor (IL-1RacP + ST2)	TRPA1, TRPV1	NP2	allergic contact dermatitis
IL-4 and IL-13	Th2 cells, ILC2s, basophils	Il-4Rα, IL-13Rα1	TRPA1, TRPV1	NP1, NP2, NP3	atopic dermatitis, chronic idiopathic pruritus
Poly I: C, Imiquimod	pathogens, drug	TLR3, TLR7			psoriasis, xerosis (dry skin)
BAM8-22 peptide	keratinocytes	MrgprC11	TRPA1, TRPV1	NP2	xerosis (dry skin)
Chloroquine	medicine in circulation	MrgprA3	TRPA1	NP2	drug-induced itch
β-alanine	medicine in circulation	MrgprD		NP1	drug-induced itch

BAM8-22, bovine adrenal medulla 8–22 peptide; CQ, chloroquine; DRG, dorsal root ganglion; ET-1, endothelin-1; IL-31, interleukin-31; 5-HT, serotonin.

1.2.4.2 Central mechanisms

From the periphery, the itch signal is transmitted to the central nervous system by activating second-order neurons that have their cell body in the spinal cord. From sensory neurons, the signal is transmitted to the spinal dorsal horn by releasing the neurotransmitters glutamate,

gastrin-releasing peptide (GRP), substance P, and brain natriuretic peptide (BNP). Glutamate, GRP, and substance P partially contribute to histamine-independent itch, while histamine-evoked itch is mediated by glutamate with a lesser role of GRP (Akiyama et al., 2014). BNP is essential for itch transmission of histamine-dependent and histamine-independent itch. Receptor for substance P, neurokinin1 (NK1) reduces the itch. Thus, all ascending axonal projections to the thalamus and parabrachial nucleus express NK1 receptors (E. Carstens & Akiyama, 2016; E. E. Carstens et al., 2010). A mix of different antagonists to NK1, GRP, and glutamate receptors inhibit scratching behavior. However, AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) antagonist alone is enough to block histaminergic itch (E. Carstens & Akiyama, 2016; E. E. Carstens et al., 2010).

The inhibitory neurotransmitters GABA (γ-aminobutyric acid) and glycine are mediators of the inhibition of itch. The genetic ablation of inhibitory interneurons resulted in an abnormal increase in itch behavior. In mice lacking *Bhlhb5* (a specific class of inhibitory interneurons expressing the transcription factor *Bhlhb5* which is crucial for the inhibition of itch), excessive scratching was significantly attenuated, and skin lesions were improved (E. Carstens & Akiyama, 2016; E. E. Carstens et al., 2010). This also helped to know about the role of inhibitory neurons in controlling itch.

Concerning the supraspinal transmission of itch, less is known about the role of neurons in the ventrobasal thalamus or parabrachial nuclei that receive direct ascending from pruriceptive inputs. Nevertheless, several human studies have revealed that different brain regions are activated during itch. These include (1) the thalamus, primary and secondary somatosensory cortex, areas involved in recognition of and attention to itch and in the rating of localization and intensity of itch, (2) the cingulate and insular cortex, areas associated with cognition, motivation to act (scratch), and awareness of emotional state and body feeling, (3) the medial parietal cortex, posterior cingulate cortex, and precuneus, areas possibly associated with the subjective sensation of itch, and (4) motor-related areas, including the supplementary, premotor, and primary motor cortices, striatum and cerebellum, areas potentially involved in planning motor responses (e.g., scratching) to itch and affective aspects such as the desire to scratch (Mochizuki & Kakigi, 2015).

As scratching the itch produces reward, it is interesting to mention that scratching activates the same areas that are activated during reward signaling in the brain, including the midbrain striatum, medial prefrontal cortex, anterior cingulate cortex, and orbitofrontal cortex. Brain region and its different part association with several functions are shown in below Figure 1.8.

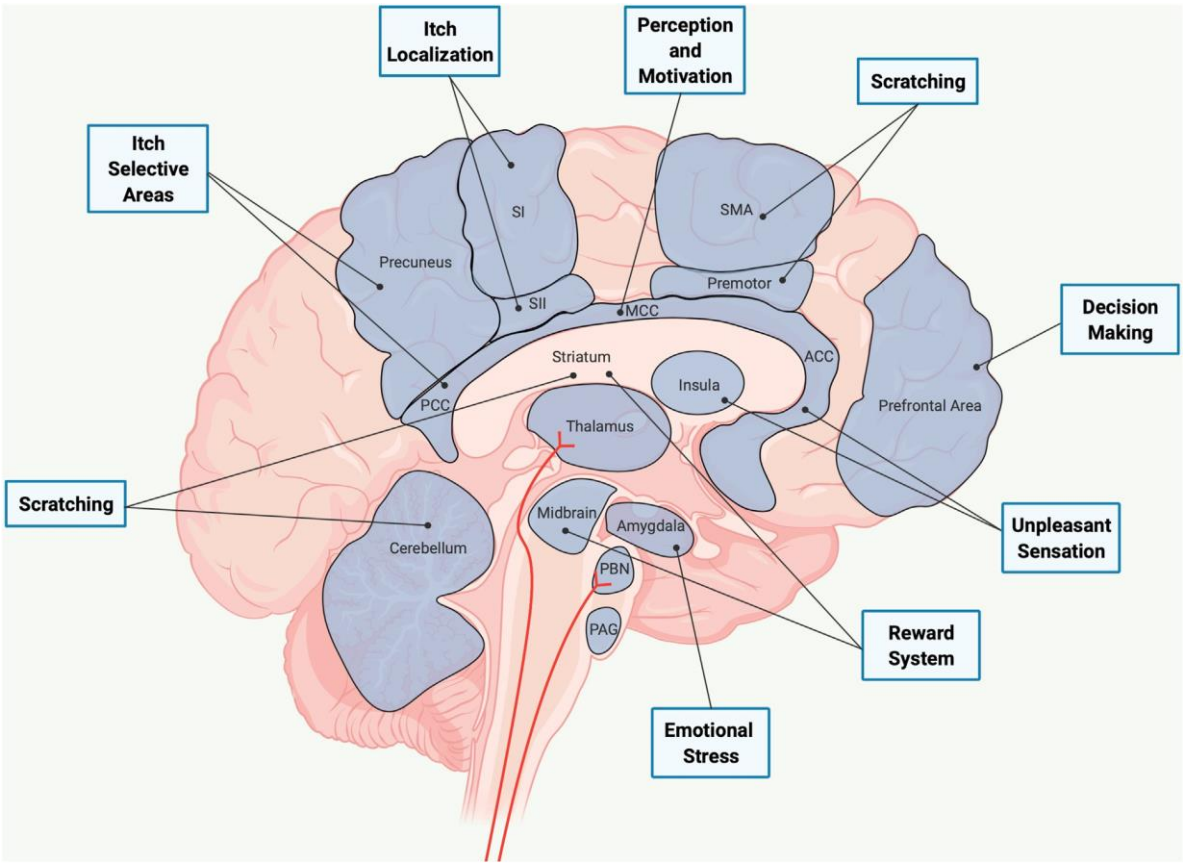

Figure 1.8 Brain regions associated with itch localization, perception, and motivation (Sutaria et al., 2022). PAG, Periaqueductal gray matter; PBN, parabrachial nucleus; PCC, posterior cingulate cortex; SI, primary somatosensory cortex; SII, secondary somatosensory cortex; SMA, supplementary motor area; MCC, midcingulate cortex.

People mimic scratching behavior and feel itchy and scratch themselves when observing others scratching in front of them. This kind of itch is called a contagious itch (Misery et al., 2018). It was also observed in monkeys (Feneran et al., 2013) and rodents (Sanders, Fast, et al., 2019). Interestingly, the histamine-induced itch area is the same brain area as the contagious itch (Holle et al., 2012).

1.2.4.3 Other pathophysiology mechanisms involved in itch

The skin is the body's largest organ and serves as the physical and immunological first line of defense in contact with the external environment. As a result, effective defense mechanisms have evolved to maintain the homeostasis and integrity of the skin (Guimarães et al., 2021).
The essential function of the skin is to provide an effective barrier between the internal and external environments. Thus, the skin acts as an interface between the organism and its external environment and provides both protection and support to the organism. In addition, it provides a physical permeability barrier, hindering excessive water and electrolyte loss in and out, and protects from external chemical, microbial, and mechanical insults. Thus, skin plays an important role in thermoregulation, the absorbance of ultraviolet radiation, sensation, and sociosexual communication (Nguyen & Soulika, 2019; Yang et al., 2020).
The epidermal barrier provides an outside-inside barrier that protects against mechanical, chemical, and microbial injury through the formation of terminally differentiated keratinocytes, a process termed keratinization. The epidermal barrier serves three primary functions: 1) limiting passive water loss, 2) restricting environmental chemical absorption, 3) preventing microbial infection (Chambers & Vukmanovic-Stejic, 2020).
During keratinization, epidermal progressively mature from the basal epidermal layers to form flattened cells of the stratum corneum. After mitosis in the basal layer, keratinocytes differentiate and migrate toward the stratum corneum through the epidermis. The differentiation process yields several keratinocyte layers within the epidermis: the stratum basale, spinosum, granulosum, and corneum (Goleva et al., 2019).
Stratum corneum is the primary mediator of the epidermal permeability barrier, accounting for over major skin functionality. Therefore, properly developing and maintaining the stratum corneum is essential for its remarkable ability to defend the body against chemical and microbial attacks and dehydration (Nguyen & Soulika, 2019).
A major defensive function of the skin is maintaining homeostasis by preventing the uncontrolled loss of water, ions, and serum proteins. The stratum corneum uses diverse strategies to maintain epidermal integrity, including enzymatic reactions, commensal bacterial colonization, immune signaling, antimicrobial lipids and peptides, low pH, and

natural moisturizing factors. The complex tissue of the stratum corneum supports the execution of these strategies. It comprises corneocytes and a matrix of intercellular lipids, with both components derived from the terminal differentiation process of keratinocytes (Nguyen & Soulika, 2019; Yang et al., 2020).

An intact skin barrier is important to limit passive water diffusion through the skin, causing the skin to dehydrate. While accumulating evidence supports a permeability barrier dysfunction in AD. In addition, an abnormal expression of epidermal differentiation-related molecules, such as collagen, filaggrin, loricrin, and involucrin, has been demonstrated in AD patients and mouse models. These molecules are expected to affect permeability barrier homeostasis (Gallegos-Alcalá et al., 2021).

Environmental exposure exacerbates epidermal permeability-depends on individual genetics and is believed to contribute to this disease process. The weakened skin barrier is more susceptible to topical infections that trigger the immune response (Luger et al., 2021). Disruption of barrier function in atopic dermatitis is multifactorial, including genetic mutations such as filaggrin mutations and physical damage from scratching that affects the regulation of immune response and inflammatory processes (Luger et al., 2021). Filaggrin is one of the important epidermal proteins for producing a natural moisturizing factor and is essential for maintaining stratum corneum hydration (Nguyen & Soulika, 2019). Genetic mutations of the filaggrin gene are found in many patients with moderate to severe disease and can be associated with early-onset AD. Also, the deficit in filaggrin production results in aberrant keratinocyte differentiation and insufficient skin lipid content (Y. Kim & Lim, 2021). Also, skin lipids (e.g., ceramides, free fatty acids, and cholesterol) are key for maintaining the epidermal barrier function. Thus, they prevent transepidermal water loss and penetration of irritants, allergens, and microbes (Luger et al., 2021).

In skin structural cells and specialized immune cells work together to maintain barrier immunity. Keratinocytes, the main cells in the epidermis, form a physical barrier through tight junctions and produce antimicrobial peptides. Fibroblasts in the dermis maintain skin integrity by secreting collagen and elastin, while also regulating immune responses. Adipocytes in the subcutaneous fat layer that modulate inflammation and immune cell function. Mononuclear phagocytes, including Langerhans cells, dermal macrophages, and dendritic cells, contribute to immune surveillance and antigen presentation. Resident memory T cells, residing in the

epidermis and dermis, provide rapid immune responses upon re-exposure to pathogens. Together, these cells form a complex network that defends against pathogens, regulates inflammation, and promotes tissue maintenance (Nguyen & Soulika, 2019).

While skin barrier disruption triggers keratinocytes to send proinflammatory and pruritogenic signals through the epidermal alarmins IL-33 and thymic stromal lymphopoietin (TSLP), causing further tissue damage, driving type 2 inflammatory cells and activating type 2 innate lymphoid cells resident in the skin. These lymphoid cells produce IL-5 and IL-13, which activate eosinophils and T helper 2 (Th2) cells (Langan et al., 2020).

Th2 cells release IL-4, IL-5, IL-13, and IL-31, activating B-cells to produce IgE molecules. Additionally, epithelial cell-derived IL-33, TSLP, and type 2 cytokines can activate itch-sensory neurons to induce pruritus. The dynamic interplay between epithelial barrier dysfunction (Hrestak et al., 2022), type 2 immunity, and pruritus is schematically shown in Figure 1.9 below.

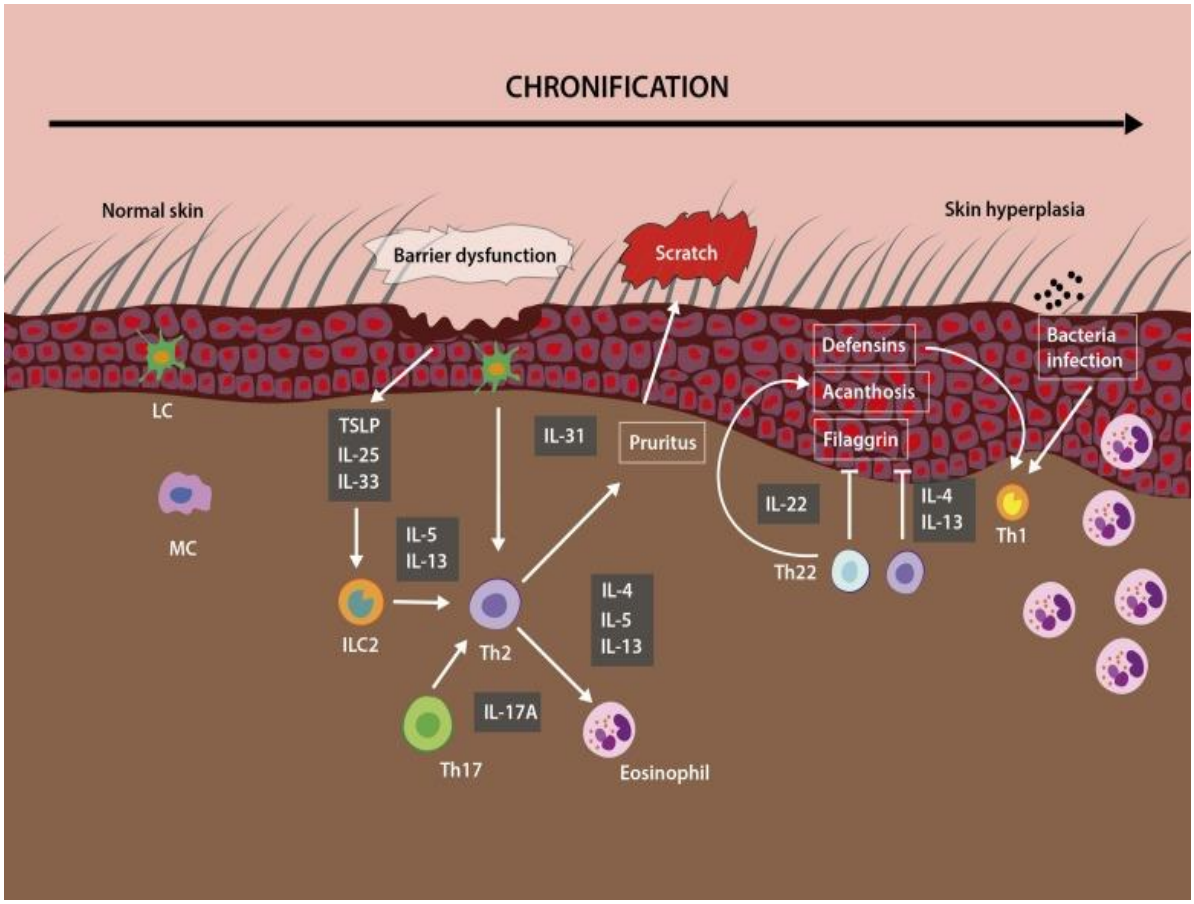

Figure 1.9 Immune responses involved in the chronification of lesions from normal skin to AD lesions. Scratching resulting from pruritus (the main symptom of AD) makes keratinocytes react by triggering cytokines important for inflammation, including TSLP (thymic stromal lymphopoietin), IL-33, and IL-25. IL-33 activates innate lymphoid cells 2 (ILC2) and Th2

lymphocytes. The release of IL-31 stimulates even more the pruritus. IL-17, produced from Th17 cells, also contributes to the pathogenesis of AD. Also, Langerhans cells present the allergen to naive T cells, causing polarization to a Th-2 phenotype which secretes cytokines such as IL-4 and IL-13. In response to IL-22, keratinocytes proliferate, resulting in diffuse epidermal hyperplasia. Eosinophils accumulate in chronic atopic skin. Chronicity leads to a progressive increase of keratinocyte-derived and various immune-cell-derived cytokines. MC: mast cells; LC: Langerhans cells; ILC2: Innate lymphoid cell 2: Interleukin; IL: T helper; Th. Above figure has been obtained from source (Daltro et al., 2020).

During early or acute pathogenesis of AD, Langerhans cells present the allergen to naive T cells, causing polarization to a Th2 phenotype which secretes cytokines, such as IL-4 and IL-13, that stimulate B cells to become plasma cells and secrete allergen-specific IgE (Kader et al., 2021). In addition to cytokines produced from Th1 and Th2 cells, IL-17, produced from Th17 cells, has been reported to contribute to the pathogenesis of AD (Daltro et al., 2020).
The release of IL-31 stimulates even more pruritus. In response to IL-22, keratinocytes proliferate, resulting in diffuse epidermal hyperplasia. In chronic AD skin lesions, a Th1/Th0 dominance has been described with increased interferon-gamma (IFN-γ) production, IL-6, IL-12, and granulocyte-macrophage colony-stimulating factor. In addition, eosinophils accumulate in chronic atopic skin (Daltro et al., 2020).

1.2.5 Mouse models for the itch

With the recent progress of uncovering several possible mechanisms of itch, an additional revolution came in the field of itch due to the use of animal models. To distinguish itch-related behavior (scratching behavior) from other behaviors, such as pain and grooming, scratching by the hind paw is used as an index of itch (Kuraishi et al., 1995). However, it must also be mentioned that it was hard to discriminate between rodents' itch and pain behavior. Generally, algogenic substances injected into the nape of the back of mice also caused a scratching behavior (Shimada & LaMotte, 2008). Therefore, to distinguish itch from pain, a “cheek injection model in mice” was introduced (Spradley et al., 2012). In this model, pruritogens and algogenic substances were injected into the cheeks. Mice responded to pruritogens by evoking scratching with the hind paw and to algogenic molecules by evoking pain through facial wiping with the forelimb (Spradley et al., 2012). Still, it has also been

revealed that the cheek skin was less sensitive than the nap neck skin in responding to pruritogens (Spradley et al., 2012). Therefore, to define and understand the mechanism of itch, rodent models help measure scratching behaviors and study pruritogen actions through genetic manipulations (LaMotte et al., 2011). Further, these mouse models can be divided into two types of itch, acute and chronic itch.

1.2.5.1 Acute itch mouse models

Several mouse models have been developed to study the mechanism of acute itch. The acute itch can be triggered using different pruritogens that can directly activate sensory neurons or non-neuronal cells such as mast cells. Histamine can be injected to mice to trigger acute itch (Laidlaw et al., 2002). Many other compounds or allergens, such as C48/80, can also trigger acute itch, which is initiated through non-neuronal cells, i.e., mast cell degranulation) (Dondalska et al., 2020). Along with the "cheek injection models of mice" as mentioned earlier (Spradley et al., 2012), mouse models for acute itch in eyes were also developed, first in guinea pigs (Laidlaw et al., 2002) and later in mice (Tang et al., 2019).

Mouse models have also been developed to understand the role of histamine receptors in acute itch, for example, the histamine receptor 1 (Inagaki et al., 1999; Nakano et al., 2008; Sugimoto et al., 1998). Recently, a new mouse model for acute itch showed that the ST-1006 H4R selective agonist could induce acute itch intradermally (Ehling et al., 2018).

1.2.5.2 Chronic itch mouse models

Chronic itch is associated with several disorders, including atopic dermatitis, psoriasis, kidney disease, and cutaneous T-cell lymphoma. Various chronic itch mouse models have been developed to understand clinical itch conditions better. Chronic itch models have also been developed for the cutaneous condition in which skin dehydration is accompanied by persistent itching, known as xerosis. These could be due to excessive washing of skin or possibly genetic defects that may lead to skin barrier impairment. The skin barrier protects from intra-epidermal water losses. A dry-skin mouse model was developed in which the skin on the back of mice was shaved and then dehydrated using a mixture of acetone and ether

(Miyamoto et al., 2002). Later, the dry-skin mouse model was also modified to induce dry skin at the hind paw (Akiyama et al., 2010).

Other chronic itch models include toxic contact chronic dermatitis induced by diphenylcyclopropenone (Seike et al., 2005), chronic allergic contact dermatitis induced by 2,4-dinitro-1-fluorobenzene or oxazolone (B. Liu et al., 2013; Saint-Mezard et al., 2003) and the atopic dermatitis NC/Nga genetic line (Grimstad et al., 2009). This was first developed in 1997 (Matsuda et al., 1997) and showed atopic dermatitis-like skin lesions and spontaneous scratching. Similarly, another dermatitis model was also produced by treating mouse skin with allergens such as ovalbumin or squaric acid dibutylester to develop itchy skin with inflammation (Jin et al., 2009). Several genetic mouse models were produced to understand the mechanisms of chronic itch and the roles of different cytokines, receptors, and ion channels, such as IL-31 transgenic mice, to study the IL-31 role (Dillon et al., 2004). Cathepsin E knockout mice were genetically produced to evaluate the role of cathepsin E concerning chronic itch (Tsukuba et al., 2003). These models have been valuable tools for investigating the pathogenesis of chronic itch and its associated mechanisms. Recent advances in mouse genetic engineering have accelerated our understanding of the biological significance of targeted genes in vivo (Sundberg et al., 2020).

Generally, these itch mice models can be assessed through several features related to itch, including;

1) Gross phenotype assessment through behavioral assays, for example, scratching or grooming behaviors and itch barrier dysfunction. 2) Serum profiling, for example, IgE (total or specific). 3) Histology and immunophenotyping. 4) Transcriptome, for example, single-cell sequencing. 5) Electrophysiological Recordings: This technique involves recording electrical activity from nerve fibers or neurons in itch signaling. It can help identify the neural pathways and mechanisms underlying itch sensation. For example, microneurography can be used to record activity from peripheral nerves in the skin. 6) Pharmacological Interventions: Various compounds, such as anti-itch drugs or neurotransmitter modulators, can be administered to mice to assess their effects on itch behavior. This can help understand the role of specific molecules or pathways in itch transmission, processing, etc. (D. Kim et al., 2019).

Along with other mouse models for itch, genetic mouse models are time-consuming, expensive, and costly to generate. However, they can provide several benefits in understanding itch pathogenesis and developing novel therapeutic agents (shown in **Table 1.3**). These include;

1) Elucidating gene-specific functions: they allow to manipulate the expression of specific genes associated with itch-related pathways. By studying mice with altered expression of specific genes, researchers can gain insights into the gene-specific functions and their contributions to itch pathogenesis.

2) Investigating mechanisms: genetic manipulation in mice provides a valuable tool to examine the underlying mechanisms of itch. Genetic changes that affect the itch sensation, neural pathways, neurotransmitters, and immune responses can be studied by altering the expression of itch-related genes.

3) Crossbreeding and comorbidity studies: genetic mice can be crossed with other mouse strains or models to study complex interactions and comorbidities associated with itch. Introducing genetic modifications related to itch into mice with other conditions or strains can help explore the relationship between itch and other diseases, such as atopic dermatitis, psoriasis, or chronic pain.

4) Drug screening and development: genetic mouse models can help to validate potential therapeutic targets for itch treatment. This validation of target molecules aids in drug screening, developing, and optimizing novel therapeutic agents.

5)Translational research: genetic mouse models can help to bridge the gap between preclinical research and clinical application. Findings from mouse studies can guide the design and implementation of clinical trials for itch treatments in humans. In addition, the knowledge gained from mouse models provides a foundation for developing therapeutic strategies, optimizing dosages, and identifying the treatment's potential side effects or limitations (Ehling et al., 2018; D. Kim et al., 2019; LaMotte et al., 2011; Nakajima et al., 2019; Nunomura et al., 2019).

Table 1.3 Characteristics of genetically engineered mouse models for itch adapted from (Jin et al., 2009; D. Kim et al., 2019; Nakajima et al., 2019)

Model	Gene or inducer	Transgene expressing cells	Phenotype					
			AD-like dermatitis	Prurits	Xerosis	Increase in IgE/IgG1 levels	TH2 cytokine/ chemokine (IL-4, IL-13, and TSLP)	Microbial community alterations
Flaky tail; ft mice	*Filaggrin (Flg)* and Tmem79 recessive loss of function	-	+ (acute, chronic)	+	+	+	+	+
Flgft mice	*Flg* recessive loss of function	-	±	+	+	ND	ND	ND
Ma/ma mice	*Tmem79* recessive loss of function	-	+ (acute, chronic)	+	+	+	+	ND
***Flg*-/-Tg mice**	FLG targeted deletion	-	-	+	+	+	ND	ND
***Asprv1*-/-Tg mice**	Skin-specific retrovirus-like aspartic protease-targeted deletion	Ubiquitous	-	-	+	-	-	ND
***Prss8*-/-Tg mice**	*CAP1/Prss8 targeted deletion*	Keratin-14+cells	-					
Blmh-/-Tg mice	Bleomycin hydrolase–targeted deletion	Ubiquitous	-					
Casp14-/-Tg mice	Caspase-14–targeted deletion	Ubiquitous	+	-	+	-	-	+
Klk7 Tg mice	KLK7 overexpression	Ubiquitous	-	+	+	ND	ND	ND
Spink5 Tg mice	Serine protease inhibitor Kazal-type 5 targeted deletion	Ubiquitous	+	ND	+	ND	ND	ND
Cdsn-/-Tg mice	Corneodesmosin-targeted deletion	Ubiquitous	+	ND	+	ND	ND	ND
Cldn1-/-Tg mice	Claudin-1–targeted deletion	Ubiquitous	-	-	+	ND	ND	ND
Apoc1 Tg mice	Apolipoprotein C1 overexpression	Ubiquitous	+ (acute, chronic)	+	+	+	+	ND
NC/Nga mice	Spontaneous development; unknown	-	+	+	+	+	+	+

	genetic defect on chromosome 9 loci							
Adam17 Tg mice	*Adam17*-targeted deletion	Sox9-expressing cells	+	+	+	+	+	+
IL-4 Tg mice	IL-4 overexpression	Keratin-14+cells	+ (acute, chronic)	+	+	+	+	ND
IL-5 Tg mice	IL-5 overexpression	Keratin-14+cells	±	+	-	ND	ND	ND
IL-13 Tg mice	IL-13 overexpression	Keratin-5+cells	+ (acute, chronic)	+	+	+	+	ND
IL-31 Tg mice	IL-31 overexpression	Ubiquitous	+ (chronic)	+	-	-	ND	ND
IL-33 Tg mice	IL-33 overexpression	Keratin-14+cells	+ (acute, chronic)	+	+	+	+	ND
TSLP Tg mice	TSLP overexpression	Keratin-5+cells	+	+	+	+	+	ND
STAT6VT mice	STAT6 overexpression	T and B lymphocytes	+	+	ND	ND	+	ND

1.2.6 Therapeutic strategies for the itch

Acute itch being simpler compared to chronic itch, appears easier to treat and manage. On the other hand, chronic itch is more complex as it may be based on more mediators, receptors, channels, and yet unknown factors. This makes treating and managing chronic itch more challenging and demanding. Itch is problematic when there is no visible lesion on the skin. Only skin examining remains insufficient to assess itch as itch could have a systemic cause. Therefore, it is vital to identify itch at the cutaneous level and through the wide range of other known causes. As itch causes are multiple, there cannot be a standard method or recommendation for itch treatment. The first step for effective treatment and management of itch is to identify the precise cause of the itch and determine the type of itch in each particular patient (Leslie, 2016).

The first treatments for itch are topical therapies. They include coolants, inhibitors, and local anesthetics, dependent on the type of itch. In the second option, with the advancement of research, many proteases, peptides, receptors, and their functions are known, and they can

also be used as therapeutic targets. For the treatment of itch, several itch mediators and sensitized neurons (in PNS or CNS) can be targeted (Raap et al., 2011). The commonly well-known treatment of itch used so far is antihistamines, which can aid patients in comfortable sleep and break the itch-scratch cycle. While, as already mentioned, chronic itch is involved in an independent histamine pathway, it might be treated differently using either mu-opioid antagonists or kappa opioid agonists (Mores et al., 2019) along with other possible treatments like phototherapy and antidepressants (**Figure 1.10**).

Similarly, such treatments as antihistamines can help patients sleep comfortably and break the itch-scratch cycle. Moreover, it is essential to know about the patient's medical history. Possible factors that can be important include age, use of recent medicines, the intensity of the itch, and many other points in questionnaires to assess the itch and recommend the best available treatment and possible way for patients to manage their itch (Weisshaar et al., 2019).

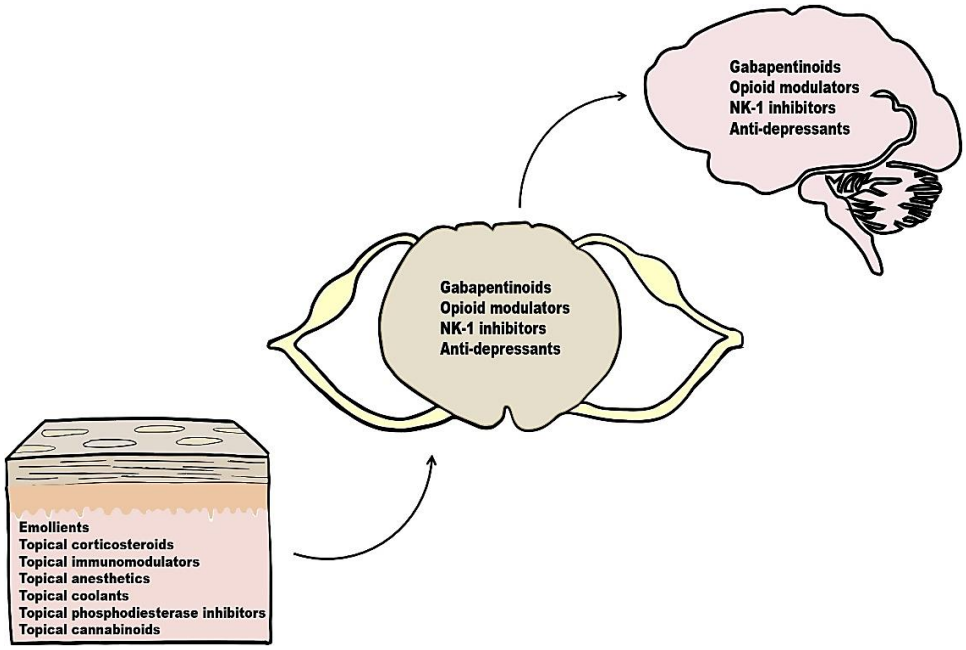

Figure 1.10 Site of action of drugs that inhibit pruritus. Drugs developed to inhibit pruritus can work at the level of the skin, spinal cord, or central nervous system (*NK-1 = neurokinin receptor*). Above figure has been obtained from source (Golpanian et al., 2020).

1.2.6.1 Topical therapies

Topical therapy treatments are considered the first-line treatments for mild itch and also generally for acute itch cases. It could be effective when the itch is localized, and other treatment options may have side effects or be harmful according to the initial situation of patients.

Coolants: the temporary relief from itch can be achieved through menthol (C10H20 O) in an aqueous cream mixture. The usage of menthol l is due to its properties such as cooling, analgesic, and antiseptic effects. Other coolants include calamine which contains zinc and ferric oxides. Also, camphor has been used for centuries for the treatment of itch. Therefore, it could come in topical and systemic therapy approaches (Weisshaar et al., 2012).

Emollients and soaps: Moisturizing creams are important to repair the skin barrier, especially in dry skin. Dry skin impairs the skin barrier and can induce scratching, allowing pathogens and allergens to enter the skin. In addition, transepidermal water loss (TEWL) can also impair the epidermal skin barrier and thus induce itch, which can be intensified during the night due to more TEWL losses. (Ali & Yosipovitch, 2013; Alpizar et al., 2013).

Local anesthetics: Capsaicin is a compound extracted from chili peppers and used as a pain reliving medication. As explained earlier, Capsaicin acts as an antipruritic agent through TRPV1, which is involved in itch. Through the depletion of substance P and unmyelinated C-fiber desensitization, capsaicin can help to relieve the itch. Capsaicin can be used for several itch disorders, including notalgia, paresthetica (a condition where the skin of the upper back becomes itchy), and postherpetic neuralgia. One topical treatment for 30 – 60 min produces pain relief with minor side effects. Though its high concentration, topical capsaicin 8% for the treatment of neuropathic itch was found effective (Lo Vecchio et al., 2018).

Glucocorticoids: Glucocorticoids are anti-inflammatory agents. Glucocorticoids are effective on different skin inflammatory conditions with high and moderate potency (Weisshaar et al., 2019). In addition, clinical data suggest they help treat itch-associated diseases like prurigo nodularis (Weisshaar et al., 2019). Due to glucocorticoids being effective against inflammation, it is known that they control inflammation by inhibiting cytokines. In this way, they can control inflammation and itch associated with skin inflammation.

Immunomodulators: Calcineurin Inhibitors, mainly including Tacrolimus and pimecrolimus, reduce itch in inflammatory skin disease (Kaufmann et al., 2006). These inhibitors successfully itch associated diseases such as prurigo nodularis and others. These inhibitors regulate the activation of T-cells and inhibit cytokine release. Calcineurin Inhibitors have anti-inflammatory and antipruritic effects(Ständer et al., 2006).

Prostanoid Inhibitors; Acetylsalicylic acid has an antipruritic effect as it inhibits prostaglandin E2 production, a well-known itch enhancer (Yosipovitch et al., 2001). However, with new European guidelines for chronic itch, experts cannot recommend topical acetylsalicylic acid treatment for chronic itch (Weisshaar et al., 2019).

Antihistamines and antidepressants drugs: Topical treatment of antihistamines has inconsistency and lacks proper functioning, but still, for example, doxepin an antidepressant drug (orally), can be used as topically 5% in the form of cream. It has been used successfully to treat atopic dermatitis. It has the main characteristics of acting as anti-H1 and anti-H2 and is considered adequate in different cases such as nummular and contact dermatitis. However, as mentioned earlier, due to limitations in the usage of antihistamines as a topical treatment of itch, it shows ineffective results in several other conditions (Patel & Yosipovitch, 2010).

1.2.6.2 Systemic therapies

Systemic antihistamines; are well-known and most commonly used drugs for treating itch. They are used for both the dermatological and non-dermatological causes of itch. 1st generation Anti-histamine can cause sedation effects; these include-chlorpheniramine, diphenhydramine, hydroxyzine, and promethazine. They can bind to H1, dopamine and serotonin receptors, and muscarinic and alpha-adrenergic receptors (Zuberbier et al., 2014). Due to their sedative effect, they are recommended for use at night when they can help not only comfortable sleep but also help to break the itch-scratch cycle in several types of dermatoses. On the other hand, 2nd generation antihistamines such as cetirizine, levocetirizine, desloratadine, ebastine, fexofenadine, and loratadine are effective for longer duration and have a less sedative effect.

Glucocorticoids; Generally, patients with inflammatory problems related to itch, such as urticaria, dermatitis, and bullous pemphigoid can be treated with glucocorticoids. Prednisolone is commonly used as a systemic corticosteroid against inflammatory itch but is recommended to use for a short time to avoid potential adverse effects (Patel & Yosipovitch, 2010).

Gabapentinoids; Gabapentin and pregabalin are structure analogs to γ-aminobutyric acid (GABA) neurotransmitters and can be effective against neuropathic itch and pain (depending on the patient for pain). They were initially known as anti-epileptic drugs but are also used as a successful treatment for chronic neuropathic itch (Martinelli-Boneschi et al., 2017). Pregabalin is similar to the structure and function of gabapentin and is a more recent compound. It has fewer side effects, and patients are treated with lower doses than gabapentin. Both gabapentin and pregabalin have side effects of weight gain, drowsiness, ataxia, blurred vision, and constipation. The dosage of both drugs is adjusted according to the patient's condition, itch intensity, and other factors (Weisshaar et al., 2019).

Antidepressants; are used for the treatment of several types of itch. These drugs, such as mirtazapine, can modulate the serotonergic and adrenergic systems and reduce itch by inhibiting the reuptake of serotonin or noradrenalin. However, combining with gabapentin or pregabalin can be more effective in treating cutaneous T-cell lymphoma (Ständer & Grundmann, 2012).

Opioid modulators; can be used against an imbalance of the opioid system that causes an itch sensation. Mu opioid receptor (MOR) antagonists and κ-opioid receptor (KOR) agonists have demonstrated positive results against itch (Dawn & Yosipovitch, 2006). The other opioid antagonists, naltrexone and naloxone, have also shown beneficial results in treating atopic dermatitis and uremic pruritus (B. Lee & Elston, 2019).

Neurokinin-1 Inhibitors, Substance P, is a neuropeptide that induces itch and pain after activating the neurokinin type 1 receptor. One drug to target neurokinin type 1 receptor is Aprepitant, an oral antiemetic drug that blocks substance P action on neurokinin type 1 receptors. Aprepitant is effective against pruritus associated with prurigo nodularis and lymphoproliferative diseases. (Tsianakas et al., 2019).

Immunosuppressants; are used orally for the treatment of inflammatory itch. These are corticosteroids, cyclosporine A, methotrexate, azathioprine, and mycophenolate mofetil. (Weisshaar et al., 2019).

Ultraviolet phototherapy; can be used to treat chronic itch. Studies have shown that ultraviolet B (UVB) radiation alone or with ultraviolet A (UVA) radiation is effective against itch caused by chronic kidney disease. Moreover, it also has a beneficial effect on itch related to the following diseases psoriasis, atopic eczema, and cutaneous T-cell lymphoma (Wallengren, 2010; Weisshaar et al., 2019).

Behavioral-modification therapies; comprise patient education regarding coping mechanisms to handle stress to interrupt the itch-scratch cycle. In patients with coexisting depression, psychotherapy in combination with psychotropic medication can be helpful even in treating chronic itch of different etiology (Weisshaar et al., 2019).

Holistic approaches; *view* something as a whole interconnected entity, understanding the bigger picture. These approaches are also used to control the itch. As itch is aggravated by stress, some holistic mindfulness techniques can benefit patients (Golpanian et al., 2020). This mindfulness techniques approach can be meditation, yoga, psychoeducation, cognitive behavior therapy, acupuncture (Schut et al., 2013).

Several topical and systemic treatment therapies are discussed above. Due to the involvement of multifactorial mechanisms, the best treatment can be chosen according to the patient's conditions out of several possible therapies. The treatment of itch, therefore, varies from case to case. With further advancement of our understanding and new techniques, we may have more improved and advanced treatments in the near future.

1.3 Collagens

In this part, I want to introduce briefly the collagens family and then focus on the Collagen VI, its role in skin and nerve tissues, which are relevant to my project.

The term collagen derives from the Greek words for 'glue' and 'to produce (gen, to generate)'. It has been considered biological glue due to its known function of holding cells together. Collagens are fibrous proteins and the principal structure protein in the ECM. They are the

most abundant proteins in both vertebrates and invertebrates. They constitute around 30% of total proteins in mammals (Ricard-Blum, 2011). They are quite rigid, inextensible, fibrous proteins that are principal components of connective tissues in mammals, including tendons, cartilage, bones, teeth, skin, and blood vessels. They have been known for a very long time.

Collagens are major components of the extracellular matrix (ECM), a highly dynamic material that undergoes a continuous turnover in order to maintain the biological and structural integrity of cells and tissues. ECM is also responsible for playing both structural and signaling roles. Although the topological and biochemical composition of the ECM in each tissue is unique, many components are common for all different connective tissues. The ECM comprises various types of proteins and polysaccharides, which are secreted by local cells and assembled into unique structures in the extracellular space. The most abundant class of ECM components are the structural proteins, which consist of collagens and elastin. The major roles of the ECM are to provide physical support for cells and regulatory signals that modulate cellular growth, metabolism, differentiation, migration, proliferation, and survival during physiological and pathophysiological conditions (Lamandé & Bateman, 2020).

The importance of the ECM in normal development is demonstrated by numerous examples of embryonic lethality or functional disorders caused by experimental deficiency or mutation, as well as in a multitude of genetic diseases (Lamandé & Bateman, 2020; Naba et al., 2016; Shao et al., 2020). In next part of this thesis, I will mainly emphasize on the collagens and its associated diseases.

1.3.1 The collagen family

The collagen family comprises 28 members in vertebrates with given Roman counting (I-XXVIII). Collagen has three critical features that distinguish them and keep them under the umbrella of the collagen family (Ricard-Blum, 2011). These features are 1) they have an amino acid repeated sequence [Gly–X–Y]. 2) X is often replaced by proline, and Y is often replaced by hydroxyproline. 3) They form a unique quaternary collagen structure with a right-handed triple helix, assembled by three left-handed identical polyproline-II α-chains. Even simple multicellular organisms express prototype genes of collagens. In vertebrates, they are the main components of connective tissues, making substantial proportion of the total protein

(around 30%) in the human body and substantial majority of the dry weight of human skin. It is approximately 80% of the organic matter of bone to 90% of tendon and corneal tissue. It gives us an idea of how extensive and essential the collagen family is in the animal kingdom. Collagen family members were discovered based on their blueprint sequence [Gly–X–Y]n (Sorushanova et al., 2019). However, tissue distribution and function of many types of collagens are still not entirely revealed. Also, in the collagen family, all types are not equally distributed in different tissue; basically, collagen I, II, and III have a major share in the family. It comprises 80-90% of total body collagen (Shoulders & Raines, 2009; Sorushanova et al., 2019). All types of collagens, their chains, and their tissue distribution are given in **table 1.4.**

Table 1.4 Collagen family characteristics and tissue distribution adapted from (Sorushanova et al., 2019) .

Collagen Type	Chains	Structure	Distribution
I	α1(I),α2(I)	Fibrillar	Skin, tendon, ligament, cornea, dura mater of brain and spinal cord, bone
II	α1(II)	Fibrillar	Cartilage, tendon, intervertebral disc
III	α1(III)	Fibrillar	Dermis, aorta, uterus, tendon, intestine, blood vessels, liver
IV	α1(IV), α2(IV), α3(IV), α4(IV), α5(IV), α 6(IV)	Non-fibrillar meshwork	Basement membranes
V	α1(V), α2(V), α3(V), α4(V)[1]	Fibrillar	Placental/embryonic tissue, dermis, bone, cornea
VI	α1(VI), α2(VI), α3(VI), α4(VI)[2], α5(VI), α 6(VI)	Beaded filament	Uterus, dermis, cartilage, muscle
VII	α1(VII)	Anchoring fibrils	Skin, amniotic membrane, cornea, mucosal epithelium
VIII	α1(VIII), α2(VIII)	Nonfibrillar, hexagonal lattice	Heart, brain, liver, lungs
IX	α1(IX), α2(IX), α3(IX)	FACIT[3]	Cartilage, tendon
X	α1(X)	Nonfibrillar, hexagonal lattice	Cartilage
XI	α1(XI), α2(XI), α3(XI)	Fine fibrils similar to those of collagen V	Cartilage, intervertebral disc
XII	α 1(XII)	FACIT,	Dermis, tendon, cartilage
XIII	α1(XIII)	Transmembrane	Endothelial cells, epidermis
XIV	α1(XIV)	FACIT	Dermis, tendon, cartilage

XV	α1(XV)	Multiplexin (multiple triple-helix domains with interruptions)	Placenta, kidney, heart, ovary, testis
XVI	α1(XVI)	FACIT,	Heart, kidney, muscle
XVII	α1(XVII)	Transmembrane	Hemidesmosomes (skin), specialized epithelia
XVIII	α1(XVIII)	Multiplexin	Kidney, liver
XIX	α1(XIX)	FACIT	Interneurons, hippocampal synapses formation, basement membranes, muscle cell
XX	α1(XX)	FACIT	Corneal epithelium, embryonic skin, cartilage, tendon
XXI	α1(XXI)	FACIT	Blood vessel walls, secreted by smooth muscle cells
XXII	α1(XXII)	FACIT	Tissue junctions
XXIII	α1(XXIII)	Transmembrane	Tumors (prostate)
XXIV	α1(XXIV)	Fibrillar, fibril associated	Brain, kidney, liver, lungs
XXV	α1(XXV)	Transmembrane	Brain, heart, testis, eyes
XXVI	α1(XXVI)		Ovary and testis
XXVII	α1(XXVII)	Thin nonstriated fibrils	Bones, cartilage
XXVIII	α1(XXVIII)	Beaded filament forming	Basement membrane of Schwann cells, the peripheral nervous system

[1] The α4(IV) chain is solely synthesized by Schwann cells.
[2] The α4(VI) chain does not exist in humans.
[3] Fibril-associated collagens with interrupted triple helices (FACIT)

1.3.2 Structure and functions of collagens

Collagens consist of three polypeptide chains, called α chains. Collagen α chains vary in size. The three α chains can be either identical to form homotrimers or different to form heterotrimers. The three α chains of fibril-forming collagens coil together to form a collagen triple helix and form a rope-like structure with a molecular weight ≈of 300 000 g/mole, length of 280 nm, and a diameter of 1.4 nm. Further, for stabilizing these helix intermolecular hydrogen bonds are important. Two types of hydrogen bonds play a key role in stability of the collagen triple helix: one between the amine group of a glycyl residue and the carboxyl group of the residue in the second position of the triplet; a second hydrogen bond involves a water

molecule interacting with the hydroxyl group of hydroxyproline in the third position. The right-handed triple helix is assembled by three left-handed identical polyproline-II α-chains, which is again the unique feature of collagens. After coiling three left-handed identical polyproline-II α-chains, they form a structure called procollagen. Further, the procollagen peptidase trims the terminal sides of procollagen, and the remaining structure is called tropocollagen. This tropocollagen can self-assemble and form collagen fibril which can further self-assemble and form collagen fiber (**Figure 1.11**). Out of these steps, procollagen formation occurs in the Golgi apparatus, while the remaining step occurs outside the fibroblast. For example, a two-dimensional network formed by type IV collagen, characterized by its unique structural flexibility is a defining feature of the basal lamina(Abreu-Velez & Howard, 2012; Sorushanova et al., 2019).

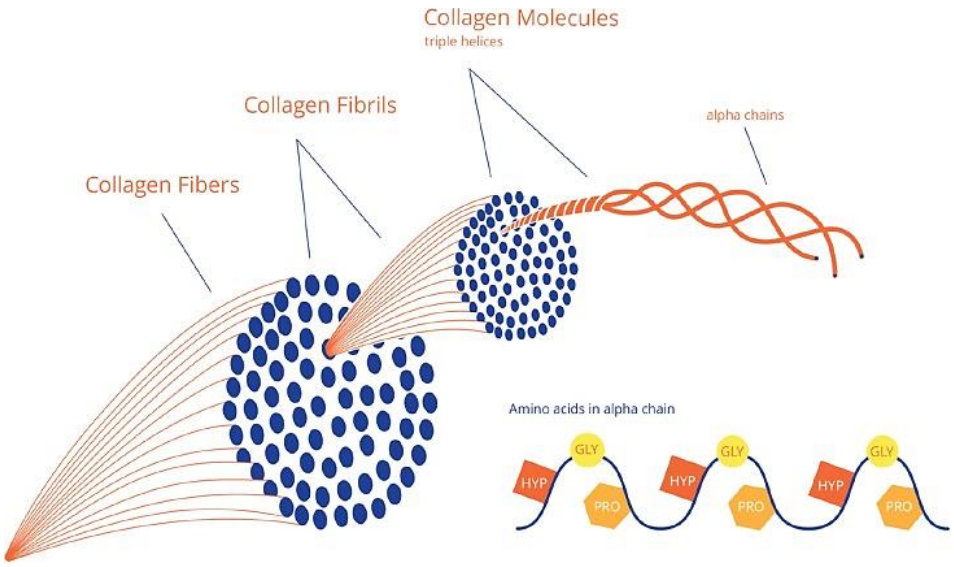

Figure 1.11 Multiple collagen fibrils form collagen fibers. Amino acids are shown in the alpha chain proline (PRO), glycine (GLY), and hydroxyproline (HYP). Above figure has been obtained from source (Nijhuis et al., 2019).

Collagens can be divided into several subfamilies depending on their domain structure and their macromolecular assembly. These subfamilies include fibrillar collagens, fibril-associated collagens with interrupted triple helices (FACIT) collagen, network-forming collagens, transmembrane collagens, multiplexin, anchoring fibrils, and beaded-filament forming collagens (Onursal et al., 2021) Here below I have discussed few subfamilies of collagen and some of their important functions:

Fibrillar collagens: Fibrillar collagens, such as types I, II, III, V, XI, XXIV, and XXVII are structural proteins extensively studied for their diverse roles in various tissues. Collagens type I, III, and

V are widespread, while collagen II is primarily found in cartilage alongside collagen XI, which is important to regulate fibril assembly in tendons. Collagen XXIV serves as a marker for differentiation of osteoblast and bone formation while collagen XXVII organizes the pericellular matrix in the growth plate. In tissues, these collagens self-assemble into fibrils with various diameters and form diverse three-dimensional structures. Fibrillar collagens are stabilized by covalent cross-links, additionally glycation also contributes to cross-link formation. Post-translational modifications such as hydroxylation and glycosylation, are key features of these collagens. Mutations in collagen genes are associated with various disorders, for example Ehlers-Danlos syndrome and other skeletal disorders. Understanding and exploring the fibrillar collagens provides insights into tissue development, mechanical properties, and implications for diverse pathological conditions (Bella & Hulmes, 2017; Karamanos et al., 2021).

Fibril-associated collagens with interrupted triple helices (FACITs): FACITs include collagen IX, XII, XIV,XVI, XIX, XX, XXI and XXII. FACITs have interruptions in the triple helical domain and found at the surfaces of collagen fibrils (Kadler et al., 2007). XIV collagen is prevalent in skin, tendon, cornea, and articular cartilage. Collagen XIV collagen is often found particularly in areas of high mechanical stress. Collagen XIV collagen is widely expressed throughout development, maturation, and aging in connective tissues. Collagen XIV contributes to organize and stabilize ECM by stabilizing collagen fibrils and anchoring microfibrils to the basement membrane (Ansorge et al., 2009). It also play role in mediating intracellular signaling affecting cell adhesion, proliferation (Sand & Karsdal, 2016).

Network-forming collagens: Collagens IV, VIII, and X are network forming collagen. They make distinct network with different combination of α chains. Collagen IV forms networks in basement membranes and contributes to the structural integrity and stability of these membranes. Collagen VIII synthesized by endothelial and smooth muscle cells, regulate corneal endothelial cell fate (Hwang et al., 2020). It is also implicated in atherosclerosis and fibrosis. Collagen X regulates the growth plate and is crucial for endochondral ossification in articular cartilage (Melrose et al. 2016).

Transmembrane collagens: These transmembrane collagens including XIII, XVII, XXIII and XXV play diverse roles in tissue development, integrity, cancer progression, and

neurodegenerative disorders. Collagen XIII is involved in the development, differentiation, and maintenance of musculoskeletal tissues and blood vessels, as well as the formation and function of the neuromuscular system. Collagen XVII is primarily expressed in basal keratinocytes and is crucial for the integrity of the epidermal-dermal junction and serves as a key player in maintaining skin integrity and associated structures (Natsuga et al., 2019; Nyström & Kiritsi, 2021). Collagen XXIII is found on the surface of basal keratinocytes and involved in cancer cell adhesion and metastasis. Collagen XXV is synthesized by neurons and is found in Alzheimer's amyloid plaques. It also promotes myoblast fusion and muscle formation (Jones et al., 2020).

Multiplexin: This category comprising collagen XV and XVIII. Collagen XV has connecting striated collagen fibers beneath the basement membrane and plays a role in regulating cell adhesion and migration. Collagen XVIII is essential for maintaining basement membrane integrity and is involved in regulating cell survival, stem or progenitor cell maintenance and differentiation, as well as inflammation, along with particular crucial role in eye development (Seppinen & Pihlajaniemi, 2011).

Beaded-filaments forming collagens: Collagen VI forms beaded filaments with a periodicity of 100 nm and plays a critical role in skeletal muscle. It is also essential for the integrity and function of the neuromuscular junction and is present in both the peripheral and central nervous systems (Gregorio et al., 2018). More detail about structure, assembly and its expression are explained in next section of this thesis.

1.3.3 Human diseases due to collagen mutations

Over 1000 mutations have been characterized in 22 genes encoding polypeptide chain

Table 1.5). Previously, most mutations were associated with only five genes, *COL1A1, COL1A2, COL3A2,* and *COL4A5*. The majority of these mutations were single-base substitutions that either change the codon of a critical amino acid or lead to abnormal RNA splicing. At the same time, other mutations include gene deletions, insertions, duplications, and complex rearrangements. The vast majority of the single base substitutions identified thus convert a codon for the obligatory glycine in a Gly-X-Y- triplet to a bulkier amino acid. It may either completely prevent the folding of the triple helix beyond this point or may generate interruption. The triple helix of most collagens is propagated from the C terminus to the N terminus, so a glycine mutation nearer to the C-terminal end of a triple-helical domain may produce a more severe phenotype than an N-terminal mutation (Myllyharju & Kivirikko, 2001). Mutations could also affect the extracellular matrix by decreasing the amount of secreted collagen, leading to the impaired molecular and supramolecular assembly through the secretion of mutant collagen. That could lead to endoplasmic reticulum stress and the unfolded protein response (Bateman et al., 2009).

Table 1.5 Diseases caused by mutations in genes for collagens adapted from (Lamandé & Bateman, 2020; Ricard-Blum, 2011) and https://omim.org/

Gene Symbol	Key diseases	OMIM
COL1A1	Osteogenesis imperfecta, Caffey disease; Ehlers–Danlos syndrome, arthrochalasia type 1	166200, 166210, 259420, 166220, 114000, 130060
COL1A2	Osteogenesis imperfecta, Ehlers–Danlos syndrome, arthrochalasia type; Ehlers–Danlos syndrome, Cardiac valvular type	166200, 166210, 259420, 617821, 225320
COL2A1	Stickler syndrome type I, nonsyndromic ocular, spondyloepiphyseal dysplasia congenita, kniest dysplasia, achondrogenesis, and Legg-Calve-Perthes disease etc	108300, 609508, 183900, 156550, 608805, 200610, 609162, 604864, 151210, 616583, 184250, 271700, 132450, 150600
COL3A1	Ehlers–Danlos syndrome, vascular type	130050
COL4A1	Brain small vessel disease, angiopathy, hereditary, with nephropathy, porencephaly; hemorrhage, schizencephaly	607595, 175780, 614519, 26160
COL4A2	Porencephaly; hemorrhage, intracerebral (susceptibility)	614483, 614519
COL4A3	Alport syndrome, autosomal recessive and autosomal dominant, hematuria, benign familial	203780, 104200, 141200
COL4A4	Alport syndrome, autosomal recessive, and autosomal dominant	203780, 104200
COL4A5	Alport syndrome X-linked	301050
COL4A6	Leiomyomatosis, diffuse with Alport syndrome; deafness X-linked	308940, 300914
COL5A1	Ehlers–Danlos syndrome	130000
COL5A2	Ehlers–Danlos syndrome	130010
COL6A1	Ullrich congenital muscular dystrophy; Bethlem myopathy	254090, 158810
COL6A2	Ullrich congenital muscular dystrophy; Bethlem myopathy; myosclerosis, autosomal recessive	254090, 158810, 255600
COL6A3	Ullrich congenital muscular dystrophy; Bethlem myopathy; myosclerosis; autosomal recessive; dystonia	254090, 158810, 255600, 616411
COL6A5	Neuropathic chronic itch	611916
COL7A1	Epidermolysis bullosa dystrophica, autosomal recessive and autosomal dominant; nail disorder, nonsyndromic congenital,	226600, 131750, 607523
COL8A2	Corneal endothelial dystrophies	136800
COL9A1	Stickler syndrome, type IV; epiphyseal dysplasia, multiple epiphyseal dysplasia	614134, 614135
COL9A2	Epiphyseal dysplasia, multiple, Stickler syndrome type V	600204, 614284
COL9A3	Epiphyseal dysplasia, multiple,	600969
COL10A1	Metaphyseal chondrodysplasia, Schmid type	156500
COL11A1	Stickler syndrome, type II; Marshall syndrome; fibrochondrogenesis	604841, 154780, 228520
COL11A2	Autosomal dominant and autosomal recessive; deafness, autosomal recessive; fibrochondrogenesis	184840, 215150, 609706, 614524
COL12A1	Bethlem myopathy; Ullrich congenital muscular dystrophy	616471, 616470
COL13A1	Myasthenic syndrome, congenital,	616720
COL15A1	Knobloch syndrome	267750
COL17A1	Epidermolysis bullosa, junctional, non-Herlitz type; epithelial recurrent erosion dystrophy	226650, 122400
COL18A1	Knobloch syndrome	267750

COL25A1	Fibrosis of extraocular muscles, congenital,	616219
COL27A1	Steel syndrome	615155

*Online Mendelian Inheritance in Man (OMIM): An Online Catalog of Human Genes and Genetic Disorders

As mentioned earlier, the animal models have greatly improved our understanding of the cause and progression of human genetic diseases. In addition, they have proven to be a valuable tool for discovering targets for therapeutic drugs. Most available animal models have been developed in mice. They present some aspects of the particular disease. A few examples of animal models for collagen-related genetic diseases are shown in **Table 1.6** below.

Table 1.6 Frequently used animal models of collagen-related genetic diseases (Gatseva et al., 2019).

Types of collagens	Affected gene	Animal model	Disease phenotype (or human equivalent)
Collagen IV	*Col4a1*	Mouse	Cerebrovascular disease, intracerebral haemorrhage, Kidney disease, Myopathy Eye defects
	Col4a1/Col4a2 double null	Mouse	Embryonically lethal, growth retardation, vascular defects
	Col4a1, Col4a2	Drosophila	Intestinal defects, myopathy
	Cola4a1, Col4a2	C. elegans	Embryonically lethal
	Col4a2	Mouse	Cerobrovascular, ocular, renal, and muscle defects
	Col4a3	Mouse	Autosomal recessive and dominant AS
	Col4a3 and Col4a4 double null	Mouse	The juvenile form of AS
	Col4a4	Mouse	Autosomal recessive AS
	Col4a5	Mouse	X-linked AS
	Col4a5	Zebrafish	Defective retinal axon guiding
	Col4a6	Zebrafish	Defective axon guiding, cerebellar granule cells defects
Collagen VI	*Col6a1*	Mouse, Zebrafish	Bethlem myopathy. Mitochondrial dysfunction, defective autophagy, fiber necrosis and osteoarthritis, abnormal collagen fibrillogenesis, CNS defect, Bethlem myopathy, myosclerosis
	Col6a3	Mouse, Zebrafish	Ullrich syndrome (in-frame deletion), Dominant mild myopathy with decreased muscle mass, Bethlem myopathy
	Col6a4	Zebrafish	Abnormal motoneuron axon growth
Collagen VII	*Col7a1*	Mouse	Recessive dystrophic epidermolysis bullosa
Collagen XV	*Col15a1*	Mouse, Drosophila, Zebrafish	Mild skeletal myopathy, Cardiomyopathy, Vascular dysfunction, Defects in nerve development and myelination, Neuronal function defects, cardiomyocyte, skeletal muscle defects, Defective development; motor axon guidance defects, and muscle atrophy
Collagen XVII	*Col17a1*	Mouse, Zebrafish	Non-Herlitz epidermolysis bullosa, growth retardation, enamel hypoplasia, Junctional epidermolysis bullosa, neuronal defect
Collagen XVIII	*Col18a1*	Mouse	Knobloch syndrome, human pigment dispersion syndrome, hydrocephalus, kidney defect, adipocyte differentiation

	Col18	C. elegans, mouse	Knobloch syndrome, human pigment dispersion syndrome, hydrocephalus, kidney defect, adipocyte differentiation defect-metabolic defect

* Alport Syndrome = AS: genetic disorder characterized by kidney disease, hearing loss, and sometimes eye abnormalities.

1.4 Collagen VI

This part will focus on a unique member of the collagen superfamily, collagen VI. Collagen VI has a particular beaded filament structure and is found in the extracellular matrix of almost all tissues. It is found in the basement membrane in several tissues such as skin, kidneys, nerves, blood vessels and muscle, where its role is in anchoring the basement membrane into the surrounding extracellular matrix. Also, collagen VI interacts with many other ECM components and surface receptors in different tissues and acts as a structure and signaling protein. Studies of this molecule also have revealed its involvement in a vast range of tissues and its association with several pathological conditions linked with human disorders (Cescon et al., 2015; Di Martino et al., 2023; Lamandé & Bateman, 2018).

1.4.1 Collagen VI genes

Collagen VI is encoded by five different genes, *COL6A1, COL6A2, COL6A3, COL6A5, and COL6A6*, which encode five separate peptide chains. Another gene *Col6a4* is found in the rodent genome but is broken in 2 transcribed, nonprocessed pseudogenes, *COL6A4P1* and *COL6A4P2* in human. The collagen VI genes are present at three distinct chromosomal loci in mammalian genomes. The *COL6A1* and *COL6A2* are tandemly arrayed in chromosome 21 in humans (and chromosome 10 in mice). *COL6A3* is arrayed on chromosome 2 in humans (and chromosome 1 in mice). *COL6A4P2, COL6A5* and *COL6A6* are located in a conserved and syntenic region found in all mammals, except for some primate species, including humans, on chromosome 3q, while *COL6A4P1* is found on the p arm of chromosome 3 and facing the opposite direction because of a pericentric inversion occurred disrupting the complex in two parts (Fitzgerald et al., 2013).

Interrogation of the genome of mice showed that these three genes (*Col6a4*, *Col6a5*, and *Col6a6*) of collagen VI are intact, and genes are located in tandem in the same orientation and order (5' to 3'): *Col6a4>Col6a5>Col6a6* on chromosome 9. The *Col6a4, Col6a5, Col6a6*, and several flanking genes (sh3bp5, capn7, pik3r4, and atp2c1) are represented, and arrows over each gene representing box indicate the orientation of each gene (Gara et al., 2008) shown in **Figure 1.12** below.

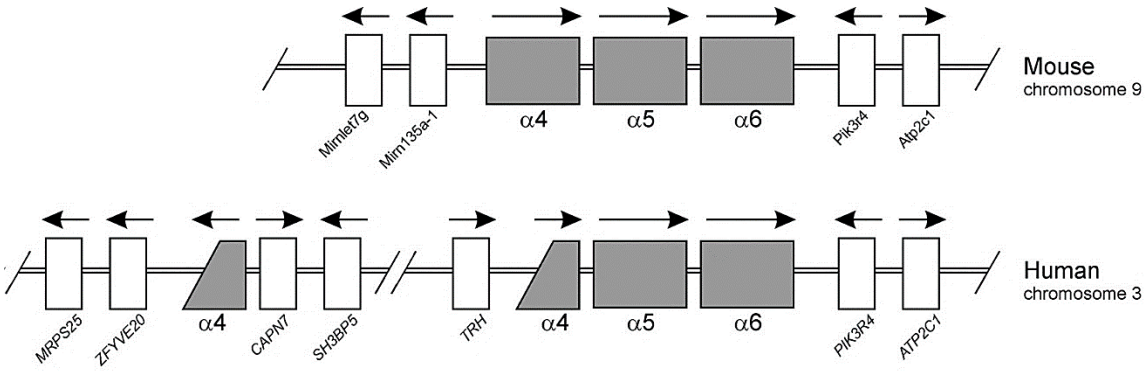

Figure 1.12 Loci for collagen VI (*Col6a4*, *Col6a5, and Col6a6*) in the mouse and human genomes (Gara et al., 2008). The orientation of the genes is indicated by arrows collagen VI exerts a broad range of physiological functions in different tissues, particularly abundant in skeletal muscle, peripheral nerves, and connective tissues.

1.4.2 Assembly of collagen VI

There are three major chains of collagen VI: alpha-1 (VI), alpha-2 (VI), and alpha-3 (VI) chains, which are encoded by the genes *COL6A1, COL6A2, and COL6A3,* respectively (Di Martino et al., 2023). The alpha-1 (VI) and alpha-2 (VI) chains have molecular masses of about 130 and 150 kilo Dalton (kDa), respectively, and contain one N-terminal (N1) and two C-terminal (C1 and C2) von Willebrand factor A (non-collagenous) domains (vWF-A). The alpha-3 (VI) chain is approximately three times larger in weight (250–300 kDa) with multiple alternatively spliced variants. It consists of ten N-terminal von Willebrand factor A domains (N10-N1), two C-terminal von Willebrand factor A domains (C1 and C2), and three particular domains in the C-terminus (C3-C5), including one unique domain (C3), one fibronectin type III domain, and one Kunitz domain (C5). So, the alpha-1 (VI) and alpha-2 (VI) chains are the smallest, and the alpha-3 (VI) chain of collagen VI is the largest shown in **Figure 1.13** (Williams et al., 2021).

Other three subunits of collagen VI are much larger than the alpha-1 (VI) and alpha-2 (VI)chains. These are alpha-4 (VI), alpha-5 (VI), and alpha-6 (VI) chains encoded by the *Col6a4, COL6A5* and *COL6A6* genes. These three chains have a similar size as the alpha-3 (VI) chain of collagen VI ranging from approximately 220 kDa to over 300 kDa, also shown in **Table 1.7** (Cescon et al., 2015; Gregorio et al., 2018; Williams et al., 2021).

Table 1.7 Collagen VI chains in mouse and human adapted from (Cescon et al., 2015; J. Wang & Pan, 2020; Williams et al., 2021).

Collagen VI Chain	Gene		Chromosome	
	Mouse	**Human**	**Mouse**	**Human**
Alpha-1 (VI)	*Col6a1*	*COL6A1*	10	21q22.3
Alpha-2 (VI)	*Col6a2*	*COL6A2*	10	21q22.3
Alpha-3 (VI)	*Col6a3*	*COL6A3*	1	2q37
Alpha-4 (VI)	*Col6a4*	*COL6A4**	9	3q21
Alpha-5 (VI)	*Col6a5*	*COL6A5*	9	3q21
Alpha-6 (VI)	*Col6a6*	*COL6A6*	9	3q21

These three chains have a short common triple-helical domain, seven vWF-A (N7-N1) domains in the N-terminus, two vWF-A domains (C1 and C2), and a unique domain in the C-terminus. In the case of the alpha-4 (VI) chain, it contains a partial Kunitz domain in the C-terminus, and the alpha-5 (VI) chain carries another vWF-A domain (C4) and a unique domain (C5) (Cescon et al., 2015; Williams et al., 2021).

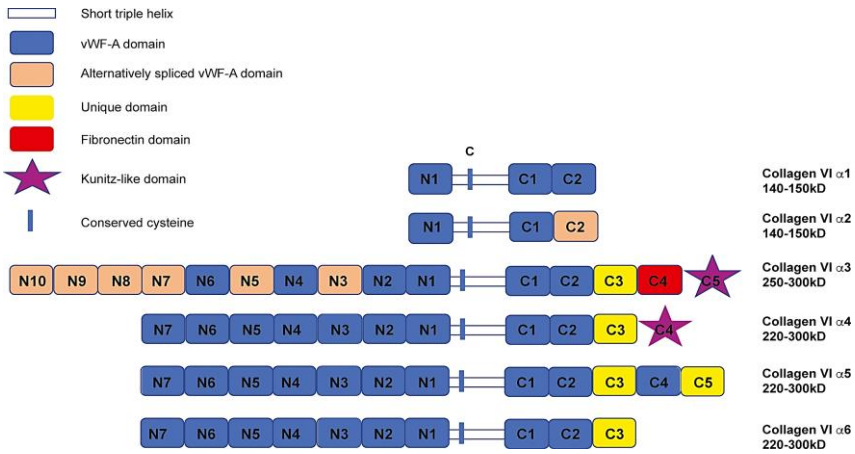

Figure 1.13 Schematic representation of domain structure of collagen VI (Williams et al., 2021)**.** All collagen VI chains comprise a relatively short central collagenous triple helix domain (THD), flanked by large N and C-terminal globular regions homologous to Willebrand factor type A (vWF-A) domains. The α3, α4, α5, and α6 chains are larger than α1 and α2, feature with variable number of von Willebrand factor type A (vWF-A). The α3 chain contains three additional domains at the carboxyl-terminal end. The α4, α5, and α6 chains share a high degree of similarity with α 3 chain.

The assembly of collagen VI is a complicated process, making it unique among the collagens. Studies have shown that alpha-1 (VI), alpha-2 (VI), and alpha-3 (VI) chains of collagen VI mentioned above are assembled intracellularly into collagen triple-helical monomers with a ratio of 1: 1: 1 each. First, these collagen VI monomers align into antiparallel, overlapping dimers held together by disulfide bonds. Then the anti-parallel dimers align further through disulfide bonds to form a tetramer, before excretion, by covalent association with their end. After that, these tetramers are secreted to the extracellular matrix and aggregate to form beaded microfibrils in an end-to-end association by interacting with the N-terminal and C-terminal globular domains with each other and the short triple helical domains (shown in **Figure 1.14**). Right after the secretion of tetramers in the extracellular, the maturation of collagen VI microfibrils (fibril processing) in the extracellular matrix begins (Lamandé & Bateman, 2018, 2020; Nerger et al., 2022).

Furthermore, after microfibril assembly, it proceeds the proteolytic process of the collagen alpha-3 (VI) chain (such as the C-5 or the C2-C5 domains). Although the detailed process of collagen VI subunits protein domains interaction involved in the assembly is still unknown. Nevertheless, it has been determined that the collagen alpha-3 (VI) chain has a central role in collagen VI assembly (Cescon et al., 2015; Fitzgerald et al., 2008).

The alpha-5 (VI) and alpha-6 (VI) chains can be substituted in humans with alpha-3 (VI). Furin-like PPC (proprotein convertase) and BMP-1 (bone morphogenetic protein 1) are involved in releasing endotrophin-containing fragments (Endotrophin is a collagen type VI–derived peptide, mediates metabolic dysregulation, inflammation, and fibrosis). The cleavage sites are between the C4 and C5 domains and the C1 and C2 domains (J. Wang & Pan, 2020), as shown in **Figure 1.14**.

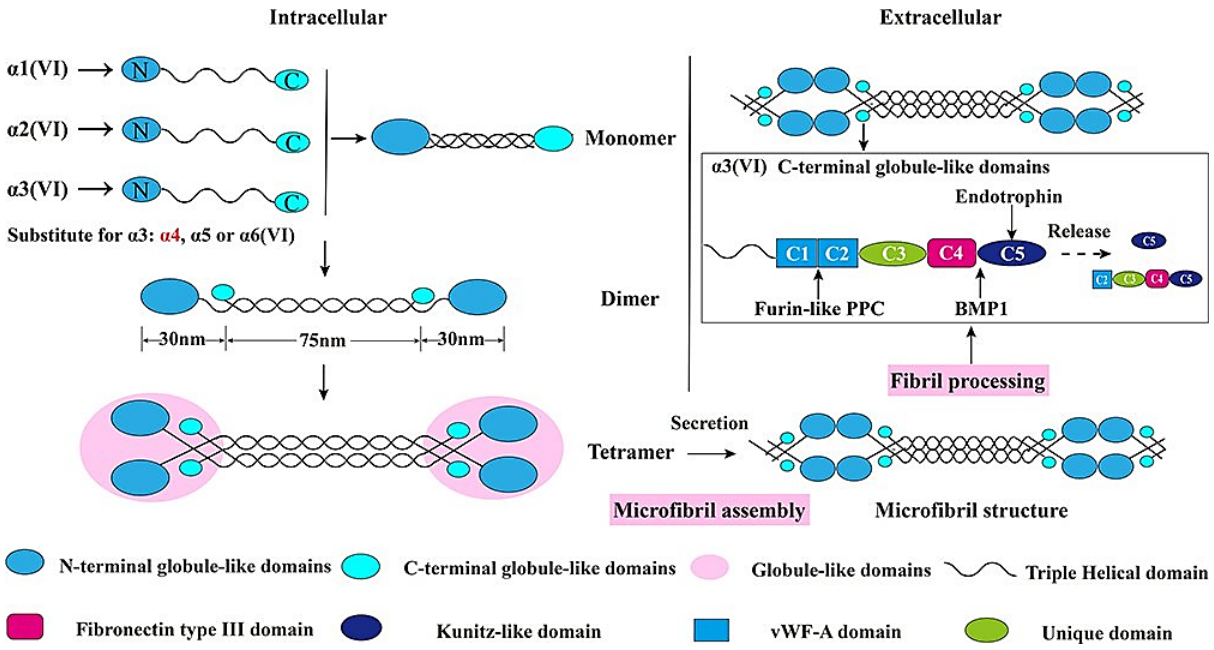

Figure 1.14 Schematic diagram of collagen VI assembly process. The maturation of collagen VI microfibrils in the extracellular matrix is a continuous process involving removing the C-terminal domains of the collagen alpha-3 (VI) chain. The alpha-5 (VI) and alpha-6 (VI) chains can be substituted for alpha-3 (VI) chains in humans. vWF-A, von Willebrand factor type A; Furin-like PPC, furin-like proprotein convertase; BMP-1, bone morphogenetic protein 1. Above figure has been obtained from source (J. Wang & Pan, 2020).

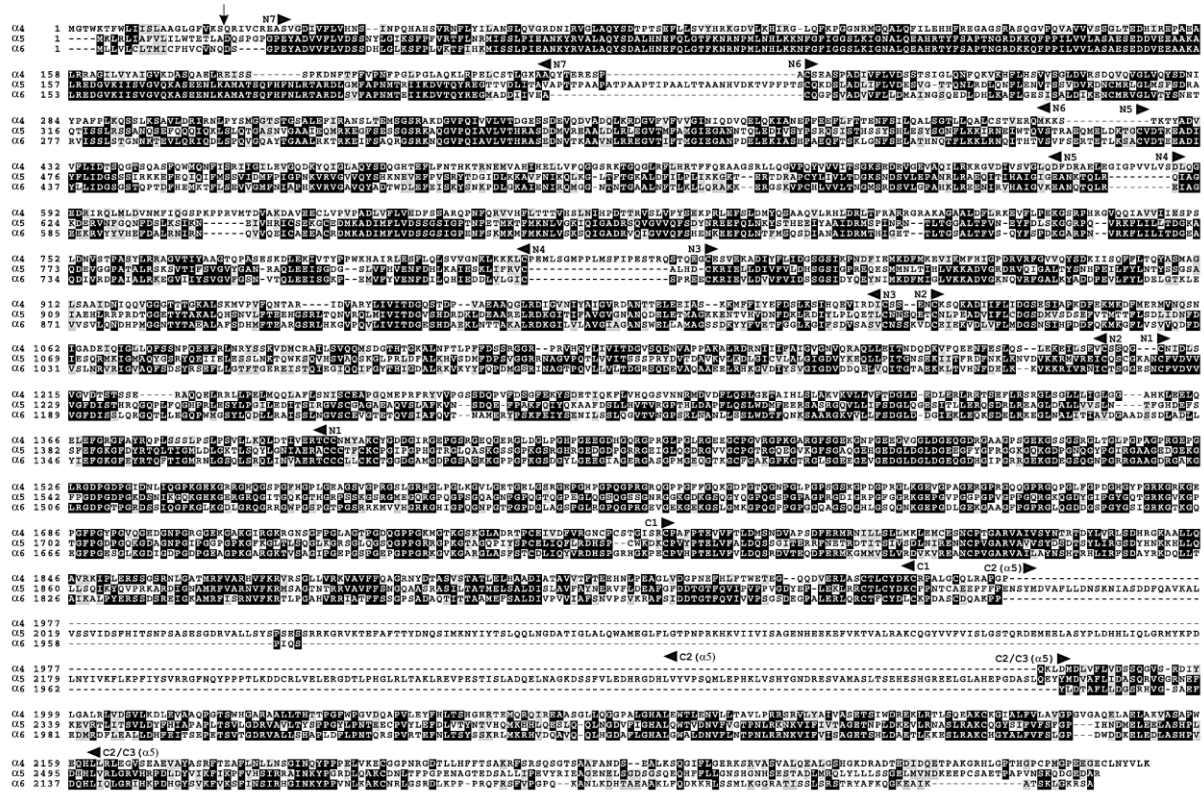

Figure 1.15 Alignment of amino acid sequences of murine collagen VI α4, α5, and α6 chains. The amino acid sequences were deduced from the cDNA sequences deposited in the database under accession numbers AM231151–AM231153, AM748256–AM748258, and AM748259–AM748262, respectively. The arrow marks the potential signal peptide cleavage sites. Arrowheads indicate the boundaries of the domains. Above figure has been obtained from source (Gara et al., 2008).

The domain structures of collagen alpha-4 (VI), collagen alpha-5 (VI), and collagen alpha-6 (VI) are very similar to that of the collagen alpha-3 (VI) alignment of these three chains, as shown in **Figure 1.15**. The overall identity at the amino acid level is highest between the collagen alpha-5 (VI) and alpha-6(VI) chains (44.7%). And the lowest identity is between the collagen alpha-4 (VI) and collagen alpha-5 (VI) chains (28.0%) (Gara et al., 2008).

1.4.3 Expression of collagen VI and links to diseases

Collagen VI is expressed in several extracellular matrices, including tendon, muscle, cartilage, lung adipose tissue, and skin, as well as in the central and peripheral nervous systems (Cescon et al., 2015). It exerts a broad range of physiological functions in these tissues. However, it is mostly expressed in a distinct anatomical niche, for example, in and adjacent to basement membranes of myofibres and intramuscular nerves. Fibroblasts are the major characterized

source of collagen VI and are the principal source in skeletal muscle and the dermis. Additionally, less well-studied sources of collagen VI are astrocytes and macrophages. Cytokines produced by activated macrophages, such as IL-4, IL-10, and TGF-β1, induce expression of collagen VI, with TGF-β1 exhibiting the most significant regulatory role. Macrophages secrete collagen VI protein abundantly, depending on their activation mode, cell density, and stage of differentiation. Interestingly, despite the abundant expression of collagen VI by differentiated macrophages, these cells could not assemble collagen VI into beaded filaments, as observed in fibroblasts (Williams et al., 2021).

In skin: Collagen VI being abundantly expressed by skin fibroblasts, skin biopsies, and in vitro primary skin fibroblast cultures have been widely used to characterize diseases such as Bethlem myopathy and Ullrich Congenital Muscular Dystrophy. Several skin abnormalities, such as keloids, dry skin, and follicular keratosis, have been described in individuals with mutations in the *COL6A1, COL6A2*, or *COL6A3* genes (Lettmann et al., 2014). Although collagen VI is absent in the epidermis, it shows a broad distribution in the dermis, including associated vasculature and nerve fibers, hair follicles, and hypodermis; it is particularly abundant in the basement membrane at the epidermal-dermal junction. Contrary to the broader distribution of the alpha-1 (VI), alpha-2 (VI), and alpha-3 (VI) chains in the skin, alpha-5 (VI) is localized in the papillary dermis, whereas alpha-6 (VI) is found around the blood vessels (Sabatelli et al., 2011). More details about the alpha-5 (VI) chain is mentioned in the next part of the thesis.

Another study by Chen et al demonstrated the involvement of Collagen VI in hair growth and maturation. Interestingly, although lack of collagen VI delays hair growth under physiological conditions, wound-induced hair regrowth is remarkably enhanced in *Col6a1−/−* mice. This process has been shown to involve the activation of the Wnt/β-catenin signaling pathway (Chen et al., 2015). It is also observed that collagen VI was highly expressed at the edges of keloid scars and in healing wounds (Theocharidis & Connelly, 2019). In a study of murine model, it is found that alpha-1 (VI), alpha-2 (VI), and alpha-3 (VI) chains are strongly expressed throughout the dermis and epidermis (Lettmann et al., 2014). Lettmann et al. (2014) also investigated collagen VI expression following local administration of bleomycin, increased alpha-3 (VI) chain expression was observed throughout the dermis, and alpha-5 (VI) chain

expression was also increased in the perivascular regions. Several studies have shown that collagen VI is expressed in the central and peripheral nervous systems (Castagnaro et al., 2021; Cescon et al., 2015; Lettmann et al., 2014).

Collagen VI in CNS: Several studies have shown that collagen VI is expressed in the central and peripheral nervous systems (Castagnaro et al., 2021; Cescon et al., 2015; Lettmann et al., 2014). Collagen VI deposition in the brain was initially found in meningeal cells (Cescon et al., 2015). It was found that collagen VI was linked to neuroprotective against cellular stress and neurodegeneration.

This neuroprotective role of collagen VI was assessed through two different studies. 1) The study found that both mRNA and protein was elevated in brain samples of Alzheimer's disease mutation. Also neuronal culture derived from Col6a1 -/- mice showed increased apoptosis as compared to wild type mice when they were treated with amyloid-β (Aβ)- a peptide that plays a central role in the development of Alzheimer's disease. This finding suggested the neuroprotective role of collagen VI against Alzheimer's disease (J. S. Cheng et al., 2009). In another study also showed the neuroprotective role of collagen VI against stressors. Researcher found that collagen VI in culture media rescued neurons from UV (ultra violet) induced apoptosis and limited the dendrites shrinkage (I. H. Cheng et al., 2011). Both these studies suggest the neuroprotective function of collagen VI against amyloid-β (Aβ) toxicity and external stressors.

Moreover, collagen VI mutations are also involved in CNS diseases such as dystonia and progressive myoclonus epilepsy syndrome (**Figure 1.16**). Dystonia (*DYT27*, OMIM #616411); is a neurological disorder characterized by involuntary muscle contractions. The mutations were identified in exons 36, 41, and 42 of *COL6A3*, encoding for the C1 (exon 36) and C4 (exons 41 and 42) domains at the C-terminal end of the collagen VI alpha-3 chain (Zech et al., 2015). While in the case of myoclonus epilepsy syndrome, a type of seizure that causes sharp, uncontrollable muscle movements, ataxia, cognitive defects, and early death (Karkheiran et al., 2013). *COL6A2* gene mutations were found to be linked to progressive myoclonus epilepsy syndrome. Interestingly, *COL6A2* gene involvement was previously linked to febrile seizures and idiopathic generalized epilepsy (H. S. Kim et al., 2007). Some polymorphisms of collagen VI genes were recently identified as rare risk variants for schizophrenia and bipolar disorder

(Salvoro et al., 2018). More recently, behavioral abnormalities associated with a distinctive deficit in dopaminergic signaling in the prefrontal cortex in *Col6a1*$^{-/-}$ mice were found. Also defects in attentional control abilities in patients bearing collagen VI mutations were identified, this suggests the collagen VI critical role in the CNS in sustaining dopamine and prefrontal cortex-regulated tasks in mice and humans (Gregorio et al., 2018).

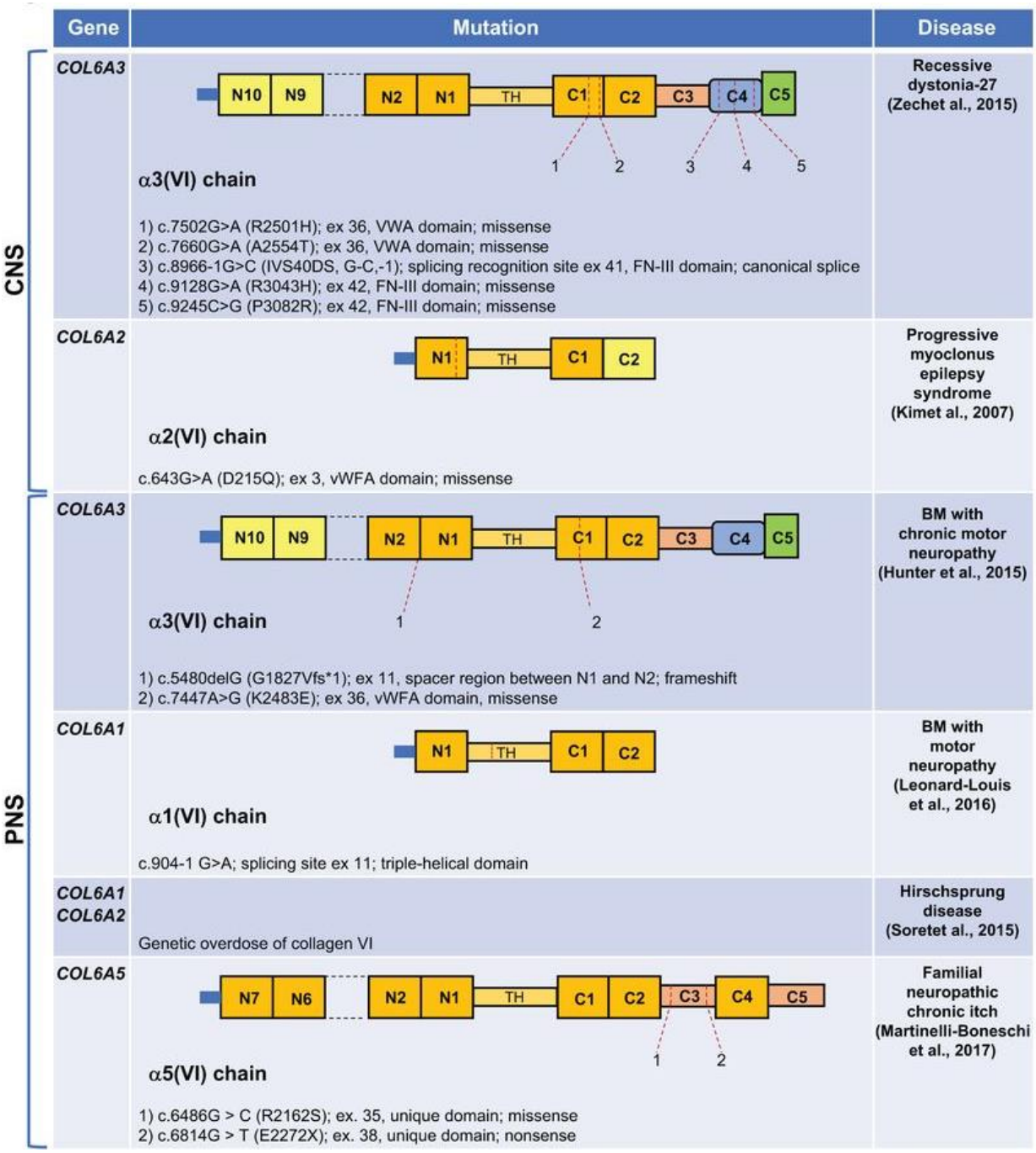

Figure 1.16 Collagen VI mutations linked to human nervous system diseases. A summary of the mutations in COL6 genes that were described to be linked to human disorders affecting the CNS and the PNS. Above figure has been obtained from source (Gregorio et al., 2018)**.**

Collagen VI in PNS: Because there are several nervous system dysfunctions linked to collagen VI mutations, an exceeding characterization of collagen VI localization and function was achieved in the PNS. In the PNS, collagen VI is abundantly expressed by Schwann cells and also present in the connective tissue of the endo-, peri- and epineurium (Chen et al., 2014). Furthermore, in the past role of collagen, VI has been shown in Schwan cell differentiation into myelinating cells (Vitale et al., 2001). It appeared that collagen VI plays an important role in maintaining PNS myelination and function in the sciatic nerves in wild-type and mutant (*Col6a1−/−*) mice. In contrast, without collagen VI altered sensory transmission, decreased conduction velocities, and affected motor and sensory function are associated. The same study also described another important function of collagen VI in peripheral nerve regeneration is its facilitation of macrophage recruitment and polarization during the process of peripheral nerve regeneration (Chen et al., 2014). Moreover, several evidence linked mutations in collagen VI genes and peripheral neuropathic conditions **Figure 1.16.**

Another study described a case of a woman diagnosed with distal hereditary motor neuropathy based on clinical and EMG (Electromyography) data. This patient carried a heterozygous variant of the *TRPV4* gene with a new heterozygous mutation in the *COL6A1* gene (Leonard-Louis et al., 2016). Similarly, Soret and colleagues studied Hirschsprung disease, characterized as the absence of nerve cells in the large intestine resulting in functional obstruction. Remarkably, increased deposition of collagen VI (due to an overexpression of *COL6A1/2* genes) was found around the enteric ganglia of Hirschsprung disease patients (Soret et al., 2015).

Besides the above considerations about the potential presence of peripheral nerve defects in collagen VI-related myopathies, a separate whole-exome sequencing study identified two rare *COL6A5* variants in different families affected by chronic neuropathic itch and characterized by reduced intradermal nerve fiber density. In addition, data analyses revealed markedly reduced α5 (VI) expression (Martinelli-Boneschi et al., 2017).

Still, a precise association of collagen VI with myelinating diseases or neuropathies is expected according to phenotypic studies in mice in the future. Limited studies have been performed on very few available mice model of collagen VI, some of these mice models and their studied

mutation and phenotypic characteristics are shown below. (see **Table 1.8** for phenotypes observed in a few mice models of collagen VI

Table 1.8 Phenotypes observed in murine models of collagen VI disorders adapted from (Williams et al., 2021).

Model	Effect of Mutation	Key phenotypes
Col6a1-/-	-Lack of α1(VI) polypeptide synthesis, -No triple helix collagen VI produced	-An early-onset myopathy -Impaired peripheral nerve regeneration -Altered neuromuscular transmission,
Col6a1GT/GT	Collagen VI fiber size variation	-Impaired muscle growth, -Reduced number of myofibers
Col6a2-/-	Preventing normal folding and microfibril assembly	-Trabecular bone mass reduced with increased osteoclast differentiation
Col6a3 hm/hm	Very low level of a non-functional α3(VI)	-Mild myopathic features
Col6a3+/d16	Mutant Collagen VI secreted as tetramer but no microfibrils assembled	-Mild myopathic features, abnormalities in muscle and tendon, and compromised muscle functions

At the same time, *COL6A6* is suspected to be involved in collagen VI-related myopathies, early-onset atopic dermatitis, and ossification of the posterior longitudinal ligament of the thoracic spine in Chinese populations. Very recently, it was found that *COL6A6* caused retinitis pigmentosa in patients with autosomal dominant transmission (Vaclavik et al., 2022). However, in my thesis, I will focus mainly on *COL6A5* (collagen VI alpha 5) with more detail, which is a core part of my thesis.

1.5 Collagen VI alpha 5

Collagen VI alpha 5 was initially named *COL29A1* based on the next collagen gene name available in the collagen family and without recognizing its association with collagen VI genes. Later, research, particularly by Gara et al. and Fitzgerald et al. in 2008, clarified the genetic landscape, highlighting *COL6A5* gene affiliation with the collagen VI cluster on chromosome 3p, recognized as a paralog of Collagen VI genes and named *COL6A5* (Fitzgerald et al., 2008; Gara et al., 2008). The link, therefore, lies in the evolution of understanding from the misdesignation of *COL29A1* to the correct identification of *COL6A5* as part of the collagen VI

family. The human *COL6A5* gene has its ortholog in rodents, including rats, mice, and rabbits (Haq et al., 2019).

In humans, *COL6A5* gene is composed of 39 exons. The gene encodes a protein of 2611 amino acids (given below in **Figure 1.17**) with a predicted molecular mass of 287 kDa. The N terminus is predicted to contain a signal peptide cleavage site between amino acids 18 and 19. The signal peptide is followed by seven VWA domains, a 336-amino acid collagen triple helix, and three more VWA domains. On either side of the C3 domain are regions that are 134 and 129 amino acids in size that do not show homology with any other protein being unique to the collagen VI alpha-5 chain (Fitzgerald et al., 2008).

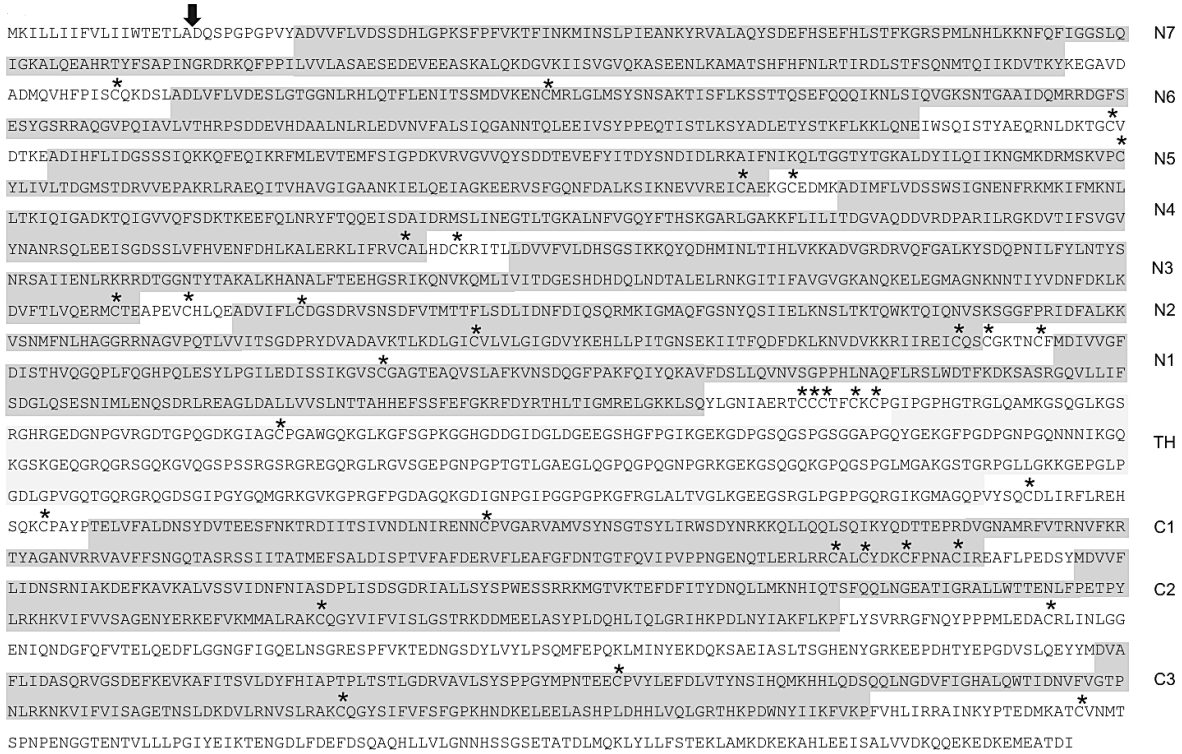

Figure 1.17 Amino acid sequence and domain structure of the α5 collagen VI chain. protein sequence of collagen VI alpha-5 with seven N-terminal (N1–N7) and three C-terminal (C1–C3) VWA domains (light gray) and the triple helical domain (dark gray) highlighted. Asterisks indicate cysteines. The signal peptide cleavage site between amino acids 18–19. The amino acid sequence is derived from the *COL6A5* DNA sequence. Above figure has been obtained from source (Fitzgerald et al., 2008).

Interestingly, the mouse *Col6a5* gene is composed of 41 exons shown below in **Figure 1.18**. However, when comparing human and mouse collagen VI alpha-5 chain, higher divergence is found at the C terminus of the alpha-5 chain and alignment of amino acid sequences of human

and mouse collagen VI a5 chains, which in addition shows alternative splicing and could represent an adaptation to a need to replace the collagen VI alpha-4 chain in Hominidae (Gara et al., 2008).

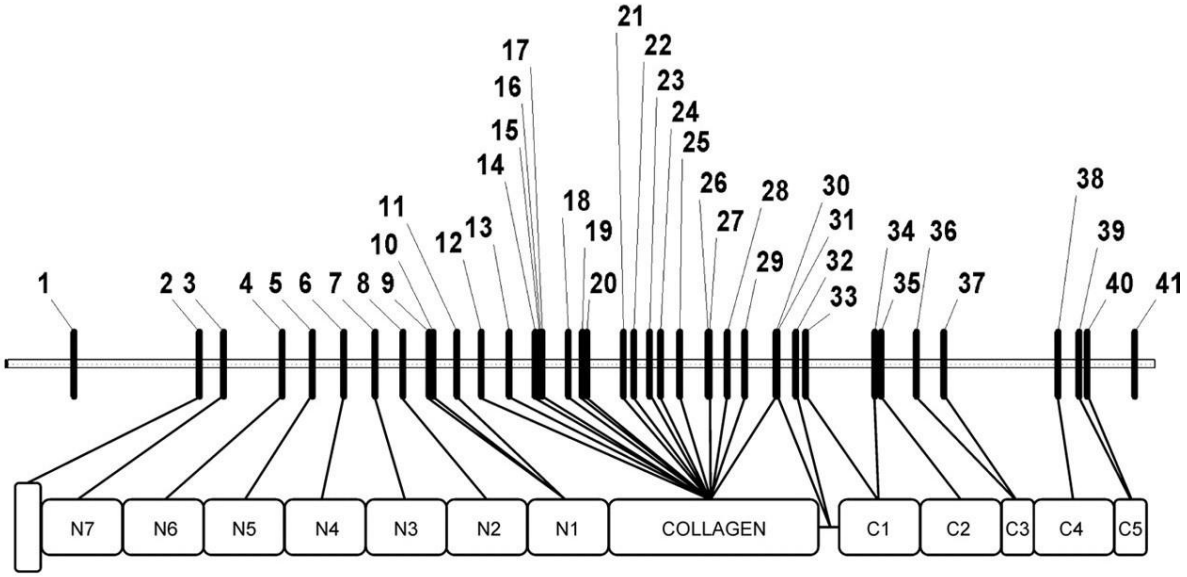

Figure 1.18 Organization of the murine *Col6a5* gene. The numbers indicate the positions of exons. The protein domain structure is given below, and the corresponding exons are indicated by lines adapted from (Gara et al., 2008).

1.5.1 Collagen VI alpha 5 expression and distribution

COL6A5 expression and its distribution in different tissue have been documented since 2007 (Fitzgerald et al., 2008; Gara et al., 2011; Söderhäll et al., 2007). These studies showed apparent differences in COL6A5 expression in different mouse and human tissues. In addition, Fitzgerald and colleagues have shown that the ovary and liver of adult mice express *Col6a5* mRNA while it was absent in human tissues (Fitzgerald et al., 2008).

In mice, *Col6a5* transcripts are present in most tissues examined, including the lung, heart, kidney, spleen, muscle, ovary, uterus, brain, skin, liver, sternum, and calvaria (skull) (Fitzgerald et al., 2008; Gara et al., 2008). In addition, a recent transcriptomic study in mice has revealed that *Col6a5* is expressed at low levels in skin and DRGs. *Col6a5* transcripts were found to be expressed 31-fold more and 13-fold more in DRG vs skin in males and females, respectively (Mecklenburg et al., 2020). At the protein level, COL6A5 was detected in skeletal muscle, diaphragm, bronchi, kidney, ovary, testis, and blood vessels with a home-made

antibody (Gara et al., 2011). Still, the analysis of COL6A5 protein expression in mice is challenged by the lack of valid commercial COL6A5 antibodies.

In humans, among 17 tissues examined, *COL6A5* mRNA was detected mainly in the skin, lung, testis, colon, placenta, and small intestine (Gara et al., 2008; Söderhäll et al., 2007) (shown in **Table 1.9**). A recent single-cell transcriptomics study showed that *COL6A5* expression is restricted to the papillary dermis in the skin. A new study also demonstrated that at atopic dermatitis lesions, *COL6A5* fibroblast expression was increased (He et al., 2020). At the protein level, COL6A5 is primarily expressed in the skeletal muscle and at the myotendinous junction and co-localizes with laminin, a basement membrane marker (Sabatelli et al., 2012). In the skin, COL6A5 protein expression was found at the dermal-epidermal junction (Martinelli-Boneschi et al., 2017). A year later, another study also confirmed that COL6A5 expression was restricted to papillary dermal fibroblasts by labeling the basal layer of the epidermis of the female breast skin (Philippeos et al., 2018).

Table 1.9 Immunohistochemical analysis of the expression of collagen VI chains in adult mice adapted from (Gara et al., 2011).

Tissue	Collagen VI alpha 5 expression
Brain	–
Eye	+
Heart	+
Lung	–
Liver	–
Spleen	–
Intestine	++
Sternum	–
Skeletal muscle	+
Kidney	++
Ovary	+++
Testis	++
Skin	++
Blood vessels	++
Vertebral cartilage	–
Knee cartilage	–

‘–’ no expression; ‘+’, weak; ‘++’ medium; ‘+++’, strong

1.5.2 Nucleotide variants found in *COL6A5* associated to several disease

In diverse diseases, single nucleotide gene variants in *COL6A5* have been identified as risk factors (**Table 1.10**). Söderhäll and colleagues reported that eight collagen variants were associated with atopic dermatitis using a family-based association analysis performed with European cohorts of 199 families, including 60% German and other countries. They pointed out the potential role of collagen variant in epidermal integrity and function (Söderhäll et al., 2007). Another genome-wide analysis study constructed DNA pools from 75 subjects with atopy and asthma, 75 subjects with atopy and without asthma, and 75 control subjects without atopy or asthma. This study reported a polymorphism associated with atopy for one of the collagen variants described by Söderhäll et al. as well as a new variant (Castro-Giner et al., 2009). Another study was performed in which four of the previously described eight variants were analyzed. This study comprised 465 patients with atopic dermatitis and 350 healthy controls along with 215 patients with chronic obstructive pulmonary disease and 130 children with asthma, with a majority of German patients. No significant relationships was found between the four variants and atopic dermatitis or asthma or chronic obstructive pulmonary disease (Harazin et al., 2010).

***Table 1.10 COL6A5* variants found associated with diseases**

Variants associated	Disease	Additional Information	References
rs13095825 rs16845861 rs10212372 A36603217 rs10934938 rs4688761 A36637742 rs9883988	Atopic dermatitis	Role of collagen variant in epidermal integrity and function	(Söderhäll et al., 2007)
rs10934938 rs7629719	Atopy and asthma	Identification of a new variant, associated with atopy	(Castro-Giner et al., 2009)
rs13095825 rs16845861 rs10212372 rs9883988	Asthma in children	No significant relationships found between the variants and disease	(Harazin et al., 2010)
rs13095825 rs16845861 rs10212372 A36603217 rs10934938 6s4688761 rs9883988	Eczema	No significant evidence between variants and atopic dermatitis	(Naumann et al., 2011)
rs1191735 rs322117	Childhood allergy	Varient causal roles in asthma and environmental exposures was found	(Bornelöv et al., 2013)
rs11917356	Hypertension	Correlation of variant with hypertension risk was found	(Yasukochi et al., 2017)
rs115375867 rs368345789	Neuropathic itch	Identified rare variants of *COL6A5*, revealing the association with familial neuropathic chronic itch	(Martinelli-Boneschi et al., 2017)
rs13062453 rs1497305 rs77123808	Lung cancer	Identified COL*6A5* variants and their association with lung cancer	(Duan et al., 2020)
rs115375867	Chiari malformation type 1	variant found in rare patients with Chiari malformation type 1	(Urbizu et al., 2021)

Naumann and colleagues analyzed a large group, including 1687 German patients with eczema and 2387 population control subjects, a collection of 274 German families with eczema disease. They investigated 7 SNPs out of the 8 previously described 8 variants and reported no significant evidence between *COL6A5* variants and atopic dermatitis (Naumann et al., 2011). Two years later, it has been reported that variations in *COL6A5* might play causal roles in asthma and environmental exposures. This study has used a novel bioinformatics approach combining feature selection and machine learning that interplay genetic and environmental factors in allergic diseases. It has been proposed that combinations of gene variants , including variants of *COL6A5,* and environmental factors affect the risk of developing childhood allergic diseases (Bornelöv et al., 2013).

In 2017, a longitudinal exome-wide association study performed among Japanese showed that the *COL6A5* novel SNP rs11917356 was significantly correlated with hypertension risk through the effect of systolic blood pressure (Yasukochi et al., 2017). The same year, another study identified two rare variants of *COL6A5*, revealing the association between the *COL6A5* gene and familial neuropathic chronic itch (Martinelli-Boneschi et al., 2017). This same mutation has recently been found in rare patients with Chiari malformation type 1 (a neurodevelopment is characterized by downward displacement of the lower part of the cerebellum into the base of the skull through which the spinal cord passes (Urbizu et al., 2021). In addition, new research was conducted using single-cell RNA sequencing on skin biopsy specimens with 5 atopic dermatitis patients and 7 healthy controls. It demonstrated that at atopic dermatitis lesions, COL6A5 fibroblast expression increased, suggesting its potential pathogenic role in atopic dermatitis (He et al., 2020).

Recently a study on the Chinese Han population has identified 3 *COL6A5* SNPs that confer susceptibility to lung cancer. As all the significant SNPs have resided in the intron region of the *COL6A5* gene, which possesses regulatory functions in pre-mRNA processing, considered that alternation at these polymorphic sites might modulate the expression efficiency of *COL6A5* mRNA, contributing to the abnormal pattern of COL6A5, and thereby influencing the individual susceptibility to lung cancer among individuals (Duan et al., 2020).

1.6 *COL6A5*-p.Glu2272* mutation

1.6.1 The human neuropathic itch associated with *COL6A5*-p.Glu2272* mutation

Along with other *COL6A5*-associated diseases, Martinelli-Boneschi and colleagues' study identified a mutation in the *COL6A5* gene associated with chronic neuropathic itch (Martinelli-Boneschi et al., 2017). Previously, a study showed that it has the highest expression below the dermal-epidermal junction and around the vessels of the reticular dermis (Sabatelli et al., 2011). Martinelli-Boneschi and colleagues found, by whole exome sequencing, that two families and a diabetic patient carried the nonsense *c.6814G>T (*p.Glu2272**)* variant. Further segregation analysis was made for the p.Glu2272* mutation and revealed its association with itch. Moreover, *COL6A5*-p.Glu2272* mutation patients' fibroblasts were used to confirm the null alleles test. RT-PCR analysis showed that patients carrying *COL6A5*-p.Glu2272* mutation nonsense variant have functional haploinsufficiency due to RNA decay. Also, patients carrying *COL6A5*-p.Glu2272* mutation had low COL6A5 protein expression in the skin. From this evidence, *COL6A5*-p.Glu2272* mutation association with idiopathic itch was concluded and later inferred as neuropathic itch.

The *COL6A5*-p.Glu2272* mutation was reported in two families shown in family 1 and family 2 below (Figure 1.19) (Martinelli-Boneschi et al., 2017).

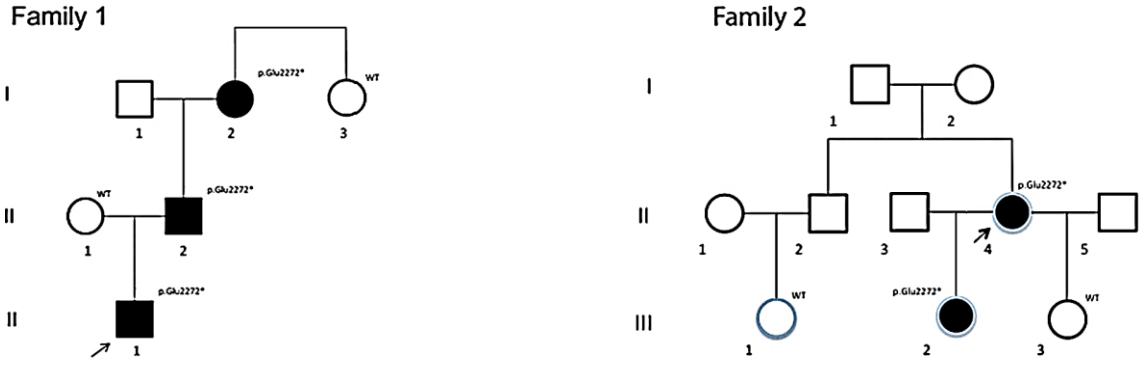

Figure 1.19 Family pedigrees and segregation of COL6A5 mutations. Families 1 and 2: segregation of p.Glu2272* variant with chronic itch (dark symbols); light blue borders: JHS/EDS-HT patients. It has been adapted from (Martinelli-Boneschi et al., 2017).

However, the patient's skin biopsy showed normal morphology of the epidermis, dermal connective tissue, and skin adnexa. This can be seen in **Figure 1.20** below. Of note, this same mutation has also been found in rare patients with Chiari malformation type 1 (Urbizu et al., 2021).

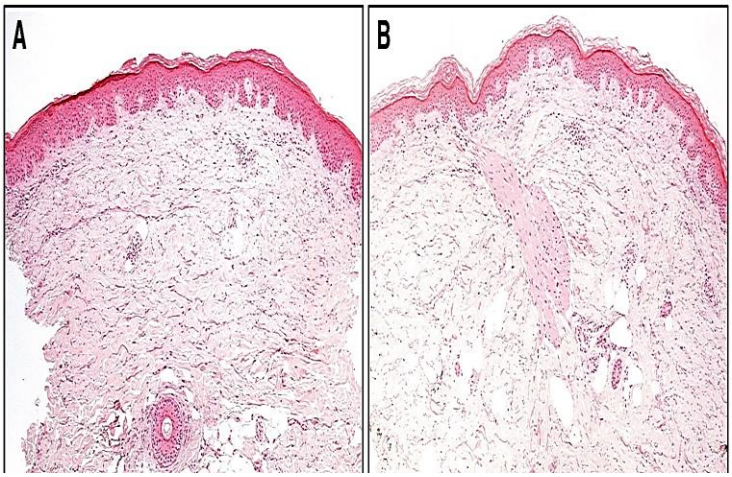

Figure 1.20 Skin biopsy sections in itch patients carrying COL5A6 variants. Family 1 skin biopsy data of two patients, including Patient II:2 and III:1. Above figure has been obtained from source (Martinelli-Boneschi et al., 2017).

1.6.2 Treatments for neuropathic itch

Treating neuropathic itch is difficult; antihistamines, corticosteroids, and most pain medications are largely ineffective. Current treatment recommendations include local or systemic administration of inhibitors of neuronal excitability to reduce scratching.

Then the critical question is how to decide on treatment for neuropathic itch even when the origin of the itch is unknown, for example, in the case of an idiopathic chronic itch. It is important to confirm the diagnosis and identify the condition's underlying cause and paraclinical examinations. These examinations may include skin biopsies to measure the density of intraepidermal nerve fibers (IENF), as well as electromyography, sural nerve conduction studies, Quantitative Sensory Testing (QST), and Magnetic Resonance Imaging (MRI). Therefore, comprehensive dermatological, neurological examination and paraclinical examinations provide further insight into the diagnosis and etiology of neuropathic itch.

These tests aid in identifying specific nerve damage or dysfunction and ultimately inform the development of an effective treatment plan for patients (Misery et al., 2014).

Also, Misery and colleagues proposed the decision tree shown in **Figure 1.21.** At first, managing neuropathic pruritus involves prioritizing aetiological treatment, including avoiding factors that increase skin dryness and temperature, using emollients, and avoiding skin irritants and spicy foods. Menthol, polidocanol, and palmitylethanolamide are suggested soothing compounds. Cognitive-behavioral therapy, relaxation exercises, and psychological support can be useful in breaking the cycle of itching and scratching. It is essential to evaluate the burden and psychosocial consequences of neuropathic pruritus in the clinical setting shown in **Figure 1.21** in the decision tree. This tree is based on the differentiation between localized pruritis, which predominantly requires local treatments, and generalized pruritus, which requires systemic treatments (Misery et al., 2014).

Localized neuropathic pruritis (local treatments); Topical lidocaine and capsaicin are promising treatments for localized neuropathic pruritus. Lidocaine has been reported to be efficient in nostalgia paraesthetica, and capsaicin antagonizes the TRPV1 cation channel, a major transducer of pruritic stimuli. Capsaicin-induced neurogenic inflammation reduces pruritus by inducing the reversible retraction of epidermal nerve fibers, but its initial burning sensation limits its use. Blinded randomized controlled trials have demonstrated the efficacy of 0.025% topical capsaicin in nostalgia paraesthetica and brachioradial pruritus. A new capsaicin 8% dermal patch has also been shown to be effective against pruritus in postherpetic neuralgia. Topical preparations containing tacrolimus, pimecrolimus, menthol, or camphor may also be useful (Misery et al., 2014). **Table 1.11**

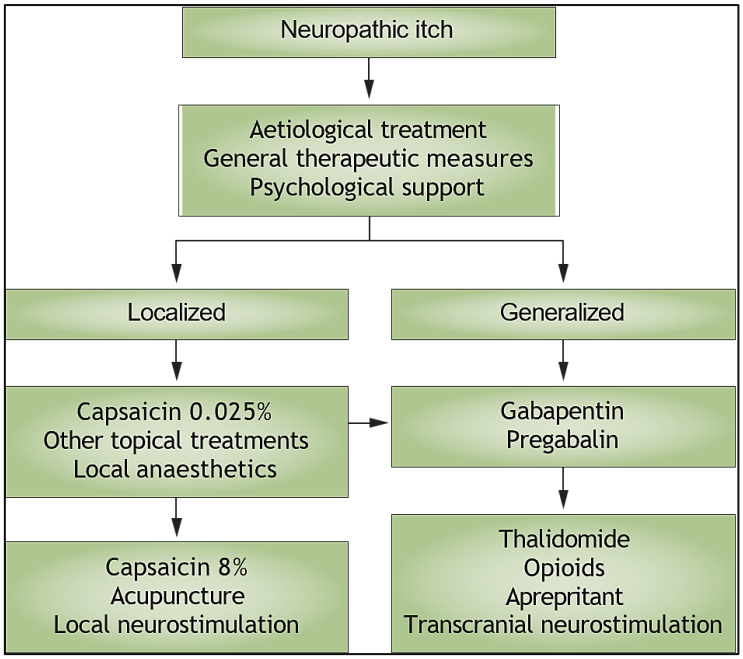

Figure 1.21 Decision tree for the treatment of neuropathic pruritus. Aetiological treatment of pruritus is seldom possible; instead, the choice of therapeutic approach is usually based on the size and localization of the pruritus. Above figure has been obtained from source (Misery et al., 2014).

Generalized neuropathic pruritus (systemic treatments); Gabapentin and pregabalin are commonly used to treat neuropathic pruritus, but its use is limited due to its side effects.

Antihistamines are generally not effective in neuropathic pruritus. There is no conclusive evidence for the efficacy of opioid-receptor antagonists or κ-opioid-receptor agonists. Cyclosporin A could be beneficial in treating neuropathic pruritus, but no clinical studies have assessed its efficacy. Aprepitant, a neurokinin-1-receptor antagonist, is a promising second-line drug that could be used in many cases of neuropathic pruritus (Misery et al., 2014).

A list of systemic treatments for neuropathic pruritus is shown be in **Table 1.12.**

Table 1.11 Topical therapies for Neuropathic itch has been obtained from (Sutaria et al., 2022).

Class	Treatment	Dosage	Adverse effects
Calcineurin inhibitor	Tacrolimus	0.03%- 0.10%	Transient burning
Anesthetics	Pimecrolimus	1%	Local irritation, temporary decreased sensation
	Pramoxine	1%	
	Lidocaine (cream)	2.5%-5%	
	Lidocaine (patch)	5%	
	Prilocaine	2.5%	
	Ketamine-amitriptylinelidocaine	10%-5%-5%	
Capsaicin	Capsaicin (cream)	0.025%-1%	Transient pain or burning
	Capsaicin (patch)	8%	
Coolants	Menthol	1%-3%	Skin irritation (high doses)
	Camphor	0.5%	
Tricyclic antidepressants	Doxepin	5%	Transient burning, drowsiness
Botulinum neurotoxin	Botulinum neurotoxin	2-10Unit	Dryness, pain, burning at injection site

Table 1.12 Systemic therapies for Neuropathic itch has been obtained from (Sutaria et al., 2022).

Class	Treatment	Dosage	Adverse effects
Antidepressants	Amitriptyline	25-75 mg QHS	Drowsiness, dry mouth, dizziness, blurred vision
	Doxepin	10-50 mg QHS	
Anticonvulsants	Gabapentin	100-1200 mg TID	Drowsiness, weight gain, lower extremity edema, ataxia
	Pregabalin	25-100 mg TID	
	Carbamazepine	200-1200 mg QD	GI distress, drowsiness, dizziness, myelosuppression

QD; every day, QHS; every night, TID;3 times daily and GI; gastrointestinal.

In a study by (Martinelli-Boneschi et al., 2017), it was observed that patients with the *COL6A5* p.Glu2272* mutation from two different families were treated with generalized gabapentin and pregabalin after an initial assessment. These medications were prescribed for the management of chronic neuropathic itch in both families, precisely described below.

Family 1 has *COL6A5*-p.Glu2272* mutation in its 3 members, including 2 males and 1 female.

All members were given gabapentin 900 mg/day, and they got relief from the itch. The same dose was adequate for one diabetic patient in this family, which suggests neuropathic itch treatment was not interfered with by diabetes in this study.

Family 2 has *COL6A5*-p.Glu2272* mutation in 2 female members. One female member was given gabapentin 1200 mg/day and got relief from the itch. The second female member received pregabalin 300 mg/day and got relief from the itch.

From this it can be observed that even in a few patients who carry *COL6A5*-p.Glu2272* mutation, the dose and treatment vary as mentioned above. Therefore, the effectiveness of these treatment over large and diverse populations would require more treatment improvements and the finding new factors and causes.

For example, a study analyzed a group of 197 French patients who were admitted to a hospital to manage their chronic pruritus between 2008 and 2018 without skin disease. Out of these 36.5% (72 patients) had neuropathic itch and 20.8% (41 patients) had unknown chronic pruritus. Although neuropathic patients were often treated with gabapentinoids but still 46.8% had partial efficacy and 38.3 % patient showed no efficacy (Robert et al., 2020) also shown in **Table 1.13.**

Table 1.13 Efficacy of treatments on pruritus adapted from (Robert et al., 2020)

Treatment	Patients n (%)	Efficacy (%)		
		Complete efficacy	Partial efficacy	No efficacy
Gabapentinoids	65 (33.0)	14.9	46.8	38.3
Antidepressants	54 (27.4)			
Serotonin and norepinephrine reuptake inhibitor (venlafaxine)	9 (4.6)	0	25.0	75.0
Serotonin reuptake inhibitor	23 (11.7)	7.7	69.2	23.1
Non-selective monoamine reuptake inhibitor	6 (3.0)	50.0	25.0	25.0
Tetracyclic	13 (6.6)	0	40.0	60.0
Tricyclic (amitriptyline)	3 (1.52)	0	100	0
Emollient	50 (25.4)	12.1	66.7	21.2
Topical steroids	40 (20.3)	20.6	67.6	11.8
Sedating antihistamines	32 (16.2)	0	55.0	45.0
Non-sedating antihistamines	18 (9.1)	10.0	50.0	40.0
Anti-pruritic emollient	30 (15.2)	20.0	53.3	26.7
Naltrexone	8 (4.1)	0	50.0	50.0
UVB therapy	8 (4.1)	0	71.4	28.6
UVA therapy	6 (3.0)	0	50.0	50.0
Methotrexate	5 (2.5)	20.0	60.0	20.0
Topical tacrolimus	3 (1.5)	0	0	100
Thalidomide	2 (1.0)	0	100	0
Prednisone	2 (1.0)	0	100	0
Cyclosporine	1 (0.5)	0	0	100

UV: ultraviolet

Overall, this study highlights the diverse causes of chronic pruritis without skin diseases and the limited effectiveness of current symptomatic treatments (Robert et al., 2020).

However, the recent new mouse models (such as this thesis study) and advancements in understanding the molecular and cellular mechanisms involved in chronic pruritis provide hope for the improved management of pruritus in the future.

1.7 Thesis Project

Genetic study of two families identified a rare *COL6A5* variant co-segregating with the chronic itch in five affected members and absent in non-affected members. These two families carried the nonsense c.6814G>T (p.Glu2272*) variant. These patients carrying the *COL6A5*^{E2272*} mutation had low COL6A5 protein expression in the skin. However, the mechanisms for the link between *COL6A5*-p.Glu2272* mutation and chronic neuropathic itch are unclear.

The aim of my Ph.D. project was to generate a mouse model for this mutation and characterize this model, mainly for itch phenotype. Therefore, in the project, I focused on the following objectives:

1. To establish the first mutant mouse model, *Col6a5*^{E2302*}, which corresponds to the human *COL6A5*-p.Glu2272* mutation using CRISPR/Cas9 technology.
2. To characterize the effects of this mutation at the cellular and molecular levels.
3. To characterize the mutant mouse model (*Col6a5*^{E2302*}) for chronic itch using behavioral testing. These investigations aim to understand better the genotype-phenotype association and the role of *COL6A5* mutations in chronic itch.

Schematic illustration of different parts of this thesis project is shown below in **Figure 1.22**

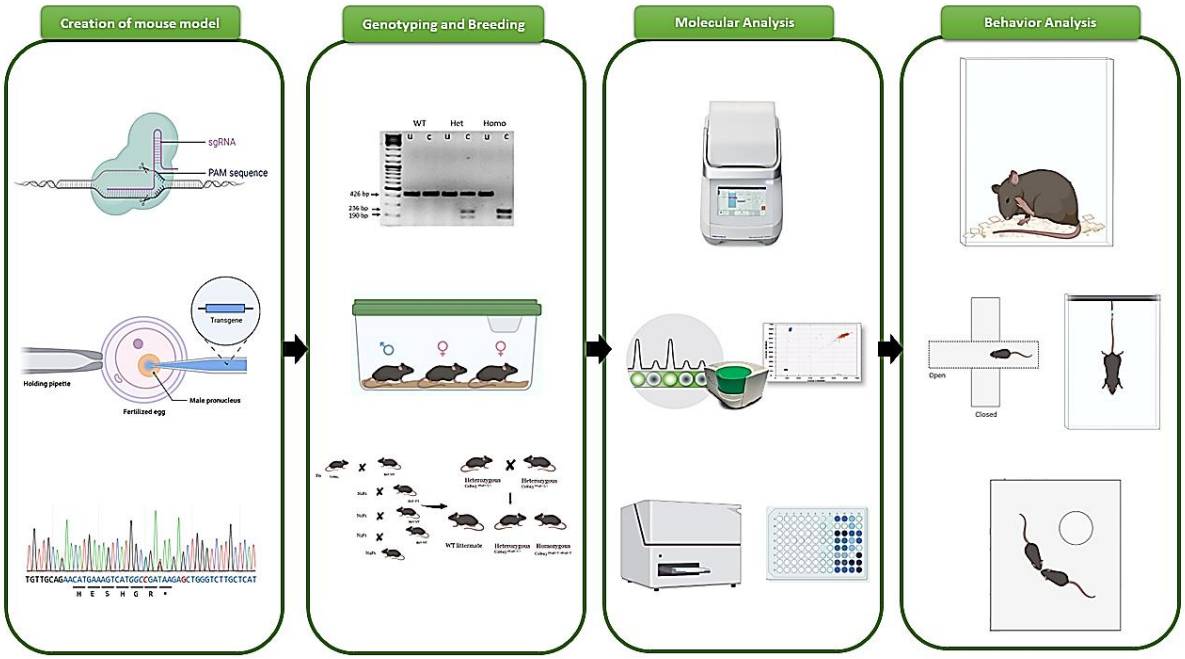

Figure 1.22 Schematic illustration of different parts of this thesis project. It includes the creation of mouse model to genotyping and breeding for making cohorts for experimental study. The molecular and behavioral study is performed on these experimental cohorts of mice.

The first aim of this project was to create a chronic neuropathic itch mutant mouse model *Col6a5*^{E2302*}corresponding to human mutation *COL6A5*^{E2272*}. This was done with the help of the Mouse clinical Institute to generate the first animals carrying the E2302* mutation. Then, I had to perform the characterization after I managed five backcrosses with C57BL/6CNr genetic background to remove any undetectable CrispR/Cas9 off-targets. Later I proceeded with cellular and molecular characterization of the mutation. Then, I conducted a detailed behavioral phenotyping analysis of the mutant mice for scratching and other behavior tests.

Chapter 2: Results

In this part **chapter 2 Results,** it contains two parts.

1) the **manuscript in preparation** (**section 2.1** of this chapter 2); which contains key material & methods part as well as key results and

2) **supplementary methods and results** (**section 2.2** of this chapter 2); this part has supplementary methods I used during my project and achieved some supplementary results included this part.

2.1 The *COL6A5*-p.Glu2272* mutation induces chronic itch in mice

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Keywords: COL6A5, chronic neuropathic itch, pruritus, CRISPR-Cas technology, scratching, spontaneous itch

2.1.1 Abstract

Pruritus is a common irritating sensation that provokes the desire to scratch. Environmental and genetic factors, altering barrier skin dysfunction, or hypersensitivity of sensory nerves, contribute to the onset of pruritus. However, the itch can become a major burden when it becomes chronic, like in neuropathic itch. The rare Collagen VI alpha 5 (*COL6A5*) gene variant p.Glu2272* was recently identified in two families and an independent patient with chronic neuropathic itch. These patients showed reduced COL6A5 expression in the skin and normal skin morphology. However, little progress has been made until now toward understanding the relationships between this mutation and chronic itch. Therefore, we developed the first mouse model that recapitulates *COL6A5*-p.Glu2272* mutation using the CRISPR-Cas technology and characterized this new mouse model. The mutant mRNA, measured by RT-ddPCR, was expressed at normal levels in dorsal root ganglia and decreased in skin. The functional exploration showed changes in the behavior of control individuals kept with mutant carriers and confirmed the effect in the mutant mice with some sex dysmorphology. Spontaneous scratching was detected in male and female mutants, with increased anxiety-like behavior in female mutants. These results suggest that the *COL6A5*-p.Glu2272* mutation found in patients contributes to chronic itch and probably induces additional behavioral changes. The *COL6A5*-p.Glu2272* mouse model could elucidate the pathophysiological mechanisms underlying *COL6A5* role in neuropathic itch and help identify potential new therapeutic targets.

2.1.2 Introduction

Itch (pruritus) is a common, irritating, unpleasant sensation that elicits the desire or reflex to scratch (Weisshaar et al., 2019). Clinically, the itch can be characterized as acute or chronic when it lasts more than 6 weeks (Yosipovitch et al., 2018). A European study revealed that the prevalence of acute itch in the general population is 8%, while it is 13% for chronic itch. Moreover, chronic itch with lifetime prevalence is 22% in the general population, indicating that more than 1 in 5 people experience chronic itch once in their life (Weisshaar et al., 2019). Moreover, the chronic itch greatly impacts the quality of life. Chronic itch is considered

neuropathic when neuronal damage is responsible for the symptom (Stumpf & Ständer, 2013). Damages at any site of the somatosensory system, including peripheral nerve fibers and ganglia, and the central nervous system, including the spinal cord, brainstem, thalamus, and cortex, may lead to neuropathic itch (Misery et al., 2014). Several conditions affecting the nervous system are associated with neuropathic itch. These include small fiber neuropathy (SFN), metabolic (e.g., diabetes), infections, and autoimmune and genetic diseases (Oaklander, 2011). The epidemiological studies investigating the prevalence and incidence of neuropathic itch have estimated that 8%-19% of chronic itch cases have a neuropathic origin (Meixiong et al., 2020; Pereira et al., 2021).

In recent years new insights have been gained. In particular, rare collagen VI alpha 5 (*COL6A5*) gene variants were identified in patients suffering from neuropathic itch (Martinelli-Boneschi et al., 2017). *COL6A5* is a member of the collagen protein superfamily. Collagens contain domains with VWA motifs that form filaments and are mainly associated with protein-ligand interactions for organizing tissue architecture and cell adhesion. COL6A5 is expressed in epithelial tissues, including the lungs and gastrointestinal tract (Fitzgerald et al., 2008), with a high expression at the dermal-epidermal junction (Philippeos et al., 2018) and around the vessels of the reticular dermis (Sabatelli et al., 2011). Previously, Martinelli-Boneschi and colleagues identified a heterozygous c.6814G>T transversion in the *COL6A5* gene of chronic neuropathic itch patients, resulting in a p.Glu2272-to-Ter (E2272X) substitution. Autosomal dominant transmission of chronic neuropathic itch was reported in 5 patients from 2 unrelated families (families 1 and 2) using whole-exome sequencing (Martinelli-Boneschi et al., 2017). These patients carrying the p.Glu2272* nonsense mutation also showed reduced COL6A5 expression in the skin. However, due to the complex nature of neuropathic itch, progress in understanding the mechanisms leading to chronic itch in these patients has not been made yet.

In order to investigate the consequence of this mutation, we developed a new mouse model for the *COL6A5*-p.Glu2272* mutation using the CRISPR-Cas9 technology. Our data provide evidence for augmented spontaneous scratching, grooming behavior and increased anxiety. This report demonstrates that the *COL6A5*-p.Glu2272* mutation contributes to itching, with emotional consequences.

2.1.3 Material and methods

2.1.3.1 Animal research

Animal research was performed in agreement with the EC directive 2010/63/UE86/609/CEE, in compliance with the animal welfare policies of the French Ministry of Agriculture (Art. R. 214-107 and 214-122). Animal were bred and maintained in our animal facility which is accredited by the French Ministry for Superior Education and Research and the French Ministry of Agriculture (agreement #C67-218-40) following the French Law (Decree n° 2013-118 01 and its supporting annexes entered into legislation on 01 February 2013) relative with the protection of animals used in scientific experimentation. All experiments were done in agreement with the local ethical committee. Studies are reported in accordance with the ARRIVE 2.0 guidelines. Mouse breeding and behavior experiments were conducted in SPF (Specific Pathogen Free) conditions in our animal facility at PHENOMIN-ICS, following the 3R principle. Mice were kept grouped in two to five animals in each cage. Mice were kept in the controlled light cycle at 12 h light and 12 h dark (light turned on at 7 AM and off at 7 PM through an automated control system). Mice were kept under a controlled temperature of 21 ± 1°C and humidity of 55 ± 10% and had free access to food (standard chow) and autoclaved tap water. For all experiments, mice were transferred to the experimental facility of ICS two weeks prior to behavior tests so that they have some time for habituation to the experimental facility of ICS. Animals were transferred to the experimental room 30 min before each experimental test.

2.1.3.2 Generating the *COL6A5*-p.Glu2272* mice model using CRISPR/Cas9

The mutant mouse line *Col6a5*^{em1YahICS}, also named *Col6a5*^{E2302*}, for the human *COL6A5*-p.Glu2272* mutation, was generated in ICS using CRISPR-Cas9 technology. In the mouse, the mutant codon is located in exon 38 (Ensembl ID ENSMUSE00000891350.1; **Figure 2.7**). The guide RNA had to drive the double strand break generated by the Cas9 protein very close to the site of insertion of the selection mutation (leading to a STOP codon). We used the CRISPOR online software (http://crispor.tefor.net/crispor.py) to select high specificity-score sgRNAs

with a low number of predicted off-target sequences. This guide RNA, AGTCATGGCAGAGAAGAACT, had a specificity score of 52 (internal name gR52). The guide RNA was validated *in vitro* for its ability to drive DSB (Double Strand Break) on a PCR fragment containing the targeted sequence in the presence of Cas9 protein. A donor single-stranded oligonucleotide (donor ssODN) was designed that bares the expected mutation (G>T, corresponding to the rs11537567 human variant) as well as 2 silent mutations (A>G and A>C). Its sequence was AGCCACGTCCATGTAATATTCTTGAAGAGAAGCATCCCCAGGCT-CATGAGCAAGACCCAGCTCTTATCGGCCATGACTTTCATGTTCTGCAACAGAATAACTTTAGTCACTA GTGGTGAAAGGGTTAACTTG. Together, these 2 mutations generate a new HaeIII restriction site. In addition, a third A>G mutation was introduced after the new STOP codon. These 3 mutations generated 3 mismatches in the donor DNA that allowed to avoid new double strand breaks after the occurrence of homology directed repair.

The CRISPR guide efficiency was tested *in vitro* using a Sureguide kit (Agilent Technologies 5190-7716). In the presence of the Cas9 protein, the PCR product, including the region of interest, should be cut. A guide is validated if it cuts the target PCR fragment. A mix containing the Cas9 mRNA, the guide RNA and the ssODN was microinjected in fertilized C57BL/6N oocytes, and PCR screened the newborn offspring. The microinjection of a mix of gR52 guide RNA (12 ng/µl), spCas9 mRNA (25 g/µl), and 10 ng/µl ssODN in the male pronuclei of 355 C57BL/6N fertilized oocytes led to 28 pups. PCR followed by HaeIII restriction digests, screened the pups. Primers F1 (AATGGAAATAATTCTGCACCAAGTG) and R1 (TAAGACAGAGGTCAGTGGAGCTGGG) were used to amplify a PCR fragment of expected size 426 bps. In the presence of the mutations of interest, 2 fragments of 236 bps and 190 bps were detected after HaeIII restriction digest. All undigested PCR products from pups showing HaeIII digests were subsequently sequenced by Sanger sequence. The insertion of the new STOP codon was confirmed by Sanger sequencing. Eleven F0 pups (>39 %) had the expected mutations. Five F0 animals were bred with wild type (wt) C57BL/6N mice to generate F1 founders. F1 mice were analyzed for germline transmission by Sanger Sequencing to establish the *Col6a5*$^{em1\text{-}E2302*}$ mouse line. All founders gave heterozygous pups. Two lines were fully established and cryopreserved, and one was further studied, as shown in the present paper.

2.1.3.3 Determination of genotype

Crude genomic DNA was extracted from mouse tail samples through Direct PCR Lysis Reagent-Tail (Viagen Biotech, Cat # 101-T) according to the manufacturer's instructions. Subsequently, the Phusion Hot Start II High-Fidelity DNA polymerase kit (Thermo Scientific) was used with primers specific for the wt (+) and mutant (*Col6a5*^{E2302*}) alleles. Moreover, PCR reaction containing the following: 500 ng genomic DNA extracted from a wt or mutant mouse, 4 µl 5×Phusion HF Buffer, 0.4 µl dNTP mix (dATP, dCTP, dGTP, dTTP at 10 mM, Thermo Scientific), 0.2 µl each primer at 0.5 µM and H2O in a total volume of 20 µl. Using a T100 thermocycler (Bio-Rad), PCR was performed with the following thermal condition: 96°C for 5min followed by 30 cycles of 96°C for 8s, 62°C for 10s and 68°C for 45s, and then annealing temperature as 68°C for 5min with final elongation step for 5min at 72°C. Next, 10 µl of PCR outcome was used to digest with enzyme HaeIII 0.2 µl, 10X Buffer of volume 2 µl, and remaining H2O to make a total volume of 20 µl for each reaction and kept at 37°C incubator for 15min. 2% agarose gel was run for both digested and non-digested PCR outcomes and differentiated between wt and mutant mice. Our point mutation (PM) containing sequence of DNA was digested by HaeIII restriction enzyme into further 236 bps and 190 bps *Col6a5*^{E2302*}allele along with 426 bps wt allele. In contrast, the non-digested = uncut PCR outcome gave only a wt allele of 426 bps. Mice genomic DNA showing only one band of the wt allele (+) of 426 bps in digested PCR outcomes were identified as wild-type littermate mice (wt lit). When both the wt allele band of 426 bps and the 236 bps & 190 bps PM alleles (mut) in digested PCR outcome, mice were heterozygous. Similarly, when mice genomic DNA showed no wt allele of 426 bps and only two bands of 236 bps and 190 bps of PM allele in digested PCR outcome they were genotyped as homozygous mice.

2.1.3.4 Analysis of transcript expression level by Droplet digital PCR (ddPCR)

We determined *Col6a5* mRNA expression using Real-Time droplet digital PCR (RT-ddPCR) on 42-week aged mice that underwent the previous behavioral characterization. Eight control (wt), 5 heterozygotes (hets) and 6 homozygotes (homs) males, plus 10 wt, 7 hets and 6 homs

females were used. Dorsal root ganglions (DRGs) were collected from wt and mutant mice and frozen in liquid nitrogen. Later, samples were lysed in TRIzol Reagent through magnetic beads containing Precellys®CK14 tubes. Total RNA was collected and purified using RNeasy Mini Kit (Qiagen) according to the protocol of the manufacturer company. Afterward, cDNA was synthesized by using the cDNA synthesis Kit "SuperScript™VILO™cDA Synthesis Kit" (Invitrogen). Then, ddPCR was performed for mRNA amplification in the volume of 20µL reactions for each sample. According to the manufacturer's recommendation, 250 nM specific primers, 125 nM of each probe, 1× ddPCR Supermix for Probes, and 50 ng DNA were used. PCR machine was run with the thermal condition as following: 10 min at 95°C, 40 cycles of 20s at 95°C, 30s at 59.2°C, 2min at 72°C, and 10min at 98°C. Droplet Digital PCR System (Bio-Rad) was used for droplet generation and quantification. Data were further analyzed using QuantaSoft Software (Bio-Rad). The sequences of primers and probes are shown in **Table 2.2**

2.1.3.5 Evans Blue dye penetration assay for skin permeability

To examine the skin barrier integrity of mice, the Evans blue dye assay was performed on 42-week aged mice that underwent the previous behavioral characterization. This assay allows to detect skin barrier impairment as described previously (Zhang et al., 2018). Ten wt, 9 hets and 10 homs males, with 14 wt, 12 hets and 11 homs females were used. Just after euthanasia with cervical dislocation, the back of each individual was shaved, and Evans blue dye (Sigma, Ref# E2129) was applied as 1% in PBS and 50 µl/mouse. After two hours, the skin was collected and homogenized in formamide (Sigma, Ref#7503). Subsequently, Evans blue dye extracted from skin samples was quantified by recording each sample by optical density (OD) at 620nm with a microplate reader.

2.1.3.6 Functional exploration and assessment

The behavioral characterization was performed on two experimental cohorts of males and females, ages 6 to 32 weeks. We used 13 B6N wt from the commercial breeders, 10 wt littermates, 12 hets and 10 homs males to evaluate scratching and grooming behaviors. For the female, 13 B6N wt, 14 wt littermates, 12 hets and 11 homs females were used. The same

cohorts or parts of these cohorts were used for recording anxiety- and despair-like behaviors and social behavior. The experimenter was blind to the mouse genotype.

2.1.3.7 Scratching and grooming behaviors

Scratching and grooming behaviors were recorded during different sessions at 6, 12, 18, 26 and 32 weeks of age. To assess behaviors, mice were given 2 weeks in the experimental facility for adaption before assessment. For each session, mice were transferred 30 min prior to the beginning of the observations in the experimental room. Further, one day before the scratching observation, mice were placed for 30 min in transparent plastic experimental boxes for habituation. On the day of the experiment, mice were given 10 min in transparent plastic boxes for habituation before the evaluation began. Behind the transparent plastic boxes, a mirror was also positioned to assess mice behavior from the front and back view, as mentioned previously (Shimada & LaMotte, 2008). Subsequently, mice behavior was video-recorded in the plastic box for 30 min as previously reported (T. Liu et al., 2016; Shimada & LaMotte, 2008; Y. G. Sun & Chen, 2007). While analyzing the videos, different parameters were scored, including scratching time (i.e. the time of lifting of the hind paw to the region of the body that is scratched (back and face) and returning to the cage floor) **(Figure 2.8)**. While grooming behavior was analyzed for whipping mice with forelimbs and licking their body and tail (Shimada & LaMotte, 2008). All scratching and grooming tests were performed between 8:00 AM and 2:00 PM.

2.1.3.8 Exploration and anxiety-like behaviors

Anxiety-related behavior was evaluated at 36 weeks of age on the same animals as those used for scoring scratching and grooming behaviors through an elevated plus maze experiment as previously described (Dubos et al., 2018). For locomotor and exploration activity, we also performed an open field test (Dubos et al., 2018). In an open-field experiment, a square apparatus (Panlab Harvard apparatus IR ACTIMETER, Bioseb, Vitrolles, France) containing all required sensors was used, and a polypropylene sheet covered the arena. Light for the open-field experiment was measured and kept at 150 lux in the center of the arena. Mice were placed at the periphery of the open field apparatus and were allowed to explore the arena

freely for 30 min. The experiment was performed in a closed room without any experimenter disturbance. The automated system measured the total distance, number of rearings, and time spent in center and peripheral regions with video tracking and infrared sensors. Mice activity was recorded with a video tracking system (Ethovision, Noldus, France) during this session of 30 min.

Further, anxiety-like behavior was assessed with the elevated plus maze. The elevated plus maze apparatus was placed at a height of 50 cm above the floor. It was made of PVC and completely automated (Imetronic, Pessac, France). It has two enclosed arms with dimensions (30 X 5 X 15 cm) and two open arms with dimensions (30 X 5 cm). The apparatus has infrared sensors to detect different parameters for anxiety, such as the number of entries in open arms, time spent in the open or closed arms, and the number of attempts made by mice etc. Mice were placed on a central platform and their exploration of the maze was recorded for 5 min.

2.1.3.9 Despair-like behavior

Despair-like behavior was evaluated at the age of 38 weeks on the same animals as those used for scoring scratching, grooming, and anxiety-like behaviors through the tail suspension test as described previously (Dubos et al., 2018). In this experiment, mice were suspended with the help of tape and hanged for 6min, and immobility time was monitored using video recording. An increased immobility time is indicative of a despair-like phenotype.

2.1.3.10 Social behavior

Social behavior was determined at the age of 38-weeks on the same cohorts and before scoring of despair-like behavior. The Stoelting system (Dublin, Ireland) was composed of three successive identical chambers (20 cm × 40 cm × 22 cm) with (5 cm × 8 cm) openings to allow access between the chambers. The protocol used was similar to the previously described (Arbogast et al., 2016; Duchon et al., 2011). We used adult C57BL/6N mice as stranger mice (unfamiliar mice). The age and sex of stranger mice and the mice tested for their social behavior were the same. Stranger mice were kept separately to avoid any olfactory or visual contact with test mice. Before the day of the experiment, stranger mice were habituated in

wire cages for 10min for 3-5 days until they felt comfortable staying in wire cages. The experiment was divided into three phases. In the first phase, test mice were placed in the middle chamber and allowed to habituate for 10min. In the second phase (social exploration), the test mouse was enclosed in the central box, an unfamiliar mouse (stranger 1 or S1) was placed randomly in one of the wire cages, and on another side, an empty wire cage was placed. The doors were re-opened, and the test mouse was allowed to explore the entire social test box for 10 min. Time spent in each chamber, the number of entries into each chamber and the time spent sniffing each wire cage were recorded. In the third phase (social discrimination), a new, unfamiliar mouse (stranger 2 or S2) was placed by replacing the empty wire cage, and the test mouse was allowed to explore for 10 more min. During this time, the test mouse could explore or sniff the already-investigated mouse (S1) and the novel unfamiliar mouse (S2). The entire social test box was washed with tap water and dried with absorbent paper between each test to remove odors.

2.1.3.11 Statistical analysis

The results are expressed as mean ± standard error of the mean (SEM) for each experimental group. Student's t-test (two-tailed) was used to compare the two groups' differences. In addition, multiple groups were compared using one-way or two-way repeated measures analysis of variance (ANOVA) with a Tukey post-hoc test where appropriate. Data was analyzed by using GraphPad Prism 9 software. For all analyses, a p-value was considered significant as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

2.1.4 Results

2.1.4.1 Generation of the *COL6A5*-p.Glu2272* mice model

The *COL6A5*-p.Glu2272* mouse model for the human mutation c.6814G>T (ID rs115535867) p.Glu2272* was generated by homologous directed repair through a CRISPR/Cas9 approach in the exon 38 (**Figure 2.1A**). The *Col6a5*^{em1} (E2302*) carrier mice line displayed the mutation as shown by both PCR analysis and Sanger sequencing (**Figure 2.1B-D**). Mutant heterozygous mice were inter-crossed to obtain wt, heterozygous and homozygous littermates. The *Col6a5* transcript expression level was evaluated in DRGs isolated from individuals with different

genotypes through RT-ddPCR, and the results are shown in **Figure 2.1E**. *Col6a5* transcripts from the wt allele were detected in control littermate mice of both sexes. In homozygous mice, only the mutant transcripts were found expressed. In heterozygous mice, both wt and mutant

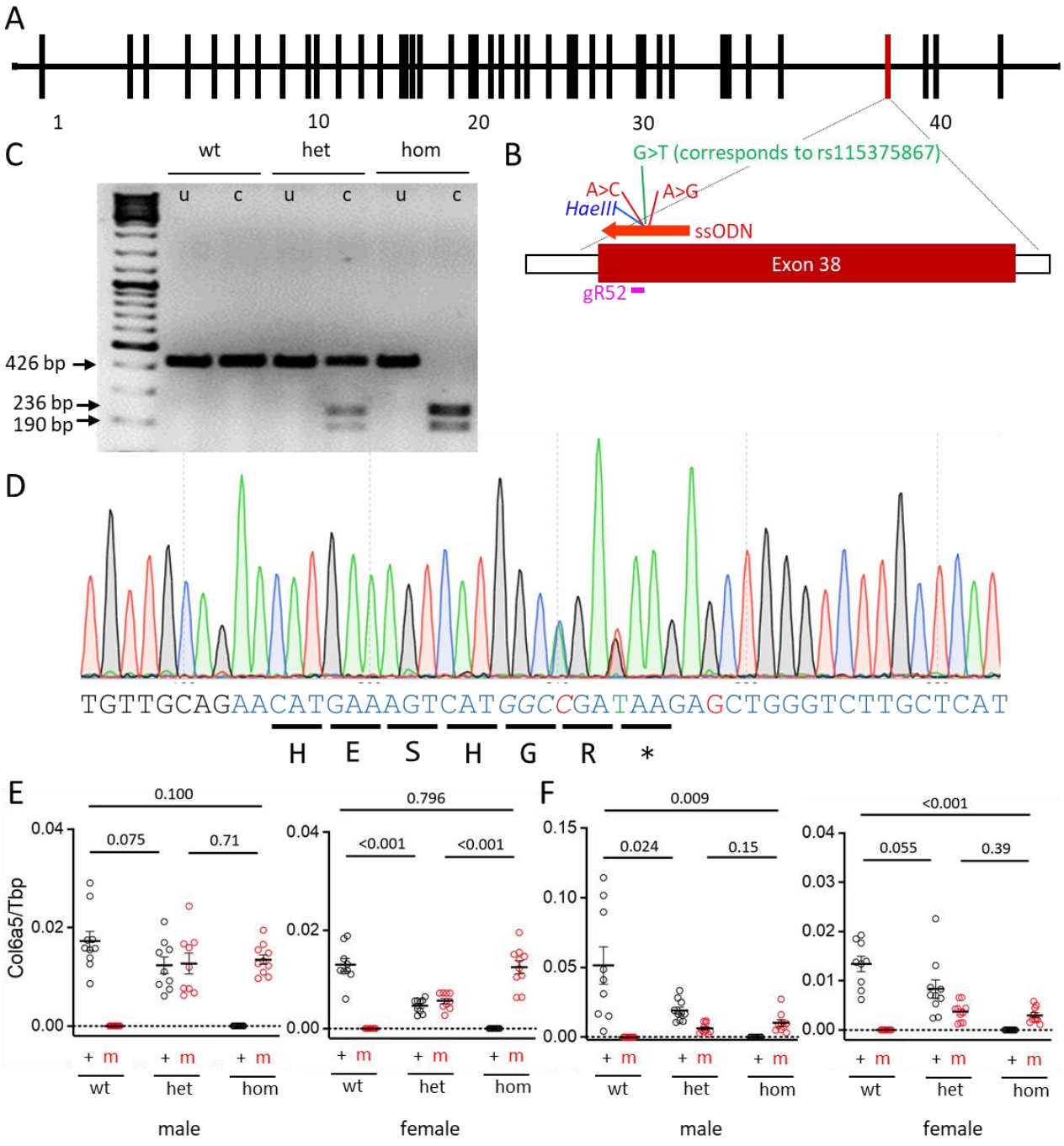

Figure 2.1 Generation and validation of the *COL6A5*-p.Glu2272* mouse model. A) Organization of the murine *Col6a5* gene. The numbers indicate the position of exons. The target exon (exon 38) is shown in red color. B) A donor single stranded oligonucleotide

(ssODN) was designed. This donor bares the expected mutation (G>T, corresponding to the rs11537567 human variant) as well as 2 silent mutations (A>G and A>C). These 3 mutations also create 3 mismatches so that no new DSBs (double-stranded breaks) could occur after. Using CRISPOR online software, high specificity-score sgRNAs (gR52) were selected with low predicted off-target sequences. C) The DNA sequence containing point mutation (PM) was digested by HaeIII enzyme "c (cut)" into further 236 bps and 190 bps PM allele along with 426 bps wt allele. In comparison, the non-digested "u(un-cut)" PCR outcome only gave wt allele of 426 bps. D) Through Sanger sequencing, PM mutation was confirmed and is shown with the stop codon (*). The wild-type (+) and mutant allele (mut) mRNA expression in DRG (E) or skin (F) isolated from *Col6a5* littermates (from 5 to 10 individuals per group) with the three genotypes: wt, heterozygous (het), and homozygous (hom) mice. *Col6a5* mRNA expression was normalized to *Tbp* expression as a control. In the DRG, Homozygous mutant mice expressed *Col6a5* mutant transcripts at levels comparable to "+" transcripts in wt mice, and heterozygous mice expressed comparable levels of each allele. F) The wt and mutant allele (Mut) mRNA expression in the skin of wt littermate, heterozygous (Het), and homozygous (Homo) mice for *Col6a5*^{em1}(E2302*). Mutant mice have significantly decreased *Col6a5* mRNA expression in the skin. Males, n=9-10/group; females, n=9-10/group. Student's t-test.

alleles were expressed at comparable levels in both sexes. On the other hand, in skin, mRNA expression was decreased significantly in mutants as compared to controls (**Figure 2.1F**). This observation was similar to that for patients with chronic neuropathic itch harboring the p.Glu2272* mutation: COL6A5 expression was reduced in the papillary dermis and around the dermal vessels compared to controls (Martinelli-Boneschi et al., 2017). Nevertheless, it is important to indicate that there was no significant sex difference in the reduction of *Col6a5* expression in the skin. *Col6a5* transcript expression levels were similarly decreased in both sexes. Yet, the expression of *Col6a5/Tbp* in the skin was noticeably lower in wt females vs males, with a value of 0.014 compared to 0.05 in wt males, implying that *Col6a5/Tbp* expression in wt females is approximately 3.57 times lower than in wt males. This baseline difference in *Col6a5* expression between wt males and females may result in a more pronounced impact of the mutation in females.

2.1.4.2 Increased skin permeability in *COL6A5*-p.Glu2272* mutant mice

To assess the impact of the mutation on the skin barrier, we investigated skin permeability in wt and mutant animals using the Evans blue dye assay. We observed that both heterozygous

and homozygous females showed a significant increase in skin permeability compared to wt females (**Figure 2.2**). However, no difference was found between mutant and wt male mice. Control wt females had lower skin permeability compared to wt males. Altogether, these results indicate a sexually dimorphic effect of the p.Glu2272* mutation on skin permeability in mice.

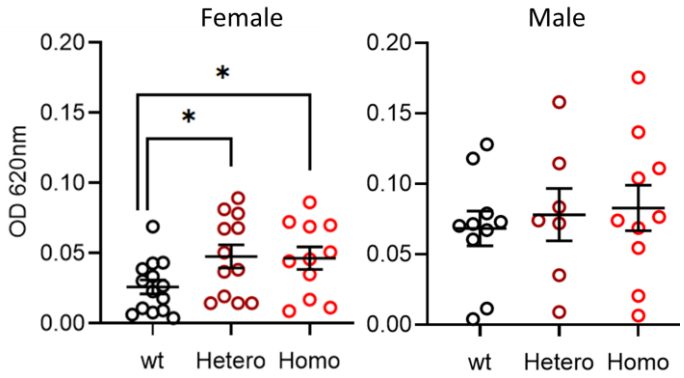

Figure 2.2 Increased skin permeability in *COL6A5*-p.Glu2272* mice. The permeabilization assay done on skin, on heterozygous (Hetero) and homozygous (Homo) skin mice showed significant differences in female mutants compared to wt littermates (wt) (n=11-14 /group; unpaired t test,*P < 0.05). No genotype difference was found in males (n=7-10 /group). Wt females had lower skin permeability compared to wt males (unpaired t test, P=0.002).

2.1.4.3 *COL6A5*-p.Glu2272* mice displayed spontaneous scratching behavior

The patients with the *COL6A5*-p.Glu2272* mutation complained of spontaneous chronic itch appearing at ages ranging from 5 to 40 years (Martinelli-Boneschi et al., 2017). To investigate spontaneous itch in mutant mice, we scored their scratching behavior (time of scratching and number of bouts during 30 min) at different ages, starting from 6 weeks until 32 weeks (**Figure 2.3**). Because scratching behavior was previously shown to be contagious and imitative in mice (Yu et al., 2017), we defined two different types of controls: a first set made of B6N unrelated wt aged-match controls (B6-Unr), that were kept in separate cages to prevent any physical or visual contact with the mice of the mutant line; and a second set of controls were the wt littermates (B6-Lit) of the mutant mice bred in the same cage with mutant carriers. The comparison of the B6-Unr and B6-Lit showed that B6-Unr female mice had an effect of age and an interaction between age and genotype but no genotype effect (two-way RM

ANOVA, P=0.01 and P=0.006, respectively). Interestingly, B6-Lit males had increased scratching in littermates as compared to unrelated B6 (two-way RM ANOVA P=0.032 for genotype). On sex-grouped animals, we observed a comparable scratching behavior in B6-Unr and B6-Lit (two-way RM ANOVA P=0.015 for age). Altogether, we could not detect a strong effect between the two groups of control by analyzing at each time point.

We tested whether the *Col6a5*^{em1}(E2302*) mutant mice showed altered scratching at 6, 12, 18, 26, and 32 weeks. For this, we observed the time spent scratching of the four groups, i.e. the 2 controls as well as heterozygotes and homozygotes. Because we observed some variation of scratching over the weeks, we also calculated the Area Under the Curve (AUC) for further statistical analysis. An increase in the time spent scratching over the weeks was unraveled when comparing heterozygote or homozygote females with both wt control groups (One-way ANOVA with Tukey's multiple comparisons test; $F(3, 46)=12.27$; $P<0.0001$; **Figure 2.3** left panels). No difference was observed between Het and Hom mutant females. An effect on scratching time was also detected in Het males ($F(3,40)=17.68$; $P<0.0001$), with increased time of scratching, and the increase was slightly lower in male homozygotes compared to heterozygotes. When considering the two sexes together, the increase of scratching in mutants versus the two wt controls was more significant ($F(3,90)=23.36$; $P<0.0001$), that may be due to the increased number of individuals as compared to single-sex analysis.

Further, we compared the number of bouts per 30 min with a similar analysis and calculation of the AUC (**Figure 2.3**, right panel). Briefly, the number of bouts was increased in heterozygotes and homozygote mutant females as compared to the 2 wt controls ($F(3,46)=14.36$; $P<0.0001$). The genotype effect was also observed in the males ($F(3,41)=15.57$; $P<0.0001$) and when both sexes were analyzed together (sex-grouped, ($F(3,91)=25.37$; $P<0.0001$). Overall, despite the lack of patients described with homozygous mutation, homozygous mice showed similar scratching behavior as heterozygous mice. These results obtained on the *Col6a5*^{em1}(E2302*) mouse model confirmed the dominant effect of the mutation. A relation of causality can now be drawn with one copy of the *COL6A5*-p.Glu2272* mutation being enough to produce the scratching phenotype.

2.1.4.4 *COL6A5*-p.Glu2272* mice showed increased grooming behavior

Induction of chronic itch in mice also led to increased grooming behavior (X. D. Wang et al., 2018). Therefore, we decided to investigate the spontaneous self-grooming behavior without administering any external inducer. Grooming behavior was recorded for 30min through video.

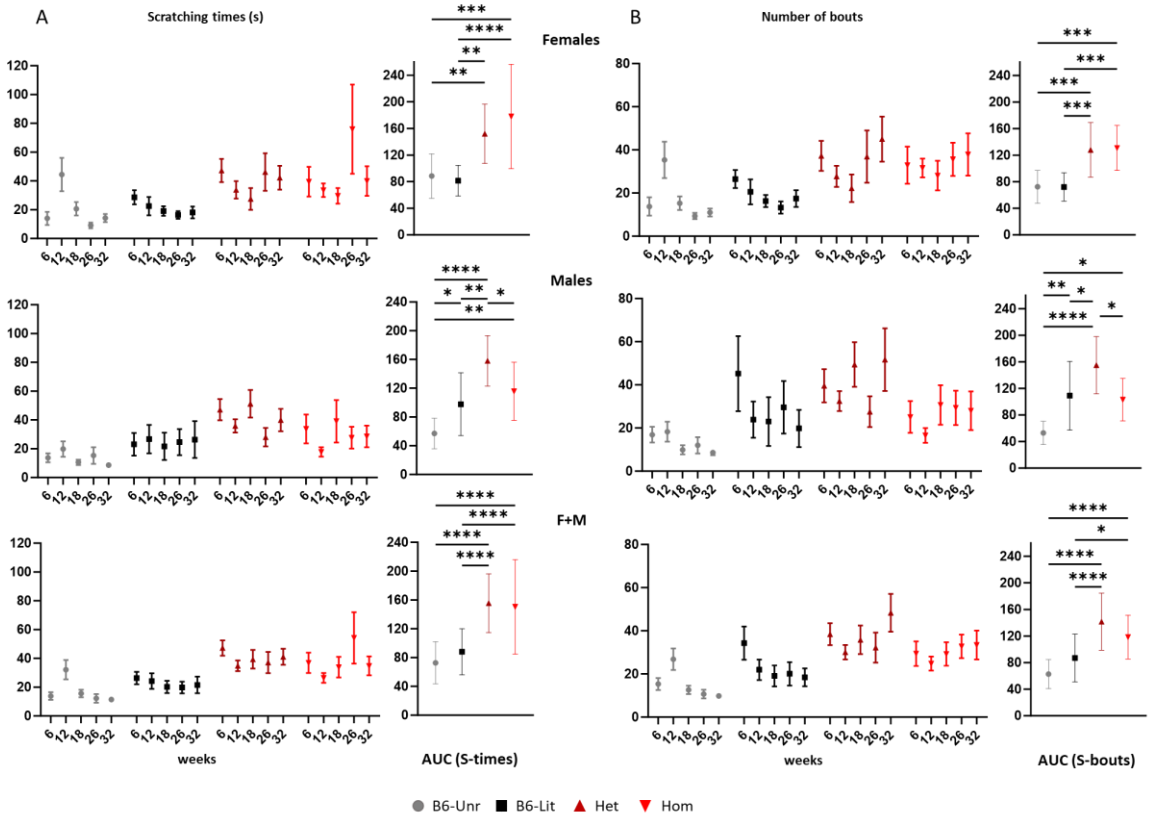

Figure 2.3 *COL6A5-p.*Glu2272* mice showed increased spontaneous scratching behavior. Scratching times (s) (A) and number of bouts (B) were scored at 6, 12, 18, 26 and 32 weeks of age on individuals, from both sexes of unrelated B6N wt (B6-Unr) mice and of the different littermates, either wt (B6-Lit), heterozygous (Het) or homozygous littermate (Hom) genotypes, derived from *Col6a5*^{em1}(E2302*) heterozygous crosses. Overall, the mutant mice, either het or hom, showed significantly increased scratching, as evaluated by calculating the Area Under the Curve (AUC) for the scratching times (left) or the number of bouts (right) and determined by One-way ANOVA with Tukey's multiple comparisons test. (Male, n=9-13 /group; Female, n=11-14 /group). Data presented as mean +/- sem. P-value as indicated with * $P < 0.05$, ** $P < 0.01$, and *** $P< 0.001$ and **** $P<0.00001$.

recording (**Figure 2.4**). Increased grooming was detected in homozygous females (One-way ANOVA; F(3, 46)=3.773; P=0.0167) and males (One-way ANOVA; F(3, 41)= 3,300; P=0.0297). Combining the two sexes showed more significant differences (One-way ANOVA; F(3, 91)=4.820; P=0.004). Overall, the data demonstrate that the p.Glu2272* mutation leads to augmented grooming.

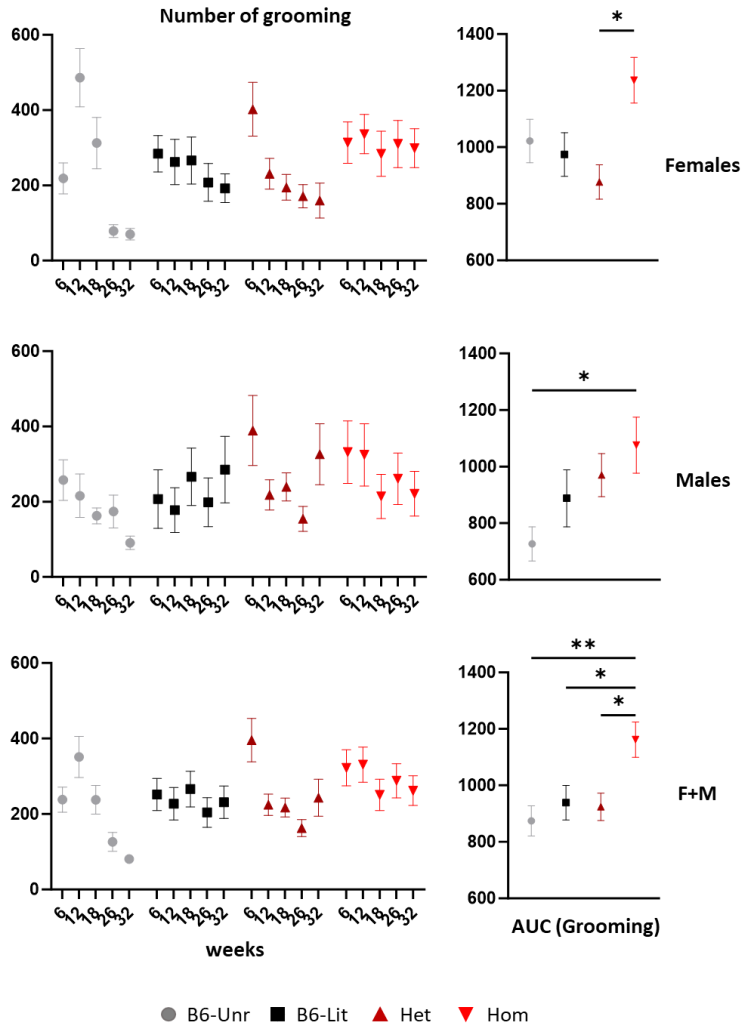

Figure 2.4 Increased grooming behavior in *COL6A5*-p.Glu2272* homozygote mice. Spontaneous self-grooming behavior was evaluated in B6N unrelated wt (B6-Unr), wt littermate (B6-Lit), heterozygote (het) and homozygote mutant mice over 6 weeks to 32 weeks of age by session of 30min (left panel). The Area Under the Curve (AUC) of grooming along the period showed that homozygotes displayed a significant increase, compared to het littermate in females and B6-Unr in males. When comparing males and females together the significance was observed between homozygotes and all the other genotypes. Male, n=9-13 /group; Female, n=11-13 /group. A p-value was considered as significant as *p < 0.05 and **p < 0.01.

2.1.4.5 Increased anxiety and despair-like behaviors in *COL6A5*-p.Glu2272* mice

Chronic itch is clinically correlated with mood disorders such as anxiety and depression (Long et al., 2022; X. D. Wang et al., 2018). Such itch-associated mood disorders have already been studied to characterize the affective consequences of chronic itch in mice (X. Zhao et al., 2018). Therefore, we also investigated these disorders in the mutant mice and performed anxiety and despair-like behavioral tests. We tested the mutant mice in the elevated plus maze (EPM) to assess anxiety-like behavior. We evaluated three different parameters of the EPM, 1) % of open arms entries, 2) % of the time in open arms, and 3) the number of attempts (**Figure 2.5**). We observed that male mutant mice showed no difference in all three parameters. On the other hand, female homozygous mice showed significant anxiety-like behaviors by reduced entries into open arms and less time spent in open arms than their wild-type counterparts.

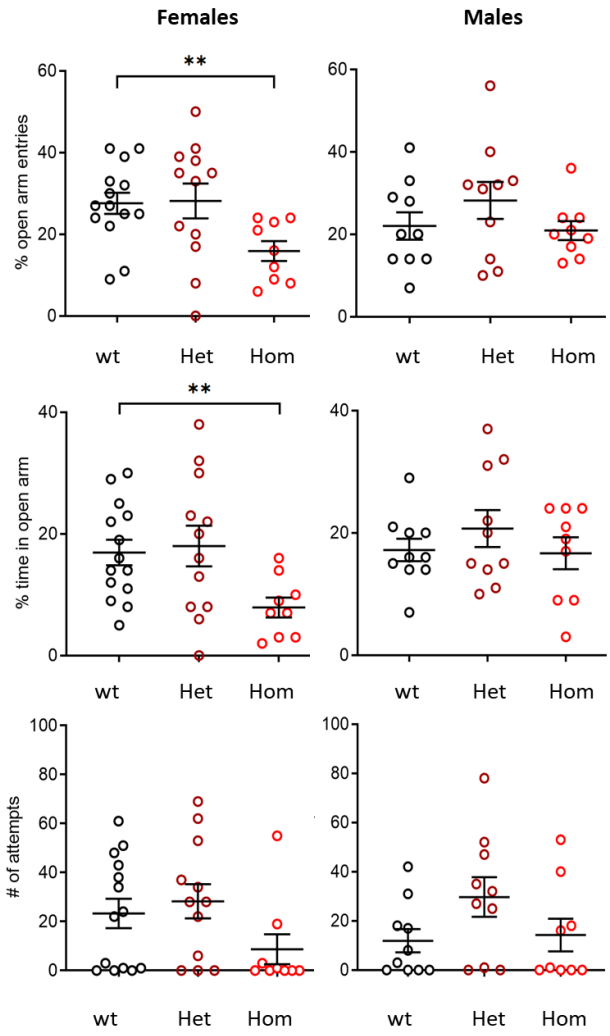

Figure 2.5 Increased anxiety-like behavior in *COL6A5*-p.Glu2272* homozygous females. Mutant and wt littermates control mice were assessed in the elevated plus maze test through three different parameters. A) % of open arms entries, B) % of the time in open arms, and C) the number of attempts. It was observed that female homozygous mice showed significant anxiety-like behavior by reduced entries into the open arm and spent less time in open arms as compared to wt mice. Overall results showed that homozygous mice have an anxiety-like behavior phenotype compared to wild-type mice. Male, n=9-10 /group; Female, n=9-14 /group. wt littermate (wt), heterozygous (Hetero), and homozygous (Homo) mice were compared. A p-value was considered as significant as *$p < 0.05$ and **$p < 0.01$.

We also tested the mutant mice for their behavior in the open field test measured to assess the basic status of mice. There was no difference between mutant and wt mice for any of the parameters studied which were the time spent in the center, rearing number, and total distance traveled (**Figure 2.9**). Overall, these results indicate that the p.Glu2272* mutation induced more anxiety in females as measured in the EPM.

In chronic pruritus, the “itch-scratch-itch” cycles may lead to a depressive state which was also studied in mice previously (X. D. Wang et al., 2018). To investigate the despair-like behavior in <p.Glu2272* mutant mice, we tested them in the tail suspension test (**Figure 2.10**). No difference was found between genotypes (One-way ANOVA).

2.1.4.6 *COL6A5*-p.Glu2272* mice show no deficit in social preference but male mutant displayed altered social discrimination

We have also investigated the social behavior of mutant mice by using the three-chamber test. In the first experiment, it was found that mice of all three genotypes, wt, heterozygous and homozygous mice, had a higher sniffing time toward a stranger mouse (S1) than in the empty compartment (E) (**Figure 2.11**). This showed that mutant animals had a social preference behavior comparable to their wt counterparts. In the second part of the test, the preference for social novelty was measured by placing another novel stranger mouse (S2) in the empty compartment and comparing the interactions of the test mouse with the familiar stranger (S1) mouse and a novel stranger (S2) mouse. We observed that wt male mice showed more sniffing time towards the novel stranger mouse. In contrast, mutant males did not show any preference for a novel stranger (**Figure 2.6**), suggesting that the mutant males have a deficit in social discrimination. For females, none of the genotypes preferred the novel stranger (S2) vs the familiar stranger (S1), precluding any conclusion. Overall, the results from the two phases of the three-chamber test indicate no alteration of social exploration in the mutant males and females and a deficit in social discrimination in the mutant males.

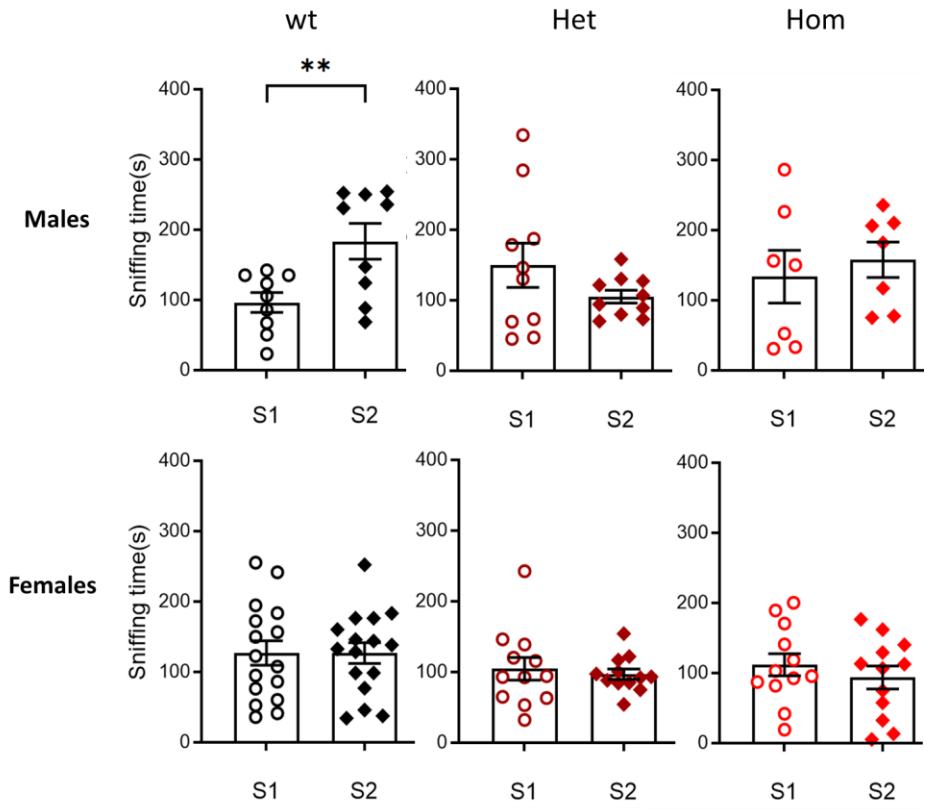

Figure 2.6 *COL6A5*-p.Glu2272* male mice showed a deficit in social discrimination. In the three-chamber test, the preference for social novelty was measured by placing a new stranger mouse in the empty compartment and comparing the interactions of the test mice with the familiar stranger 1 (S1) mouse and the novel stranger 2 (S2) mouse. Male wt littermates (wt) mice showed more sniffing time towards the novel stranger S2, while mutant males did not. All of the female mice groups were impaired for discriminating social novelty. Heterozygous (Het) and homozygous (Hom) mice. Males, n=7-10 /group; females, n=12-16 /group. A p-value was considered as significant as **$p < 0.01$.

2.1.5 Discussion

We successfully generated the *Col6a5*^{E2302*} mutant mouse model corresponding to the human *COL6A5*-p.Glu2272* mutation using the CRISPR/Cas9 technology. We used this genetic model we confirmed the genotype-phenotype association of this mutation with chronic itching in mice. We explored molecular aspects of this mutation and its consequences on other behaviors. We found that the wt and mutant *Col6a5* transcripts were expressed at similar levels in DRGs. However, mutant transcripts were expressed at lower levels in the skin, in accordance with the decreased level of COL6A5 expression found in patients' fibroblasts of the affected families in the clinical study (Martinelli-Boneschi et al., 2017). We also found enhanced skin permeability in mutant female mice. Further, we observed spontaneous itch

in adult p.Glu2272* mutant mice, providing evidence of long-lasting itch with consequences on emotional behaviors.

Mutant female mice showed enhanced skin permeability compared to their wt littermates, which could be due to several factors. For instance, a study in Human reported that in skin dermis higher concentration of collagen in the skin was correlated with the thickness of the skin. Another study reported the differences in skin structure in male and female mice (Azzi et al., 2005). These observations were also confirmed in other studies (Arai et al., 2017; Calabro et al., 2011). Interestingly, this article (Arai et al., 2017) demonstrated that the expression of *Col1a1, Col1a2,* and *Col3a1* was higher in the skin of male mice than in female mice, while *Col5a1* and *Col4a1* were expressed similarly in both sexes. Our results show a similar expression of wt *Col6a5* transcripts in male and female skin. We can hypothesize that being expressed close to the skin barrier, a change in COL6A5 structure may affect the permeability of the skin. This is an interesting hypothesis to follow, considering the association of COL6A5 polymorphisms with atopic dermatitis (Szalus et al., 2023) and an additional hypothesis based on COL6A5+ dermis fibroblasts contributing to skin inflammation (Li et al., 2023).

The clinical study by Martinelli-Boneschi and colleagues showed that all individuals in the two families with the p.Glu2272* mutation showed chronic itch at various ages. Family 1 had three patients, including one female and two males, and family 2 had two female patients. In all five patients, the onset age for the appearance of spontaneous chronic itch was different but shared similar characteristics. In family 1, two male and one female patients reported onset age for chronic itch as 30, 33, and 40 years respectively. In family 2, the two patients reported age onsets for itch 5 and 8 years (Martinelli-Boneschi et al., 2017). Considering these early and adult ages for the onset of itch, in this study, we observed the scratching behavior of mutant mice over an extended period from early age to late age (6 weeks to 32 weeks). When the analysis started, at six weeks of age, the mice were still considered at a young age. Though many of the physiological systems were mature, the immune and nervous systems continued to be settled within the next 2 weeks. Indeed, before 8 and 11 weeks of age, the mouse systems were under ongoing maturation, marking by the end of rapid changes in mass and cell number in the mouse central nervous system and getting the various brain structures to

reach their adult-like states during this age (Fu et al., 2013). Therefore, in this study, mice scratching behavior was observed not at a specific age but for an extended period. In our study, we have monitored for extended and continuous period as compared to other chronic itch model studies where often the tests are only done for a few weeks. For example, Ueda and colleagues produced a chronic itch mice model and observed scratching behavior till 8 weeks (Ueda et al., 2006). To gain a deeper understanding of spontaneous chronic itch, we argue that it is crucial to conduct analysis over an extended period of time. For instance, another study was performed for spontaneous chronic itch till 14 to 20 weeks of age (Z. Q. Zhao et al., 2013). For this reason, our study's approach of conducting a more prolonged and continuous assessment of genetically induced spontaneous chronic itch is more valuable in this context, enabling a more comprehensive exploration of chronic itch.

Recently, mood impairment was reported for the first time in male mice with chronic itch induced by a repetitive treatment with acetone and diethyl ether followed by water which models dry skin, the AEW model (X. Zhao et al., 2018). Also, anxiety-like behaviors were detected in mice with histamine or serotonin-induced acute itch (Sanders, Sakai, et al., 2019). When analyzed for mood alterations, only the female *Col6a5*^{E2302*} homozygotes showed anxiety in the elevated plus maze but no despair-like phenotypes, while males were not affected. Interestingly, both *Col6a5*^{E2302*} heterozygous and homozygous male mutants showed a deficit in social preference in the three-chamber test. This defect in discrimination may be due to attention deficit as a result of increased itch. Further exploration of the emotional and social behaviors of *Col6a5*^{E2302*} mutant mice is needed to decipher whether these altered behaviors are a direct or indirect effect of increased itch or a secondary effect of the *Col6a5* mutation itself.

This is the first study to report a mouse model of chronic itch developed from a mutation in a collagen gene identified in chronic neuropathic itch patients. Previously, another mutation, the L811P mutation in the *SCN11A* gene, has been identified in patients with chronic itch and shown to cause chronic itch in the *Scn11a*^{L811P} mouse model (Ebbinghaus et al., 2020; Salvatierra et al., 2018). Our mouse model provided useful insight for investigating the pathophysiology of human mutation *COL6A5*-p.Glu2272* as well as chronic neuropathic itch. Further, this study provided evidence that chronic neuropathic itch can be associated with

other behavioral phenotypes, affecting anxiety and sociability. Together, these findings may open novel avenues for the study of chronic neuropathic itch and its underlying mechanisms and help identify new therapeutic targets for treating chronic itch.

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COMPETING INTEREST

None

2.1.8 Author Contribution declaration

The conceptualization of the study was done by GL, CGR and YH; The data curation and formal analysis by AR, CGR, YH; the funding Acquisition by GL, YH; the investigation by AR based on methodology developed by AR, CG, MCB, and YH: the project was administrated by YH with resources from MCB; The supervision and validation was done by CGR, YH; The original draft was prepared by AR, CGR, YH; The Review & Editing of the final manuscript was done by all authors.

2.1.9 References

Arai KY, Hara T, Nagatsuka T, et al (2017) Postnatal changes and sexual dimorphism in collagen expression in mouse skin. PLoS ONE 12:1–21. https://doi.org/10.1371/journal.pone.0177534

Arbogast T, Ouagazzal A-M, Chevalier C, et al (2016) Reciprocal Effects on Neurocognitive and Metabolic Phenotypes in Mouse Models of 16p11.2 Deletion and Duplication Syndromes. PLoS Genet 12:e1005709. https://doi.org/10.1371/journal.pgen.1005709

Azzi L, El-Alfy M, Martel C, Labrie F (2005) Gender differences in mouse skin morphology and specific effects of sex steroids and dehydroepiandrosterone. Journal of Investigative Dermatology 124:22–27. https://doi.org/10.1111/j.0022-202X.2004.23545.x

Calabro K, Curtis A, Galarneau J-R, et al (2011) Gender variations in the optical properties of skin in murine animal models. Journal of Biomedical Optics 16:011008. https://doi.org/10.1117/1.3525565

Dubos A, Meziane H, Iacono G, et al (2018) A new mouse model of ARX dup24 recapitulates the patients' behavioral and fine motor alterations. Human Molecular Genetics 27:2138–2153. https://doi.org/10.1093/hmg/ddy122

Duchon A, Pothion S, Brault V, et al (2011) The telomeric part of the human chromosome 21 from Cstb to Prmt2 is not necessary for the locomotor and short-term memory deficits observed in the Tc1 mouse model of Down syndrome. Behavioural Brain Research 217:271–281. https://doi.org/10.1016/j.bbr.2010.10.023

Ebbinghaus M, Tuchscherr L, von Banchet GS, et al (2020) Gain-of-function mutation in SCN11A causes itch and affects neurogenic inflammation and muscle function in Scn11a+/L799P mice. PLoS ONE 15:1–17. https://doi.org/10.1371/journal.pone.0237101

Fitzgerald J, Rich C, Zhou FH, Hansen U (2008) Three novel collagen VI chains, α4(VI), α5(VI), and α6(VI). Journal of Biological Chemistry 283:20170–20180. https://doi.org/10.1074/jbc.M710139200

Fu Y, Rusznák Z, Herculano-Houzel S, et al (2013) Cellular composition characterizing postnatal development and maturation of the mouse brain and spinal cord. Brain Structure and Function 218:1337–1354. https://doi.org/10.1007/s00429-012-0462-x

Li Z, Cao T, Li Q, et al (2023) Cross-disease characterization of fibroblast heterogeneities and their pathogenic roles in skin inflammation. Clinical Immunology 255:109742. https://doi.org/10.1016/j.clim.2023.109742

Liu T, Han Q, Chen G, et al (2016) Alloknesis , and Spinal Astrocyte Activation in Male Mice. 157:806–817

Long Q, Jin H, You X, et al (2022) Eczema is a shared risk factor for anxiety and depression: A meta-analysis and systematic review. PLoS ONE 17:. https://doi.org/10.1371/journal.pone.0263334

Martinelli-Boneschi F, Colombi M, Castori M, et al (2017) COL6A5 variants in familial neuropathic chronic itch. Brain 140:555–567. https://doi.org/10.1093/brain/aww343

Meixiong J, Dong X, Weng HJ (2020) Neuropathic Itch. Cells 9:5–7. https://doi.org/10.3390/cells9102263

Misery L, Brenaut E, Le Garrec R, et al (2014) Neuropathic pruritus. Nature Reviews Neurology 10:408–416. https://doi.org/10.1038/nrneurol.2014.99

Pereira MP, Wiegmann H, Agelopoulos K, Ständer S (2021) Neuropathic Itch: Routes to Clinical Diagnosis. Frontiers in Medicine 8:. https://doi.org/10.3389/fmed.2021.641746

Philippeos C, Telerman SB, Oulès B, et al (2018) Spatial and Single-Cell Transcriptional Profiling Identifies Functionally Distinct Human Dermal Fibroblast Subpopulations. Journal of Investigative Dermatology 138:811–825. https://doi.org/10.1016/j.jid.2018.01.016

Sabatelli P, Gara SK, Grumati P, et al (2011) Expression of the collagen VI α5 and α6 Chains in normal human skin and in skin of patients with collagen VI-related myopathies. Journal of Investigative Dermatology 131:99–107. https://doi.org/10.1038/jid.2010.284

Salvatierra J, Dong X, Bosmans F (2018) A disease mutation reveals a role for Na V 1.9 in acute itch The Journal of Clinical Investigation. J Clin Invest 128:5434–5447

Sanders KM, Sakai K, Henry TD, et al (2019) A subpopulation of amygdala neurons mediates the affective component of itch. Journal of Neuroscience 39:3345–3356. https://doi.org/10.1523/JNEUROSCI.2759-18.2019

Shimada SG, LaMotte RH (2008) Behavioral differentiation between itch and pain in mouse. Pain 139:681–687. https://doi.org/10.1016/j.pain.2008.08.002

Söderhäll C, Marenholz I, Kerscher T, et al (2007) Variants in a novel epidermal collagen gene (COL29A1) are associated with atopic dermatitis. PLoS Biology 5:1952–1961. https://doi.org/10.1371/journal.pbio.0050242

Stumpf A, Ständer S (2013) Neuropathic itch: diagnosis and management

Sun YG, Chen ZF (2007) A gastrin-releasing peptide receptor mediates the itch sensation in the spinal cord. Nature 448:700–703. https://doi.org/10.1038/nature06029

Szalus K, Zysk W, Gleń J, et al (2023) The Associations of Single Nucleotide Polymorphisms of the COL3A1, COL6A5, and COL8A1 Genes with Atopic Dermatitis. Journal of Personalized Medicine 13:. https://doi.org/10.3390/jpm13040661

Ueda Y, Inoue T, Rahman MA, et al (2006) A new chronic itch model accompanied by skin lesions in hairless mice. International Immunopharmacology 6:1609–1615. https://doi.org/10.1016/j.intimp.2006.06.002

Wang XD, Yang G, Bai Y, et al (2018) The behavioral study on the interactive aggravation between pruritus and depression. Brain and Behavior 8:1–9. https://doi.org/10.1002/brb3.964

Weisshaar E, Szepietowski JC, Dalgard FJ, et al (2019) European S2k Guideline on Chronic Pruritus. Acta dermato-venereologica 99:469–506. https://doi.org/10.2340/00015555-3164

Yosipovitch G, Rosen JD, Hashimoto T (2018) Itch: From mechanism to (novel) therapeutic approaches. Journal of Allergy and Clinical Immunology 142:1375–1390. https://doi.org/10.1016/j.jaci.2018.09.005

Yu YQ, Barry DM, Hao Y, et al (2017) Molecular and neural basis of contagious itch behavior in mice. Science 355:1072–1076. https://doi.org/10.1126/science.aak9748

Zhang Y, Hwang BJ, Liu Z, et al (2018) BP180 dysfunction triggers spontaneous skin inflammation in mice. Proceedings of the National Academy of Sciences of the United States of America 115:6434–6439. https://doi.org/10.1073/pnas.1721805115

Zhao X, Yu C, Ye F, et al (2018) Chronic itch impairs mood and HPA axis function in mice: Modulation by CRFR1 antagonist. Pain 159:2201–2213. https://doi.org/10.1097/j.pain.0000000000001319

Zhao ZQ, Huo FQ, Jeffry J, et al (2013) Chronic itch development in sensory neurons requires BRAF signaling pathways. Journal of Clinical Investigation 123:4769–4780. https://doi.org/10.1172/JCI70528

2.1.10 Supplementary materials, tables and Figures

Table 2.1 Sequence of Col6a5 primers used for genotyping.

Primer	Sequence
Forward	AATGGAAATAATTCTGCACCAAGTG
Reverse	TAAGACAGAGGTCAGTGGAGCTGGG

Table 2.2 Sequence of Col6a5 and Tbp probes and primers used for RTddPCR.

Primer /Probe	Sequence
Col6a5-Forward primer sequence (5'-3')	AGGAAAGCCACGTCCATGTAATA
Col6a5-Reverse primer sequence (5'-3')	GGAAATGACCGGGAATCTGTAG
wt-*Col6a5*-probe	AGTCATGGCAGAGAAGAACTGGGT
Mut-*Col6a5*-probe	AAGTCATGGCCGATAAGAGCTGGG
Tbp - Forward primer sequence (5'-3')	CTACCGTGAATCTTGGCTGTAA
Tbp -Reverse primer sequence (5'-3')	GTTGTCCGTGGCTCTCTTATT
Tbp-Probe	AATCCCAAGCGATTTGCTGCAGTC

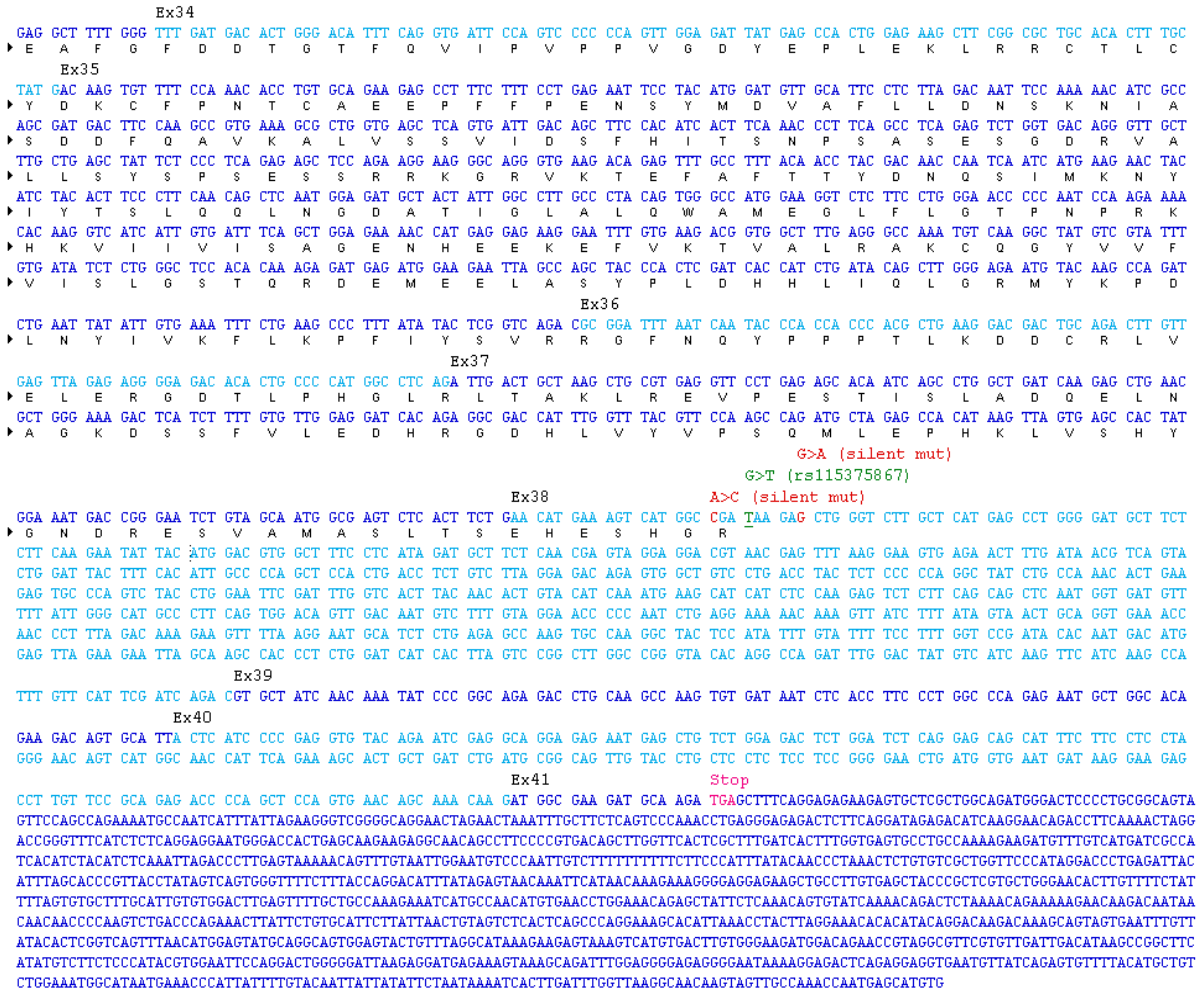

Figure 2.7 mRNA sequence detail of target exons 38. The sequence of exon 38 and surrounded exons are shown. In addition, Exon 38 target mutation and silent mutation position showed.

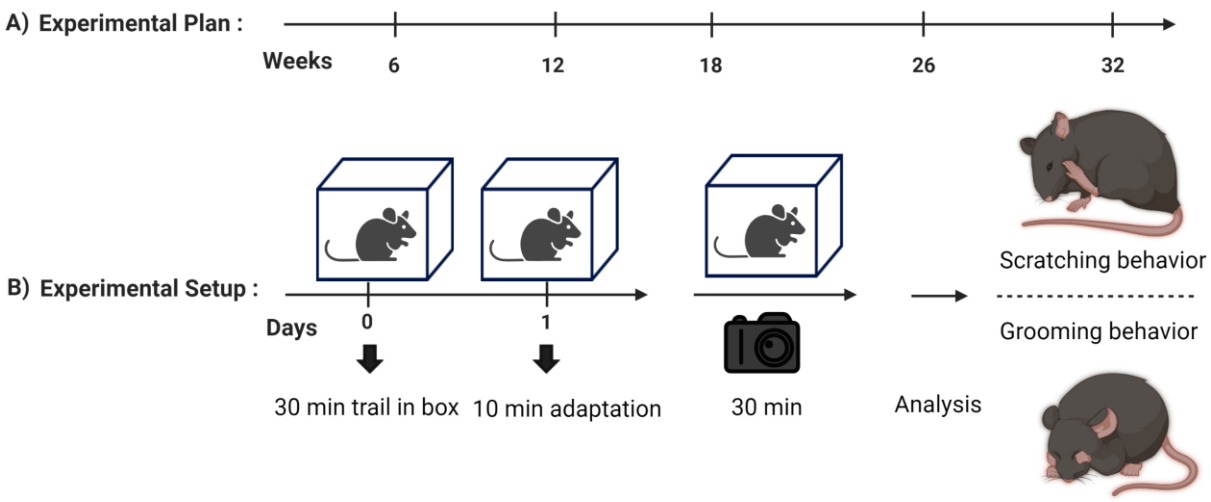

Figure 2.8 Experimental study of scratching behavior and control groups. (A) The whole experiment started when mice were 6 weeks old and ended after 32 weeks. (B) The experimental setup shows that on day 0, mice were given 30 min in the experimental box for adaptation. On experimental day 1, mice were given 10 min adaptation time followed by 30 min of the experiment.

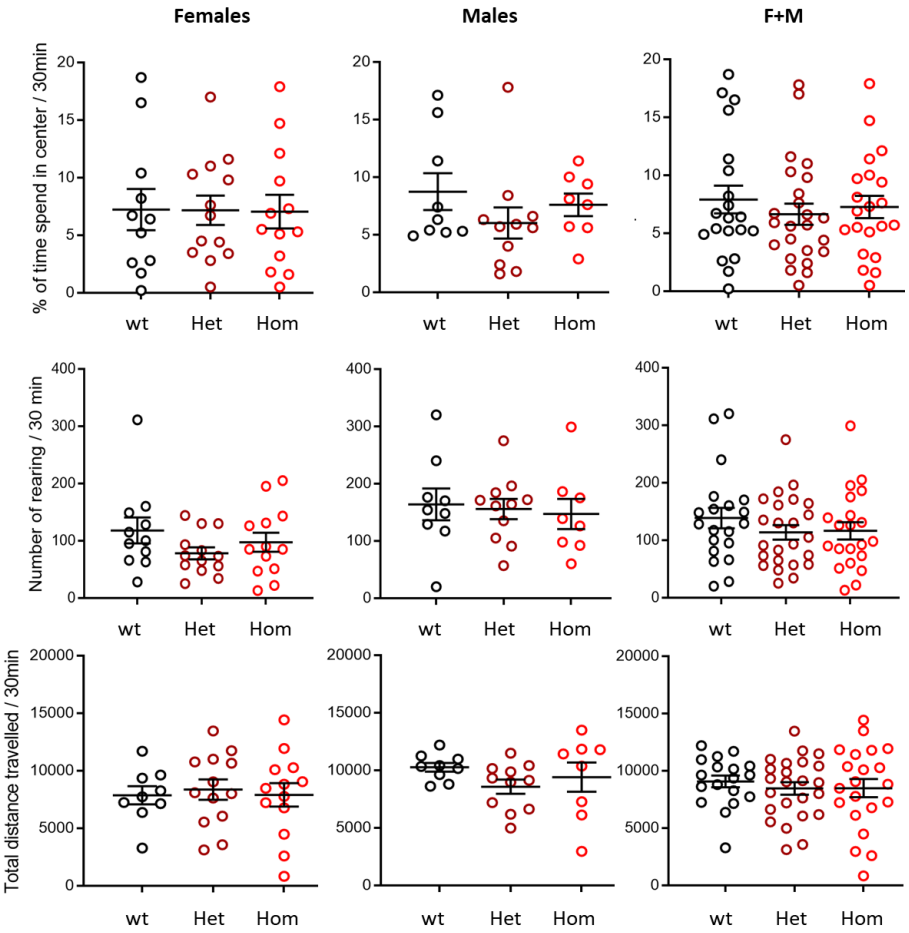

Figure 2.9: Normal locomotor activity of *COL6A5*-p.Glu2272* mice. The open-field test showed no difference between mutant and wt mice. This indicates that mutant mice had normal locomotor activities. The wt littermate (wt), heterozygous (Het), and homozygous (Hom) mice were compared. Male, n=8-11 /group; Female, n=11-13 /group.

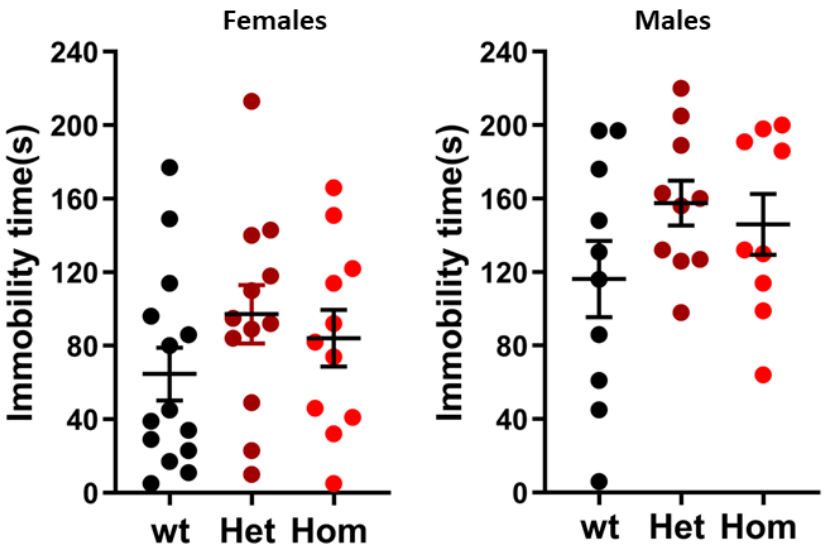

Figure 2.10 : No significant phenotype found in *COL6A5*-p.Glu2272* mice in the tail suspension test. Heterozygous (Het) or homozygote (Hom) mutant mice showed mice no difference in immobility time compared to their wt littermates. Mice, n=9-14 /group. Females: one-way ANOVA (F(2, 34) = 1.223; P=0.3071) and males: one-way ANOVA(F(2,26) =1.632;P=0.2150).

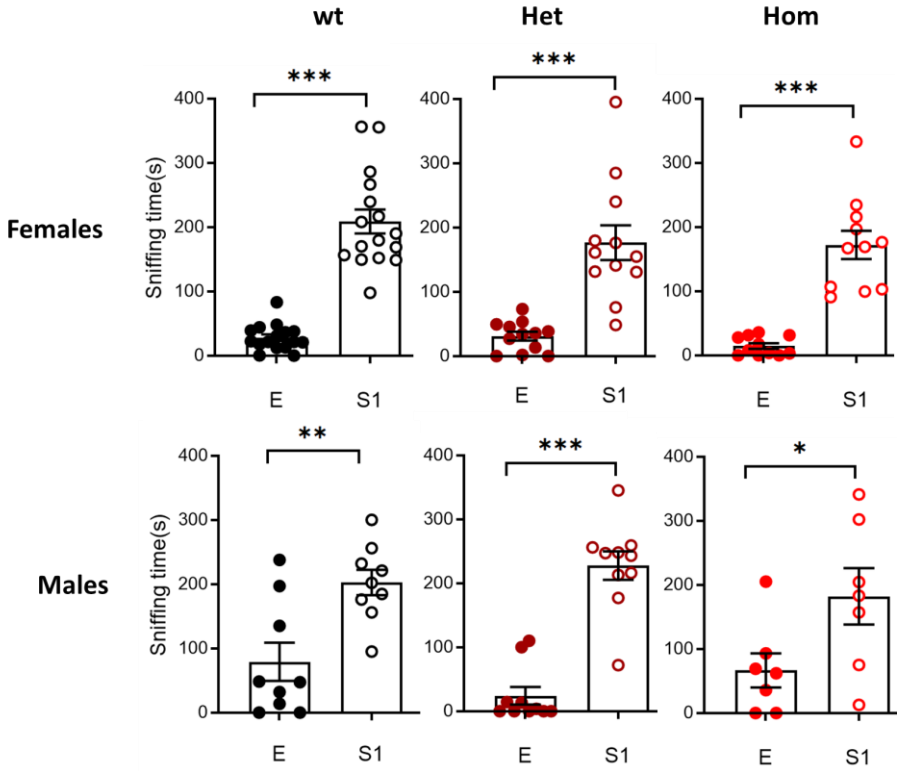

Figure 2.11 *COL6A5*-p.Glu2272* mice showed no change in social preference. In the three-chamber test, mice of all genotypes, including wt littermate (wt), heterozygous (Het), and homozygous (Hom), showed significantly more sniffing time towards stranger 1 (S1) mice as compared to the empty cage (E). Males, n=7-10 /group; females, n=12-16 /group. A p-value was considered as significant as *p < 0.05, **p < 0.01, and ***p < 0.001.

2.2 Supplementary methods and results

This part has supplementary methods and results achieved in this project which are not included in manuscript for performed during this study. However, it should be noted that creation of this *Col6a5*^{E2302*}mice through innovative CRISPR/Cas9 technology was contributed by Marie-Christine Birling's lab, specializing in the Genetic Engineering and Genome Editing Department at ICS. Marie-Christine Birling's lab demonstrates proficiency in sgRNA selection, donor oligonucleotide design, and precise genome editing methodologies. With a dedicated commitment to high specificity and minimal off-target effects, the lab's meticulous approach has facilitated the creation of this mouse model. Furthermore, I followed the protocol provided by Marie-Christine Birling's lab to confirm the point mutation in this mouse model, conducting PCR and genotyping to establish breeding setups for further characterization of this model. I performed molecular and behavioral characterization of these mice including, ddPCR, skin permeability test, behavioral study etc.

2.2.1 Supplementary method

2.2.1.1 Creation of *Col6a5*^{E2302*} mutant mice using CRISPR/Cas9 technology

CRISPR stands for clustered regularly interspaced palindromic repeats (CRISPR)-associated protein 9 (Cas9). CRISPR/Cas9 systems found within bacterial and archaeal organisms as a defense against viruses and foreign plasmids. The function of the CRISPR pathway is to target nucleases to invasive DNA, creating a potentially double-strand break (DSB). Shortly after the function of this pathway was recognized, the type II CRISPR/Cas9 system from the bacterium Streptococcus pyogenes (SpCas9) was repurposed to create a simple yet powerful molecular tool that could be programmed to target nucleases to a specific genomic sequence. It is simple, easy, and quick to implement. To create such a tool, the endogenous CRISPR pathway was reduced to two principal components: 1) the Cas9 nuclease and 2) a guide RNA (gRNA), also referred to as single gRNA (sgRNA). The Cas9 nuclease and sgRNA form a Cas9, which can bind and cut a specific DNA target in a whole genome. In order to be cleaved, a target must possess two specific sequences. First, the gRNA requires 17–21 RNA-to-DNA homology bases,

called the protospacer. Second, the Cas9 protein requires a short protospacer adjacent motif (PAM) to bind to the target DNA. If homology exists between the gRNA and the genomic target, Cas9 will cleave both target DNA strands, creating a double strand break (DSB) at this precise location in the genome (**Figure 2.12**). Cas9 nucleases sit specifically at 3' nucleotides from the 5' side of the PAM. Then the cell will repair this DSB by two major systems: the error-prone non-homologous end joining resulting in random insertion or deletions (indels) disrupting the target sequence or the homologous directed repair pathway where point mutation (PM) and knock-in (KI) can be made by providing a homologous DNA repair template as a single-stranded DNA oligo-deoxynucleotides (ssODN) identical to the broken region and containing the PM to be introduced (Cong et al., 2013; Guitart et al., 2016).

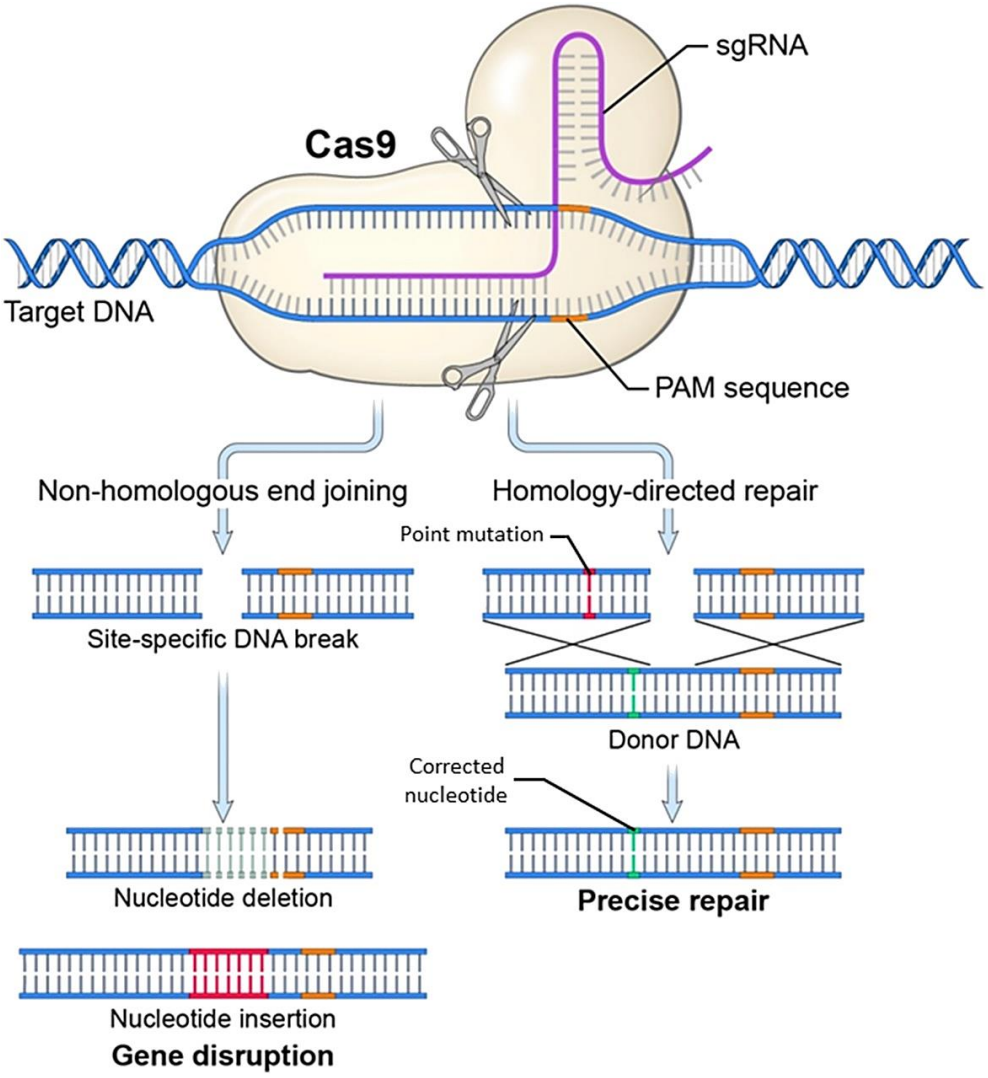

Figure 2.12 CRISPR-induced NHEJ and HDR. Upon Cas9-induced DNA DSB, the cell repairs the DSB by either NHEJ or HDR. In NHEJ, random nucleotide insertions and deletions occur as the

cell ligates the DNA DSB, resulting in gene disruption. In HDR, the DSB is repaired using an externally supplied homologous DNA as a template for copying. The nucleotide sequence of the donor template is copied into the targeted site, resulting in a directed, precise repair. Cas9, CRISPR-associated protein 9; CRISPR, clustered regularly interspaced palindromic repeats; DSB, double-stranded break; HDR, homology-directed repair; NHEJ, nonhomologous end-joining; PAM, protospacer adjacent motif; sgRNA, single guide RNA. Above figure has been obtained from source (Guitart et al., 2016).

Using the above working principle of CRISPR/Cas9 systems, mutant mice were generated through the analysis of several parameters and steps.

2.2.1.2 sgRNA and donor oligonucleotides design

The CRISPOR online software (http://tefor.net/crispor/crispor.cgi) was used, developed by the French TEFOR infrastructure, to select sgRNAs. First, the C57BL/6NCr mouse *Col6a5* sequence was downloaded from the Ensembl database (http://www.ensembl.org/index.html) and put the targeted exon (*Col6a5* exon 38) sequence into CRISPOR software. Then the species genome and protospacer adjacent motif (PAM) was selected to submit. Finally, all the possible sgRNA with specificity scores and off-targets were obtained. High specificity score sgRNA with low off-targets were selected to use (Figure 2.13).

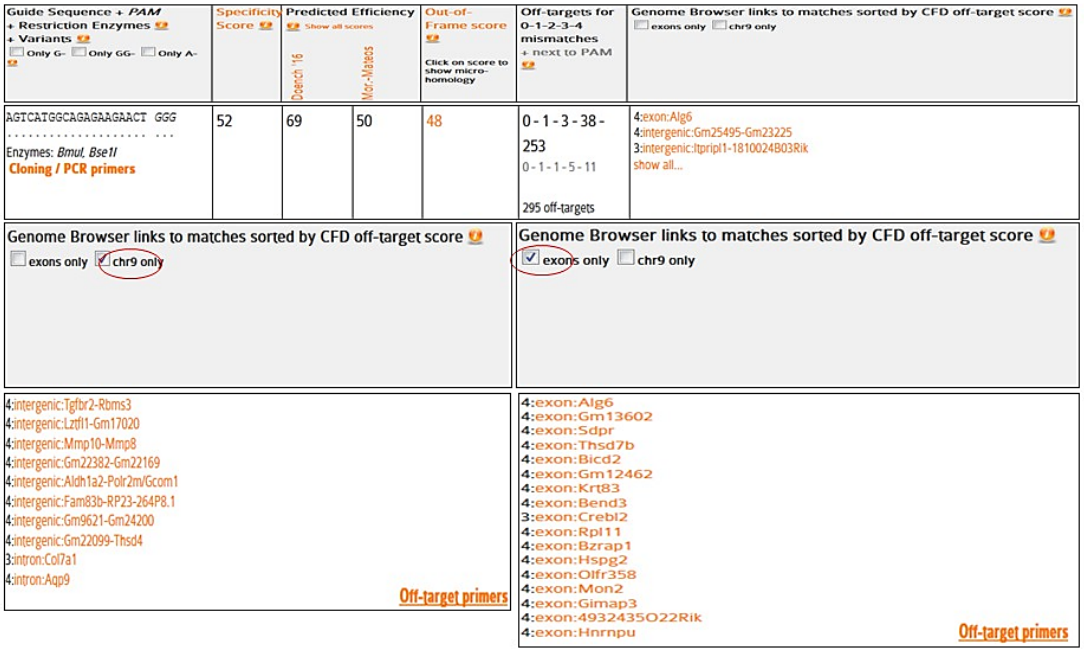

Figure 2.13 sgRNA design and selection. Using the CRISPOR software, the target exon and *Col6a5* sequence were submitted from the Ensembl database along with the species genome

and PAM. It was identified that gRNAs with high specificity scores and a low number of off targets. Therefore, the gR52 was selected and used for strategy.

Sequences for donor templates with homology arms at least 60 nucleotides in size and flanking the intended point mutation were designed. To prevent re-cutting of the modified allele by Cas9, a silence mutation in PAM loci was designed, GAA change to GAG. Furthermore, another silence mutation de-signed into ssODN, AGA, changed to CGA for introducing restriction digest site to aid with subsequent animal genotyping (Figure 2.14). Single-stranded oligo-deoxynucleotide donor sequences were ordered using GATC-Biotech services.

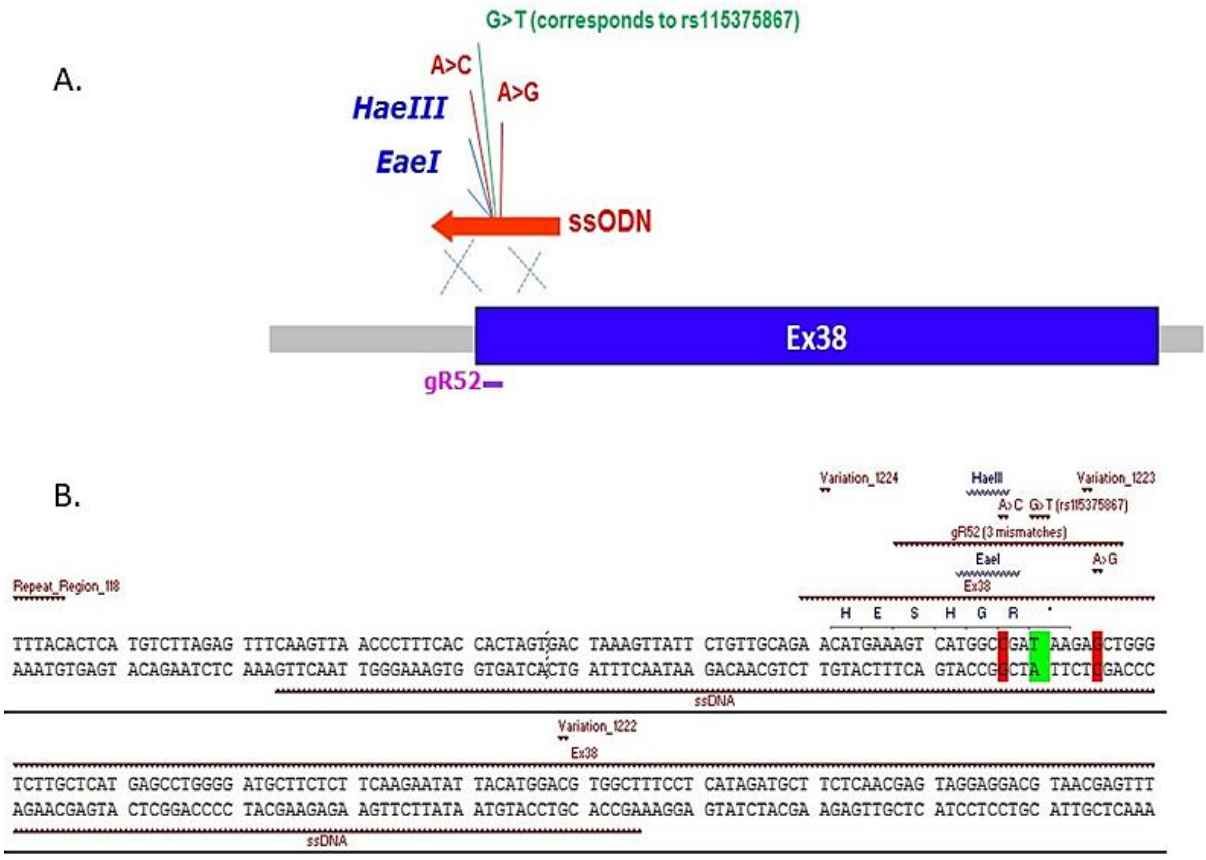

Figure 2.14 Illustration of a proposed strategy A) introducing STOP codon (p.Glu2272*, human coordinate) by CRISPR/Cas9 approach and homology-directed repair (HDR) with two silent mutations. **B)** Allele sequence after HDR was introduced. A donor single-stranded oligonucleotide (ssODN) was designed. This donor bares the expected mutation (G>T, corresponding to the rs11537567 human variant) as well as 2 silent mutations (A>G and A>C). So, after HDR, no new DSBs could be cleaved. Together, these 2 mutations generate a new HaeIII enzyme diagnostic restriction site.

2.2.1.3 Cas9 Synthesis and sgRNA transcription in Vitro

In the first step, The Cas9 vector (T7-Cas9 wt cloned in pUC57) with T7 promoter was linearized with AccI as a template, using in vitro transcription with T7 polymerase. Next, Cas9 mRNA was transcribed using mMESSAGEmMACHINE T7 Ultra Kit according to the manufacturer's manual (Life Technologies). 1 µg plasmid containing sgRNA scaffold (C5648) was linearized with BgII restriction enzyme. According to the protocol provided, the linearized plasmid was purified with the Nucleospin Gel and PCR Clean-up (Macherey Nagel). gDNA was obtained by PCR amplification with primers. The gDNA has to be purified with the Nucleospin Gel and PCR Clean-up (Macherey Nagel). After that, it was used to transcribe sgRNAs using the Megashort script T7 kit. In the end, Cas9 mRNA and sgRNAs were purified with the PCR Clean up (Macherey Nagel) and eluted in TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, Invitrogen) for microinjections.

2.2.1.4 Off-targets Primer Design and Optimization of the Assay Conditions

The primer sequences for off-targets analysis were selected using Vector NTI 5.0 software, similarly as before for the design of primers for the mutation on the *Col6a5* gene.

2.2.1.5 Check in Vitro sgRNA Validity

Before injecting sgRNAs in eggs, it is important to test their validated efficiency. SgRNA was tested on the targeted DNA PCR product in vitro. First, the sequence surrounding the target was amplified using the optimized protocol. Later, 2µL targeted DNA at 50ng/µl was mixed with 1µl Cas9 protein at 100ng/ µl, 1 µl sgRNA at 100ng/ µl, 2 µl NEB buffer 2.1, and remaining H2O was added to make it a total volume of 20 µl. For a control, the Cas9 protein was not inserted. The reactions incubate in a thermocycler 30min at 30°C, then 15min at 65°C. The reaction was loaded on a 2.5% agarose gel. The correct sizes after Cas9 cleavage allow for validation of the functionality of the tested guide. Only sgRNAs showing a cut (even partial) were injected into eggs.

2.2.1.6 Pronuclear Microinjections of Zygotes

All mice were kept at an automated control system for a temperature of 21 °C on the light cycle of 12/12 h light-dark cycle (7:00 am - 07:00 pm) in the SPF facilities. Sexually immature female C57BL/6CNr mice (4-5 weeks old) were super-ovulated by intraperitoneal injection of 5 IU eCG followed by 2.5 IU hCG interval 48 h and mated overnight with C57BL/6NCr male mice that were aged less than 10 weeks. Zygotes were collected after 20 h of hCG injection by oviductal flashing, and pronuclei-formed zygotes were put into the M2 medium (Sigma M-7167). Microinjection was performed using a microinjector (Eppendorf Femtojet 4i) equipped microscope. RNA solution was injected into the cytoplasm and the pronucleus of each zygote using continuous pneumatic pressure. After the injection, embryos were in vitro cultured in the M16 medium (Sigma M-7292) at 37 °C in a 5% CO2 incubator. The survivors of the injected embryos were implanted into the oviducts of pseudo-pregnant CD1 mice.

2.2.1.7 PCR Amplification and Sequencing

As defined above, each project's PCRs run, and an aliquot was analyzed on an agarose gel using optimized conditions. Potential PCR products were sent for Sanger sequencing by GATC-Biotech sequencing services. Sequence information was obtained from GATC-Biotech Sequencing services. Protocol and cyclic condition were the same as mentioned in the manuscript under genotyping method.

2.2.1.8 Analysis of Off Targets

CRISPOR is an online software (http://tefor.net/crispor/crispor.cgi) that allows to predict of possible off-target sites within the rest of the mouse genome based on the sequence of each of the sgRNA recognition sequences chosen. The potential off-target sequences around the mouse genome were selected that contained the minimal numbers (2 to 3) of mismatch regions and the same chromosome with the targeted region and used CRISPOR software. PCR primers were designed using Vector NTI 5.0 software that flanked off-target sites at an optimal distance of 100-150 base pairs to give PCR product lengths between 400-500 base pairs to aid in subsequent sequencing. Purified PCR products were sequenced by GATC-Biotech sequencing services using each of the PCR primers used for the PCR reaction. Finally,

the software MEGA X analyzed sequence information obtained from GATC-Biotech Sequencing services.

2.2.1.9 Molecular and cellular characterization of *Col6a5*^{E2302*} mutant mice

2.2.1.10 Skin observation for dermatitis

Mice were kept in the light cycle controlled at 12 h light and dark cycle with an automated system. For mice skin observation, temperature and humidity were also controlled as follows: temperature of 21 ± 1°C and humidity of 55 ± 10% provided by the ICS experimental facility. With keeping stable condition, mice were also observed physically every week, if there were any skin lesions or scars on the skin and more frequently observation were made for those mice with increasing dermatitis skin. If dermatitis skin was found more increasing, then with the recommendation of a veterinary doctor, spray “dermidine (MP abo)” was treated on mice skin. Mice were sacrificed if their skin was not healed.

2.2.2 Behavioral characterization of *Col6a5*^{E2302*} mutant mice

2.2.2.1 Live mouse tracking of behavior

This method of tracking mice's real-time behavior helps to assess the impact of environmental factors or genetic mutations on complex traits such as decision-making and social interactions. This method was previously performed using live tracker apparatus (de Chaumont et al., 2018). Briefly, for this experiment, pair of female mice of the same genotype were selected for each experiment. Mice were moved to the experiment room 30 min before two hours before the experiment. Mice were given 30 min adaptation time in an experimental cage with an enriched environment. Mice were allowed to move around freely, explore, drink, or eat food. Mice were given two hours for this particular experiment and tracked during this time. The recording was made with a speed of 30 frames per second. After each experiment, experimental cages were cleaned and dried appropriately. New cage bedding, food, and

water were kept each time to avoid odor interference for each experiment. After each experiment, LMT automatically extracted data collected and plotted graphically.

2.2.3 Supplementary results

2.2.3.1 Molecular and cellular characterization of the *Col6a5*^{E2302*} mice model

To characterize *Col6a5*^{E2302*} mice model, it was ensured to have enough mice available for all experiments with all genotypes, including wt, heterozygous, and homozygous mutant mice. Heterozygous mutant mice were intercrossed as breeding trios to produce homozygous mice. wt and mutant mice were differentiated through wt and mutant alleles, as shown in the schematic diagram below, **Figure 2.15**.

PCR outcome was expected to have the size of 426 bps for the wt allele and for mutant allele containing point mutation (*) after digestion with HaeIII enzyme formed two fragments of 236 bps and 190 bps shown in **Figure 2.15**(**B**). As expected in the case of wt mice samples, only one band was detected on gel with the size of 426 bps with and without digestion of restriction enzyme HaeIII. Similarly, in the case of heterozygous mice, the allele of 426 bps and fragments of mutant allele 236 bps and 190 bps were detected. Also, in the case of homozygous mice samples, we detected two fragments of 236 bps and 190 bps after the digestion of the HaeIII enzyme, as shown previously in (**Figure 2.1 C**). Based on these results, mice were separated and tagged for all experiments. Although the *Col6a5* mutation was found in humans as heterozygous but still produced homozygous mice to observe the homozygous phenotype for *Col6a5* as well. Whether homozygous mice are lethal or not. Homozygous mutants were healthy and normal as wt littermate and heterozygous mutant mice. Overall, three cohorts were produced for behavior and molecular study.

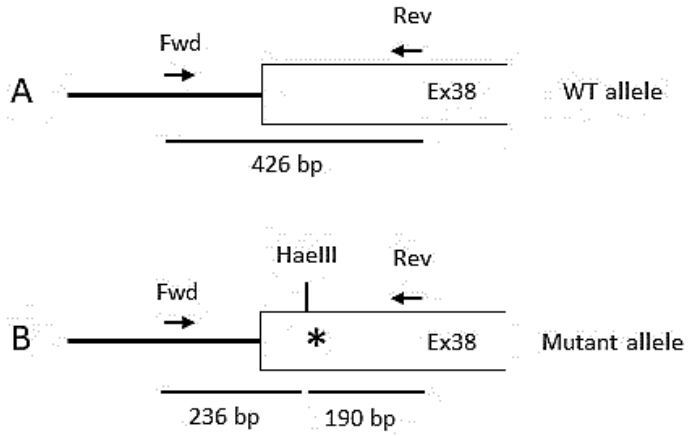

Figure 2.15 Schematic diagram of wt and Mutant allele. **A)** Diagram show part of target exon 38 containing wt allele with a full length of 426 bps. **B)** Mutant allele containing point mutation was digested through the HaeIII enzyme, forming two fragments of 236 bps and 190 bps.

According to the nature of this project, it was essential to observe any skin changes occurring among different mice cohorts. It was found that some mice have skin problems (skin dermatitis-like lesions/wounds). These skin changes (lesions) appeared in mice at 22 weeks of age. Treatment started as soon as the lesion increased and became a wound. Almost all mice either recovered by the end of 33 weeks of age or were euthanized. The dermatitis antiseptic spray (dermidine, MP labo) was treated one time per day. After treating the wound, if it became severe, mice were euthanized. If the wound started to reduce the severity and the mice had some recovery, then mice were kept under observation with continuous treatment. However, non of these mice with a lesion or apparent wound was used for behavior experiments. Instead, they have been excluded from experiments.

There was no skin problem for wild type mice in all three cohorts. In the 1st cohort, 50% of male heterozygous mice had to sever skin lesions on the skin that converted into wounds later. These wounds were on the abdomen of mice; treatment was started with an antiseptic spray every day. However, 10% of mice recovered completely, while 10% died naturally, and 30% were euthanized due to having less chance of recovery **(Table 2.3)**. While female heterozygous mice have fewer skin problems, only 10% have severe skin lesions, and due to lack of recovery after the treatment, all were euthanized. In the case of homozygous mice, no skin changes were observed in female homozygous mice. Whereas 14.28% of male homozygous mice have shown mild skin lesions, all recovered completely after the treatment. About 42.85% of male homozygous mice have shown severe skin lesions; even with

treatment, non of these mice could recover. It was realized that the 2nd cohort lacked skin problems as seen in the 1st cohort. In the 2nd cohort, only 25% of male heterozygous mice have shown sever skin lesions. All of these mice were treated and recovered completely within two to three weeks. On the contrary, the 3rd cohort of mice showed no skin changes or lesions. And all mice were healthy to be used for experiments.

These observations showed that all affected mice were male except 10% of heterozygous females. Moreover, some wounds were so severed that it was hard to recover. After careful observation was made for 2nd and 3rd cohorts, it was concluded that these skin wounds were not due to the skin problem but mainly to a fight between male mice. All mice with severe symptoms had skin problems in the abdomen (which was the clue that the wound occurred during the fight between mice). Also, 1st cohort mice were not grouped very early, which probably caused them to fight with each other. Based on these observations, it was concluded that skin problems in these mice were not due to collagen mutation.

Table 2.3 Skin observation of different cohorts of mice.

			The severity of skin lesion					
Cohort	**Genotype**	**Gender**	**Mild**	**Moderate**	**Severe**	**Natural death**	**Euthanized**	**Recovered after treatment**
1	Wild type	M	-	-	-	-	-	-
		F	-	-	-	-	-	-
	Heterozygous	M	-	-	50%	10%	30%	10%
		F	-	-	10%	-	10%	0%
	Homozygous	M	14.28%	-	42.85%	42.85%	0%	14.28%
		F	-	-	-	-	-	-
2	Wild type	M	-	-	-	-	-	-
		F	-	-	-	-	-	-
	Heterozygous	M	-	-	25%	0%	0%	100%
		F	-	-	-	-	-	-
	Homozygous	M	-	-	-	-	-	-
		F	-	-	-	-	-	-
3	Wild type	M	-	-	-	-	-	-
		F	-	-	-	-	-	-
	Heterozygous	M	-	-	-	-	-	-
		F	-	-	-	-	-	-
	Homozygous	M	-	-	-	-	-	-
		F	-	-	-	-	-	-

2.2.3.2 Skin permeability of wt mice

To test wt mice male and female for skin permeability test, it was realized that they significantly differ in Evans blue dye absorption (Figure 2.16). This difference suggested a difference in mice skin between the two genders. This also helped us to not combined data for male and female *Col6a5*^{E2302*} mutant mice due to gender differences in skin wt mice. As previously described, male C57Bl/6 mice have a significantly thicker dermis and tissue depth than female mice (Calabro et al., 2011).

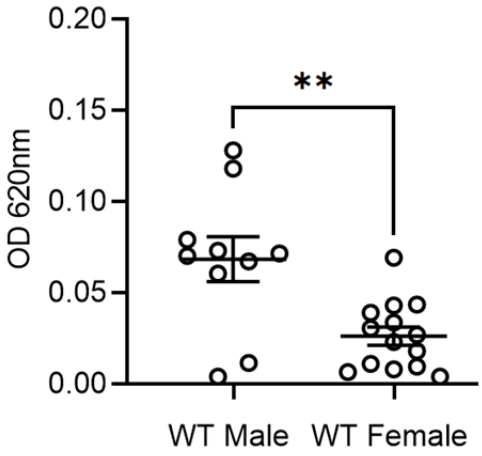

Figure 2.16 Skin permeability of wt male mice compared to wt Female mice. Evans blue dye assay was performed to test skin permeability. When Evans blue dye was treated on mice's skin, it was found that both wt Male and wt female mice showed significant differences in OD at 620nm. Male, n=10/group; Female, n=14/group. A p-value was considered as significant as **$p < 0.01$.

This study showed that wt male mice have significantly higher OD values than wt female mice. This means wt male mice have significantly higher skin permeability than wt female mice, which is the reason not to combine male and female mice. But it is interesting that due to skin thickness differences between gender still, wt male mice still have significantly increased skin permeability compared to wt female mice.

2.2.3.3 Scratching behavior of *Col6a5*^{E2302*} mutant mice

To investigate spontaneous itch in mutant mice, each mouse scratching behavior was scored at different ages, from 6 weeks until 32 weeks (**Figure 2.17**). Generally, mice do more facial scratching (Wimalasena et al., 2021), so facial and back scratches were analyzed separately.

In case of facial scratching, the overall comparison of mice of the four genotypes (B6N wt, wt littermates, heterozygous and homozygous) showed a genotype effect and no age effect in males (two-way RM ANOVA, $P<0.0001$ for genotype). The post-hoc analysis indicated that heterozygous mutant male mice showed a significant increase in scratching behavior at 6, 18, and 32 weeks of age compared to B6N wt mice. Also, heterozygous mutant male mice showed a significant difference between 6 and 18 weeks of age compared to wt littermate mice. There was a genotype effect in females (two-way RM ANOVA, $P=0.003$ for genotype). Mutant females showed a significantly increased scratching at the ages of 26 and 32 weeks compared to B6N wt females. Results showed that male mutant mice have early age, particularly at the age of 6 and 18 weeks, significant facial scratching compared to wild type mice. In contrast, female mutant mice showed facial scratching behavior at age 26 and 32 weeks compared to wild type mice, as shown in **Figure 2.17 (A)**. Conversely, the sex-grouped mutant mice showed significantly increased facial scratching at ages 6, 18, 26, and 32 weeks. Taken globally, this suggests that mutant mice had a chronic facial scratching phenotype at adult age from 6 weeks to 32 weeks.

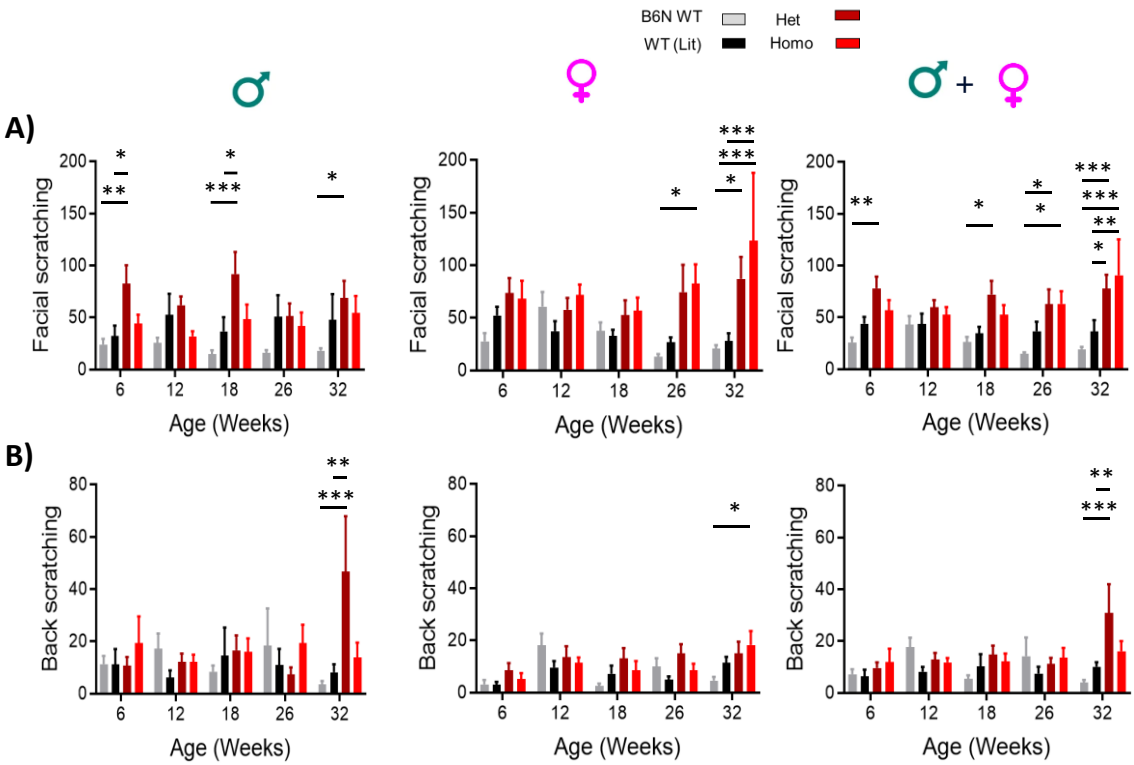

Figure 2.17 Facial and back scratching *Col6a5*^{E2302*} mutant mice. A scratching behavior test was performed on commercial C57BL/6N (B6N wt), wt littermates (wt lit), heterozygous (Het) and homozygous (Homo) mice. **A)** Mutant mice showed significantly facial increased scratching at 6, 18, 26, and 32 weeks. **B)** Mutant mice showed significantly increased scratching at the age of 32 weeks. Male, n=10-13 /group; Female, n=11-13 /group. A p-value was considered as significant as *p < 0.05, **p < 0.01, and ***p < 0.001.

In contrast to facial scratching, back-scratching, the scratching of mice on their back or abdomen had a different pattern (**Figure 2.17 B**). Male mutant mice showed no effect of age or genotype interaction between age and genotype. While female mutant mice showed the effect of age and interaction between age and genotype (two-way RM ANOVA, P=0.0003 and P=0.038, respectively). Interestingly, the sex-grouped mutant mice affected genotype and interaction between age and genotype (two-way RM ANOVA, P=0.036 and P=0.025, respectively). Furthermore, male, female, and sex-grouped mutant mice significantly increased in back scratching at adult age at 32 weeks only. This showed the difference between facial and back-scratching and their age pattern in mutant mice. In conclusion, the mutant mice had a more obvious overall phenotype for facial scratching than back-scratching.

Further on facial scratching, the overall comparison of mice of the two genotypes (B6N wt and wt littermates) was made, shown graphically below in **Figure 2.18**. Male B6N wt showed

an effect of interaction between age and genotype (two-way RM ANOVA, P=0.013). Interestingly, female B6N wt mice were affected by genotype and age (two-way RM ANOVA, P=0.019 and P=0.001, respectively). Together, the sex-grouped mutant mice had the effect of an interaction between age and genotype (two-way RM ANOVA, P=0.016). From these results genotype effect in female B6N wt as compared to wt littermate suggested using control B6N wt mice is important for study for better assessment of scratching behavior.

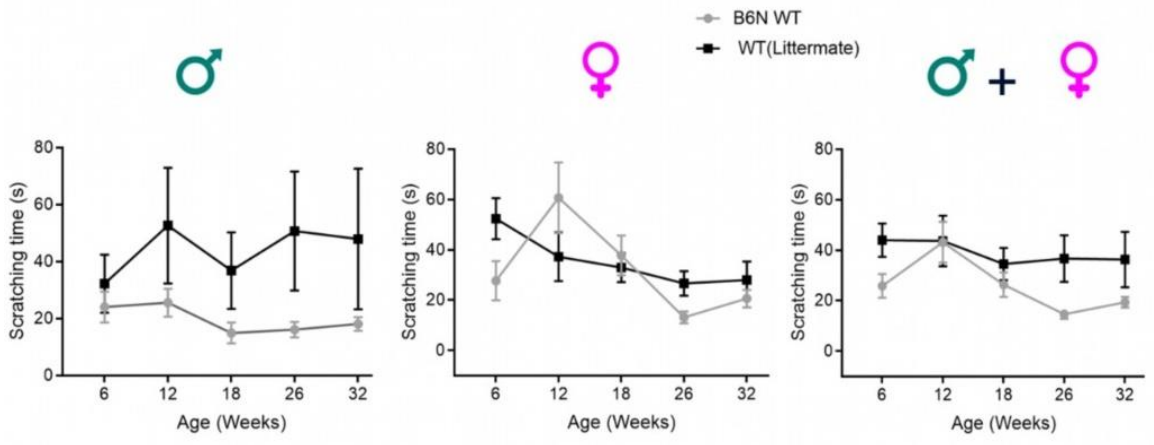

Figure 2.18 Facial Scratching of wt littermate compared to B6N over 6 weeks to 32 weeks of age. We compared the scratching behavior between B6N wt and wt littermate mice scratching. Male, n=10-13/group; Female, n=13-14/group.

Again, in the case of back-scratching, the comparison of mice of the two genotypes (B6N wt and wt littermates) was also made, shown graphically below in **Figure 2.19**. Male B6N wt showed no effects of age and genotype. Interestingly, female B6N wt mice had an effect of age and an interaction between age and genotype (two-way RM ANOVA, P< 0.0001 and P=0.004, respectively). Together, the sex-grouped mutant mice had no effect on age and genotype. The results shown that both B6N wt and wt littermates have similar back-scratching behavior with no genotype effect in both genders.

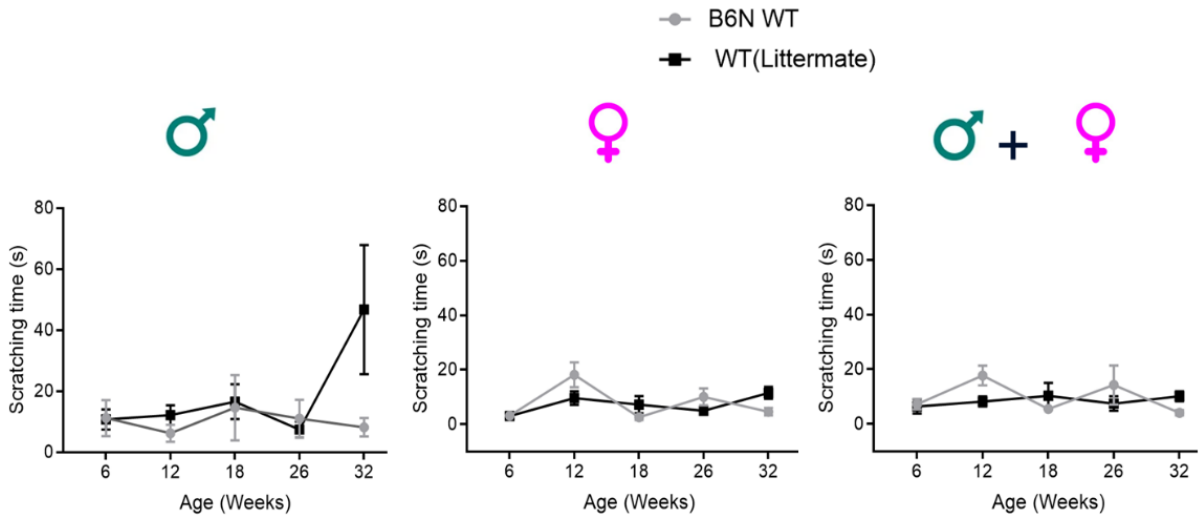

Figure 2.19 Back Scratching of wt littermate compared to B6N over 6 weeks to 32 weeks of age. The scratching behavior was compared between B6N wt and wt littermate mice scratching. It was found no significant different between both wt mice. Male, n=10-13 /group; Female, n=13-14 /group.

Next, it was investigated whether wt B6N has some effect or not in overall phenotype assessment. The data in **Figure 2.20** showed that by excluding the commercial wt B6N mice from the analysis, male mutant mice showed the effect of genotype (two-way RM ANOVA, P=0.031), while female mutant mice showed the effect of both genotype and age (two-way RM ANOVA, P=0.031 and P=0.031 respectively). And sex-grouped mutant mice had a stronger effect of genotype (two-way RM ANOVA, P=0.004).

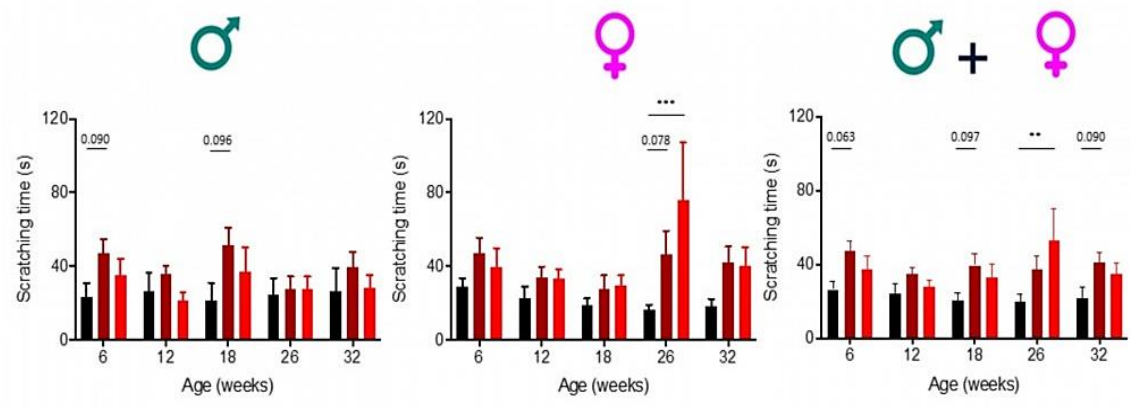

Figure 2.20 Whole body scratching of *Col6a5*^{E2302*} mutant mice compared to wt littermate. Scratching behavior test was performed on wt littermate (wt lit), heterozygous (Het) and homozygous (Homo) mice. Mutant mice showed significantly increased scratching at the age of 32 weeks. Male, n=10-13 /group; Female, n=11-13 /group. A p-value was considered as significant as **p < 0.01 and ***p < 0.001.

It was also seen that male and female mice mutant mice showed significant differences at the same age of 18 weeks, but in sex-grouped mutant mice lost the phenotype. Overall, from these analyses, it was proposed that B6N wt was better control for this study. As the B6N pattern also showed reduced scratching at a later age, for example, 26 and 32 weeks, which provided a better comparison for mutant mice with B6N wt.

2.2.3.4 Grooming behavior of *Col6a5*^{E2302*} mutant mice

In addition, grooming behavior was also observed in mutant mice for both facial grooming and back grooming (Shown in **Figure 2.21**).

In the case of facial grooming, the results showed the effects of genotype and age in male mutant mice (two-way RM ANOVA, P=0.003 and p < 0.0001, respectively). Interestingly, female mutant mice affected genotype, age, and interaction between age and genotype (two-way RM ANOVA, P=0.003, p < 0.0001 and P=0.002, respectively). Together, the sex-grouped mutant mice have an effect of genotype, age and interaction between age and genotype effect (two-way RM ANOVA, p < 0.0001, p < 0.0001, and P=0.0003, respectively). Also, mutant mice showed significantly increased facial grooming behavior at age 6 weeks. Facial grooming was reduced from the age of 12 weeks to 32 weeks.

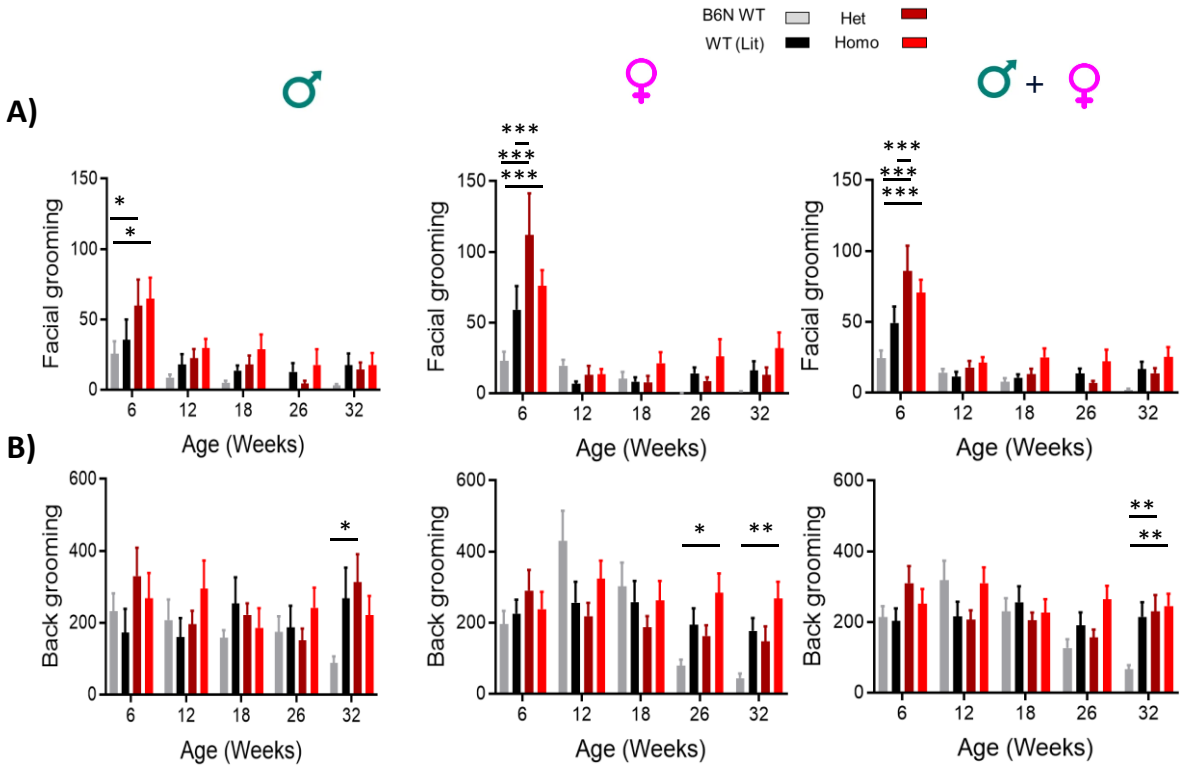

Figure 2.21 Facial and back grooming *Col6a5*^{E2302*} mutant mice. Mice spontaneous and self-grooming was tested without external inducers. For that, mice were tested for 30min and analyzed the grooming behavior through video recording. **A)** It was observed that mutant mice showed a significantly increase in facial grooming compared to wt control at 6 weeks of age. **B)** It was observed that mutant mice showed a significant increase in grooming compared to wt control at 32 weeks of age. B6N wt, wt littermate (wt Lit), heterozygous (Het), and homozygous (Homo) mice were compared. Male, n=9-13 /group; Female, n=11-13 /group. A p-value was considered as significant as *p < 0.05, **p < 0.01, and ***p < 0.001.

The result of facial grooming (**Figure 2.21**) showed that mutant mice have significantly increased facial grooming at the age of 6 weeks compared to wt control. Also, overall grooming time decreased from 12 weeks to 32 weeks. Therefore, it suggested that these mice might have spent more time on back grooming from 12 weeks to 32 weeks of age. Results showed that female mutant mice had effect in age and interaction of age and genotype (two-way RM ANOVA, p < 0.0001 and P=0.0008, respectively). Moreover, the sex-grouped mutant mice have an effect of age and interaction between age and genotype effect (two-way RM ANOVA, P=0.0024 and P=0.0007, respectively). From these, mutant mice showed significantly increased grooming at 26 to 32 weeks of age compared to the wt control.

For facial grooming, the overall comparison of mice of the two genotypes (B6N wt and wt littermates) was made, shown graphically below in **Figure 2.22**. Male B6N wt showed the effect of age and genotype (two-way RM ANOVA, P=0.001 and P=0.037, respectively). While female B6N wt mice had the effect of age, genotype, and an interaction between age and genotype (two-way RM ANOVA, P=0.032 P < 0.0001 and P=0.006 respectively). Together, the sex-grouped mutant mice also had the effect of age, genotype and also effect of an interaction between age and genotype (two-way RM ANOVA, P=0.0019 P < 0.0001 and P=0.028 respectively). From these results, B6N wt mice showed genotype effect in the case of facial grooming of mice compared to wt littermates.

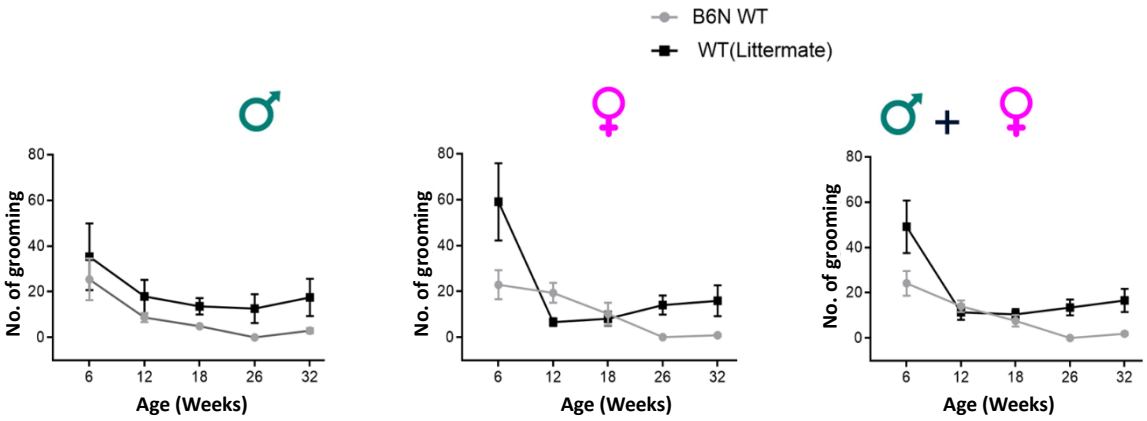

Figure 2.22 Facial Grooming wt littermate compared to B6N wt over 6 weeks to 32 weeks of age. The scratching behavior was compared between B6N wt and wt littermate mice scratching. It was found no significant different between both wt mice. Male, n=10-13 /group; Female, n=13-14/group.

Similar to facial grooming, in the case of back grooming, the overall comparison of mice of the two genotypes (B6N wt and wt littermates) was made, shown graphically below in **Figure 2.23**. Male B6N wt showed an effect of an interaction between age and genotype (two-way RM ANOVA, P=0.031). While female B6N wt mice had an effect of age and an interaction between age and genotype (two-way RM ANOVA, P < 0.0001 and P=0.005, respectively). Together, the sex-grouped mutant mice also had the effect of age and also effect of the interaction between age and genotype (two-way RM ANOVA, P=0.0003 and P=0.003, respectively).

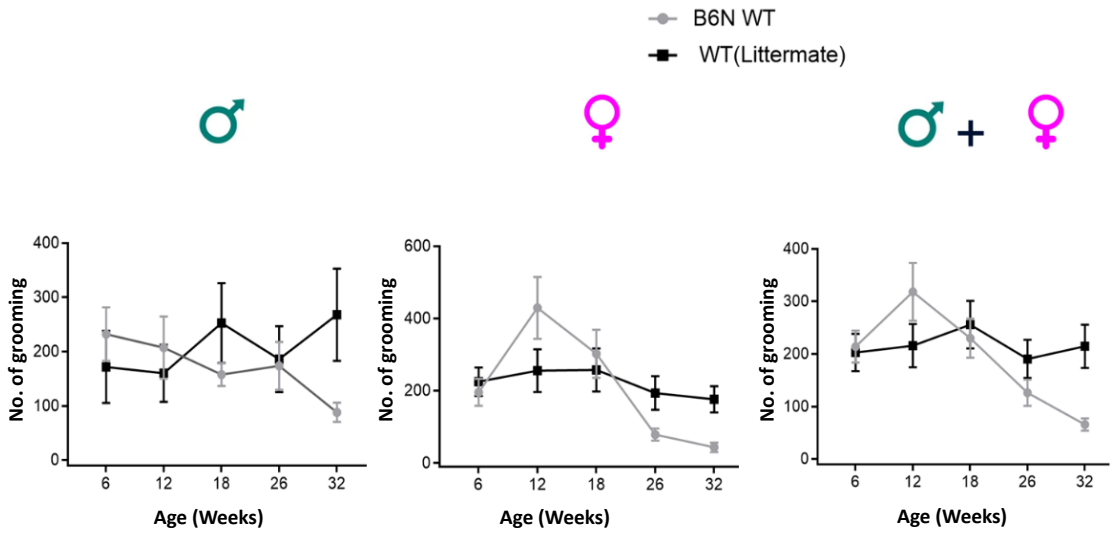

Figure 2.23 Back grooming wt littermate compared to B6N wt over 6 weeks to 32 weeks of age. The scratching behavior was compared between B6N wt and wt littermate mice scratching. It was found no significant different between both wt mice. Male, n=10-13 /group; Female, n=13-14/group.

From these results, B6N wt mice showed no genotype effect in the case of back grooming of mice as compared to wt littermate. These results also confirmed wt B6N mice are essential control in this study to assess the phenotype.

2.2.3.5 Live mouse tracking of *Col6a5*^{E2302*} mutant mice

Live mouse tracking (LMT) helps observe mice behavior in enriched environment with freely available food and water mice. It is a more reliable system to annotate automatically what mice are doing in their environment and their social interactions, as described previously (de Chaumont et al., 2018). Through this automated system, live mouse tracking is recorded and analyzed for a single animal and a small group of mice simultaneously, as shown in **Figure 2.24** below.

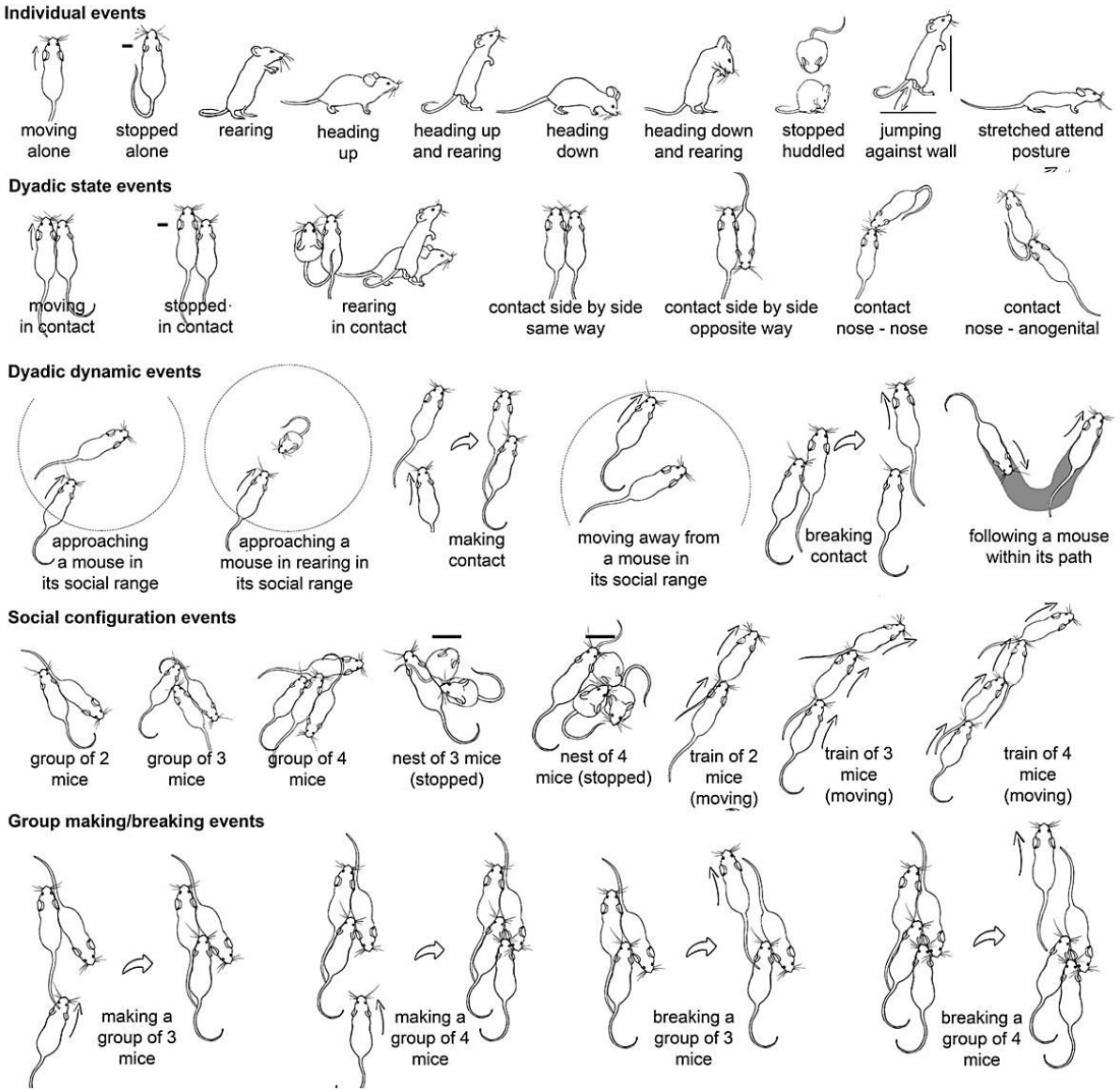

Figure 2.24 Live mouse tracking different reference behavior. A list of extracted behaviors, classified into five major groups: individual, social dyadic, dyadic dynamic, configuration, and group-making and breaking events (dashes indicate that the animals are not moving). Above figure has been obtained from source (de Chaumont et al., 2018).

In my project, I used this test to assess the interaction of female-female mice, which was observed as mounting behavior in the home cages of mice. For that reason, only female-

female mice pairs (couple or duo) were selected, and importantly, each pair of mice was chosen from their home cage so that mice should not be strangers to each other in live tracking cages.

Through the automated LMT system, three major categories were assessed A) Contacts, 2) social interactions, and 3) individual social avoidance, as shown in **Figure 2.25.** Heterozygous mutant female-female mice showed a significantly increased number of contacts, oral-oral contacts, side-by-side contacts, side-by-side contacts in the opposite way, the number of social approaches, and the number of moving contacts as compared to wt-wt female mice. And interestingly, homozygous mutant female-female showed significantly increased oral-oral contacts, oral-genital contacts, side-by-side contacts, social approaches and number of approaches contacts compared to wt-wt females. Moreover, homozygous mutant female-female showed a significantly decreased number of isolated moves compared to wt-wt females (**Figure 2.25**). Overall, these findings showed that mutant female-female mice interact and approach other female mice more than the wt females. At the moment, the reasons for this altered behavior are unknown. The study may be completed by including more animals per group, and tests are required at the hormonal level.

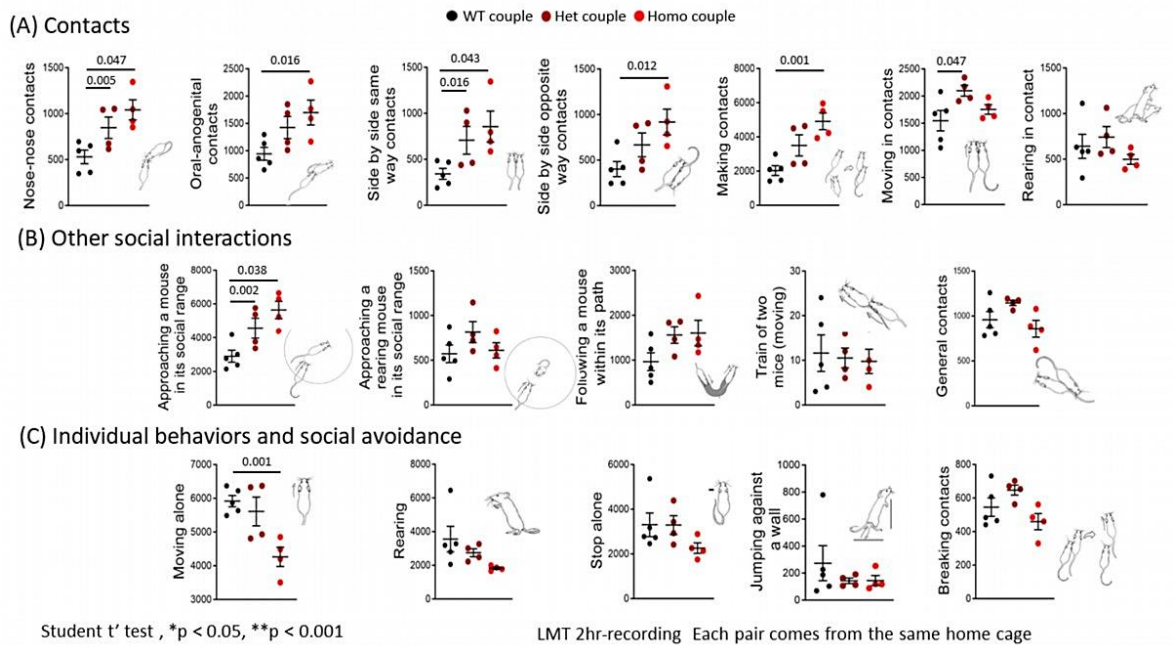

Figure 2.25 Live mouse tracking (LMT) behavior test of female-female couple mice. wt littermate- wt littermate (wt-wt) (in black dot), heterozygous- heterozygous (Het- Het) (dark red dot), and homozygous (Hom- Hom) (in light red dot) mice were compared. Each dot

represents one pair of mice. A p-value was considered as significant as *p < 0.05, **p < 0.01, and ***p < 0.001.

According to LMT parameters, it was found that while moving in enrich environment, *Col6a5*^{E2302*}mutant couple have significantly increased nose-nose contacts, oral-anoggenital contacts, not only side by side but also opposite side contacts, making contacts and moving contacts as compared to wt couples. While rearing contacts were not found significantly different between mutant and wt couples, shown in **Figure 2.25**. It suggested mutant couples made significant contacts with each other and came closer to each other as was expected and while having contact and rearing might distract contact behavior. Due to that reason, there was no significant rearing in contact between mutant and wt couples.

Next, these mice social interactions were assessed. Significantly increased social interaction was found parameters in mutant couple mice only when a mouse approached in a social range of its couple as compared to wt couple mice shown in **Figure 2.25 B.**

Further, the individual behavior of each couple was also assessed. Interestingly, the mutant couple showed significantly decreased moving alone behavior compared to wt couple mice. While there was no significant was found in other individual behavior such as rearing, stop alone (the mouse was static), and jumping against the wall. Lastly, social avoidance was assessed, and no significant difference was found between the mutant couple and wt couples in case of breaking contact to avoid their partner **Figure 2.25 C**.

Overall, the result suggested that mutant couple female mice have significantly increased contacts while moving, social interaction when a mouse was approaching in a social range, moving alone (at individual level), and no difference in avoidance behavior compared to wt couple female mice. This interesting behavior that might be related to some potential role of col6a5 mutation at the hormonal level were observed. Recently study also showed that *Col6a5* potential role in ovaries and gonadal fat pads in the hyperandrogenic mouse model (L. F. Sun et al., 2021). Or at the neuronal level, mutation may have a potential effect in hypothalamic neurons, which are responsible for the same-sex mounting behavior of mice, as described by (Karigo et al., 2021).

Chapter 3: General Discussion

3.1 Aims and achievements of the thesis

My Ph.D. thesis project focused on three main objectives:

1. To establish the first chronic itch mouse model (*Col6a5*^{E2302*}) corresponding to the human *COL6A5*- p.Glu2272* mutation.

2. To characterize the effects of *COL6A5*-p.Glu2272* mutation at the cellular and molecular levels .

3. To characterize the human *COL6A5* mutation p.Glu2272* using a mice model for chronic itch through behavioral testing. These investigations aim to understand better the genotype-phenotype association and the consequential role of *COL6A5* mutations in chronic neuropathic itch.

A summary of key results is given below in **Table 3.1**

Table 3.1 Results summary.

Results	Description
Model	The first mouse model to recapitulate the *COL6A5* mutation found in patients with chronic neuropathic itch.
Genotype-Phenotype Association	There was significant decrease in *Col6a5* expression was observed in the skin but not in DRG of the *Col6a5*^{E2302*} mutant mice
Skin Permeability	Female *Col6a5*^{E2302*} mutant mice showed a significant increase in skin permeability
Spontaneous Scratching Behavior	Both male and female *Col6a5*^{E2302*}mutant mice displayed an increase in spontaneous scratching behavior
Anxiety-Like Behavior	*Col6a5*^{E2302*} female mutant mice exhibited a significant increase in anxiety-like behavior
Despair-Like Behavior	*Col6a5*^{E2302*} mutant mice of both sexes showed an increase in despair-like behavior
Conclusion	The *Col6a5* mutation contributes to chronic itch with emotional consequences, as evidenced by the behavioral changes observed in the mutant mice

We want to highlight important discussion points derived from my achieved work through this project at molecular to phenotypic levels:

1. The few patients described by Martinelli-Boneschi and colleagues were heterozygous for the *COL6A5* variant p.Glu2272* mutation (Martinelli-Boneschi et al., 2017). Heterozygous mice were also intercrossed to assess the behavior and lethality of homozygous mice. Homozygous mice were physically normal and healthy, and their scratching behavior was similar to heterozygous mutant mice. Thus we confirmed that one copy of the *COL6A5* variant p.Glu2272* mutation is enough to produce the scratching phenotype in mouse as it is in human.
2. The mutant mouse model (*Col6a5*^{em1} (E2302*)) corresponding to the human *COL6A5* E2272* mutation mRNA was expressed in DRG and showed no major defect in this mutant transcript, at least in DRGs of mutant mice. Nevertheless, we found a significantly decreased mRNA level in mutant mice's skin.
3. Skin permeability was augmented in *Col6a5*^{E2302*} mutant female mice, suggesting that COL6A5 protein might have an important role in the skin barrier while there is no mutation effect in male mutants. Males are known to have thicker skin than females, which might have induced a lesser mutation effect. Any alternative collagen protein may have compensated a potentially defective function of *Col6a5*^{E2302*} mutant protein in the mutant males. In the future, transcriptomics or proteomics studies may examine potential transcript or protein levels compensations.
4. The study used two types of control mice to investigate contagious itch and avoid interaction between control and mutant mice. Results showed a difference between the two control mice in terms of scratching behavior. Although there was slight influence of mutant mice on control wt littermates, the use of B6N wt control is still recommended in future itch studies.
5. *Col6a5*^{E2302*}mutant mice encounter itch for the long term (chronically) as they showed increased scratching till 32 weeks of age. A comparison of scratching behaviors in mutant mouse models with spontaneous scratching to those in the *Col6a5*^{em1} (E2302*) mutant mouse line was made. Data on spontaneous scratching were analyzed from various publications, and the fold increase in scratching in mutant vs control mice

(**Table 3.2**). Scratching behavior in the *Col6a5*^{E2302*}mutants compared to controls was found to be consistent with previously published results.

6. A phenotype of anxiety- and despair-like behaviors was found in *Col6a5*^{E2302*}mutant mice. However, it is not unknown whether these are consequences of the itch itself, scratching behavior that chronically interferes with their normal life and potentially activates the immune system, or whether their anxiety and despair-like state would contribute towards more scratching. More experiments are needed to the links between the two phenotypes.
7. The behavior of female-female mounting was observed in mice, which might have a functional role in social hierarchy establishment/maintenance. Furthermore, studies suggest that levels of pro-social motivation influence female-female interactions and may involve affiliative interactions. Additionally, the thesis presented that mice with a mutation of *Col6a5* have increased social interaction with female partners, especially in making nose-to-nose or oral congenital contacts, indicating that the mutation strongly affects female-female interaction. However, further research is needed to establish the role of chronic itch, hormonal changes, and neural circuits' alteration in female-female interaction.

In detail these points are discussed below.

3.2 A single copy of *COL6A5*-p.Glu2272* is sufficient to produce a scratching phenotype

Two families with heterozygous genetic mutations p.Glu2272* in *COL6A5* corresponding to itch phenotype were reported previously (Martinelli-Boneschi et al., 2017). More recently, the Spanish family, including five individuals with Chiari Malformation Type 1 (CM-1) carrying the same heterozygous mutations p.Glu2272* in *COL6A5*, was reported (Urbizu et al., 2021). Both studies mentioned that patients carried heterozygous mutations p.Glu2272* in *COL6A5*. This study also produced the first mutant mouse model (*Col6a5*^{em1} (E2302*)) corresponding to the human *COL6A5*- p.Glu2272* mutation as well heterozygous. Heterozygous mice were intercrossed to assess the behavior and lethality of homozygous mice though in patients, non of them were homozygous for this mutation. Both homozygous and heterozygous mutant mice had no obvious abnormal phenotypes. They were fertile and had normal life spans. This

suggests that one copy of the *COL6A5*-p.Glu2272* mutation is enough to produce the scratching phenotype.

3.3 *Col6a5* mRNA expression of the *Col6a5*^{E2302*} mutant mice

In the initial study that reported the *COL6A5*-p.Glu2272* mutation in chronic itch patients, the analysis of cDNA from the patient's fibroblasts in culture showed only the wt allele's expression suggesting a nonsense-mediated mRNA decay of the mutant mRNA (Martinelli-Boneschi et al., 2017). The nonsense-mediated mRNA decay pathway exists in eukaryotes to reduce errors of some genes by eliminating mRNA transcripts that contain premature stop codons. This usually results in reduced protein expression. Nonsense-mediated mRNA decay could affect various cellular surveillance pathways (Pawlicka et al., 2020). However, in *Col6a5*^{E2302*} mutant mice, the mutant transcript expression in DRGs was found comparable to that of the wt allele. *Col6a5* mRNA was also found expressed in mouse DRG in other studies (Gara et al., 2008; Söderhäll et al., 2007), which is consistent with this project's results. Recent transcriptomic data also confirmed our results, showing that *Col6a5* mRNA was expressed in DRG and skin (Mecklenburg et al., 2020). Also, the *Col6a5*-p.Glu2272* mRNA expression in *Col6a5*^{E2302*}mutant mice skin significantly decreases compared to wt mice. This is consistent with previous studies, which argued that epidermis of individuals with AD had decreased the expression of collagen VI α5 chain (COL6A5), which contains a von Willebrand factor type A domain (vWA). This protein is responsible for binding other proteins and ligands and is essential for maintaining the cohesion of keratinocytes. Consequently, a reduction in COL6A5 could decrease the integrity of the epidermis, leading to increased penetration of antigens through the skin (Johanek et al., 2008b).

3.4 Skin difference in male and female mice

To assess skin permeability, the Evans blue dye assay was used as proposed previously (Zhang et al., 2018). This thesis results showed an increased OD value, suggesting that the skin of female mutant mice is more permeable than wt females, while in male mice, no significant difference of OD value was found**.** Also, it was observed that the OD values in the skin of wt

females were lower compared to wt male mice (**Figure 2.16**). Why female mice have more skin permeability could have several reasons and though not fully understood.

Nevertheless, Calabro et al. (Calabro et al., 2011) showed that male mice have a thicker dermis layer of skin than female mice. This thicker dermis could obstruct Evans dye from passing through the dermal-epidermal junction. However, in female mice as dermis layer is not as thick as male mice; could have potential role in skin permeability. To assess this further experiments are required to know the role of each skin layers in skin permeability including epidermis, dermis and hypodermis.

To compare these results of gender difference with skin permeability, it was referred to the results obtained by a similar study (Zhang et al., 2018) in which Evans blue dye was also treated topically in mice skin. Nevertheless, unfortunately, Zhang et al. performed the same experiment without differentiating the male and female mice rather presented the combined data of both genders. In contrast, other studies have performed this test by intravenous injecting Evans blue dye in the tail of mice, not topically over the skin.

3.5 wt littermate and B6N wt scratching and grooming behavior differences

Because we hypothesized that control mice housed with mutant mice could show imitative scratching mimicking the mutant mice, we used two wt control group of wt mice mainly for the scratching and grooming study including wt littermate and B6N wt from an independent colony. We aimed to avoid visual or physical contact of control mice with mutant mice. Indeed Yu et al (Yu et al., 2017) reported previously that increase itch occurs in mice based on imitative scratching in normal mice by observing excessive scratching in genetically modified mice. This phenomenon was already reported in non-human primates observing normal scratching behavior (Feneran et al., 2013; Nakayama, 2004) as well as humans (Lloyd et al., 2013). Our findings are to previous finding where wt mice housed with those experiencing chronic itch showed higher spontaneous scratching behavior, our study adds valuable insights by examining the specifics of this behavior, particularly in relation to gender differences and grooming behavior.

Our observation that both male wt mice (B6N and wt littermate) exhibited two distinct trend lines for facial scratching (which contributes major portion of scratching data in our study) (Figure 2.18) that did not overlap with each other suggests that there may be an influence of the presence of mutant mice on the scratching behavior of male wt mice. our results are align with the previous study (Yu et al., 2017).

On the other hand, our finding that female mice did not show the same clear separation in trend lines between wt littermate and B6N wt for facial scratching indicates that there may be variations in how gender influences contagious itch behavior (Figure 2.19**)**. This is an interesting aspect to explore further, as it suggests that the mimicking the scratching may differ between male and female mice.

Furthermore, our observation that grooming behavior doesn't appear to be effected by keeping wt mice and mutant mice together in this study, but there might be a subtle influence of mutant mice on the grooming behavior of wt littermates, provides additional depth to our understanding of how social interactions and visual cues among mice may affect different behaviors. We proposed to use B6N wt mice as controls for future itch studies, especially when studying spontaneous itch.

3.6 Comparison of the scratching results from this work to previously published work in genetic mouse models with spontaneous scratching

In this chapter, we aimed to compare the scratching results from this work to scratching results previously published in genetic mouse models with spontaneous scratching. For this purpose, publications describing spontaneous scratching in mutant mouse models were searched. For simplicity and clarity, studies that contained only results based on scratching induced by chemicals, like histamine or serotonin, were not included. Rather publications describing spontaneous scratching without any experimental triggering except the mutation are used. Also, only papers were used in which the scratching data on control mice (wild type, wt or floxed) were shown, as one purpose was to discuss on the fold increase in scratching in mutant vs control mice. **Table 3.2** recapitulates scratching behaviors in the *Col6a5*^{E2302*} mutant mice and in mutant lines previously described for spontaneous scratching. It is

organized in (1) mouse models for point mutations found in patients with chronic itch and (2) other mouse models. For some mouse lines, a complementary information is written below the table as appropriate.

Table 3.2 Comparison of the data from this work to previously published mouse models with spontaneous scratching

Publication	Mutation	Mouse age	Scratching in control mice	Fold scratching over controls
Mouse models for point mutations found in patients with chronic itch				
This thesis work	Col6a5-E2272*	6 weeks	B6N WT 14/30 min	3.4
			WT litterm.‡ 26/30 min	1.8
		32 weeks	B6N WT 11.5/30 min	3.6
			WT litterm. 22/30 min	1.9
Ebbinghaus et al. 2020	Scn11a-L811P/wt (NaV1.9 sodium channel)	3-5 month	4.2/60 min -> 3.1/30 min	0.7*
		9-12 month	3.7/60 min -> 1.8/30 min	3.7
Salvatierra et al. 2018	Scn11a-L811P	2-3 month	5/30 min	4.4
Other mouse models				
Buhl et al. 2020	Protease-activated receptor-2 (Par2) overexpressed in keratinocytes (Grhl3-positive cells)	12 weeks	35/30 min	4.6
Nunomura et al. 2019	Ikk2 serine-threonine kinase cKO in fibroblasts of facial skin (Nes-Cre cells)	4 weeks	0.2/min -> 6/30 min	5.3
		8 weeks	0.15/min -> 4.5/30 min	7.7
Zhang et al. 2018	NC16A-truncated Col17 (BP180)	8 weeks	0.5/15min -> 1/30min	24
		12 weeks	2/15 min -> 4/30 min	10
Liu et al. 2010	Vesicular glutamate transporter type 2 (VGlut2) cKO in Nav1.8 neurons	1-2 month	55/30 min	6.9

Col6a5^{em1} (E2302*)). * stands for the stop mutation at residue E2272 found in the chronic itch patients. In this thesis work, two types on wild-type (wt) control mice were recorded for spontaneous scratching, commercial C57BL/6N wild-type (B6N wt) mice that were maintained in separated cages from mutant mice, and wt littermates issued from the same heterozygous breeding trios as the mutant heterozygous mice for which scratching data are shown in this **Table 3.2.** Scratching data are shown as scratching time in seconds per 30 min

observation. The fold scratching over controls is shown for heterozygous sex-grouped mutant mice.

Scn11a-L811P/wt (NaV1.9 channel) mouse line. The de novo pL811 mutation was identified in a female patient with severe pruritus without family history. The patient reported a partial loss-of-pain sensation. Gabapentin treatment resulted in a drastic decrease in discomfort and healing of the lesions provoked to previous scratching. The data by (Ebbinghaus et al., 2020) and by (Salvatierra et al., 2018) have been generated by independent teams. The 0.7* for fold scratching over controls (Ebbinghaus et al., 2020) indicates no significant difference in scratching at 3-5 months of age.

Ikk2 cKO in fibroblasts of facial skin (Nes-Cre cells) mouse line. (Nunomura et al., 2019)Reported inactivation of the floxed *Ikk2* gene in face dermal fibroblasts. They did not mention any data on *Ikk2* gene inactivation or decreased expression in the peripheral or central nervous system.

NC16A-truncated Col17 (BP180) mouse line. Although the sign of itch were appeared in 2 weeks but this study investigated on 8 weeks and 12 weeks age to highlight the complexity and underlying mechanism of itch. (Zhang et al., 2018)

Our this present study on *Col6a5*^{em1} (E2302*) line that analyzed both female and male mutants the information for the sex of animals analyzed in the other articles was not mentioned in articles.

For the *Col6a5*^{em1} (E2302*) line, scratching data are shown as scratching time in a given 30 min duration. Conversely, some publications showed data in scratching bouts. The comparison of the scratching bouts in the different articles indicates that the numbers of scratching bouts/30 min ranged from 1 to 55 in control mice. In these publications, increased scratching in the mutants ranges from 3.7 to 24 fold depending on the effect of mutation and age of mice. Interestingly, (Ebbinghaus et al., 2020) found an age-dependent increase in scratching behavior when 3-5 months of age were compared to 9-12 months. The 9-12 month-old mutants showed a 3.7-fold increase in scratching, while there was no phenotype in the 3-5 month-old mutants, indicating scratching augmented with age. Notably, Zhang et al. also found an increased scratching behavior in 12-week-old mutants compared to 8-week-

old mutants. However, the fold increase vs controls was lower in the 12-week-old mutants due to elevated scratching in 12-week-old controls as compared to 8-week-old controls. Overall, the findings on scratching behavior in the *Col6a5*^{E2302*}mutants compared to B6N controls agree with previously published results.

3.7 The role of potential factors in modulating itch phenotype in Mice

In this part of discussion, we explored various factors that could potentially modulate the itch phenotype in mice. These potential factors are following:

1. Genetic factors: Differences in collagen gene expression levels, particularly *COL6A5, COL6A6, COL7A1, and COL17A1*, in the skin may contribute to variations in itch severity. Collagen in human skin can have either a fibrillar or non-fibrillar supramolecular structure, depending on the type. Of the 28 types of collagens present in the skin, the fibrillar collagen types are I, III, and V. And the non-fibrillar collagens are types IV, VI, VII, XIV, XVI and XVII, as noted by (Potekaev et al., 2021). These collagens, IV, VI, VII, and XVII, are also important for the dermal-epidermal junction of the skin, as indicated by (Potekaev et al., 2021; Theocharidis & Connelly, 2019).

The expression of COL6A5 in the skin is less than that of COL7A1. Nevertheless, the expression of COL6A5 is higher than the expression of COL6A6 in the skin (Potekaev et al., 2021). It could be the reason that the phenotype of COL6A5 was not so severe apparently and due to the lack of blisters in human patients and so in this mouse model. A recent publication Daniela Schmidt et al 2022 found spontaneous scratching in a mouse model of COL7A1 mutation (Col7a1flNeo/flNeo mice) (Schmidt et al., 2022). Data presented by Schmidt et al showed that mutant mice have spontaneous scratching (39.3 6± 2.2 bouts/30min) compared to controls (11.6 6 ± 1.4 bouts/30min) with a 3.3 fold increase. Though data presented on both genders, the number of mice is less (6 control 8 mutant) shown only on a single timeline at 2-4 months. While this thesis study on *Col6a5*^{em1} (E2302*) line also showed a similar result of spontaneous itch at same age with data on both genders with Males (n=10-13 /group) and Females (n=13-14 /group). From this, it can be assessed that the expression of COL6A5 is lower than that of

COL7A1 and COL17A1, but still, the *Col6a5*^{em1} (E2302*) line showed a significant increase in spontaneous itch, and mice have prominent phenotype at a late age timeline.

Contrary to the higher expression of COL7A1 and COL17A1, COL6A6 has lower expression in the skin, as shown (Potekaev et al., 2021)Unfortunately, no mouse model study has been available in the case of COL6A6 mutation till now. Interestingly, whole-exome sequencing data identified that the COL6A6 variant is associated with atopic dermatitis (AD) in Koreans (Heo et al., 2017; Johanek et al., 2008b).

Previously, Söderhäll et al. reported a decrease in the expression of COL6A5 in the outer epidermis of AD patients. Similarly, a recent study also showed that COL6A6 expression decreased in the epidermis and dermis of AD patients (Johanek et al., 2008b).

The decrease in COL6A5 expression could result in a decline in epidermal integrity, allowing for the penetration of antigens through the skin. Consequently, individuals with AD may become more prone to allergic reactions and skin infections. Notably, *COL6A6* exists in the same genetic locus as *COL6A5*. In addition, COL6A6 also contains a vWA domain and is a vital basal lamina component, binding epithelial cells to fibronectin. Hence, it is very likely that the reduction of COL6A5 (Martinelli-Boneschi et al., 2017) may have a similar effect as the reduction of COL6A6 in atopic dermatitis patients (Johanek et al., 2008b).

(Potekaev et al., 2021) has not reported exact source and parameters of calculation they made for measuring the expression of different collagen level in skin. As it depends on several factors such as age of cohorts, biopsy samples, population variability, genetic diversity in population, disease prevalence in population, culture and behavior difference and most importantly study design. Without complete detail its hard to assess exactly comparison of COL6A5 with other skin related collagen. Therefore, more comparative studies are required in this field.

It was planned and performed western blot and analyzed the expression of Col6a5 protein in *Col6a5*^{em1} (E2302*), particularly in the skin and DRG (data not included in this thesis). Unfortunately, out of available commercial antibodies, all were not tested yet for western blot and were not available for full length of Col6a5 variant 289.5 kDa. Only one antibody (COL6A5 Rabbit Polyclonal Antibody, Origene, CAT#: TA333468) was available with

immunogen sequence homology of 86% for mouse, and all others were not tested for western blot and also have immunogen sequence homology of 76% or less. Although it has immunogen sequence homology of 86% for mouse, it has a predicted size of 62 kDa and was not for detecting full-length protein. Mostly commercial antibodies are still available for human tissues. Hopefully, with the availability of new antibodies for mice tissues, this *Col6a5*^{em1} (E2302*) line would be helpful to investigate a of expression of Col6a5 and its role in skin integrity.

Also, it was of great interest to assess Col6a5 expression at the different timelines of age in these mice to find any possible correlation with mice scratching behavior. It was also worth to visualize the localization of Col6a5 in skin and DRG especially. It would surely provide more fine detail about colocalization with other components, distribution in different tissues as well as cellular structure aspect of Col6a5.Hopefully, with the availability of new antibodies for mice tissues, this *Col6a5*^{em1} (E2302*) line would be helpful to investigate a of expression of Col6a5 and its role in skin integrity.

2. Early life experiences: Human research has already shown (Walsh et al., 2019) that prenatal anxiety and depression could increase the risk 2-fold in offspring for a mental disorder (e.g., attention deficit hyperactivity disorder or anxiety), and this effect extends from childhood through adolescence. It suggested that not only the prenatal period of offspring is crucial but also maternal childhood. A study has shown that emotional neglects during her (mother) childhood are associated with neural changes such as the amygdala and medial prefrontal regions in her offspring shortly after birth (Hendrix et al., 2021). Both brain regions (amygdala and medial prefrontal cortex) are involved in chronic itch (Sanders & Akiyama, 2018). This suggests that the early life experiences of mice and their parents are important in this study.

3. Environmental factors: Another important thing to care about to avoid any false results was the handling of the mice it required extra care because the stress could change the result during this sensitive test of scratching. Similarly, other environmental factors, such as noise, light, and urine smell, could also change the results.

4. Cage composition and number of mice per cage: Here it is interesting to assess that the trios of heterozygous mutant mice for breeding were used to make the scratching test cohort. Moreover, these trios for scratching test mice had one wt male and two heterozygous females. These two heterozygous females would also be going through chronic itch

(scratching) and other phenotypes such as anxiety and depress-like behaviors. These parents (heterozygous mice) may have transferred neuronal changes in the amygdala region associated with itch. Not only this, but offspring can also mimic the scratching behavior of parents. That could be one reason that wt littermate are not the best control for this study.

Additionally, mice scratching behavior could also be affected by the number of mice per cage. For example, after weaning, one wt littermate and 4 mutant mice in a same cage could have different behavior compared to 4 wt littermates with 1 mutant mouse in one cage. Also, the total number of mice in each cage could cause the difference. However, it was not possible to have each cage with an equal number of wt littermate and mutant mice in each cage or fix the number of mice in each cage due to the availability of mice of different genotypes and the extra cost of cages.

5. Brain regions and itch-related behaviors: The amygdala is involved in the emotional process and rewards. Findings suggest a role for the amygdala in the processing of itch. Chronic itch is associated with increased anxiety and other mood disorders. In turn, stress and anxiety exacerbate itch, leading to a vicious cycle that affects patient behavior (scratching) (Sanders & Akiyama, 2018). These studies strengthen our results of chronic itch in this *Col6a5*^{em1} (E2302*) mouse model and the phenotype of anxiety- and despair-like behaviors as they are linked with each other. Other animal studies also provided evidence of the involvement of several anxiety-related structures (amygdala) in the processing of spontaneous itch. In mice, study had shown that when amygdala neurons were activated through optogenetic or chemo-genetic resulted in spontaneous scratching and grooming behaviors directed all over the body (Samineni et al., 2021). Similarly, this study of the *Col6a5*^{E2302*} mouse model also showed spontaneous grooming and itching all over the body. This gives a clue that the brain region amygdala, is involved in this sort of scratching behavior and is a neuropathic itch. Not only amygdala, another important brain region, hippocampus activation appears to go hand-in-hand in most studies of itch, suggesting that the memory of previous itch experiences may be a significant factor in itch-related anxiety (Sanders & Akiyama, 2018). This study further enhanced our findings and also explained wt littermate results in our study. It was suspected that wt littermates mimic their parent and cage fellows and may also remember their past behaviors (such as scratching).

6. Hair cycle stages: Another key factor 'skin hairs' can also play unique and essential functions in buffering external forces and maintaining body temperature. Hair follicles support the mechanical strength of the skin by their deeply invigilated structure into the dermis, and the skin barrier functions as a route of sebum to the skin surface. Hair cycle stages consist of key stages of growth (anagen), regression (catagen), and quiescence (telogen) (Lin et al., 2022). Studied showed that the number of weeks are important for the hair growth cycle. In wt normal mice, catagen and telogen follicles coexisted at 6 weeks of age, and all were telogen at 7 weeks. Telogen and anagen follicles coexisted at 8 and 9 weeks and all were anagen at 10 weeks (Tasaki et al., 2016). This suggested that generally, mice have hair cycle transition (catagen-telogen) from 6-7 weeks and 8-9 weeks transition (telogen-anagen). These transitions in the hair cycle may affect the sensitive behaviors of mice, such as grooming and scratching. However, a recent publication shows only data till 14 weeks for the hair cycle (Lin et al., 2022) and it is supposed that after 12 weeks of age hair cycle has stability due to a lack of available data. In the case of scratching behavior, hairs can play a role in mechanical itch. Also, the hair cycle has a role in activating several pathways and immune cells, which could affect the scratching behavior of mice. In this study, *Col6a5*^{E2302*}mice showed increased spontaneous itch at 6 weeks and 8 weeks (data not shown), while it looks like the phenotype disappeared at the age of 12 weeks. It might be the reason that hair cycle transitions, as mentioned earlier at the age of 6 and 8 weeks, may influence the results of scratching data. However, after 12 weeks, due to the stability of the hair cycle and progression of chronic itch appearance of phenotype at the age of 18, 26, and 32 weeks is more convincing data in our study. To support this, literature suggest the hair cycle, and hair follicles have potential a role in generating itch-inducing substances, and stress can further enhance this process. This mechanism contributes to the itching sensations experienced in conditions like psoriasis, with neuroendocrine factors, pruritogens, and inflammatory mediators all playing a part in the itch-scratch cycle (Leon et al., 2019).

7. Exploring scratching patterns and daytime itch behavior: Another aspect of this scratching behavior was to find any pattern in these mice. Unfortunately, any specific scratching pattern among each genotype or gender or any other specific scratching pattern was not found during the 30 min. Similarly, another recent publication (Honda et al., 2022) also reported that they could not find any pattern for scratching even in 1 hour, which endorses our study. However,

they explore an interesting thing the number of scratching bouts per hour in the light period was less than that in the dark period. The average number of scratching bouts in the dark period was higher in females than in males among wt mice (Takanami et al., 2023). This suggests that the scratching experiment should also monitor at night in our study. In this study, 24-hour scratching behavior was not monitored for two major reasons. 1) publication showed (Honda et al., 2022) during the night time, mice showed more prominent gender differences in scratching behavior 2) in humans, generally chronic itch patients show more scratching at night, and for mice day and night are reversed as compared to humans, as mice sleep during the day and are more active during night time. Therefore, it was preferred to perform scratching during the daytime.

3.8 Anxiety and despair-like behaviors in chronic itch

Recently concern about depression and anxiety in atopic dermatitis has been increasing. A study among adults in the United States (cohort included 2893 adults, 602 with atopic dermatitis found that adults with atopic dermatitis had significantly higher anxiety and depression scores (Nicholas & Drucker, 2019). Similarly, in another study performed on a cohort of 1017 adults; having moderate-to-severe atopic dermatitis. About 46.6% reported having moderate-to-severe depression, and 60.9% reported having anxiety. This suggested that comorbidities, including depression and anxiety, are significantly associated with mental health (Kwatra et al., 2021).

The population-based study focused on the prevalence of acne in a representative sample of the French population aged between 15 and 24 years. Approximately 30% of 15-19-year-olds and 17% of 20-24-year-olds reported the relationship between acne and feelings of stress, fatigue upon waking, and sleep disorders. They found that people with acne (n=1,375) experience more stress those without acne. Specifically, 18% of people with acne reported experiencing daily stress (compared to 13.9% of the control group) (Misery et al., 2015).

Recently, a meta-analysis to study the association of eczema with depression and anxiety included diverse data from Asia, Europe, and North America with a cohort of 141,910 patients with eczema and 4,736,222 control. The results suggested that eczema is associated with an increased risk of developing depression and anxiety (Long et al., 2022).

Very few studies have been performed on rodents to demonstrate anxiety and depression-like symptoms and negative emotions in relation to atopic dermatitis. Yeom M et al. showed that atopic dermatitis induces anxiety and depressive-like behavior. Although the exact mechanism of atopic dermatitis and negative emotions are not yet fully understood, they still shed some light on the topic. The finding demonstrated that AD-like skin lesion elicits anxiety- and depressive-like phenotypes that are associated with neuroplasticity-related changes in reward circuitry, providing a better understanding of AD-associated emotional impairments (Yeom et al., 2020, 2022).

Among these studies, only one study has been performed on chronic itch mouse model and also extended their investigation to include chronic depressive mouse model (X. D. Wang et al., 2018). Wang and colleagues demonstrated the interconnected aggravation of chronic itch and depression (X. D. Wang et al., 2018).

These current evidence supports our approach to performed experiments related to anxiety and depression in mutant mice. However, to obtain deeper understanding, further experiments are required to assess the timeline or uncover mechanisms of anxiety- and depressive-like symptoms in these mice. Despite providing relief of itch at first, continuous scratching, as in the case of chronic itch, can lead to an increase in the intensity of the itch. Patients are often unable to stop scratching despite adverse consequences. This appears to be due to the rewarding properties of scratching. Besides itch relief, scratching evokes a pleasurable feeling, particularly in chronic itch patients (Mochizuki & Kakigi, 2015). In addition, studies have addressed that this pleasurable experience is related to the brain's reward system implicated in controlling reward and motivated behaviors. The interactive aggravation of pruritus and depression is well-known, but an appropriate experimental model that could mimic this behavioral phenomenon is still lacking. Thus, a systematic animal behavioral investigation was carried out in this study.

Central to the brain's reward system is the mesolimbic dopamine system, arising from the ventral tegmental area and substantia nigra of the midbrain and projecting to target limbic regions, including the nucleus accumbens and dorsal striatum. It is recognized that, apart from regulating reward and motivated behaviors, the mesolimbic dopamine reward circuit also contributes importantly to mediating negative emotional states such as anxiety,

depression, and stress (Nestler & Carlezon, 2006). Recent neuroimaging results show that depressive symptoms are related to reduced activation of the brain's reward circuitry in depressed patients (Satterthwaite et al., 2015). An animal study has also shown that social defeat stress-induced behavioral changes (e.g., depression) are associated with the altered mesolimbic dopamine system in animals (Yeom et al., 2020, 2022).

Interestingly, the study used three groups of mice, including control, losers (chronically defeated), and winners (chronically aggressive), and analyzed their brain transcriptomes after 20 days of agonistic interactions. The brain regions were dissected and compared between groups. In the striatum, 20 collagen genes in the losers (chronically defeated) changed their expression under agonistic interactions. *Col6a5* gene was downregulated in the striatum (reward processing region) (Smagin et al., 2019). This also gives evidence that Col6a5 has been linked to anxiety, depressive-like phenotype, and a chronic itch. More research is required to find the importance of Col6a5 in the striatum.

Another independent study was performed to examine alterations in splenic function (spleen: the largest peripheral immune organ) and gene expression in mice with depressive-like behavior. It was found that splenic function and immunity in the mice with depressive-like behavior were markedly impaired. A total of 53 genes exhibited a differential response in the mice with depressive-like behavior, 11 of which were more notable. Of these 11 genes, Col6a5 was highly upregulated (10-fold). In contrast, other genes were up or down regulated approximately 2-3 fold only (Zhan et al., 2017). This genetic insight suggests a potential link between depressive-like behavior and specific gene expression changes, shedding light on another aspect of the complex relationship between anxiety, depression, and genetic factors in *Col6a5*^{E2302*}mice.

3.9 Female-female mounting behavior in mice: Exploring affiliative interactions

In our study, we observed mutant female mice engaging in mounting behavior similar to male mice. This female-female mounting behavior raised questions about its significance and underlying factors. To gain insight, we observed this female-female mounting behavior

through live mouse tracking (LMT) study on female mice, considering its relevance to chronic itch.

Female mounting behavior in mice, though not related to reproduction like male mounting, appears to play a role in establishing and maintaining the social hierarchy among females (Williamson et al., 2019). This behavior may be influenced by pro-social motivation and stress responses, potentially mediated by the hypothalamic-pituitary-adrenal (HPA) axis (Aoki et al., 2010; Varholick et al., 2019).

Our study revealed increased social interaction, especially nose-to-nose and oral congenital contacts, in *Col6a5*^{E2302*} mutant female mice (**Figure 2.25**), particularly among homozygous mutants. However, further research is needed to have some definitive conclusions. It remains unclear whether anxiety or the direct/indirect effects of chronic itch primarily drive female-female social interaction. Brain regions related to itch-scratch cycles and reward circuits overlap, suggesting a potential connection (Setsu et al., 2020).

Despite these insights, our study has limitations, including a small group size for live tracking and the need for ethical committee approval to assess mounting behavior.

Ultrasonic vocalization (USV) production may serve as a proxy for social and sexual motivation, but its communicative significance and relation to behaviors like itch and pain remain unclear. Future research could explore real-time measurements and manipulations of reward region neurons to understand how USV changes affect social behavior during various stresses, including chronic itch, anxiety, or depression, and vice versa.

Our study sheds light on unique female-female social interactions and mounting behaviors in mutant mice, likely influenced by pro-social motivation and stress responses, but further research is needed to fully understand their significance in the context of chronic itch.

Chapter 4: Conclusion and perspectives

4.1 Conclusion and perspectives

This study successfully demonstrate that the first mouse model for *COL6A5*-p.Glu2272* mutation identified in neuropathic itch recapitulates the human main feature, i.e. chronic itching. To date, this is the second mouse model for chronic neuropathic itch based on a mutation found in patients following the model on the *Scn11a-L811P* (NaV1.9 channel) mutation published by (Salvatierra et al., 2018) and by (Ebbinghaus et al., 2020), and the first model based on a mutation in a collagen gene. This *Col6a5*^{E2302*}mouse model was used to explore the genotype-phenotype association. Our results showed no significant mRNA difference in *Col6a5* expression in DRG of *Col6a5*^{E2302*}mutant mice. Skin permeability was increased in female mutant mice. The behavior studies showed elevated spontaneous scratching behavior in male and female mutants, increased anxiety-like behavior in female mutants, and despair-like behavior in sex-grouped mutants. These results demonstrate that the *COL6A5*-p.Glu2272* mutation found in patients contributes to chronic itch with emotional consequences. Now, the *Col6a5*^{em1} (E2302*) line represents a good model to go further in the understanding of *COL6A5* mutations in chronic Itch.

4.2 Intraepidermal nerve fiber density (IENFD)

Martinelli-Boneschi and colleagues found that magnetic resonance imaging (MRI) of the spinal cord and brain was normal in the patients with the *COL6A5*-p.Glu2272* mutation. They also found reduced intraepidermal nerve fiber density (IENFD) in 4 out of 5 patients in families 1 and 2 (Martinelli-Boneschi et al., 2017). The assessment of the IENFD allows the morphological quantification of the unmyelinated C-fibers crossing the basement membrane of the epidermis. This diagnostic tool has gained interest in recent years in the diagnostics of neuropathic pruritus since, next to central processes, a dysfunction of the small unmyelinated C-fibers plays a central role in neuropathic pruritus. Furthermore, a recent study on 142 patients with generalized chronic itch (60 females, median age: 62.5 years) showed that their itch was associated with small-fiber neuropathy with IENFD at <30% of the normative cut-off value (Pereira et al., 2021). Therefore, the analysis of IENFD in the *Col6a5*^{E2302*} mouse model will provide extra information on small fiber neuropathy in these mice.

4.3 Microneurography

Slowly conducting peripheral afferent C-nerve fibers are important in the sensations of itch. Single nerve fiber recordings (microneurography) of human skin nerves have already provided detailed information on the peripheral input leading to itching sensations. Technology improvement has extended microneurography to studying different nerve types (Ottaviani et al., 2020). Along with microneurography technique improvement and the evolution of experimental research models through genetic engineering, pre-clinical studies focused on the study of specific genetic alterations induced primarily in small animals such as mice and rats. At the same time, the innovation in technical equipment and the improved miniaturization of acquisition devices helped the way for electrophysiological recording in such animal models. Through this, the researchers gained powerful tools to explore the interactions between the CNS and PNS by studying the alterations in nerve traffic recording after different stimuli or genetic conditions. The core concept of this technique is to record the electrical currents flowing through a nerve, analyzing this signal by characterizing the activations (spikes) or groups of activations. The differences in spikes provide information on single C-fiber associated with itch (acute or chronic). As our *Col6a5*^{E2302*} mouse model represents one of the few models for chronic neuropathic itch, it would be worth getting insight from this unique technology. In the Second year of my Ph.D. project, I planned to send the mutant mice to our project partner Neuroscience Technology (NT) Company, in Barcelona. However, due to the COVID-19 pandemic, the plan could not be executed, and then the company closed later.

4.4 Exploring the intensity of spontaneous itch and grooming in *Col6a5*^{E2302*} mutant mice model

Chronic neuropathic itch is a severe unpleasant sensation triggered by nerves and becomes persistent over time. Therefore, it is important to assess it in the mutant mice through various behavioral features.

The itch can be assessed based on a scoring system from mild to severe in patients. However, in rodents, the intensity of the itch feeling cannot be measured, and most studies count

scratching behavior as a primary sign reflecting itch. It may be scratching bouts or scratching duration. However, most studies on scratching behavior in rodents did not explain the intensity of itch, which is a key factor in chronic itch. So long, scratching behavior was described previously through low-frame video recording (LaMotte et al., 2011). Most studies follow a similar method of recording videos and counting scratching bout or scratching duration.

Conversely, some recent studies using machine learning and automated detection of behaviors still focused on improving the accuracy and specificity of scratching behavior (Bohnslav et al., 2021; Park et al., 2019). However, these and many other studies did not focus on the type of scratching behavior and other delicate information that can distinguish each scratching bout and give a more informative view on the intensity of scratching behavior. Recently, a study explained the nature of scratching bouts and their different patterns and intensity when video recorded at high frame speed (Wimalasena et al., 2021). This new approach could be used to characterize the *Col6a5*^{E2302*}mutant mice. Then, it could be applied, and based on the speed and acceleration of each scratching bout, the intensity of scratching can be analyzed. It might be critical to record specific intensity ranges of scratching behavior, which might help us to distinguish the nature of the itch type. For example, chronic neuropathic itch bout has unique nature as compared to atopic dermatitis or acute itch. Observing the nature of bouts in our chronic itch mouse model would be interesting. However, unfortunately, this study was published very recently, in October 2021 (Wimalasena et al., 2021), and, therefore, could not be applied during my Ph.D. project. In addition, the grooming pattern, intensity, and nature of grooming due to chronic itch could be assessed similarly to scratching bouts in the mutant mice.

4.5 Analysis of experimentally induced itch and grooming behaviors in *Col6a5*^{E2302*}mutant mice

The *COL6A5*-p.Glu2272* patients have similar features of itch, but the age for the appearance of those features differed among patients from 5 years to 40 years (Martinelli-Boneschi et al., 2017). Therefore, it will be worth evaluating itching and grooming behaviors along ageing. We

will get more insight on itch, determine if the phenotype worsen with ageing in the mutant mice.

For inducing chronic itch, a study has shown that treating with a compound such as oxazolone (0.5%) can induce chronic itch within 15 days in mice (Moosbrugger-Martinz et al., 2017) (Moosbrugger-Martinz et al., 2017). Previously, it was also found that the oxazolone effect was correlated with its affinity for the $\alpha_2\delta$ subunit of voltage-gated Ca^{2+} channels. Moreover, the expression of the $\alpha_2\delta$-1 subunit in DRG after repeated oxazolone application was significantly higher than in control mice. The up-regulation of the $\alpha_2\delta$-1 subunit in DRG has a role in its pruritic activity (Tsukumo et al., 2011). In addition, another well-known compound, vitamin D3 or its low-calcemic analog calcipotriol MC903 was used as an inducer of chronic itch (Donglang et al., 2021). Using such compounds may help to see more enhanced scratching effects in our genetic model.

Similarly, an augmented grooming behavior was observed in the 2,4-dinitrofluorobenzene-induced chronic itch mouse model (X. D. Wang et al., 2018). Wang and colleague recorded grooming behavior in these experiments following a 30% sucrose solution splashing. In the future, it will be interesting to investigate this more substantial grooming effect in mutant mice over a separate cohort of mice.

4.6 Additional tests for social behaviors

The social behavior of mice is important for analyzing their normal social activities and the potential effect of the mutation. I performed the three-chamber behavior test in my thesis to assess their sociability and social novelty. However, additional social behavior tests are required due to the complexity of social behaviors, and investigating these behaviors in mice is still challenging. Along with the technical limitations of data gathering, analyzing social behaviors requires access to observing a group of individuals in an enriched environment. Also, the social behavior recording during short periods can affect the social behavior of mice. Therefore, mice social behavior can now be tested in a group of mice using live mouse tracking with an enriched environment and free access to water and food. These dynamic environment studies can assess mouse social behavior better and accurately. More dynamic group study of mice was possible during my Ph.D. project. However, it required a

radiofrequency identification tag and ethical approval which I had unfortunately limited time to perform along with the age of mice.

It would be interesting to have a behavior test for 24 hours during the light and dark periods. Some studies have shown the difference of scratching behavior of mice during light and dark cycles. But keeping mice isolated for 24 hours for scratching behavior required ethical approval, which was a hurdle. A separate cohort with 24 hours recording of scratching data might be interesting in the future. Alternatively, automated scratching behavior recording for 24 hours in their home cages may have some exciting results.

Further to confirm neuropathy in these mice we may plan to preform:

1) Electrophysiological testing: This involves recording the electrical activity of nerves as mentioned earlier in microneurography and can be helpful to measure nerve conduction velocity, which can be used to assess neuropathy in mice.
2) Behavior tests: some behavior tests to assess the mice ability to sense and respond to different stimuli, such as touch, heat, and cold. This can be used to measure changes in sensitivity that are indicative of neuropathy.
3) Morphological analysis: This involves examining the structure of the peripheral nerves and can be used to detect changes in nerve fiber density, diameter, and myelination, which are also characteristic of neuropathy.
4) Histological analysis: By examining the tissue samples of peripheral nerves and can be used to identify any abnormalities, such as deterioration or shrinkage in the myelin sheath and surrounding cells as well as any changes in the number and distribution of nerve fibers, which can be used to assess neuropathy.
5) Biochemical analysis: Measuring changes in the levels of various proteins associated with neuropathy, including nerve growth factor, glial cell-derived neurotrophic factor, and inflammatory cytokines etc.

These are some experiments suggested to confirm further that these mice have neuropathy. These experiments can provide indications of neuropathy, but a definitive diagnosis typically necessitates multiple pieces of evidence and contemplation of alternative nerve damage causes. By combining several experiments, we could build a more comprehensive

understanding of the underlying mechanisms of neuropathy in relation to Col6a5 in these mice and developed more effective treatments for this disorder in the future.

4.7 Concluding remarks

The *Col6a5*^{E2302*} mouse model for *COL6A5*-p.Glu2272* mutation, generated and characterized in this thesis work, showed an elevated spontaneous scratching behavior. Other mutations have been found in the patients described in the publication by Martinelli and colleagues, although this *COL6A5*-p.Glu2272* mutation was the only one commonly found. The present thesis work demonstrates that the *COL6A5*-p.Glu2272* mutation found in patients contributes to chronic itch. Also, it would be a valuable model to assess mechanisms, genetic study, and bone density-related research pertaining to Chiari malformation type 1 disease as 26% of Chiari malformation type 1 patient also carry *COL6A5*-p.Glu2272* mutation (Urbizu et al., 2021). Some key features that can be studied on this mouse model could be embryonic, fetal and craniofacial development by histologic analyses, neuroimaging and micro-computerized tomographic imaging (MCT). Additionally, neurological signs associated to Chiari malformation type 1 diseases primary involve abnormalities in reflexes, muscle strength, and coordination. Therefore, conducting several behavioral tests to evaluate motor function and coordination in these mice for example rotarod test, grip strength test, balance beam test, and footprint analysis etc. could be advantageous. Also, this model can also be used as a biomarker study for idiopathic itch and Chiari malformation type 1 in the form of SNP (rs115375867) or finding new diagnostic markers. It could also be used for the functional study of COL6A5 in several tissues such as ovaries, fat, and lungs etc. Furthermore, this genetic model may further explore the mechanisms of COL6A5 and the role of COL6A5 in reward-mediated neuronal circuits involved in chronic itch to develop and improve new treatments.

Chapter 5: References

Abreu-Velez, A., & Howard, M. (2012). Collagen IV in normal skin and in pathological processes. *North American Journal of Medical Sciences*, *4*(1), 1. https://doi.org/10.4103/1947-2714.92892

Akiyama, T., Carstens, M. I., & Carstens, E. (2010). Enhanced scratching evoked by PAR-2 agonist and 5-HT but not histamine in a mouse model of chronic dry skin itch. *Pain*, *151*(2), 378–383. https://doi.org/10.1016/j.pain.2010.07.024

Akiyama, T., Tominaga, M., Takamori, K., Carstens, M. I., & Carstens, E. (2014). Roles of glutamate, substance P, and gastrin-releasing peptide as spinal neurotransmitters of histaminergic and nonhistaminergic itch. *Pain*, *155*(1), 80–92. https://doi.org/10.1016/j.pain.2013.09.011

Ali, S. M., & Yosipovitch, G. (2013). Skin pH: From basic science to basic skin care. *Acta Dermato-Venereologica*, *93*(3), 261–267. https://doi.org/10.2340/00015555-1531

Alpizar, Y. A., Gees, M., Sanchez, A., Apetrei, A., Voets, T., Nilius, B., & Talavera, K. (2013). Bimodal effects of cinnamaldehyde and camphor on mouse TRPA1. *Pflugers Archiv European Journal of Physiology*, *465*(6), 853–864. https://doi.org/10.1007/s00424-012-1204-x

Ansorge, H. L., Meng, X., Zhang, G., Veit, G., Sun, M., Klement, J. F., Beason, D. P., Soslowsky, L. J., Koch, M., & Birk, D. E. (2009). Type XIV Collagen Regulates Fibrillogenesis. *Journal of Biological Chemistry*, *284*(13), 8427–8438. https://doi.org/10.1074/jbc.M805582200

Aoki, M., Shimozuru, M., Kikusui, T., Takeuchi, Y., & Mori, Y. (2010). Sex differences in behavioral and corticosterone responses to mild stressors in ICR mice are altered by ovariectomy in peripubertal period. *Zoological Science*, *27*(10), 783–789. https://doi.org/10.2108/zsj.27.783

Arai, K. Y., Hara, T., Nagatsuka, T., Kudo, C., Tsuchiya, S., Nomura, Y., & Nishiyama, T. (2017). Postnatal changes and sexual dimorphism in collagen expression in mouse skin. *PLoS ONE*, *12*(5), 1–21. https://doi.org/10.1371/journal.pone.0177534

Arbogast, T., Ouagazzal, A.-M., Chevalier, C., Kopanitsa, M., Afinowi, N., Migliavacca, E., Cowling, B. S., Birling, M.-C., Champy, M.-F., Reymond, A., & Herault, Y. (2016). Reciprocal Effects on Neurocognitive and Metabolic Phenotypes in Mouse Models of 16p11.2 Deletion and Duplication Syndromes. *PLOS Genetics*, *12*(2), e1005709. https://doi.org/10.1371/journal.pgen.1005709

Azzi, L., El-Alfy, M., Martel, C., & Labrie, F. (2005). Gender differences in mouse skin morphology and specific effects of sex steroids and dehydroepiandrosterone. *Journal of Investigative Dermatology*, *124*(1), 22–27. https://doi.org/10.1111/j.0022-202X.2004.23545.x

Bateman, J. F., Boot-Handford, R. P., & Lamandé, S. R. (2009). Genetic diseases of connective tissues: Cellular and extracellular effects of ECM mutations. *Nature Reviews Genetics*, *10*(3), 173–183. https://doi.org/10.1038/nrg2520

Bella, J., & Hulmes, D. J. S. (2017). Fibrillar Collagens. In D. A. D. Parry & J. M. Squire (Eds.), *Fibrous Proteins: Structures and Mechanisms* (Vol. 82, pp. 457–490). Springer International Publishing. https://doi.org/10.1007/978-3-319-49674-0_14

Berger, T. G., & Steinhoff, M. (2011). Pruritus and renal failure. *Seminars in Cutaneous Medicine and Surgery*, *30*(2), 99–100. https://doi.org/10.1016/j.sder.2011.04.005

BISHOP, G. H. (1948). The skin as an organ of senses with special reference to the itching. *The Journal of Investigative Dermatology*, *11*(2), 143–154. https://doi.org/10.1038/jid.1948.79

Bohnslav, J. P., Wimalasena, N. K., Clausing, K. J., Dai, Y. Y., Yarmolinsky, D. A., Cruz, T., Kashlan, A. D., Chiappe, M. E., Orefice, L. L., Woolf, C. J., & Harvey, C. D. (2021). DeepEthogram, a machine learning pipeline for supervised behavior classification from raw pixels. *eLife*, *10*. https://doi.org/10.7554/elife.63377

Bornelöv, S., Saäf, A., Meleń, E., Bergstrom̈, A., Moghadam, B. T., Pulkkinen, V., Acevedo, N., Pietras, C. O., Ege, M., Braun-Fahrlaṅder, C., Riedler, J., Doekes, G., Kabesch, M., Van Hage, M., Kere, J., Scheynius, A., Soder̈häll, C., Pershagen, G., & Komorowski, J. (2013). Rule-based models of the interplay between genetic and environmental factors in childhood allergy. *PLoS ONE*, *8*(11), 1–12. https://doi.org/10.1371/journal.pone.0080080

Bueller, H. A., Bernhard, J. D., & Dubroff, L. M. (1999). Gabapentin treatment for brachioradial pruritus. *Journal of the European Academy of Dermatology and Venereology*, *13*(3), 227–228. https://doi.org/10.1111/j.1468-3083.1999.tb00890.x

Buteau, A., & Reichenberg, J. (2018). Psychogenic Pruritus and Its Management. *Dermatologic Clinics*, *36*(3), 309–314. https://doi.org/10.1016/j.det.2018.02.015

Calabro, K., Curtis, A., Galarneau, J.-R., Krucker, T., & Bigio, I. J. (2011). Gender variations in the optical properties of skin in murine animal models. *Journal of Biomedical Optics*, *16*(1), 011008. https://doi.org/10.1117/1.3525565

Canziani, B. C., Uestuener, P., Fossali, E. F., Lava, S. A. G., Bianchetti, M. G., Agostoni, C., & Milani, G. P. (2018). Clinical Practice: Nausea and vomiting in acute gastroenteritis: Physiopathology and management. *European Journal of Pediatrics*, *177*(1), 1–5. https://doi.org/10.1007/s00431-017-3006-9

Carstens, E., & Akiyama, T. (2016). Central Mechanisms of Itch. *Current Problems in Dermatology (Switzerland)*, *50*, 11–17. https://doi.org/10.1159/000446011

Carstens, E. E., Carstens, M. I., Simons, C. T., & Jinks, S. L. (2010). Dorsal horn neurons expressing NK-1 receptors mediate scratching in rats. *NeuroReport*, *21*(4), 303–308. https://doi.org/10.1097/WNR.0b013e328337310a

Castagnaro, S., Gambarotto, L., Cescon, M., & Bonaldo, P. (2021). Autophagy in the mesh of collagen VI. *Matrix Biology*, *100–101*, 162–172. https://doi.org/10.1016/j.matbio.2020.12.004

Castro-Giner, F., Bustamante, M., Ramon González, J., Kogevinas, M., Jarvis, D., Heinrich, J., Antó, J. M., Wjst, M., Estivill, X., & De Cid, R. (2009). A pooling-based genome-wide analysis identifies new potential candidate genes for atopy in the European Community Respiratory Health Survey (ECRHS). *BMC Medical Genetics*, *10*, 1–9. https://doi.org/10.1186/1471-2350-10-128

Cescon, M., Gattazzo, F., Chen, P., & Bonaldo, P. (2015). Collagen VI at a glance. *Journal of Cell Science*, *128*(19), 3525–3531. https://doi.org/10.1242/jcs.169748

Chambers, E. S., & Vukmanovic-Stejic, M. (2020). Skin barrier immunity and ageing. *Immunology*, *160*(2), 116–125. https://doi.org/10.1111/imm.13152

Chen, P., Cescon, M., & Bonaldo, P. (2015). Lack of Collagen VI Promotes Wound-Induced Hair Growth. *Journal of Investigative Dermatology*, *135*(10), 2358–2367. https://doi.org/10.1038/jid.2015.187

Chen, P., Cescon, M., Megighian, A., & Bonaldo, P. (2014). Collagen VI regulates peripheral nerve myelination and function. *FASEB Journal*, *28*(3), 1145–1156. https://doi.org/10.1096/fj.13-239533

Cheng, I. H., Lin, Y.-C., Hwang, E., Huang, H.-T., Chang, W.-H., Liu, Y.-L., & Chao, C.-Y. (2011). Collagen VI protects against neuronal apoptosis elicited by ultraviolet irradiation via

an Akt/Phosphatidylinositol 3-kinase signaling pathway. *Neuroscience*, *183*, 178–188. https://doi.org/10.1016/j.neuroscience.2011.03.057

Cheng, J. S., Dubal, D. B., Kim, D. H., Legleiter, J., Cheng, I. H., Yu, G. Q., Tesseur, I., Wyss-Coray, T., Bonaldo, P., & Mucke, L. (2009). Collagen VI protects neurons against Aβ toxicity. *Nature Neuroscience*, *12*(2), 119–121. https://doi.org/10.1038/nn.2240

Cohen, O. S., Chapman, J., Lee, H., Nitsan, Z., Appel, S., Hoffman, C., Rosenmann, H., Korczyn, A. D., & Prohovnik, I. (2011). Pruritus in familial Creutzfeldt-Jakob disease: A common symptom associated with central nervous system pathology. *Journal of Neurology*, *258*(1), 89–95. https://doi.org/10.1007/s00415-010-5694-1

Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Hsu, P. D., Wu, X., Jiang, W., & Marraffini, L. a. (2013). Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Habib, N., … Zhang, F. (2013). Multiplex Genome Engineering Using CRISPR/Cas Systems. Science (New York, N.Y.). *Science (New York, N.Y.)*, *339*(6121), 819–823. https://doi.org/10.1126/science.1231143.Multiplex

Daltro, S. R. T., Meira, C. S., Santos, I. P., Ribeiro dos Santos, R., & Soares, M. B. P. (2020). Mesenchymal Stem Cells and Atopic Dermatitis: A Review. *Frontiers in Cell and Developmental Biology*, *8*. https://doi.org/10.3389/fcell.2020.00326

Dawn, A. G., & Yosipovitch, G. (2006). Butorphanol for treatment of intractable pruritus. *Journal of the American Academy of Dermatology*, *54*(3), 527–531. https://doi.org/10.1016/j.jaad.2005.12.010

de Chaumont, F., Ey, E., Torquet, N., Lagache, T., Dallongeville, S., Imbert, A., Legou, T., Sourd, A. M. L., Faure, P., Bourgeron, T., & Olivo-Marin, J. C. (2018). Live Mouse Tracker: Real-time behavioral analysis of groups of mice. *bioRxiv*. https://doi.org/10.1101/345132

Di Martino, A., Cescon, M., D'Agostino, C., Schilardi, F., Sabatelli, P., Merlini, L., & Faldini, C. (2023). Collagen VI in the Musculoskeletal System. *International Journal of Molecular Sciences*, *24*(6), 5095. https://doi.org/10.3390/ijms24065095

Dillon, S. R., Sprecher, C., Hammond, A., Bilsborough, J., Rosenfeld-Franklin, M., Presnell, S. R., Haugen, H. S., Maurer, M., Harder, B., Johnston, J., Bort, S., Mudri, S., Kuijper, J. L., Bukowski, T., Shea, P., Dong, D. L., Dasovich, M., Grant, F. J., Lockwood, L., … Gross, J. A. (2004). Interleukin 31, a cytokine produced by activated T cells, induces dermatitis in mice. *Nature Immunology*, *5*(7), 752–760. https://doi.org/10.1038/ni1084

Dondalska, A., Rönnberg, E., Ma, H., Pålsson, S. A., Magnusdottir, E., Gao, T., Adam, L., Lerner, E. A., Nilsson, G., Lagerström, M., & Spetz, A. L. (2020). Amelioration of Compound 48/80-Mediated Itch and LL-37-Induced Inflammation by a Single-Stranded Oligonucleotide. *Frontiers in Immunology*, *11*(September), 1–14. https://doi.org/10.3389/fimmu.2020.559589

Dong, X., & Dong, X. (2018). Peripheral and Central Mechanisms of Itch. *Neuron*, *98*(3), 482–494. https://doi.org/10.1016/j.neuron.2018.03.023

Donglang, G., Tongtong, L., Dan, C., Chan, Z., Changming, W., Guang, Y., Yan, Y., & Zongxiang, T. (2021). Comparative Study on Different Skin Pruritus Mouse Models. *Frontiers in Medicine*, *8*(February), 1–7. https://doi.org/10.3389/fmed.2021.630237

Duan, Y., Liu, G., Sun, Y., Wu, J., Xiong, Z., Jin, T., & Chen, M. (2020). Collagen type VI α5 gene variations may predict the risk of lung cancer development in Chinese Han population. *Scientific Reports*, *10*(1), 1–10. https://doi.org/10.1038/s41598-020-61614-x

Dubos, A., Meziane, H., Iacono, G., Curie, A., Riet, F., Martin, C., Loaëc, N., Birling, M. C., Selloum, M., Normand, E., Pavlovic, G., Sorg, T., Stunnenberg, H. G., Chelly, J., Humeau, Y., Friocourt, G., & Hérault, Y. (2018). A new mouse model of ARX dup24

recapitulates the patients' behavioral and fine motor alterations. *Human Molecular Genetics*, *27*(12), 2138–2153. https://doi.org/10.1093/hmg/ddy122

Duchon, A., Pothion, S., Brault, V., Sharp, A. J., Tybulewicz, V. L. J., Fisher, E. M. C., & Herault, Y. (2011). The telomeric part of the human chromosome 21 from Cstb to Prmt2 is not necessary for the locomotor and short-term memory deficits observed in the Tc1 mouse model of Down syndrome. *Behavioural Brain Research*, *217*(2), 271–281. https://doi.org/10.1016/j.bbr.2010.10.023

Ebbinghaus, M., Tuchscherr, L., von Banchet, G. S., Liebmann, L., Adams, V., Gajda, M., Hübner, C. A., Kurth, I., & Schaible, H. G. (2020). Gain-of-function mutation in SCN11A causes itch and affects neurogenic inflammation and muscle function in Scn11a+/L799P mice. *PLoS ONE*, *15*(8 August), 1–17. https://doi.org/10.1371/journal.pone.0237101

Ehling, S., Butler, A., Thi, S., Ghashghaei, H. T., & Bäumer, W. (2018). To scratch an itch: Establishing a mouse model to determine active brain areas involved in acute histaminergic itch. *IBRO Reports*, *5*, 67–73. https://doi.org/10.1016/j.ibror.2018.10.002

Feneran, A. N., O'Donnell, R., Press, A., Yosipovitch, G., Cline, M., Dugan, G., Papoiu, A. D. P., Nattkemper, L. A., Chan, Y. H., & Shively, C. A. (2013). Monkey see, monkey do: Contagious itch in nonhuman primates. *Acta Dermato-Venereologica*, *93*(1), 27–29. https://doi.org/10.2340/00015555-1406

Fitzgerald, J., Holden, P., & Hansen, U. (2013). The expanded collagen VI family: New chains and new questions. *Connective Tissue Research*, *54*(6), 345–350. https://doi.org/10.3109/03008207.2013.822865

Fitzgerald, J., Rich, C., Zhou, F. H., & Hansen, U. (2008). Three novel collagen VI chains, α4(VI), α5(VI), and α6(VI). *Journal of Biological Chemistry*, *283*(29), 20170–20180. https://doi.org/10.1074/jbc.M710139200

Fowler, E., & Yosipovitch, G. (2019). Chronic itch management: Therapies beyond those targeting the immune system. *Annals of Allergy, Asthma and Immunology*, *123*(2), 158–165. https://doi.org/10.1016/j.anai.2019.01.016

Freiha, J., Riachi, N., Chalah, M. A., Zoghaib, R., Ayache, S. S., & Ahdab, R. (2020). Paroxysmal Symptoms in Multiple Sclerosis-A Review of the Literature. *Journal of Clinical Medicine*, *9*(10), 3100. https://doi.org/10.3390/jcm9103100

Fu, Y., Rusznák, Z., Herculano-Houzel, S., Watson, C., & Paxinos, G. (2013). Cellular composition characterizing postnatal development and maturation of the mouse brain and spinal cord. *Brain Structure and Function*, *218*(5), 1337–1354. https://doi.org/10.1007/s00429-012-0462-x

Gallegos-Alcalá, P., Jiménez, M., Cervantes-García, D., & Salinas, E. (2021). The keratinocyte as a crucial cell in the predisposition, onset, progression, therapy and study of the atopic dermatitis. *International Journal of Molecular Sciences*, *22*(19). https://doi.org/10.3390/ijms221910661

Gara, S. K., Grumati, P., Squarzoni, S., Sabatelli, P., Urciuolo, A., Bonaldo, P., Paulsson, M., & Wagener, R. (2011). Differential and restricted expression of novel collagen VI chains in mouse. *Matrix Biology : Journal of the International Society for Matrix Biology*, *30*(4), 248–257. https://doi.org/10.1016/j.matbio.2011.03.006

Gara, S. K., Grumati, P., Urciuolo, A., Bonaldo, P., Kobbe, B., Koch, M., Paulsson, M., & Wagener, R. (2008). Three novel collagen VI chains with high homology to the α3

chain. *Journal of Biological Chemistry*, *283*(16), 10658–10670. https://doi.org/10.1074/jbc.M709540200

Garibyan, L., Rheingold, C. G., & Lerner, E. A. (2013). Understanding the pathophysiology of itch. *Dermatologic Therapy*, *26*(2), 84–91. https://doi.org/10.1111/dth.12025

Gatseva, A., Sin, Y. Y., Brezzo, G., & Van Agtmael, T. (2019). Basement membrane collagens and disease mechanisms. *Essays in Biochemistry*, *63*(3), 297–312. https://doi.org/10.1042/EBC20180071

Goleva, E., Berdyshev, E., & Leung, D. Y. M. (2019). Epithelial barrier repair and prevention of allergy. *Journal of Clinical Investigation*, *129*(4), 1463–1474. https://doi.org/10.1172/JCI124608

Golpanian, R. S., Kim, H. S., & Yosipovitch, G. (2020). Effects of Stress on Itch. *Clinical Therapeutics*, *42*(5), 745–756. https://doi.org/10.1016/j.clinthera.2020.01.025

Gregorio, I., Braghetta, P., Bonaldo, P., & Cescon, M. (2018). Collagen VI in healthy and diseased nervous system. *DMM Disease Models and Mechanisms*, *11*(6). https://doi.org/10.1242/dmm.032946

Grimstad, Ø., Sawanobori, Y., Vestergaard, C., Bilsborough, J., Olsen, U. B., Grønhøj-Larsen, C., & Matsushima, K. (2009). Anti-interleukin-31-antibodies ameliorate scratching behaviour in NC/Nga mice: A model of atopic dermatitis. *Experimental Dermatology*, *18*(1), 35–43. https://doi.org/10.1111/j.1600-0625.2008.00766.x

Guimarães, G. R., Almeida, P. P., de Oliveira Santos, L., Rodrigues, L. P., de Carvalho, J. L., & Boroni, M. (2021). Hallmarks of aging in macrophages: Consequences to skin inflammaging. *Cells*, *10*(6). https://doi.org/10.3390/cells10061323

Guitart, J. R., Johnson, J. L., & Chien, W. W. (2016). Research Techniques Made Simple: The Application of CRISPR-Cas9 and Genome Editing in Investigative Dermatology. *Journal*

of Investigative Dermatology, *136*(9), e87–e93. https://doi.org/10.1016/j.jid.2016.06.007

Haq, F., Ahmed, N., & Qasim, M. (2019). Comparative genomic analysis of collagen gene diversity. *3 Biotech*, *9*(3), 1–9. https://doi.org/10.1007/s13205-019-1616-9

Harazin, M., Parwez, Q., Petrasch-Parwez, E., Epplen, J. T., Arinir, U., Hoffjan, S., & Stemmler, S. (2010). Variation in the COL29A1 gene in German patients with atopic dermatitis, asthma and chronic obstructive pulmonary disease. *Journal of Dermatology*, *37*(8), 740–742. https://doi.org/10.1111/j.1346-8138.2010.00923.x

Hashimoto, T., & Yosipovitch, G. (2019). Itching as a systemic disease. *Journal of Allergy and Clinical Immunology*, *144*(2), 375–380. https://doi.org/10.1016/j.jaci.2019.04.005

He, H., Suryawanshi, H., Morozov, P., Gay-Mimbrera, J., Del Duca, E., Kim, H. J., Kameyama, N., Estrada, Y., Der, E., Krueger, J. G., Ruano, J., Tuschl, T., & Guttman-Yassky, E. (2020). Single-cell transcriptome analysis of human skin identifies novel fibroblast subpopulation and enrichment of immune subsets in atopic dermatitis. *Journal of Allergy and Clinical Immunology*, *145*(6), 1615–1628. https://doi.org/10.1016/j.jaci.2020.01.042

Hendrix, C. L., Dilks, D. D., McKenna, B. G., Dunlop, A. L., Corwin, E. J., & Brennan, P. A. (2021). Maternal Childhood Adversity Associates With Frontoamygdala Connectivity in Neonates. *Biological Psychiatry: Cognitive Neuroscience and Neuroimaging*, *6*(4), 470–478. https://doi.org/10.1016/j.bpsc.2020.11.003

Heo, W. I., Park, K. Y., Jin, T., Lee, M. K., Kim, M. J., Choi, E. H., Kim, H. S., Bae, J. M., Moon, N. J., & Seo, S. J. (2017). Identification of novel candidate variants including COL6A6 polymorphisms in early-onset atopic dermatitis using whole-exome sequencing. *BMC Medical Genetics*, *18*(1). https://doi.org/10.1186/s12881-017-0368-9

Holle, H., Warne, K., Seth, A. K., Critchley, H. D., & Ward, J. (2012). Neural basis of contagious itch and why some people are more prone to it. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(48), 19816–19821. https://doi.org/10.1073/pnas.1216160109

Honda, K., Tominaga, M., & Takamori, K. (2022). *Scratching bouts are modeled as Bernoulli trials until successful itch-extinguishing in mice* [Preprint]. Neuroscience. https://doi.org/10.1101/2022.02.03.478919

Hrestak, D., Matijašić, M., Paljetak, H. Č., Drvar, D. L., Hadžavdić, S. L., & Perić, M. (2022). Skin Microbiota in Atopic Dermatitis. *International Journal of Molecular Sciences*, *23*(7). https://doi.org/10.3390/ijms23073503

Huguen, J., Brenaut, E., Clerc, C.-J., Poizeau, F., Marcorelles, P., Quereux, G., Dupuy, A., & Misery, L. (2019). Comparison of Characteristics of Neuropathic and Non-neuropathic Pruritus to Develop a Tool for the Diagnosis of Neuropathic Pruritus: The NP5. *Frontiers in Medicine*, *6*(APR), 1–5. https://doi.org/10.3389/fmed.2019.00079

Hunter, J. M., Ahearn, M. E., Balak, C. D., Liang, W. S., Kurdoglu, A., Corneveaux, J. J., Russell, M., Huentelman, M. J., Craig, D. W., Carpten, J., Coons, S. W., Demello, D. E., Hall, J. G., Bernes, S. M., & Baumbach-Reardon, L. (2015). Novel pathogenic variants and genes for myopathies identified by whole exome sequencing. *Molecular Genetics and Genomic Medicine*, *3*(4), 283–301. https://doi.org/10.1002/mgg3.142

Hwang, J. S., Ma, D. J., Choi, J., & Shin, Y. J. (2020). COL8A2 Regulates the fate of corneal endothelial cells. *Investigative Ophthalmology and Visual Science*, *61*(11). https://doi.org/10.1167/iovs.61.11.26

Ikoma, A., Steinhoff, M., Ständer, S., Yosipovitch, G., & Schmelz, M. (2006). The neurobiology of itch. *Nature Reviews Neuroscience*, *7*(7), 535–547. https://doi.org/10.1038/nrn1950

Inagaki, N., Nakamura, N., Nagao, M., Musoh, K., Kawasaki, H., & Nagai, H. (1999). Participation of histamine H1 and H2 receptors in passive cutaneous anaphylaxis-induced scratching behavior in ICR mice. *European Journal of Pharmacology*, *367*(2–3), 361–371. https://doi.org/10.1016/S0014-2999(98)00974-1

Jin, H., He, R., Oyoshi, M., & Geha, R. S. (2009). Animal models of atopic dermatitis. *The Journal of Investigative Dermatology*, *129*(1), 31–40. https://doi.org/10.1038/jid.2008.106

Johanek, L. M., Meyer, R. A., Friedman, R. M., Greenquist, K. W., Shim, B., Borzan, J., Hartke, T., LaMotte, R. H., & Ringkamp, M. (2008a). A role for polymodal C-fiber afferents in nonhistaminergic itch. *Journal of Neuroscience*, *28*(30), 7659–7669. https://doi.org/10.1523/JNEUROSCI.1760-08.2008

Johanek, L. M., Meyer, R. A., Friedman, R. M., Greenquist, K. W., Shim, B., Borzan, J., Hartke, T., LaMotte, R. H., & Ringkamp, M. (2008b). A role for polymodal C-fiber afferents in nonhistaminergic itch. *Journal of Neuroscience*, *28*(30), 7659–7669. https://doi.org/10.1523/JNEUROSCI.1760-08.2008

Jones, V. A., Patel, P. M., Gibson, F. T., Cordova, A., & Amber, K. T. (2020). The Role of Collagen XVII in Cancer: Squamous Cell Carcinoma and Beyond. *Frontiers in Oncology*, *10*. https://doi.org/10.3389/fonc.2020.00352

Kader, H. A., Azeem, M., Jwayed, S. A., Al-Shehhi, A., Tabassum, A., Ayoub, M. A., Hetta, H. F., Waheed, Y., Iratni, R., Al-Dhaheri, A., & Muhammad, K. (2021). Current insights into immunology and novel therapeutics of atopic dermatitis. *Cells*, *10*(6). https://doi.org/10.3390/cells10061392

Kadler, K. E., Baldock, C., Bella, J., & Boot-Handford, R. P. (2007). Collagens at a glance. *Journal of Cell Science*, *120*(12), 1955–1958. https://doi.org/10.1242/jcs.03453

Kahremany, S., Hofmann, L., Gruzman, A., & Cohen, G. (2020). Advances in understanding the initial steps of pruritoceptive itch: How the itch hits the switch. *International Journal of Molecular Sciences*, *21*(14), 1–45. https://doi.org/10.3390/ijms21144883

Karamanos, N. K., Theocharis, A. D., Piperigkou, Z., Manou, D., Passi, A., Skandalis, S. S., Vynios, D. H., Orian-Rousseau, V., Ricard-Blum, S., Schmelzer, C. E. H., Duca, L., Durbeej, M., Afratis, N. A., Troeberg, L., Franchi, M., Masola, V., & Onisto, M. (2021). A guide to the composition and functions of the extracellular matrix. *FEBS Journal*, *288*(24), 6850–6912. https://doi.org/10.1111/febs.15776

Karigo, T., Kennedy, A., Yang, B., Liu, M., Tai, D., Wahle, I. A., & Anderson, D. J. (2021). Distinct hypothalamic control of same- and opposite-sex mounting behaviour in mice. *Nature*, *589*(7841), 258–263. https://doi.org/10.1038/s41586-020-2995-0

Karkheiran, S., Krebs, C. E., Makarov, V., Nilipour, Y., Hubert, B., Darvish, H., Frucht, S., Shahidi, G. A., Buxbaum, J. D., & Paisán-Ruiz, C. (2013). Identification of COL6A2 mutations in progressive myoclonus epilepsy syndrome. *Human Genetics*, *132*(3), 275–283. https://doi.org/10.1007/s00439-012-1248-1

Kaufmann, R., Bieber, T., Helgesen, A. L., Andersen, B. L., Luger, T., Poulin, Y., Al-Hafidh, J., & Paul, C. (2006). Onset of pruritus relief with pimecrolimus cream 1% in adult patients with atopic dermatitis: A randomized trial. *Allergy: European Journal of Allergy and Clinical Immunology*, *61*(3), 375–381. https://doi.org/10.1111/j.1398-9995.2005.00977.x

Kim, D., Kobayashi, T., & Nagao, K. (2019). Research Techniques Made Simple: Mouse Models of Atopic Dermatitis. *Journal of Investigative Dermatology*, *139*(5), 984-990.e1. https://doi.org/10.1016/j.jid.2019.02.014

Kim, H. S., Yim, S. V., Jung, K. H., Zheng, L. T., Kim, Y. H., Lee, K. H., Chung, S. Y., & Rha, H. K. (2007). Altered DNA copy number in patients with different seizure disorder type: By array-CGH. *Brain and Development*, *29*(10), 639–643. https://doi.org/10.1016/j.braindev.2007.04.006

Kim, H. S., & Yosipovitch, G. (2020). The skin microbiota and itch: Is there a link? *Journal of Clinical Medicine*, *9*(4). https://doi.org/10.3390/jcm9041190

Kim, Y., & Lim, K. M. (2021). Skin barrier dysfunction and filaggrin. *Archives of Pharmacal Research*, *44*(1), 36–48. https://doi.org/10.1007/s12272-021-01305-x

Kremer, A. E., Mettang, T., & Weisshaar, E. (2020). Non-dermatological challenges of chronic itch. *Acta Dermato-Venereologica*, *100*(1), 22–27. https://doi.org/10.2340/00015555-3345

Kuraishi, Y., Nagasawa, T., Hayashi, K., & Satoh, M. (1995). Scratching behavior induced by pruritogenic but not algesiogenic agents in mice. In *European Journal of Pharmacology* (Vol. 275, pp. 229–233). ELSEVIER.

Kwatra, S. G., Gruben, D., Fung, S., & DiBonaventura, M. (2021). Psychosocial Comorbidities and Health Status Among Adults with Moderate-to-Severe Atopic Dermatitis: A 2017 US National Health and Wellness Survey Analysis. *Advances in Therapy*, *38*(3), 1627–1637. https://doi.org/10.1007/s12325-021-01630-z

Laidlaw, A., Flecknell, P., & Rees, J. L. (2002). Production of acute and chronic itch with histamine and contact sensitizers in the mouse and guinea pig. *Experimental Dermatology*, *11*(4), 285–291. https://doi.org/10.1034/j.1600-0625.2002.110401.x

Lamandé, S. R., & Bateman, J. F. (2018). Collagen VI disorders: Insights on form and function in the extracellular matrix and beyond. *Matrix Biology*, *71–72*, 348–367. https://doi.org/10.1016/j.matbio.2017.12.008

Lamandé, S. R., & Bateman, J. F. (2020). Genetic Disorders of the Extracellular Matrix. *Anatomical Record*, *303*(6), 1527–1542. https://doi.org/10.1002/ar.24086

LaMotte, R. H., Shimada, S. G., & Sikand, P. (2011). Mouse models of acute, chemical itch and pain in humans. *Experimental Dermatology*, *20*(10), 778–782. https://doi.org/10.1111/j.1600-0625.2011.01367.x

Langan, S. M., Irvine, A. D., & Weidinger, S. (2020). Atopic dermatitis. In *Www.thelancet.com* (Vol. 396). http://www.homeforeczema.org/

Lauria, G., Hsieh, S. T., Johansson, O., Kennedy, W. R., Leger, J. M., Mellgren, S. I., Nolano, M., Merkies, I. S. J., Polydefkis, M., Smith, A. G., Sommer, C., & Valls-Solé, J. (2010). European Federation of Neurological Societies/Peripheral Nerve Society Guideline on the use of skin biopsy in the diagnosis of small fiber neuropathy. Report of a joint task force of the European Fe-deration of Neurological Societies and the Peripheral Ne. *European Journal of Neurology*, *17*(7), 903-e49. https://doi.org/10.1111/j.1468-1331.2010.03023.x

Lee, B., & Elston, D. M. (2019). The uses of naltrexone in dermatologic conditions. *Journal of the American Academy of Dermatology*, *80*(6), 1746–1752. https://doi.org/10.1016/j.jaad.2018.12.031

Lee, J. S., Han, J. S., Lee, K., Bang, J., & Lee, H. (2016). The peripheral and central mechanisms underlying itch. *BMB Reports*, *49*(9), 474–487. https://doi.org/10.5483/BMBRep.2016.49.9.108

Leon, A., Rosen, J. D., Hashimoto, T., Fostini, A. C., Paus, R., & Yosipovitch, G. (2019). Itching for an answer: A review of potential mechanisms of scalp itch in psoriasis. *Experimental Dermatology*, *28*(12), 1397–1404. https://doi.org/10.1111/exd.13947

Leslie, T. A. (2016). Itch Management in the Elderly. *Current Problems in Dermatology (Switzerland)*, *50*, 192–201. https://doi.org/10.1159/000446094

Lettmann, S., Bloch, W., Maaß, T., Niehoff, A., Schulz, J. N., Eckes, B., Eming, S. A., Bonaldo, P., Paulsson, M., & Wagener, R. (2014). Col6a1 null mice as a model to study skin phenotypes in patients with collagen VI related myopathies: Expression of classical and novel collagen VI variants during wound healing. *PLoS ONE*, *9*(8). https://doi.org/10.1371/journal.pone.0105686

Li, Z., Cao, T., Li, Q., Zhang, J., Du, J., Chen, J., Bai, Y., Hao, J., Zhu, Z., Qiao, H., Fu, M., Dang, E., Wang, G., & Shao, S. (2023). Cross-disease characterization of fibroblast heterogeneities and their pathogenic roles in skin inflammation. *Clinical Immunology*, *255*, 109742. https://doi.org/10.1016/j.clim.2023.109742

Lin, X., Zhu, L., & He, J. (2022). Morphogenesis, Growth Cycle and Molecular Regulation of Hair Follicles. *Frontiers in Cell and Developmental Biology*, *10*. https://doi.org/10.3389/fcell.2022.899095

Liu, B., Escalera, J., Balakrishna, S., Fan, L., Caceres, A. I., Robinson, E., Sui, A., McKay, M. C., McAlexander, M. A., Herrick, C. A., & Jordt, S. E. (2013). TRPA1 controls inflammation and pruritogen responses in allergic contact dermatitis. *FASEB Journal*, *27*(9), 3549–3563. https://doi.org/10.1096/fj.13-229948

Liu, T., Han, Q., Chen, G., Huang, Y., Zhao, L., Berta, T., & Gao, Y. (2016). *Alloknesis , and Spinal Astrocyte Activation in Male Mice*. *157*, 806–817.

Lloyd, D. M., Hall, E., Hall, S., & McGlone, F. P. (2013). Can itch-related visual stimuli alone provoke a scratch response in healthy individuals? *British Journal of Dermatology*, *168*(1), 106–111. https://doi.org/10.1111/bjd.12132

Lo Vecchio, S., Andersen, H. H., & Arendt-Nielsen, L. (2018). The time course of brief and prolonged topical 8% capsaicin-induced desensitization in healthy volunteers evaluated by quantitative sensory testing and vasomotor imaging. *Experimental Brain Research*, *236*(8), 2231–2244. https://doi.org/10.1007/s00221-018-5299-y

Long, Q., Jin, H., You, X., Liu, Y., Teng, Z., Chen, Y., Zhu, Y., & Zeng, Y. (2022). Eczema is a shared risk factor for anxiety and depression: A meta-analysis and systematic review. *PLoS ONE*, *17*(2 February). https://doi.org/10.1371/journal.pone.0263334

Luger, T., Amagai, M., Dreno, B., Dagnelie, M. A., Liao, W., Kabashima, K., Schikowski, T., Proksch, E., Elias, P. M., Simon, M., Simpson, E., Grinich, E., & Schmuth, M. (2021). Atopic dermatitis: Role of the skin barrier, environment, microbiome, and therapeutic agents. *Journal of Dermatological Science*, *102*(3), 142–157. https://doi.org/10.1016/j.jdermsci.2021.04.007

Martinelli-Boneschi, F., Colombi, M., Castori, M., Devigili, G., Eleopra, R., Malik, R. A., Ritelli, M., Zoppi, N., Dordoni, C., Sorosina, M., Grammatico, P., Fadavi, H., Gerrits, M. M., Almomani, R., Faber, C. G., Merkies, I. S. J., Toniolo, D., Cocca, M., Doglioni, C., … Lauria, G. (2017). COL6A5 variants in familial neuropathic chronic itch. *Brain*, *140*(3), 555–567. https://doi.org/10.1093/brain/aww343

Matsuda, H., Watanabe, N., Geba, G. P., Sperl, J., Tsudzuki, M., Hiroi, J., Matsumoto, M., Ushio, H., Saito, S., Askenase, P. W., & Ra, C. (1997). Development of atopic dermatitis-like skin lesion with IgE hyperproduction in NC/Nga mice. *International Immunology*, *9*(3), 461–466. https://doi.org/10.1093/intimm/9.3.461

Mazeh, D., Melamed, Y., Cholostoy, A., Aharonovitzch, V., Weizman, A., & Yosipovitch, G. (2008). Itching in the psychiatric ward. *Acta Dermato-Venereologica*, *88*(2), 128–131. https://doi.org/10.2340/00015555-0406

Mecklenburg, J., Zou, Y., Wangzhou, A., Garcia, D., Lai, Z., Tumanov, A. V., Dussor, G., Price, T. J., & Akopian, A. N. (2020). Transcriptomic sex differences in sensory neuronal populations of mice. *Scientific Reports*, *10*(1), 1–18. https://doi.org/10.1038/s41598-020-72285-z

Meixiong, J., Dong, X., & Weng, H. J. (2020). Neuropathic Itch. *Cells*, *9*(10), 5–7. https://doi.org/10.3390/cells9102263

Melrose J, Shu C, Whitelock JM, Lord MS (2016) The cartilage extracellular matrix as a transient developmental scaffold for growth plate maturation. Matrix Biology 52–54:363–383. https://doi.org/10.1016/j.matbio.2016.01.008

Misery, L., Brenaut, E., Le Garrec, R., Abasq, C., Genestet, S., Marcorelles, P., & Zagnoli, F. (2014). Neuropathic pruritus. *Nature Reviews Neurology*, *10*(7), 408–416. https://doi.org/10.1038/nrneurol.2014.99

Misery, L., Dutray, S., Chastaing, M., Schollhammer, M., Consoli, S. G., & Consoli, S. M. (2018). Psychogenic itch. *Translational Psychiatry*, *8*(1). https://doi.org/10.1038/s41398-018-0097-7

Misery, L., Wolkenstein, P., Amici, J. M., Maghia, R., Brenaut, E., Cazeau, C., Voisard, J. J., & Taïeb, C. (2015). Consequences of acne on stress, fatigue, sleep disorders and sexual activity: A population-based study. *Acta Dermato-Venereologica*, *95*(4), 485–488. https://doi.org/10.2340/00015555-1998

Miyamoto, T., Nojima, H., Shinkado, T., Nakahashi, T., & Kuraishi, Y. (2002). Itch-Associated Response Induced by Experimental Dry Skin in Mice. *Japanese Journal of Pharmacology*, *88*(3), 285–292. https://doi.org/10.1254/jjp.88.285

Mochizuki, H., & Kakigi, R. (2015). Central mechanisms of itch. *Clinical Neurophysiology*, *126*(9), 1650–1660. https://doi.org/10.1016/j.clinph.2014.11.019

Moosbrugger-Martinz, V., Schmuth, M., & Dubrac, S. (2017). A mouse model for atopic dermatitis using topical application of vitamin D3 or of its analog MC903. *Methods in Molecular Biology*, *1559*, 91–106. https://doi.org/10.1007/978-1-4939-6786-5_8

Mores, K. L., Cummins, B. R., Cassell, R. J., & Van Rijn, R. M. (2019). A review of the therapeutic potential of recently developed G protein-biased kappa agonists. *Frontiers in Pharmacology*, *10*(APR), 1–14. https://doi.org/10.3389/fphar.2019.00407

Myllyharju, J., & Kivirikko, K. I. (2001). Collagens and collagen-related diseases. *Annals of Medicine*, *33*(1), 7–21. https://doi.org/10.3109/07853890109002055

Naba, A., Clauser, K. R., Ding, H., Whittaker, C. A., Carr, S. A., & Hynes, R. O. (2016). The extracellular matrix: Tools and insights for the "omics" era. *Matrix Biology*, *49*, 10–24. https://doi.org/10.1016/j.matbio.2015.06.003

Nakajima, S., Nomura, T., Common, J., & Kabashima, K. (2019). Insights into atopic dermatitis gained from genetically defined mouse models. *Journal of Allergy and Clinical Immunology*, *143*(1), 13–25. https://doi.org/10.1016/j.jaci.2018.11.014

Nakano, T., Andoh, T., Sasaki, A., Nojima, H., & Kuraishi, Y. (2008). Different roles of capsaicin-sensitive and H1 histamine receptor-expressing sensory neurones in itch of mosquito allergy in mice. *Acta Dermato-Venereologica*, *88*(5), 449–454. https://doi.org/10.2340/00015555-0479

Nakayama, K. (2004). Observing Conspecifics Scratching Induces a Contagion of Scratching in Japanese Monkeys (Macaca fuscata). *Journal of Comparative Psychology*, *118*(1), 20–24. https://doi.org/10.1037/0735-7036.118.1.20

Natsuga, K., Watanabe, M., Nishie, W., & Shimizu, H. (2019). Life before and beyond blistering: The role of collagen XVII in epidermal physiology. *Experimental Dermatology*, *28*(10), 1135–1141. https://doi.org/10.1111/exd.13550

Naumann, A., Söderhäll, C., Fölster-Holst, R., Baurecht, H., Harde, V., Müller-Wehling, K., Rodríguez, E., Ruether, A., Franke, A., Wagenpfeil, S., Novak, N., Mempel, M., Kalali, B. N., Allgaeuer, M., Koch, J., Gerhard, M., Melén, E., Wahlgren, C. F., Kull, I., … Weidinger, S. (2011). A comprehensive analysis of the COL29A1 gene does not support a role in eczema. *Journal of Allergy and Clinical Immunology*, *127*(5), 1187-1194.e7. https://doi.org/10.1016/j.jaci.2010.12.1123

Nerger, B. A., Jones, T. M., Rose, K. W. J., Barqué, A., Weinbaum, J. S., Petrie, R. J., Chang, J., Vanhoutte, D., LaDuca, K., Hubmacher, D., & Naba, A. (2022). The matrix in focus: New directions in extracellular matrix research from the 2021 ASMB hybrid meeting. *Biology Open*, *11*(1). https://doi.org/10.1242/bio.059156

Nestler, E. J., & Carlezon, W. A. (2006). The Mesolimbic Dopamine Reward Circuit in Depression. *Biological Psychiatry*, *59*(12), 1151–1159. https://doi.org/10.1016/j.biopsych.2005.09.018

Nguyen, A. V., & Soulika, A. M. (2019). The dynamics of the skin's immune system. *International Journal of Molecular Sciences*, *20*(8). https://doi.org/10.3390/ijms20081811

Nijhuis, W. H., Eastwood, D. M., Allgrove, J., Hvid, I., Weinans, H. H., Bank, R. A., & Sakkers, R. J. (2019). Current concepts in osteogenesis imperfecta: Bone structure, biomechanics and medical management. *Journal of Children's Orthopaedics*, *13*(1), 1–11. https://doi.org/10.1302/1863-2548.13.180190

Nowak, D., & Yeung, J. (2017). *Canadian Family Physician Medecin De Famille Canadien*, *63*(12), 925–931.

Nunomura, S., Ejiri, N., Kitajima, M., Nanri, Y., Arima, K., Mitamura, Y., Yoshihara, T., Fujii, K., Takao, K., Imura, J., Fehling, H. J., Izuhara, K., & Kitajima, I. (2019). Establishment of a Mouse Model of Atopic Dermatitis by Deleting Ikk2 in Dermal Fibroblasts. *Journal of Investigative Dermatology*, *139*(6), 1274–1283. https://doi.org/10.1016/j.jid.2018.10.047

Nyström, A., & Kiritsi, D. (2021). Transmembrane collagens—Unexplored mediators of epidermal-dermal communication and tissue homeostasis. *Experimental Dermatology*, *30*(1), 10–16. https://doi.org/10.1111/exd.14180

Onursal, C., Dick, E., Angelidis, I., Schiller, H. B., & Staab-Weijnitz, C. A. (2021). Collagen Biosynthesis, Processing, and Maturation in Lung Ageing. *Frontiers in Medicine*, *8*, 593874. https://doi.org/10.3389/fmed.2021.593874

Ottaviani, M. M., Wright, L., Dawood, T., & Macefield, V. G. (2020). In vivo recordings from the human vagus nerve using ultrasound-guided microneurography. *Journal of Physiology*, *598*(17), 3569–3576. https://doi.org/10.1113/JP280077

Park, I., Lee, K., Bishayee, K., Jeon, H. J., Lee, H., & Lee, U. (2019). Machine-learning based automatic and real-time detection of mouse scratching behaviors. *Experimental Neurobiology*, *28*(1), 54–61. https://doi.org/10.5607/en.2019.28.1.54

Patel, T., & Yosipovitch, G. (2010). Therapy of pruritus. *Expert Opinion on Pharmacotherapy*, *11*(10), 1673–1682. https://doi.org/10.1517/14656566.2010.484420

Pauli-Magnus, C., Mikus, G., Alscher, D. M., Kirschner, T., Nagel, W., Gugeler, N., Risler, T., Berger, E. D., Kuhlmann, U., & Mettang, T. (2000). *Naltrexone Does Not Relieve Uremic Pruritus: Results of a Randomized, Double-Blind, Placebo-Controlled Crossover Study*.

Pawlicka, K., Kalathiya, U., & Alfaro, J. (2020). Nonsense-Mediated mRNA Decay: Pathologies and. *Cancers*, *12*(3), 1–17.

Pereira, M. P., Wiegmann, H., Agelopoulos, K., & Ständer, S. (2021). Neuropathic Itch: Routes to Clinical Diagnosis. *Frontiers in Medicine*, *8*(February), 1–8. https://doi.org/10.3389/fmed.2021.641746

Pfisterer, K., Shaw, L. E., Symmank, D., & Weninger, W. (2021). The Extracellular Matrix in Skin Inflammation and Infection. *Frontiers in Cell and Developmental Biology*, *9*(July), 1–19. https://doi.org/10.3389/fcell.2021.682414

Philippeos, C., Telerman, S. B., Oulès, B., Pisco, A. O., Shaw, T. J., Elgueta, R., Lombardi, G., Driskell, R. R., Soldin, M., Lynch, M. D., & Watt, F. M. (2018). Spatial and Single-Cell Transcriptional Profiling Identifies Functionally Distinct Human Dermal Fibroblast Subpopulations. *Journal of Investigative Dermatology*, *138*(4), 811–825. https://doi.org/10.1016/j.jid.2018.01.016

Potekaev, N. N., Borzykh, O. B., Medvedev, G. V., Petrova, M. M., Gavrilyuk, O. A., Karpova, E. I., Trefilova, V. V., Demina, O. M., Popova, T. E., & Shnayder, N. A. (2021). *Turnover and Functioning*.

Raap, U., Ständer, S., & Metz, M. (2011). Pathophysiology of itch and new treatments. *Current Opinion in Allergy and Clinical Immunology*, *11*(5), 420–427. https://doi.org/10.1097/ACI.0b013e32834a41c2

Rajagopalan, M., Saraswat, A., Godse, K., Shankar, D. S. K., Kandhari, S., Shenoi, S. D., Tahiliani, S., & Zawar, V. V. (2017). Diagnosis and management of chronic pruritus: An expert consensus review. *Indian Journal of Dermatology*. https://doi.org/10.4103/0019-5154.198036

Ricard-Blum, S. (2011). The Collagen Family. *Cold Spring Harbor Perspectives in Biology*, *3*(1), 1–19. https://doi.org/10.1101/cshperspect.a004978

Robert, M., Misery, L., & Brenaut, E. (2020). Chronic pruritus in the absence of skin disease: A retrospective study of 197 french inpatients. *Acta Dermato-Venereologica*, *100*(16), 1–5. https://doi.org/10.2340/00015555-3630

Ruppenstein, A., Limberg, M. M., Loser, K., Kremer, A. E., Homey, B., & Raap, U. (2021). Involvement of Neuro-Immune Interactions in Pruritus With Special Focus on Receptor Expressions. *Frontiers in Medicine*, *8*. https://doi.org/10.3389/fmed.2021.627985

Sabatelli, P., Gara, S. K., Grumati, P., Urciuolo, A., Gualandi, F., Curci, R., Squarzoni, S., Zamparelli, A., Martoni, E., Merlini, L., Paulsson, M., Bonaldo, P., & Wagener, R. (2011). Expression of the collagen VI α5 and α6 Chains in normal human skin and in skin of patients with collagen VI-related myopathies. *Journal of Investigative Dermatology*, *131*(1), 99–107. https://doi.org/10.1038/jid.2010.284

Sabatelli, P., Gualandi, F., Gara, S. K., Grumati, P., Zamparelli, A., Martoni, E., Pellegrini, C., Merlini, L., Ferlini, A., Bonaldo, P., Maraldi, N. M., Paulsson, M., Squarzoni, S., & Wagener, R. (2012). Expression of collagen VI α5 and α6 chains in human muscle and in Duchenne muscular dystrophy-related muscle fibrosis. *Matrix Biology*, *31*(3), 187–196. https://doi.org/10.1016/j.matbio.2011.12.003

Sadeghi, P., Papay, F. A., Vidimos, A. T., & Helm, T. N. (2004). Trigeminal trophic syndrome—Report of four cases and review of the literature. *Dermatologic Surgery*, *30*(5), 807–812. https://doi.org/10.1111/j.1524-4725.2004.30220.x

Saint-Mezard, P., Krasteva, M., Chavagnac, C., Bosset, S., Akiba, H., Kehren, J., Kanitakis, J., Kaiserlian, D., Nicolas, J. F., & Berard, F. (2003). Afferent and efferent phases of allergic

contact dermatitis (ACD) can be induced after a single skin contact with haptens: Evidence using a mouse model of primary ACD. *Journal of Investigative Dermatology*, *120*(4), 641–647. https://doi.org/10.1046/j.1523-1747.2003.12093.x

Salvatierra, J., Dong, X., & Bosmans, F. (2018). A disease mutation reveals a role for Na V 1.9 in acute itch The Journal of Clinical Investigation. *J Clin Invest*, *128*(12), 5434–5447.

Salvoro, C., Bortoluzzi, S., Coppe, A., Valle, G., Feltrin, E., Mostacciuolo, M. L., & Vazza, G. (2018). Rare Risk Variants Identification by Identity-by-Descent Mapping and Whole-Exome Sequencing Implicates Neuronal Development Pathways in Schizophrenia and Bipolar Disorder. *Molecular Neurobiology*, *55*(9), 7366–7376. https://doi.org/10.1007/s12035-018-0922-2

Samineni, V. K., Grajales-Reyes, J. G., Grajales-Reyes, G. E., Tycksen, E., Copits, B. A., Pedersen, C., Ankudey, E. S., Sackey, J. N., Sewell, S. B., Bruchas, M. R., & Gereau, R. W. (2021). Cellular, circuit and transcriptional framework for modulation of itch in the central Amygdala. *eLife*, *10*. https://doi.org/10.7554/eLife.68130

Sand, J. M. B., & Karsdal, M. A. (2016). Type XVI Collagen. In *Biochemistry of Collagens, Laminins and Elastin* (pp. 101–106). Elsevier. https://doi.org/10.1016/B978-0-12-809847-9.00016-7

Sanders, K. M., & Akiyama, T. (2018). The vicious cycle of itch and anxiety. *Neuroscience and Biobehavioral Reviews*, *87*, 17–26. https://doi.org/10.1016/j.neubiorev.2018.01.009

Sanders, K. M., Fast, K., & Yosipovitch, G. (2019). Why we scratch: Function and dysfunction. *Experimental Dermatology*, *28*(12), 1482–1484. https://doi.org/10.1111/exd.13977

Sanders, K. M., Sakai, K., Henry, T. D., Hashimoto, T., & Akiyama, T. (2019). A subpopulation of amygdala neurons mediates the affective component of itch. *Journal of Neuroscience*, *39*(17), 3345–3356. https://doi.org/10.1523/JNEUROSCI.2759-18.2019

Satterthwaite, T. D., Kable, J. W., Vandekar, L., Katchmar, N., Bassett, D. S., Baldassano, C. F., Ruparel, K., Elliott, M. A., Sheline, Y. I., Gur, R. C., Gur, R. E., Davatzikos, C., Leibenluft, E., Thase, M. E., & Wolf, D. H. (2015). Common and Dissociable Dysfunction of the Reward System in Bipolar and Unipolar Depression. *Neuropsychopharmacology*, *40*(9), 2258–2268. https://doi.org/10.1038/npp.2015.75

Schmelz, M. (2021). How Do Neurons Signal Itch? *Frontiers in Medicine*, *8*. https://doi.org/10.3389/fmed.2021.643006

Schmelz, M., Schmidt, R., Bickel, A., Handwerker, H. O., & Torebjörk, H. E. (1997). Specific C-Receptors for Itch in Human Skin. *The Journal of Neuroscience*, *17*(20), 8003–8008. https://doi.org/10.1523/JNEUROSCI.17-20-08003.1997

Schmidt, D., Díaz, P., Muñoz, D., Espinoza, F., Nystrom, A., Fuentes, I., Ezquer, M., Bennett, D. L., & Calvo, M. (2022). Characterisation of the pathophysiology of neuropathy and sensory dysfunction in a mouse model of recessive dystrophic epidermolysis bullosa. *Pain*, *163*(10), 2052–2060. https://doi.org/10.1097/j.pain.0000000000002599

Schut, C., Weik, U., Tews, N., Gieler, U., Deinzer, R., & Kupfer, J. (2013). Psychophysiological effects of stress management in patients with atopic dermatitis: A randomized controlled trial. *Acta Dermato-Venereologica*, *93*(1), 57–61. https://doi.org/10.2340/00015555-1415

Seike, M., Ikeda, M., Kodama, H., Terui, T., & Ohtsu, H. (2005). Inhibition of scratching behaviour caused by contact dermatitis in histidine decarboxylase gene knockout mice. *Experimental Dermatology*, *14*(3), 169–175. https://doi.org/10.1111/j.0906-6705.2005.00247.x

Seppinen, L., & Pihlajaniemi, T. (2011). The multiple functions of collagen XVIII in development and disease. *Matrix Biology*, *30*(2), 83–92. https://doi.org/10.1016/j.matbio.2010.11.001

Shao, X., Taha, I. N., Clauser, K. R., Gao, Y. (Tom), & Naba, A. (2020). MatrisomeDB: The ECM-protein knowledge database. *Nucleic Acids Research*, *48*(D1), D1136–D1144. https://doi.org/10.1093/nar/gkz849

Shimada, S. G., & LaMotte, R. H. (2008). Behavioral differentiation between itch and pain in mouse. *Pain*, *139*(3), 681–687. https://doi.org/10.1016/j.pain.2008.08.002

Shoulders, M. D., & Raines, R. T. (2009). Collagen structure and stability. *Annual Review of Biochemistry*, *78*, 929–958. https://doi.org/10.1146/annurev.biochem.77.032207.120833

Simone, D. A., Alreja, M., & Lamotte, R. H. (1991). Psychophysical Studies of the Itch Sensation and Itchy Skin ("Alloknesis") Produced by Intracutaneous Injection of Histamine. *Somatosensory & Motor Research*, *8*(3), 271–279. https://doi.org/10.3109/08990229109144750

Smagin, D. A., Galyamina, A. G., Kovalenko, I. L., Babenko, V. N., & Kudryavtseva, N. N. (2019). Aberrant expression of collagen gene family in the brain regions of male mice with behavioral psychopathologies induced by chronic agonistic interactions. *BioMed Research International*, *2019*. https://doi.org/10.1155/2019/7276389

Söderhäll, C., Marenholz, I., Kerscher, T., Rüschendorf, F., Esparza-Gordillo, J., Worm, M., Gruber, C., Mayr, G., Albrecht, M., Rohde, K., Schulz, H., Wahn, U., Hubner, N., & Lee, Y. A. (2007). Variants in a novel epidermal collagen gene (COL29A1) are associated with atopic dermatitis. *PLoS Biology*, *5*(9), 1952–1961. https://doi.org/10.1371/journal.pbio.0050242

Song, J., Xian, D., Yang, L., Xiong, X., Lai, R., & Zhong, J. (2018). Pruritus: Progress toward Pathogenesis and Treatment. *BioMed Research International*, *2018*. https://doi.org/10.1155/2018/9625936

Soret, R., Mennetrey, M., Bergeron, K. F., Dariel, A., Neunlist, M., Grunder, F., Faure, C., Silversides, D. W., & Pilon, N. (2015). A collagen VI-dependent pathogenic mechanism for Hirschsprung's disease. *Journal of Clinical Investigation*, *125*(12), 4483–4496. https://doi.org/10.1172/JCI83178

Sorushanova, A., Delgado, L. M., Wu, Z., Shologu, N., Kshirsagar, A., Raghunath, R., Mullen, A. M., Bayon, Y., Pandit, A., Raghunath, M., & Zeugolis, D. I. (2019). The Collagen Suprafamily: From Biosynthesis to Advanced Biomaterial Development. *Advanced Materials*, *31*(1), 1–39. https://doi.org/10.1002/adma.201801651

Spradley, J. M., Auva, D., Carstens, M. I., & Carstens, E. (2012). Opioid modulation of facial itch- and pain-related responses and grooming behavior in rats. *Acta Dermato-Venereologica*, *92*(5), 515–520. https://doi.org/10.2340/00015555-1364

Ständer, S., & Grundmann, S. A. (2012). Chronic pruritus. *Giornale Italiano Di Dermatologia e Venereologia*, *147*(2), 161–169. https://doi.org/10.1056/nejmcp1208814

Ständer, S., Schürmeyer-Horst, F., Luger, T. A., & Weisshaar, E. (2006). Treatment of pruritic diseases with topical calcineurin inhibitors. *Therapeutics and Clinical Risk Management*, *2*(2), 213–218. https://doi.org/10.2147/tcrm.2006.2.2.213

Ständer, S., Weisshaar, E., Mettang, T., Szepietowski, J. C., Carstens, E., Ikoma, A., Bergasa, N. V., Gieler, U., Misery, L., Wallengren, J., Darsow, U., Streit, M., Metze, D., Luger, T. A., Greaves, M. W., Schmelz, M., Yosipovitch, G., & Bernhard, J. D. (2007). Clinical classification of itch: A position paper of the international forum for the study of itch.

Acta Dermato-Venereologica, *87*(4), 291–294. https://doi.org/10.2340/00015555-0305

Steinhoff, M., Schmelz, M., Szabó, I. L., & Oaklander, A. L. (2018). Clinical presentation, management, and pathophysiology of neuropathic itch. *The Lancet Neurology*, *17*(8), 709–720. https://doi.org/10.1016/S1474-4422(18)30217-5

Stumpf, A., & Ständer, S. (2013). *Neuropathic itch: Diagnosis and management*.

Sugimoto, Y., Umakoshi, K., Nojiri, N., & Kamei, C. (1998). Effects of histamine H1 receptor antagonists on compound 48/80-induced scratching behavior in mice. *European Journal of Pharmacology*, *351*(1), 1–5. https://doi.org/10.1016/S0014-2999(98)00288-X

Summers, C. G., & Macdonald, J. T. (1988). Paroxysmal Facial Itch: A Presenting Sign of Childhood Brainstem Glioma. *Journal of Child Neurology*, *3*(3), 189–192. https://doi.org/10.1177/088307388800300308

Sun, L. F., Yang, Y. L., Wang, M. Y., Zhao, H. S., Xiao, T. X., Li, M. X., Wang, B. B., Huang, C., Ren, P. G., & Zhang, J. V. (2021). Inhibition of Col6a5 Improve Lipid Metabolism Disorder in Dihydrotestosterone-Induced Hyperandrogenic Mice. *Frontiers in Cell and Developmental Biology*, *9*. https://doi.org/10.3389/fcell.2021.669189

Sun, Y. G., & Chen, Z. F. (2007). A gastrin-releasing peptide receptor mediates the itch sensation in the spinal cord. *Nature*, *448*(7154), 700–703. https://doi.org/10.1038/nature06029

Sundberg, J. P., Herbert Pratt, C., Goodwin, L. P., Silva, K. A., Kennedy, V. E., Potter, C. S., Dunham, A., Sundberg, B. A., & HogenEsch, H. (2020). Keratinocyte-specific deletion of SHARPIN induces atopic dermatitis-like inflammation in mice. *PLoS ONE*, *15*(7 July), 1–28. https://doi.org/10.1371/journal.pone.0235295

Sutaria, N., Adawi, W., Goldberg, R., Roh, Y. S., Choi, J., & Kwatra, S. G. (2022). Itch: Pathogenesis and treatment. *Journal of the American Academy of Dermatology*, *86*(1), 17–34. https://doi.org/10.1016/j.jaad.2021.07.078

Szalus, K., Zysk, W., Gleń, J., Zabłotna, M., Nowicki, R. J., & Trzeciak, M. (2023). The Associations of Single Nucleotide Polymorphisms of the COL3A1, COL6A5, and COL8A1 Genes with Atopic Dermatitis. *Journal of Personalized Medicine*, *13*(4). https://doi.org/10.3390/jpm13040661

Takanami, K., Uta, D., Ichi Matsuda, K., Kawata, M., Carstens, E., Sakamoto, T., & Sakamoto, H. (2023). *Estrogens influence female itch sensitivity via the spinal gastrin-releasing peptide receptor neurons*. *118*. https://doi.org/10.1073/pnas.2103536118/-/DCSupplemental

Tang, Y., Chang, H., Chiang, C., Lai, C., Hsu, M., Han, H., Chen, L., & Lin, D. P. (2019). *Anatomy and Pathology / Oncology by Continuous Exposure to Particulate Matter 2. 5*. 4–12.

Tasaki, N., Minematsu, T., Mugita, Y., Ikeda, S. I., Nakagami, G., & Sanada, H. (2016). Telogen elongation in the hair cycle of ob/ob mice. *Bioscience, Biotechnology and Biochemistry*, *80*(1), 74–79. https://doi.org/10.1080/09168451.2015.1069693

Theocharidis, G., & Connelly, J. T. (2019). Minor collagens of the skin with not so minor functions. *Journal of Anatomy*, *235*(2), 418–429. https://doi.org/10.1111/joa.12584

Tsianakas, A., Zeidler, C., Riepe, C., Borowski, M., Forner, C., Gerss, J., Metz, M., Staubach, P., Raap, U., Kaatz, M., Urban, M., Luger, T. A., & Ständer, S. (2019). Aprepitant in anti-histamine-refractory chronic nodular prurigo: A multicentre, randomized, double-blind, placebo-controlled, cross-over, phase-II trial (APREPRU). *Acta Dermato-Venereologica*, *99*(4), 379–385. https://doi.org/10.2340/00015555-3120

Tsukuba, T., Okamoto, K., Okamoto, Y., Yanagawa, M., Kohmura, K., Yasuda, Y., Uchi, H., Nakahara, T., Furue, M., Nakayama, K., Kadowaki, T., Yamamoto, K., & Nakayama, K. I. (2003). Association of Cathepsin E Deficiency with Development of Atopic Dermatitis. *Journal of Biochemistry*, *134*(6), 893–902. https://doi.org/10.1093/jb/mvg216

Tsukumo, Y., Matsumoto, Y., Miura, H., Yano, H., & Manabe, H. (2011). Gabapentin and pregabalin inhibit the itch-associated response induced by the repeated application of oxazolone in mice. *Journal of Pharmacological Sciences*, *115*(1), 27–35. https://doi.org/10.1254/jphs.10173fp

Twycross, R. (2003). Itch: Scratching more than the surface. *QJM*, *96*(1), 7–26. https://doi.org/10.1093/qjmed/hcg002

Ueda, Y., Inoue, T., Rahman, M. A., Yatsuzuka, R., Jiang, S., & Kamei, C. (2006). A new chronic itch model accompanied by skin lesions in hairless mice. *International Immunopharmacology*, *6*(10), 1609–1615. https://doi.org/10.1016/j.intimp.2006.06.002

Urbizu, A., Garrett, M. E., Soldano, K., Drechsel, O., Loth, D., Marcé-Grau, A., Soler, O. M. i., Poca, M. A., Ossowski, S., Macaya, A., Loth, F., Labuda, R., & Ashley-Koch, A. (2021). Rare functional genetic variants in COL7A1, COL6A5, COL1A2 and COL5A2 frequently occur in Chiari Malformation Type 1. *PLoS ONE*, *16*(5 May), 1–20. https://doi.org/10.1371/journal.pone.0251289

Vaclavik, V., Tiab, L., Sun, Y. J., Mahajan, V. B., Moulin, A., Allaman-Pillet, N., Munier, F. L., & Schorderet, D. F. (2022). New COL6A6 Variant Causes Autosomal Dominant Retinitis Pigmentosa in a Four-Generation Family. *Investigative Ophthalmology and Visual Science*, *63*(3). https://doi.org/10.1167/iovs.63.3.23

Varholick, J. A., Pontiggia, A., Murphy, E., Daniele, V., Palme, R., Voelkl, B., Würbel, H., & Bailoo, J. D. (2019). Social dominance hierarchy type and rank contribute to phenotypic variation within cages of laboratory mice. *Scientific Reports*, *9*(1). https://doi.org/10.1038/s41598-019-49612-0

Vitale, P., Braghetta, P., Volpin, D., Bonaldo, P., & Bressan, G. M. (2001). Mechanisms of transcriptional activation of the col6a1 gene during Schwann cell differentiation. *Mechanisms of Development*, *102*(1–2), 145–156. https://doi.org/10.1016/S0925-4773(01)00303-3

Wallengren, J. (2010). Ultraviolet phototherapy of pruritus. *Pruritus*, *18*(5), 325–334. https://doi.org/10.1007/978-1-84882-322-8_51

Walma, D. A. C., & Yamada, K. M. (2020). The extracellular matrix in development. *Development (Cambridge, England)*, *147*(10). https://doi.org/10.1242/dev.175596

Walsh, K., McCormack, C. A., Webster, R., Pinto, A., Lee, S., Feng, T., Sloan Krakovsky, H., O'Grady, S. M., Tycko, B., Champagne, F. A., Werner, E. A., Liu, G., & Monk, C. (2019). Maternal prenatal stress phenotypes associate with fetal neurodevelopment and birth outcomes. *Proceedings of the National Academy of Sciences of the United States of America*, *116*(48), 23996–24005. https://doi.org/10.1073/pnas.1905890116

Wang, J., & Pan, W. (2020). The biological role of the collagen alpha-3 (Vi) chain and its cleaved c5 domain fragment endotrophin in cancer. *OncoTargets and Therapy*, *13*, 5779–5793. https://doi.org/10.2147/OTT.S256654

Wang, X. D., Yang, G., Bai, Y., Feng, Y. P., & Li, H. (2018). The behavioral study on the interactive aggravation between pruritus and depression. *Brain and Behavior*, *8*(6), 1–9. https://doi.org/10.1002/brb3.964

Weisshaar, E., Eckart, W. U., & Bernhard, J. D. (2015). Historical background of itch. *Handbook of Experimental Pharmacology*, *226*, 1–14. https://doi.org/10.1007/978-3-662-44605-8_1

Weisshaar, E., GrÃ¼ll, V., KÃ¶nig, A., Schweinfurth, D., Diepgen, T. L., & Eckart, W. U. (2009). The symptom of itch in medical history: Highlights through the centuries. *International Journal of Dermatology*, *48*(12), 1385–1394. https://doi.org/10.1111/j.1365-4632.2009.04117.x

Weisshaar, E., Szepietowski, J. C., Dalgard, F. J., Garcovich, S., Gieler, U., Giménez-Arnau, A. M., Lambert, J., Leslie, T., Mettang, T., Misery, L., Şavk, E., Streit, M., Tschachler, E., Wallengren, J., & Ständer, S. (2019). European S2k Guideline on Chronic Pruritus. *Acta Dermato-Venereologica*, *99*(5), 469–506. https://doi.org/10.2340/00015555-3164

Weisshaar, E., Szepietowski, J. C., Darsow, U., Misery, L., Wallengren, J., Mettang, T., Gieler, U., Lotti, T., Lambert, J., Maisel, P., Streit, M., Greaves, M. W., Carmi-Chael, A., Tschachler, E., Ring, J., & Ständer, S. (2012). European guideline on chronic pruritus: In cooperation with the European dermatology forum (EDF) and the European academy of dermatology and venereology (EADV). *Acta Dermato-Venereologica*, *92*(5), 563–581. https://doi.org/10.2340/00015555-1400

Whitehouse, J., Micheletta, J., & Waller, B. M. (2017). Stress behaviours buffer macaques from aggression. *Scientific Reports*, *7*(1), 1–9. https://doi.org/10.1038/s41598-017-10754-8

Williams, L., Layton, T., Yang, N., Feldmann, M., & Nanchahal, J. (2021). Collagen VI as a driver and disease biomarker in human fibrosis. *FEBS Journal*. https://doi.org/10.1111/febs.16039

Williamson, C. M., Lee, W., DeCasien, A. R., Lanham, A., Romeo, R. D., & Curley, J. P. (2019). Social hierarchy position in female mice is associated with plasma corticosterone levels and hypothalamic gene expression. *Scientific Reports*, *9*(1). https://doi.org/10.1038/s41598-019-43747-w

Wimalasena, N. K., Milner, G., Silva, R., Vuong, C., Zhang, Z., Bautista, D. M., & Woolf, C. J. (2021). Dissecting the precise nature of itch-evoked scratching. *Neuron*, *109*(19), 3075-3087.e2. https://doi.org/10.1016/j.neuron.2021.07.020

Yang, G., Seok, J. K., Kang, H. C., Cho, Y. Y., Lee, H. S., & Lee, J. Y. (2020). Skin barrier abnormalities and immune dysfunction in atopic dermatitis. *International Journal of Molecular Sciences*, *21*(8). https://doi.org/10.3390/ijms21082867

Yasukochi, Y., Sakuma, J., Takeuchi, I., Kato, K., Oguri, M., Fujimaki, T., Horibe, H., & Yamada, Y. (2017). Longitudinal exome-wide association study to identify genetic susceptibility loci for hypertension in a Japanese population. *Experimental and Molecular Medicine*, *49*(12), 1–10. https://doi.org/10.1038/emm.2017.209

Yeom, M., Ahn, S., Jang, S. Y., Jang, J. H., Lee, Y., Hahm, D. H., & Park, H. J. (2022). Acupuncture attenuates comorbid anxiety- and depressive-like behaviors of atopic dermatitis through modulating neuroadaptation in the brain reward circuit in mice. *Biological Research*, *55*(1). https://doi.org/10.1186/s40659-022-00396-0

Yeom, M., Ahn, S., Oh, J. Y., Kim, S. Y., Lee, H., Hahm, D. H., & Park, H. J. (2020). Atopic dermatitis induces anxiety- and depressive-like behaviors with concomitant neuronal adaptations in brain reward circuits in mice. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *98*. https://doi.org/10.1016/j.pnpbp.2019.109818

Yosipovitch, G., Greaves, M. W., & Schmelz, M. (2003). Itch. *The Lancet*, *361*(9358), 690–694. https://doi.org/10.1016/S0140-6736(03)12570-6

Yosipovitch, G., Rosen, J. D., & Hashimoto, T. (2018). Itch: From mechanism to (novel) therapeutic approaches. *Journal of Allergy and Clinical Immunology*, *142*(5), 1375–1390. https://doi.org/10.1016/j.jaci.2018.09.005

Yosipovitch, G., Sugeng, M. W., Chan, Y. H., Goon, A., Ngim, S., & Goh, C. L. (2001). The effect of topically applied aspirin on localized circumscribed neurodermatitis. *Journal of the American Academy of Dermatology*, *45*(6), 910–913. https://doi.org/10.1067/mjd.2001.117399

Yu, Y. Q., Barry, D. M., Hao, Y., Liu, X. T., & Chen, Z. F. (2017). Molecular and neural basis of contagious itch behavior in mice. *Science*. https://doi.org/10.1126/science.aak9748

Zech, M., Lam, D. D., Francescatto, L., Schormair, B., Salminen, A. V., Jochim, A., Wieland, T., Lichtner, P., Peters, A., Gieger, C., Lochmüller, H., Strom, T. M., Haslinger, B., Katsanis, N., & Winkelmann, J. (2015). Recessive Mutations in the α3 (VI) Collagen Gene COL6A3 Cause Early-Onset Isolated Dystonia. *American Journal of Human Genetics*, *96*(6), 883–893. https://doi.org/10.1016/j.ajhg.2015.04.010

Zhan, H., Huang, F., Yan, F., Zhao, Z., Zhang, J., Cui, T., Yang, F., Hai, G., Jia, X., & Shi, Y. (2017). Alterations in splenic function and gene expression in mice with depressive-like behavior induced by exposure to corticosterone. *International Journal of Molecular Medicine*, *39*(2), 327–336. https://doi.org/10.3892/ijmm.2017.2850

Zhang, Y., Hwang, B. J., Liu, Z., Li, N., Lough, K., Williams, S. E., Chen, J., Burette, S. W., Diaz, L. A., Su, M. A., Xiao, S., & Liu, Z. (2018). BP180 dysfunction triggers spontaneous skin inflammation in mice. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(25), 6434–6439. https://doi.org/10.1073/pnas.1721805115

Zhao, X., Yu, C., Ye, F., Wang, Y. G., Mei, Q. Y., Ma, Q., Cui, W. G., & Zhou, W. H. (2018). Chronic itch impairs mood and HPA axis function in mice: Modulation by CRFR1 antagonist. *Pain*, *159*(11), 2201–2213. https://doi.org/10.1097/j.pain.0000000000001319

Zhao, Z. Q., Huo, F. Q., Jeffry, J., Hampton, L., Demehri, S., Kim, S., Liu, X. Y., Barry, D. M., Wan, L., Liu, Z. C., Li, H., Turkoz, A., Ma, K., Cornelius, L. A., Kopan, R., Battey, J. F., Zhong, J., & Chen, Z. F. (2013). Chronic itch development in sensory neurons requires BRAF signaling pathways. *Journal of Clinical Investigation*, *123*(11), 4769–4780. https://doi.org/10.1172/JCI70528

Zuberbier, T., Aberer, W., Asero, R., Bindslev-Jensen, C., Brzoza, Z., Canonica, G. W., Church, M. K., Ensina, L. F., Giménez-Arnau, A., Godse, K., Gonçalo, M., Grattan, C., Hebert, J., Hide, M., Kaplan, A., Kapp, A., Abdul Latiff, A. H., Mathelier-Fusade, P., Metz, M., … Maurer, M. (2014). The EAACI/GA2LEN/EDF/WAO Guideline for the definition, classification, diagnosis, and management of urticaria: The 2013 revision and update. *Allergy: European Journal of Allergy and Clinical Immunology*, *69*(7), 868–887. https://doi.org/10.1111/all.12313

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Scientific Production

Publication

1-Ameer Rasheed, Marie-Christine Birling, Giuseppe Lauria Claire, Gaveriaux-Ruff and Yann Herault. The *COL6A5*-p.Glu2272* mutation induces chronic itch in mice (manuscript in preparation).

2- Chidiac C, Xue Y, Muniz Moreno MDM, Bakr Rasheed AA, Lorentz R, Birling MC, Gaveriaux-Ruff C, Herault Y. The Human SCN10A^{G1662S} Point Mutation Established in Mice Impacts on Mechanical, Heat, and Cool Sensitivity. Front Pharmacol. 2021 Dec 1;12:780132. doi: 10.3389/fphar.2021.780132. PMID: 34925037; PMCID: PMC8671994. (Manuscript ID: 780132).

Neuropathic pain caused by a lesion or disease of the somatosensory nervous system and has major impact on quality of life. It affects the general population and particularly prevalent among patients with neurological diseases. It is characterized by abnormal sensitivity of pain with an increase response to pain (hyperalgesia), pain in response of non-painful stimuli that are not typically painful (allodynia) and functional changes in primary sensory neurons and within the central nervous system. Primary neurons play critical role in pain transmission and perception. These primary nociceptive neurons with unique voltage gated sodium channel NAV 1.8 are critical for initiation and transmission of pain signals to central nervous system. Due to injury or damage can result alteration in sodium channels subunits within sensory neurons, which may lead to imbalance between excitatory and inhibitory signaling. These changes can influence the threshold for pain experiencing, leading to hyper or hyposensitivity to pain. Mutation in SCN10A gene which encodes NAV1.8 channel have been identified in

patients with idiopathic painful small fiber neuropathy including the SCN10A^{G1662S} gain-of-function mutation, although exact impact on pain perception remains unclear.

For better understanding the SCN10A^{G1662S} mutation role in pain perception, first mouse model (named Scn10a^{G1663S} mutant mice) was developed that carries the corresponding mutation to SCN10A^{G1662S} mutation found in SFN patients. The study extensively analyzed SCN10 expression level, intraepidermal nerve fiber density, and nociception through various behavioral tests for thermal and mechanical sensitivity.

Scn10a^{G1663S} mutants expression level was found similar in dorsal root ganglia as compared to their wild type littermates. The study investigated intraepidermal nerve fiber density in the hindpaw skin of Scn10a^{G1663S} mutants mice and found no significant as compared wild type littermates. Mutant mice exhibited an increase in sensitivity to touch through Von Frey behavior test. Notably, findings highlighted the relevance of performing phenotypical assessment in both male and female mice by revealing gender specific differences. For instance, it was found that female homozygous mutants tended to increase sensitivity in response to cold stimuli and had longer latencies in the tail flick test. While male homozygous displayed shorter latencies to radiant heat and shorter reaction latency on the 54°C hot plate. Altogether, Scn10a^{G1663S} mutant mice exhibited a consistent and moderate increase in sensitivity to behavioral tests assessing the nociception.

The Scn10a^{G1663S} mouse model serves as a valuable platform and tool for exploring the impact of SCN10A mutations in pain sensitivity. This mouse model also demonstrated that the corresponding G1662 mutation of SCN10A found in SFN patients with pain contributes to their pain symptoms.

I also contributed for this publication mainly with performing mouse perfusion experiments and also sampling for immunohistochemistry experiments.

Key aspects of publication	Description
Objective of study	To investigate SCN10A^{G1662S} mutation's role in pain sensitivity
Mouse model	Developed Scn10a^{G1663S} mouse model
Key findings	1) Scn10aG1663S mutants expression level was normal in dorsal root ganglia
	2) Intraepidermal nerve fiber density in the hindpaw skin of Scn10a^{G1663S} mutants mice and found no significant
	3) Mutants displayed increased sensitivity to mechanical stimuli
	4) Male mutant showed increased sensitivity to radiant heat
	5) Mutant females tended to be more sensitive to cold stimuli
	6) Sexual dimorphism was observed for several pain tests
Implication	SCN10A^{G1662S} mutation contributes to pain symptoms in small fiber neuropathy patients
Future prospective	Potential for further research on pain and drug-induced analgesia

Oral and poster presentations

Date	Place	Meeting	Authors	Title
05/04/2019	IGBMC Strasbourg	Second PAIN-Net annual Meeting	Ameer AbuBakr Rasheed	Development of a new mouse model for familial neuropathic chronic itching and investigation for therapeutic approach
13/09/2019	Strasbourg Doctoral School	"Back to school" ITI Neurostra	Ameer AbuBakr Rasheed, Marie-Christine Birling, Hamid Meziane, Claire Gaveriaux-Ruff, Yann Herault	Development of a new mouse model for neuropathic chronic itch due to mutation in *COL6A5*
27/09/2019	IGBMC Strasbourg	33rd International Mammalian Genome Conference	Ameer AbuBakr Rasheed, Marie-Chrisitne Birling, Hamid Meziane, Claire Gaveriaux-Ruff, Yann Herault	Development of a new mouse model for neuropathic chronic itch due to mutation in *COL6A5*
17/07/2020	IGBMC Strasbourg	Mid-thesis	Ameer AbuBakr Rasheed	Development of a new mouse model for neuropathic chronic itch due to mutation in *COL6A5*
28/10/2020	Visio	Final PAIN-Net annual Meeting	Ameer AbuBakr Rasheed	Development of a new mouse model for familial neuropathic chronic itching and investigation for therapeutic approach

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Nouvelles perspectives sur les démangeaisons chroniques : Une nouvelle enquête sur un modèle murin des conséquences de la variante génétiqueCOL6A5 p.Glu2272*.

Résumé

Les démangeaisons chroniques peuvent être causées par des dysfonctionnements des neurones sensibles aux démangeaisons qui produisent une sensation sensorielle de stimuli prurigineux. Récemment, une variante rare du gène Collagen VI α5 (COL6A5), pGlu2272*, a été identifiée dans deux familles comme étant associée à des démangeaisons neuropathiques chroniques. Pour comprendre les conséquences de la mutation *COL6A5*-Glu2272*, nous avons développé un nouveau modèle murin de démangeaison neuropathique chronique en utilisant la technologie CRISPR-Cas9. La mutation *COL6A5*-Glu2272* a diminué le transcript *Col6a5* dans la peau des souris mutantes. Nos résultats ont montré une augmentation de la perméabilité cutanée des souris mutantes femelles, augmentation du grattage spontané chez les souris mutantes, augmentation du comportement de type anxiété chez les souris mutantes femelles. Ce modèle murin améliorera notre compréhension des conséquences de la mutation *COL6A5*-Glu2272* et aidera à identifier des cibles cellulaires pour le développement traductionnel de nouveaux traitements contre les démangeaisons neuropathiques chroniqués.

Mots clés : COL6A5, démangeaison neuropathique chronique, prurit, technologie CRISPR-Cas, barrière cutanée, grattage, démangeaison spontanée.

Résumé en anglais

Chronic neuropathic itch can be caused by dysfunctions of itch-sensing neurons that produce sensory sensation of pruritogenic stimuli. Neuropathic itch is difficult to diagnose and often underdiagnosed. Recently, a rare Collagen VI α5 (*COL6A5*) gene variant*, COL6A5*-p.Glu2272*, was identified in two families as associated to chronic neuropathic itch. To understand the consequences of *COL6A5*-Glu2272* mutation, this study developed a new mouse model of chronic neuropathic itch by using the CRISPR-Cas9 technology. The mouse line showed no alteration of growth, survival, and global health state. The *COL6A5*-Glu2272* mutation decreased *Col6a5* transcript in skin of the mutant mice. Results also showed increased skin permeability of female mutant mice, increased spontaneous scratching in mutant mice and increased anxiety-like behavior in female mutant mice. This mouse model will improve our understanding about the consequences of *COL6A5*-Glu2272* mutation and will help identify cellular targets for translational development of new treatments for chronic neuropathic itch.

Keywords : COL6A5, chronic neuropathic itch, pruritus, CRISPR-Cas technology, skin barrier, scratching, spontaneous itch.