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Skin-derived mechanisms of uremic pruritus

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SKIN-DERIVED MECHANISMS OF UREMIC PRURITUS

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DEDICATION

I would like to dedicate this work to my parents and grandparents,

with love and gratitude.

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ABSTRACT

Uremic pruritus (UP) arises in end-stage renal disease (ESRD) and is not relieved by proper dialysis. While the pathogenesis of UP is not well understood, UP responds poorly to anti-histamines. We performed a case-control study to test if cutaneous protease-mediated, non-histamine itch is augmented in UP, and if UP is associated with altered epidermal and/or papillary dermal innervation. We recruited 12 hemodialysis subjects with ESRD-specific itch (cases) (Visual Analogue Scale (VAS)-average itch in the preceding week, 78/100), and 13 age- and sex-matched hemodialysis subjects without pruritus (controls) (VAS- average itch in the preceding week, 0/100; $p<0.0001$ cases vs. controls). Cowhage spicule-induced itch was induced in the back where all subjects exhibited itch, and the entire duration of itch was measured with the general Labeled Magnitude Scale. Subsequently, a punch biopsy was taken from this sensory-tested skin and multi-label immunohistochemistry was performed to measure epidermal and papillary dermal innervation. In cases vs. controls, cowhage-induced area under the curve (AUC) for itch was significantly larger (median, 25%–75%: 175.4, 101.0–252.2 vs. 42.4, 24.0–160; $p=0.04$) as was perceived peak itch intensity (53.6, 53.3–78.9 vs. 34.2, 20.9–55.6; $p=0.02$). Cases showed a significant reduction in papillary dermal nerve length

(PDNL)/mm epidermis (2295, 1659–2970 vs. 2909, 2228–3523; p=0.003), resulting from the loss of papillary dermal (PD)-calcitonin gene related peptide (CGRP) (+) nerves (p<0.0001), with preservation of %PD-substance P (+) nerves (p=0.1) and intraepidermal nerve fiber density (p=0.1). VAS-average itch in the preceding week negatively correlated with PDNL/mm epidermis (correlation coefficient (CC)=-0.53, p=0.003) and %PD-CGRP (+) nerves (CC=-0.37, p=0.03). Cowhage-induced AUC-itch negatively correlated with %PD-CGRP nerves only in cases (CC=-0.40, p=0.02). Our data suggest augmented protease-dependent signaling contributes to UP and indicate a mechanism for how PD-CGRP (+) nerve loss contributes to UP and augmented cowhage-itch: loss of an afferent skin-derived itch-inhibition signal to the spinal cord dorsal horn.

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LIST OF ABBREVIATIONS

ACh...Acetylcholine

ANP ....Atrial Natriuretic Peptide

AUC..Area Under the Curve

BAM8-22 .... Bovine Adrenal Medulla 8-22

BMC ....Boston Medical Center

BNP ....Brain Natriuretic Peptide

CC..Correlation Coefficient

CDD... Cardiodilatin

CGRP.. Calcitonin Gene Related Peptide

CMH ....Mechano- and heat-sensitive C-fibers

CMHC..Mechano-, heat- and cold-sensitive C-fibers

CMi..Mechano-insensitive C-fibers

CMiHi ....Mechano- and heat-insensitive C-fibers

CMiHis+ .... Mechano-insensitive, histamine-responsive C-fibers

C-polymodal... Polymodal C-fibers

DRG..Dorsal Root Ganglion

DOPPS .... Dialysis Outcomes and Practice Patterns Study

ESRD ....End-Stage Renal Disease

gLMS .... general version of the Labeled Magnitude Scale

GRP .... Gastrin-Releasing Peptide

HD .... Hemodialysis

CHAPTER 1. INTRODUCTION

This study investigates the cutaneous pathophysiology of uremic pruritus. The motivation for this study was the question—what causes and sustains chronic itch? This question originates from the clinical observance that chronic itch is as detrimental to patients as is chronic cancer pain. Several theories have been proposed to explain chronic itch based on the signaling pathways of specific peptides postulated in animal models to mediate itch sensation. Because chronic itch states are heterogeneous, no one signaling pathway will account for all types of clinical chronic itch. Uremic pruritus was selected as the study objective because of its clinical relevance and relative lack of efficacious clinical treatment options. Uremic pruritus arises in the setting of kidney disease. Numerous confounding metabolic abnormalities can contribute to itch in this patient population. Nevertheless, in a significant subset of subjects with kidney failure, pruritus will persist despite their adequate correction, including proper dialysis and resolution of both hyperparathyroidism and ion-derangements. We defined this persistent itch as true uremic pruritus as it serves as a model for chronic itch in the absence of cutaneous inflammation.

In Chapter 2, I briefly review the extant literature, identify gaps in our current understanding of the causes of uremic pruritus, and articulate the hypotheses. In Chapter 3, I delineate specific aims of the study. In Chapter 4, I discuss the research design and methodological strategies. In Chapter 5, I present the findings in two parts: Part 1 focuses on the statistical analysis of data from clinical questionnaires, cutaneous sensory tests and

cutaneous nerve quantifications; Part 2 provides images of multi-label immunohistochemistry with different markers in the skin and heart. Chapter 6 theorizes the findings, discusses the contributions of this work to academia and clinical practice, reflects on the limitations of the data, and pinpoints directions for future studies. Chapter 7 concludes the dissertation by summarizing the main themes of the study.

CHAPTER 2. LITERATURE REVIEW

This chapter reviews the classifications of itch and the different putative itch mediators and signaling pathways proposed to be involved in uremic pruritus. This chapter also states our hypotheses, and provides underlying evidence and rationale, for the possible causes of uremic pruritus.

2.1 Pruritus: Definition and Classification

Defined by German physician Samuel Harfenreffer more than three centuries ago (Dhand and Aminoff, 2013), pruritus is an unpleasant sensation that provokes the desire or reflex to scratch. Itch has been classified into three mechanistic categories: pruriceptive itch, neurogenic itch, and neuropathic itch (Ikoma et al., 2006). Pruriceptive itch is induced from peripheral mechanisms within a healthy nervous system. Neurogenic itch is induced from central mechanisms within a healthy nervous system. Neuropathic itch is caused by diseased neurons (Ikoma et al., 2006).

Clinically, acute itch is defined as itch that lasts less than six weeks, while chronic itch lasts more than six weeks (Dhand and Aminoff, 2013). More clinically significant, chronic itch has been divided into itch related to skin disorders, e.g. atopic dermatitis, psoriasis, and chronic urticaria, etc.; itch related to systemic disorders, e.g. chronic renal failure and chronic liver disease; itch secondary to psychological causes, e.g. delusional parasitosis; and itch related to neuropathic mechanisms, e.g. brain tumors from central

nervous system or post-herpetic itch from the peripheral nervous system. Skin-disorder chronic itch is thought to be pruriceptive with abnormal activity or sensitivity of primary afferent skin neurons (i.e., neurons in the skin that send signals into the spinal cord). Systemic-disorder chronic itch may arise from a combination of pruriceptive and neurogenic mechanisms. Uremic pruritus, a chronic itch, responds to treatment with narrow band ultraviolet light B (nbUVB), supporting a pruriceptive mechanism (Ada et al., 2005). However, uremic pruritus also can be treated by μ-opioid antagonists and κ-opioid agonists, indicating a neurogenic mechanism as well (Odou et al., 2001; Wikstrom et al., 2005).

Itch can also be sub-divided based on the efficacy of anti-histamine medication therapy into histamine-dependent and histamine independent pruritus. Histamine-dependent itch will occur in the setting of the cutaneous "axon flare", which is a triple response consisting sequentially of vasodilation, flare and wheal. Anti-histamine treatment is effective for some causes of acute itch, such as urticaria, acute insect bite reactions, and mastocytosis. However, most chronic itch conditions, including uremic pruritus, are not responsive to systemic anti-histamines, but non-histamine itch mediators for these chronic itch conditions are not well characterized.

2.2 Neuroanatomy of Cutaneous Innervation

2.2.1 Classification of Primary Afferent Nerves in the Skin

Peripheral sensory nerves are composed of A- and C-fibers. Subsets of both A-δ fibers, a

sub-type of A-fibers, and C-fibers both convey the sensation of itch. C-fibers make up about 20% of primary afferent fibers. In contrast, A-δ fibers, the other set of itch-mediating nerve fibers, account for 80% of all post-ganglionic A-fibers (Lawson, 2002).

2.2.1.1 A fibers

A fibers are classified into α, β, and δ subtypes. Skin is innervated by β and δ fibers, whereas A-α fibers mediate sensory innervation of muscles (Lawson, 2002). A-α fibers are the thickest myelinated and with the highest conductivity.

A-β fibers are moderately thick myelinated nerve fibers that innervate touch receptors. They are low threshold mechano-receptors for detection of vibration and slight indentation. They are also the nerves making up nerve end organs such as Meissner's corpuscles, Merkel cell neurite complexes, Pacinian corpuscles, and Ruffini endings. A-β mechanoreceptors also play an important role in mediating pain from non-painful stimulation (pain central sensitization) upon sensitization from capsaicin. (Baron et al., 2000).

A-δ fibers are thinly myelinated with the slowest conducive velocity among A fibers. However, they can have as long as 5cm unmyelinated branches in the skin which make them difficult to differentiate from cutaneous C-fibers (Peng et al., 1999). Both A-δ fibers

and C-fibers mediate thermal and nociceptive sensations. A-δ fibers can be subdivided into classes of fibers for cold sensitivity (A-δ cold) and nociceptive feelings. As nociceptive receptors, A-δ fibers are further divided into those with a high threshold for mechanical stimulation (mechano-insensitive) and those that are mechano-heat sensitive (Aδ-MH) (Schmelz 2011). Aδ-MHs are responsible for heat and burning pain from heat sensation, which A-δ cold fibers can suppress (Campero et al., 2009).

2.2.1.2 C Fibers

C fibers are thin and unmyelinated with slow conductivity. They are classically involved in the warm sensation (C-warm fibers) and nociceptive sensations (Schmelz 2011). They are also implicated in emotional or limbic touch in the recent literature (Essick et al., 1999). C nociceptors are further divided into polymodal C-nociceptors and mechano-insensitive C-nociceptors (Schmelz 2011). Polymodal C nociceptors respond to a variety of different types of painful modalities (Ma, 2010) and can be classified as 1) C-cold fibers that respond to innocuous cold but also to hot burning pain; 2) C2 fibers capable of detecting both heat and cold; 3) CH responding to noxious heat; 4) CMHC fibers that are sensitive to noxious mechanical, heat, and cold; and 5) CMH that respond to both mechanical stimuli and heat. Interestingly, subsets of CMH fibers also mediate histamine-independent itch (Johanek et al., 2008). Mechano-insensitive C-nociceptors have a higher activation threshold for heat and do not respond to mechanical stimuli. This subset of C fibers is also termed heat- and mechano-insensitive C fibers (CMiHi).

Mechano-insensitive histamine-responsive C fibers (CMiHis+) comprise about 10% of CMiHi fibers and are activated by histamine with durations of activation and receptive fields matching the histamine-itch duration and flare area (Schmelz et al., 1997).

2.2.2 Neuroanatomy of Itch

Itch sensation is transmitted from the skin to the spinal cord by small diameter, unmyelinated C-fibers and thinly myelinated Aδ-fibers (Ringkamp et al., 2011; Schmelz, 2010). In human skin, C-fibers that mediate pruritus are either mechano-insensitive, histamine-responsive nerves (CMiHis+) or mechano- and heat-sensitive, polymodal nociceptors (CMH) which are unresponsive or only weakly responsive to histamine (Ringkamp et al., 2011; Schmelz, 2010). Accordingly, CMiHis+ neurons mediate histamine-dependent itch while CMH neurons mediate at least some types of non-histamine dependent itch (Johanek et al., 2008).

The central nervous system pathway that mediates itch begins in the dorsal horn of the spinal cord with the synapse formed by the afferent primary cutaneous nerve (i.e., going from the skin into the spinal cord) and the second order neuron dendrites. These second order neurons either are interneurons that in turn synapse with neurons located in deeper layers of the dorsal horn or are projection neurons that send axons to the contralateral spinothalamic tract to terminate on third-order neurons in the thalamus. From the

thalamus, the signal is then projected diffusely to cortical and subcortical regions (Fig. 2-1, 2-2) (Dhand and Aminoff, 2013).

2.2.2.1 CMiHi Nerves

CMiHi nerves have higher electrical activation thresholds than polymodal C-nociceptors (Ikoma et al., 2005). When CMiHi nerves are activated in human skin, the axon reflex is observed, consistent with histamine-induced itch. However, the H1 histamine receptor, responsible for histamine-induced itch, was shown to be essential for pain mediation in a mouse model (Mobarakeh et al., 2000). CMiHi was also shown to be involved in central sensitization of pain (Schmelz, 2000). Therefore, CMiHi are involved in transmitting both histamine-dependent itch and pain.

Figure 2-1. Peripheral Anatomy of Itch (Dhand and Aminoff, 2013).
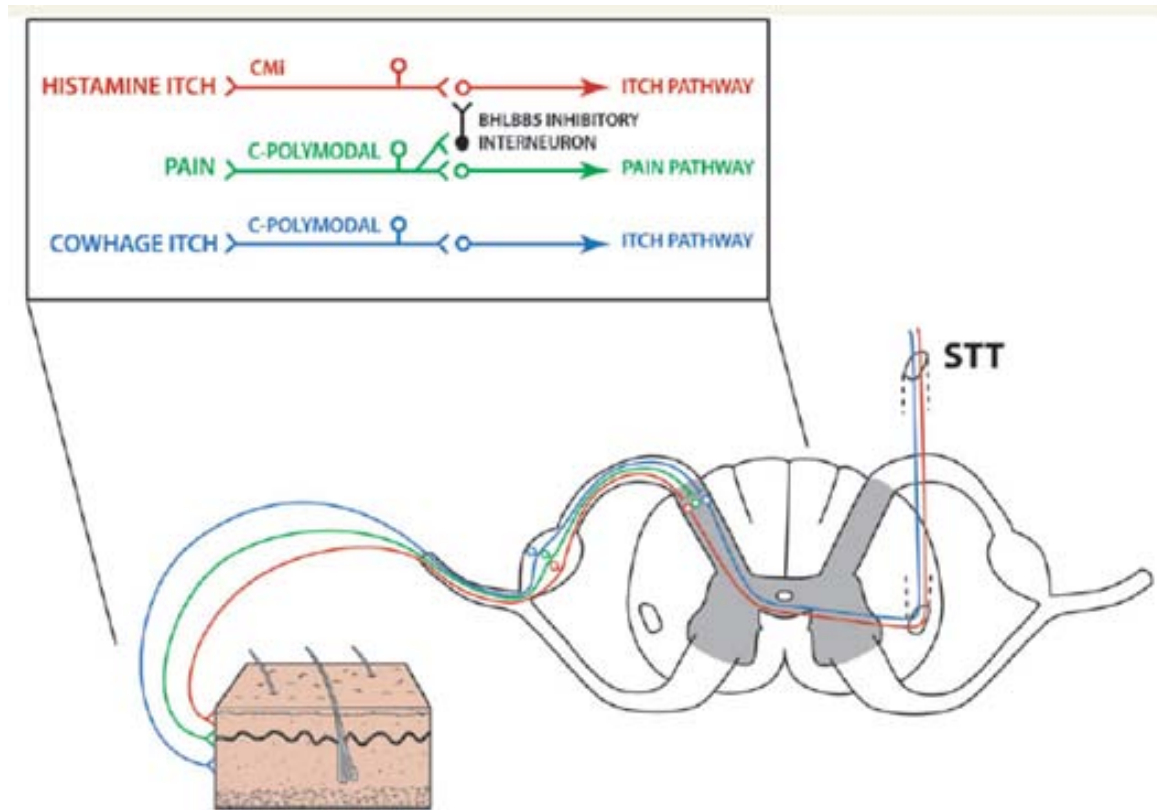
The histamine-dependent (red) and histamine-independent (blue) itch pathways and the peripheral pain pathway (green) are shown. Histamine-dependent itch is induced by cutaneous application of histamine and transmitted via cutaneous mechanically insensitive C-fibers. Histamine-independent itch can be induced by numerous cutaneous stimuli, including cowhage, and is transmitted via cutaneous polymodal C-fibers. Itch modulation by pain has be postulated in mice to be partially mediated by Bhlbb5 interneurons in the dorsal horn of the spinal cord. STT = spinothalamic tract; Bhlbb5 = transcription factor protein; CMi = mechano-insensitive C-fibers; C-polymodal = polymodal C-fibers. (Figure from Dhand and Aminoff, 2013; legend adapted from Dhand and Aminoff, 2013)

Figure 2-2. Central Anatomy of Itch (Dhand and Aminoff, 2013).

Cortical and Subcortical Structures Activated in Itch	
HISTAMINE	COWHAGE
Primary and secondary somatosensory cortex	Primary and secondary somatosensory cortex
Posterior parietal cortex	Posterior parietal cortex
Superior and middle temporal cortex	Superior and middle temporal cortex
Posterior cingulate cortex	Posterior cingulate cortex
Anterior cingulate cortex	Anterior cingulate cortex
Precuneus	Precuneus
Cuneus	Cuneus
	Insular cortex
	Claustrum
	Basal ganglia
	Putamen

Thalamic Nuclei Activated In Itch	
HISTAMINE	COWHAGE
Ventral posterior lateral	Ventral posterior lateral
Ventral posterior inferior	Ventral posterior inferior
Posterior	Posterior
	Suprageniculate
	Medial Geniculate
	Pulvinar

Second and higher order itch fibers for histamine (red) and cowhage (blue) travel in the spinothalamic tract to the thalamic nuclei (listed in the left box) usually contralateral to the stimulus. Cowhage neurons project to a broader set of nuclei than histamine neurons. In cowhage itch, thalamic neurons project to multiple cortical and subcortical structures (listed in the right box) with a broader and more diffuse pattern. These structures are usually activated in a bilateral and symmetric pattern, except that the insular cortex, claustrum, basal ganglia, and putamen have a minor emphasis contralateral to the stimulus. STT = spinothalamic tract. (Figure from Dhand and Aminoff, 2013; legend adapted from Dhand and Aminoff, 2013)

2.3 Histamine-Independent Itch: Protease Pathway

In human skin, itch independent of histamine mediation is defined as itch without an accompanying axon-reflex erythema (Ikoma et al., 2005). Cutaneously-applied proteases can generate pruritus in a histamine-independent manner. Therefore, protease signaling has emerged as an effective marker for the histamine-independent, peripheral itch-signaling neuronal pathway (Steinhoff et al., 2003).

Cowhage spicules derived from the pod of the bean plant *Mucuna pruriens*, when inserted into the skin at the level of the dermoepidermal junction, elicit an intense itch sensation admixed with milder pricking/stinging and burning sensations (LaMotte et al., 2009). The active pruritogenic component of cowhage is mucunain (Shelley and Arthur, 1955), a cysteine protease (Reddy et al., 2008). Cowhage-induced itch has been proposed as a clinically useful tool to assess histamine-independent itch (LaMotte et al., 2009; Ikoma et al., 2006). Cowhage spicules induce cutaneous sensations when the tip is inserted into the skin to a depth that terminates approximately at the dermal-epidermal junction to depths of around 200μm into the papillary dermis (Papoiu et al., 2011). Histamine-independent, cowhage-induced itch activates polymodal CMH and Aδ-fibers that project to a population of ascending spinothalamic tract (STT) neurons distinct from those activated by histamine (Davidson et al., 2007; Namer et al., 2008)

The cowhage cysteine protease mucunain activates the G protein-coupled receptors, protease-activated receptors (PARs) 2 and 4 (Reddy et al., 2008). Evidence showed that

PAR-2 and transient receptor potential vanilloid 1 (TRPV-1) are co-expressed in 90% of all TRPV-1 expressing C-fibers. PAR-2 can sensitize TRPV-1 and induce thermal hyperalgesia (Amadesi et al., 2004). PAR-2 is also involved in inflammation and subsequent hyperalgesia (Cottrell et al., 2003). PAR-2 expression is therefore involved in nerves that mediate both itch and pain.

Human epidermis expresses endogenous cysteine proteases, such as those of the cathepsin family, including cathepsin S, cathepsin V, and cathepsin L (Reddy et al., 2010). Specifically, cathepsin S was shown to be pruritic when injected into human skin (Reddy et al., 2010). While cathepsin S is an endogenous agonist of PAR-2 and 4 (Reddy et al., 2010), this (and possibly other) proteases may be mediating histamine-independent itch using receptors beyond PARs. Specifically, cathepsin S, can induce itch via activation of a different class of G protein-coupled receptors, the Mas-related G-protein-coupled receptor (Mrgpr), Mrgpr C11 (Reddy et al., 2013). In mice skin, different Mrgprs mediate distinct chemical itch sensations, and the neurons that express these receptors transmit itch sensation to the spinal cord (Dong et al., 2001). Mrgprs have human homologues, and their ligands, such as the bovine adrenal medulla 8-22 (BAM8-22) peptide [a proteolytic cleavage product of proenkephalin A], chloroquine, SLIGRL (also a ligand of PAR-2), and β-alanine, induce itch in humans (Sikand et al., 2011; Liu et al., 2009; Lembo et al., 2002). Mrgprs could potentially represent another marker for a subset of histamine-independent itch nerves.

2.4 Mechanism of Chronic Itch

Two possible pathophysiologic states have been proposed to explain chronic itch: peripheral sensitization and central sensitization. Peripheral sensitization is the decreased activation threshold and increased basal activity of cutaneous itch-signaling nerve fibers (Dhand and Aminoff, 2013). Central sensitization results from the neuroplasticity that occurs in the spinal cord and brain such that non-pruritic cutaneous stimuli are perceived as, or augment, the sensation of itch (Dhand and Aminoff, 2013).

2.4.1 Central Sensitization

Specifically, in the spinal cord, itch and pain processing can be centrally sensitized such that touch stimuli evoke itch (alloknesis) or pain (allodynia) or that punctate mechanical stimuli evoke more intense pricking pain (punctate hyperalgesia) or itch (punctate hyperknesis). Moreover, normally painful stimuli can be misinterpreted as itch in chronic itch patients (''pain alloknesis'') or normally itch-inducing stimuli can be mistaken as pain in chronic pain patients (''itch allodynia'') (Figure 2-3) (Ikoma et al., 2006).

Experiments have provided evidence supporting the role of central sensitization in itch onset. In mice, genetic ablation of the transcription factor Bhlhb5, a marker for inhibitory neurons (Figure 2-1), induced a scratching phenotype, interpreted as itch (Ross et al., 2010;). These authors showed this scratching phenotype arose due to the loss of inhibitory neurons in the dorsal spinal cord (Ross et al., 2010).

Clinical and/or experimental tests for central sensitization include application to the skin of brush-strokes, pinpricks from a graded set of weighted needles, and the application of defined thermal stimuli (Ikoma et al., 2004; Sikand et al., 2009).

Figure 2-3. Mediators and the Sensitization Patterns of Nociceptive and

Pruriceptive Neurons (Ikoma et al., 2006).
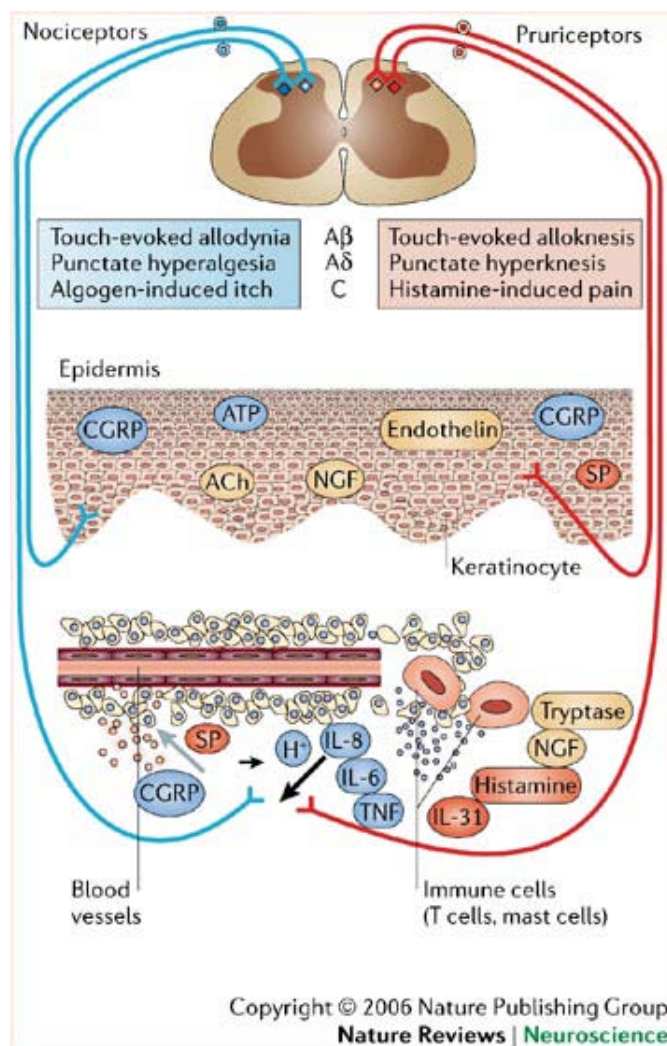
Central sensitization in the pain (blue) and itch (red) pathways. Mediators can predominantly sensitize and activate itch pathways (red) or pain pathways (blue) or both itch and pain pathways equally (yellow). In the spinal cord, for each class of cutaneous nerve, incoming painful input can generate central sensitization for pain and incoming pruritic signals can provoke central sensitization for itch. Thus, for central sensitization to touch, amyloid-β (Aβ) fibers generate allodynia for pain versus alloknesis for itch; A-δ fibers generate punctate hyperalgesia for pain versus punctate hyperknesis for itch; and for C-fibers histamine-induced pain versus algogen-induced itch. ACh, acetylcholine; CGRP, calcitonin-gene-related protein; H^+, hydrogen ion; IL, interleukin; NGF, nerve growth factor; SP, substance P; TNF, tumor necrosis factor. (Figure from Ikoma et al., 2006; legend adapted from Ikoma et al., 2006)

Brush stroke test examines for alloknesis or allodynia that are thought to be mediated by low-threshold mechanoreceptors (Aβ fibers), requiring ongoing activity of nociceptive C fibers (Ikoma et al., 2006). After single cowhage application, more than 40% of healthy subjects experienced alloknesis (Sikand et al., 2009). This suggests that cowhage may activate central mechanisms of itch sensation.

Pin-prick test examines punctate hyperalgesia or punctate hyperknesis that are thought to be induced by nociceptive C fibers and mediated by Aδ fibers (Ziegler et al., 1999). Pin-prick is sensed as mildly painful in normal controls. The pain increases as the weight load increases, with the maximum pain less than 4 on a numeric scale ranging from 0 (no sensation) to 10 (maximal sensation imaginable) (Ikoma et al., 2004). Punctate hyperknesis was seen in lesional skin in 75% of atopic dermatitis patients with itch rating ≥2 on the 0–10 scale. (Ikoma et al., 2004).

Thermal test examines for pain alloknesis that is thought to be mediated by Aδ- and C-fibers (Ikoma et al., 2006). Metal rod preheated to 49 °C is sensed as noxious pain in normal controls. The maximum pain was 3.5 on the above 0–10 scale (Ikoma et al., 2004). This noxious heat was also sensed as itch in lesional skin of atopic dermatitis subjects, with the maximum itch of 2.5 on the 0–10 scale (Ikoma et al., 2004).

2.4.2. Central Sensitization and Uremic Pruritus

Evidence suggests that central sensitization also contributes to uremic pruritus. Unlike

atopic dermatitis, the skin in uremic pruritus lacks observable inflammation. Systemic circulating itch signals and/or toxin(s) may be present, bypassing the peripheral nerves and acting directly in the dorsal horn of the spinal cord to either elicit itch or generate inappropriate itch-sensations to otherwise non-pruritic cutaneous stimuli. Opioids are one such systemic itch signaling-candidate. Mu-opioids, such as morphine, facilitate itch sensation, and kappa-opioids, such as dynorphin, reduce itch (Stander and Schmelz, 2006; Togashi et al., 2002). However, studies correlating beta-endorphin (a mu-agonist) levels with itch intensity in uremic pruritus have been mixed with one study showing a positive correlation and the other, no correlation (Odou et al., 2001; Mettang et al., 1998). Moreover, nalfurafine, a kappa agonist, showed only modest itch reduction in ESRD subjects on hemodialysis. The itch reduction in nalfurafine 5mg group was 32–37% compared to a 20% decrease in the placebo group (Wikstrom et al., 2005; Kumagai et al., 2009). As we have discussed extensively in the 2.9 Uremic Pruritus section, the role of systemic opioids in pathogenesis of uremic pruritus has not been well-established, opening the possibility for other non-opioid systemic-acting itch mediators.

Nearly all intraepidermal nerve fibers (IENFs) in human epidermis are TRPV-1 positive (i.e., capable of sensing at least pain). All markers to date of itch sensing neurons in human skin (including BNP/BNP precursor expressing nerves) terminate predominantly at the DEJ (and not in the epidermis). In some inflammatory skin conditions characterized by pruritus (such as lichen amyloidosis and nummular dermatitis), IENFs are lost despite relative preservation of the DEJ nerves (Maddison et al., 2011; Maddison

et al., 2008). Thus, pathologic itch may arise from loss of intraepidermal pain nerves with preservation of DEJ itch signaling fibers (Timmes et al., 2013).

Moreover, it is also not known whether or not papillary dermal cutaneous nerves, expressing itch-signaling markers, are involved in uremic pruritus. One study, with significant methodological issues, reported a decrease of both epidermal nerves and CGRP-positive nerves at the DEJ in human skin after nbUVB or UVB+UVA treatment for eczema (Wallengren and Sundler, 2004).

2.4.3 Peripheral Sensitization

Evidence for peripheral sensitization of protease-mediated nerve signaling in human pruritic skin diseases is lacking. In normal subjects, cowhage spicule induced-itch lasts on average about 15 minutes and has usually completely and spontaneously resolved after 30 minutes after onset (Sikand et al., 2009). In some patients with atopic dermatitis, the duration of itching lasted up to one hour (Arthur and Shelley, 1958).

2.4.4. Peripheral Sensitization and Uremic Pruritus

Clinical experience with experimental treatments for uremic pruritus indicates that peripheral sensitization of itch-signaling cutaneous nerves contributes significantly to uremic pruritus. Specifically, topical capsaicin and narrow band UVB phototherapy, treatments that target cutaneous nerves and/or keratinocytes that can directly signal to these nerves, alleviated uremic pruritus. Topical application of capsaicin opens the

TRPV-1 ion channel, and over time, the resulting influx of divalent ions leads to degeneration of the terminal ends of these axons (Sharma et al., 2013). Several small double-blinded studies have suggested that topical capsaicin-induced chemodenervation of TRPV-1 epidermal and DEJ nerves partially relieved uremic pruritus (Breneman et al., 1992; Makhlough, 2010; Cho et al., 1997). One study found a reduction from moderate-to-severe to minimal-to-none (8 of 9 patients, no placebo control) (Breneman et al., 1992). In a second study that was double-blinded and placebo-controlled, of 5 patients, 2 had complete resolution of itch and 3 had mild-to-moderate improvement. There was no change in itch on the placebo-treated area (Breneman et al., 1992). Another randomized double-blinded crossover clinical trial was performed on 34 patients and used a complicated measure for itch, but found that topical capsaicin reduced the itch from a rating of ~16 to 2.5 vs. placebo that reduced itch from ~15 to 7.2 (p<0.001) (Makhlough, 2010). Finally, in a double blinded, cross-over study, of 22 subjects treated with topical capsaicin, 19 responded to capsaicin while 5 responded to placebo (Cho et al., 1997).

Second, phototherapy utilizing narrow-band UVB is a clinically effective treatment to relieve uremic pruritus, with a reported response rate of ~50% to 80% in various studies with small sample sizes (Ee et al., 2006; Ada et al., 2005). While the mechanism(s) by which nb-UVB treats pruritus is(are) not well-understood, phototherapy with nb-UVB utilizes the wavelengths 311–313 nm to treat skin disease. These wavelengths can penetrate the superficial layers of the skin at the levels epidermis and papillary dermis. Thus, efficacy of topical capsaicin and nb-UVB suggests keratinocytes (in the epidermis)

as well as itch-sensing nerves that reside in the papillary dermis and possibly the epidermis, are targets to reduce peripheral sensitization.

While TRPV-1 expression or putative inhibition by nb-UVB does not distinguish between nerves that signal histamine and non-histamine-mediated itch, uremic pruritus responds poorly or not at all to anti-histamines (Weisshaar et al., 2003). Moreover, increased plasma histamine levels in uremic patients have been detected in some but not all studies, and no relationship was found between plasma histamine level and severity of pruritus (Weisshaar et al., 2003). Thus, clinical and experimental evidence suggests the histamine pathway is not active in this type of chronic itch.

2.5 Peptidergic Itch Markers (CGRP, SP, NPPB)

In the spinal cord dorsal horn, neurotransmitters such as calcitonin gene related peptide (CGRP), gastrin-releasing peptide (GRP), substance P (SP), and glutamate have been implicated as mediators of the first itch synapse (Davidson and Giesler, 2010). Glutamate is known to be the principle fast neuron transmitter in primary afferent neurons (Rogoz et al., 2014). Molecular markers of nociceptive neurons are divided into non-peptidergic and peptidergic (Rogoz et al., 2014). CGRP and SP are known to be the peptidergic markers of nociceptive neurons (Snider and McMahon, 1998). Recent experiments in mice raised the possibility that Brain Natriuretic Peptide (BNP) and/or its precursors, may also be novel itch mediators and so molecular markers of itch-sensing nerve pathways (Mishra and Hoon, 2013).

2.5.1 Substance P (SP)

Substance P was the first discovered neuropeptide in mammals and is a member of the tachykinin neuropeptide family. SP has been recognized as a neurotransmitter of primary afferent nerves since the 1950's, its endogenous receptor is the neurokinin 1 receptor (NK1-receptor, NK1R). SP is a neuropeptide widely distributed in the peripheral and central nervous system. In the peripheral nervous system, SP is synthesized in the cell bodies of C-fibers, and its release into the skin causes vasodilatation and increased vascular permeability.

SP is also thought to intensify itch perception. Intradermal injection of SP provokes itch as well as characteristics of neurogenic inflammation such as erythema, wheal, and flare (Hagermark et al., 1978). However, endogenous release of SP does not lead to mast cells degranulation in healthy human skin (Schmelz et al., 1999) or induce itch at physiological concentrations (Weidner et al., 2000). However, low concentrations of SP can sensitize NK1R on mast cells, leading to increased production of tumor necrosis factor (TNF-α) (Cocchiara et al., 1999). In turn, TNF-α sensitizes nociceptive nerve endings.

SP is co-localized with other neurotransmitters, such as serotonin, dopamine, or CGRP, and acts as a neuromodulator. SP is elevated in plasma in atopic dermatitis patients compared to controls and correlates with disease activity (Toyoda et al., 2002). On the other hand, SP has been found to play an important role in the induction of pain and

hyperalgesia in rodents (Laird et al., 2001), although there is little evidence for the clinical analgesic efficacy of the antagonists of its receptor NK1R (Hill, 2000). However, the NK1R antagonist Aprepitant has been shown to alleviate itch in patients with Sezary syndrome, solid tumors, or with chronic pruritus related to various systemic diseases (Duval and Dubertret, 2009; Vincenzi et al., 2010; Stander et al., 2010). Thus, SP signaling is involved in both itch and pain signaling pathways. SP might contribute to itch by increasing neuronal sensitization and through its long-term interaction with mast cells (Yosipovitch et al., 2003).

2.5.2 Calcitonin Gene Related Peptide (CGRP)

CGRP belongs to the calcitonin family of peptides, which consists of at least six members, namely, calcitonin, amylin, intermedin, adrenomedullin, and CGRP (Poyner et al., 2002). CGRP is widely distributed in the peripheral and central nervous systems, as well as cardiovascular, respiratory and gastrointestinal systems (Arulmani et al., 2004). There are two isoforms of CGRP, α-CGRP and β- CGRP (Arulmani et al., 2004). α-CGRP is preferentially expressed in sensory neurons (Rosenfeld et al., 1983). Other than acting as a neuropeptide, CGRP is a potent vasodilator (Brain et al., 1985). To date, CGRP is implicated in mediating both itch and pain.

CGRP antagonists exhibit a promising future for treating migraines (Villalon and Olesen, 2009). Although systemic infusion of CGRP has successfully induced migraine (Lassen

et al., 2002), CGRP does not cross the blood-brain barrier. The mechanisms by which CGRP antagonists treat migraines include inhibition of peripheral and central sensitization of pain and inhibiting vessel dilatation in the brain (Villalon and Olesen, 2009).

There are two forms of CGRP receptor antagonists: peptide $CGRP_{8-37}$ and non-peptide (BIBN4096BS (Olcegepant) (Doods et al., 2000) and MK-0974 (Telcagepant) (Williams et al., 2006)). New CGRP receptor antibodies are under investigation. In rats, CGRP is involved in inducing morphine tolerance in treating nociceptive pain (Powell et al., 2000). Intrathecal injection of CGRP receptor antagonists ($CGRP_{8-37}$ or BIBN4096BS) combined with morphine can counteract the tolerance seen when morphine was given alone.

In the skin, CGRP is one of the most abundantly expressed neuropeptides and often is co-localized with either SP or somatostatin (Gibbins et al., 1987). The exact role of CGRP in itch and pain remains to be elucidated. In rodents, CGRP has been implicated in itch signaling. Genetic ablation of CGRPα-expressing sensory neurons reduced sensitivity to itch that is induced by histamine and chloroquine, that activates mouse MrgprA3 and human MrgprX1 respectively, but did not impair β-alanine-induced itch that is associated with non-peptidergic sensory neurons (McCoy et al., 2013).

However, there is more evidence in rodents for CGRP in signaling pain. In rodent studies, CGRP was reported to mediate noxious heat (Mogil et al., 2005). Intrathecal injection of CGRP was reported to induce hyperalgesia and central sensitization for pain in rats (Sun et al., 2004). In mouse models of atopic dermatitis, the concentration of CGRP in the skin was decreased, at the same time SP was increased compared to controls. This observation supports the idea that CGRP mediates pain (i.e., by being reduced in a pruritus model) while SP mediates itch (ie, by being increased in a pruritus model) (Katsuno et al., 2003). Intensity of pain induced by noxious heat positively correlated with CGRP expression and sensitivity in mouse models (Mogil et al., 2005). In human studies, CGRP was significantly increased in the plasma in some chronic pain states, though no significant correlation was seen between pain rating and CGRP concentration in the plasma (Birklein et al., 2001). Thus, although CGRP may be involved in both itch and pain pathways in rodents, the data suggest CGRP has a greater role in nociceptive pain in both human and animal models (Ikoma et al., 2006).

2.5.3 Natriuretic Polypeptide Precursor B (NPPB)

Natriuretic polypeptide precursor B (NPPB) is the rodent analogue of the human Brain Natriuretic Peptide (BNP) precursor protein. In mice, NPPB was shown to be an itch-specific neurotransmitter released by primary cutaneous somatosensory nerves to active itch-signaling neurons in the dorsal horn of the spinal cord that expressed its receptor, natriuretic peptide receptor A (NprA) (Figure 2-4) (Mishra and Hoon, 2013). In mice, blockage of NPPB signaling inhibited both histamine-dependent and histamine-

independent itch (Mishra and Hoon, 2013). Therefore, while NPPB expression did not distinguish between these two signaling pathways, it nevertheless highlighted itch-specific cutaneous sensory nerves.

Figure 2-4. NPPB as an Itch-Specific Neurotransmitter (Mishra and Hoon, 2013).
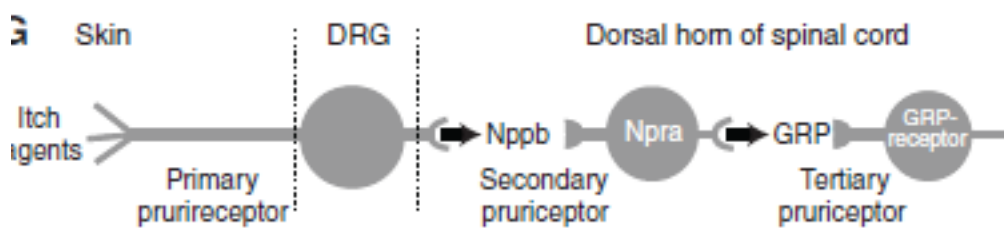
Neuroanatomical model of the itch-specific nerve pathway. Primary pruriceptive nerves release Nppb in the dorsal horn of the spinal cord to activate the Npra receptor on second order neurons. These activated second order neurons in turn utilize GRP as neurotransmitter to active third order neurons that express the GRP-receptor. Nppb: Natriuretic Polypeptide B; Npra: natriuretic peptide receptor A; GRP: Gastrin-Releasing Peptide. (Figure from Mishra and Hoon, 2013; legend adapted from Mishra and Hoon, 2013)

The human BNP gene is first translated to pre-proBNP$_{1\text{-}134}$ (AKA: pre-proNPPB$_{1\text{-}134}$), then cleavaged to proBNP$_{1\text{-}108}$ (AKA: proNPPB$_{1\text{-}108}$) within cells. Upon excretion, proBNP$_{1\text{-}108}$ is cleavaged to active BNP$_{1\text{-}32}$ (AKA: NPPB$_{1\text{-}32}$) and amino-terminal proBNP$_{1\text{-}76}$ (NT-proBNP) (Figure 2-5) (Martinez-Rumayor et al., 2008). Active BNP$_{1\text{-}32}$ and proBNP$_{1\text{-}108}$ can bind human NprA, and so are the most likely candidates to mediate itch (Stein and Levin, 1998).

Figure 2-5. Human BNP Metabolism (Martinez-Rumayor et al., 2008).
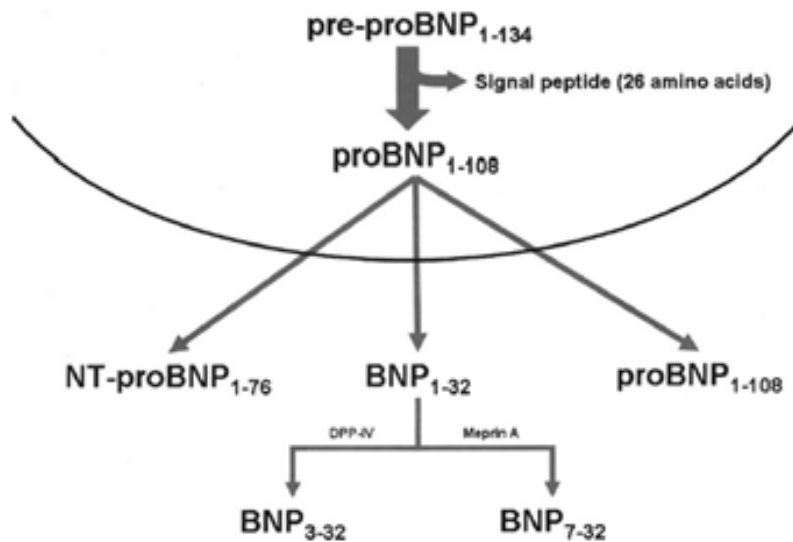
Schematic model describing the synthesis and release of BNP. BNP = B-type natriuretic peptide; DPP-IV = dipeptidyl peptidase–IV; NT-proBNP = amino-terminal pro–B-type natriuretic peptide. (Figure from Martinez-Rumayor et al., 2008; legend based on Martinez-Rumayor et al., 2008)

In humans, most BNP is excreted from heart muscle, including atria and ventricles. BNP synthesis and release from cardiac muscle is stimulated by mechanical stretching of this muscle, such as in conditions of volume overload, including congestive heart failure and kidney failure, as well as after ischemic injury, angiotensin II, and other pro-inflammatory cytokines (Martinez-Rumayor et al., 2008). Active BNP signaling functions in humans to achieve potent natriuretic, diuretic and vasodepressor effects (de Bold et al., 1981). BNP signaling as a cause of itch in humans has not been shown to date. In this regard, nesiritide, a drug with the same structure as BNP_{1-32}, caused pruritus in more than 1% of the study population during a clinical trial to treat decompensated congestive heart failure (Mills et al., 1997; http://dailymed.nlm.nih.gov/dailymed/archives/fdaDrugInfo.cfm?archiveid=6077#section-8). Moreover, anecdotal case reports exist to indicate that acute heart failure is associated with significant pruritus (Javelle et al., 2011). Finally, BNP and NT-proBNP levels in uremic subjects are variably increased due to their accumulation secondary to loss of renal excretion (specifically for NT-proBNP), cardiac co-morbidity, and volume overload (Satyan et al., 2007; Mallamaci et al., 2001; Tapolyai et al., 2013). The level of $ProBNP_{1-108}$ in end-stage renal disease (ESRD) individuals has not been addressed in the medical literature to date, but a commercially available BNP assay can detect proBNP1-108 as well (Martinez-Rumayor et al., 2008). Recently, a study found a positive correlation between blood BNP levels and itch in hemodialysis patients (Shimizu et al., 2014). However, the design and conduct of this study has flaws detracting from the strength of this finding. This study determined both pre-and post-dialysis BNP values but only reported the mean of the post-dialysis

BNP level. The article used one of these two BNP levels for multiple regression analysis but did not specify which one was utilized. The BNP level used only correlated significantly with day-time and not nocturnal itch. However, if BNP is the systemic circulating molecule that induces itch, nocturnal itch should also have correlated with BNP levels as theoretically, enough time has passed post-dialysis for BNP to re-accumulate. In addition, NPPB mRNA has been identified at low levels in human trigeminal ganglia and the BNP receptor NprA mRNA has been identified in the spinal cord dorsal horn (Goswami et al., 2014). Therefore, if BNP-expressing primary cutaneous nerves mediate itch through release into the human spinal cord, BNP/BNP precursors could then cause itch through peripheral sensitization as well.

Recently, there has been some debate over whether NPPB was expressed in mice DRG. Although Mishra and Hoon exhibited NPPB staining in mice DRG, another group was unable to replicate this finding (Mishra and Hoon, 2013; Liu et al., 2014). A new study demonstrated NPPB mRNA in mice DRG, consistent with Mishra and Hoon, suggesting that NPPB could be the primary pruriceptive peptide (Solorzano et al., 2015). The existence of primary afferent NPPB/NPPB precursor-expressing sensory nerves in human skin was unknown. We have generated preliminary data proving the existence of NPPB/NPPB precursor expressing primary cutaneous neurons in human skin with an itch-sensing phenotype and anatomic location (Chapter 5. Findings, section 5.7.1). This is in accordance with the above described RNA-sequencing study in human trigeminal

ganglia (Goswami et al., 2014). However, their contribution, if any at all, to uremic pruritus is unknown.

2.6 Transient Receptor Potential (TRP) Receptors

TRP receptors respond to 'hot' and 'cold' and represent a superfamily involved in itch and pain. To date six groups of molecules complete this superfamily: (1) the canonical (TRPC), (2) the melastatin (TRPM), (3) the polycystin (TRPP), (4) the ankyrin transmembrane protein 1 (TRPA), (5) the mucolipin (TRPML), and (6) the vanilloid (TRPV) subfamilies. All members of this superfamily are nonselective calcium-permeable sensory transduction channels (Clapham, 2003).

The first and best-known is the TRP vanilloid receptor 1 (TRPV-1), activated by capsaicin, the pungent ingredient of hot chili peppers. Endovanilloids interact with TRPV-1 by activating or sensitizing TRPV-1 directly or indirectly (Caterina et al., 1997; Hwang et al., 2000). Endovanilloids constitute a group of itch mediators, such as eicosanoids, histamine, bradykinin, ATP, and various neurotrophins (NTs) (Chuang et al., 2001; Hwang et al., 2000; Mohapatra and Nau, 2003; Shin et al., 2002).

TRPV-1 expression in humans was first described on primary nociceptive afferent neurons (Caterina et al., 1997). TRPV-1-expressing nerves make up nearly 100% of the intraepidermal nerve fibers (IENF) and ~80% of the nerves at the papillary dermis

(Simone et al., 1996; Simone et al., 1998). Importantly, both histamine-dependent and histamine-independent nerve fiber pathways express TRPV-1, so responsiveness to capsaicin cannot distinguish these different classes in human skin (Sharma et al., 2013). Topical application of TRPV-1 agonist capsaicin has been used to treat both itchy and painful skin lesions; for example, brachioradial pruritus (Zeidler et al., 2015) and postherpetic neuralgia (Backonja et al., 2010). Clinically, the first days of the therapy are accompanied by a burning sensation and neurogenic inflammation, followed by a lasting depression of pain and itch. Other than action potential generation upon TRPV-1 activation, neuropeptides are released, especially SP and CGRP (Caterina and Julius, 2001). SP triggers plasma extravasation and CGRP triggers vasodilation, respectively. Under chronic activation, sensory nerve fiber stores of neuropeptides, such as substance P, are depleted, disrupting the communication between mast cells and skin sensory neurons (Yosipovitch et al., 2003). With chronic stimulation, TRPV-1 is desensitized in a Ca^{2+}-dependent manner and leaves the nociceptive and pruriceptive neurons inactive. Moreover, the axonal transport of both neuropeptides and nerve growth factor (NGF) in the periphery is slower after chronic activation. Interestingly, TRPV-1 acts synergistically with the cowhage receptor PAR2 (Amadesi et al., 2006) and the SP receptor NK1R (Ikoma et al., 2006), which might have implications for the peripheral sensitization of itch and pain.

Intradermal injection of capsaicin is always perceived as painful (Simone et al., 1989). Intradermal injection of capsaicin can also induce pain central sensitization (Sikand et al.,

2011). In addition, loss of epidermal nerves from capsaicin application paralleled hypoalgesia (Nolano et al., 1999), indicating epidermal nerves also mediate pain. Interestingly, application of capsaicin to dermal-epidermal junction by deactivated cowhage spicules induces pruritus (Sikand et al., 2009). Moreover, itch was reported in more than 50% of subjects with topical application of capsaicin in one study (Green and Shaffer, 1993). Based on these data, TRPV1-expressing nerves that sense pain and itch are located in or near the epidermis whereas TRPV1-expressing nerves that only sense pain are located deeper in the dermis (Ma, 2010). Therefore, capsaicin receptor, TRPV-1, underlies a shared pathway of pain and itch but the ultimate sensation perceived from cutaneous activation of TRPV1-expressing nerves may depend on their anatomic location in the skin.

TRPV-1 is also expressed in human epidermal and hair follicle keratinocytes (Stander et al., 2004), as well as mast cells and dendritic cells (Bodo et al., 2004). Upon activation, TRPV-1 can induce the release of cytokines from keratinocytes, which are involved in pruritus (Southall et al., 2003). Therefore, topically applied capsaicin may not only target nerve endings but may also provoke TRPV-1-mediated signaling in other non-neuronal skin cells to counteract a pruritogenic outcome.

TRPV-1 signaling can also be modulated by skin inflammation. Skin inflammation decreases intradermal pH, and protons activate the TRPV-1 receptor. TRPV-1 stimulation

leads to the release of prostaglandin E_2 (PGE_2) and IL-8, that in turn aggravate the inflammation cascade (Southall et al., 2003).

2.7 Itch Modulation (Pain Inhibits Itch)

Pain and itch can be easily distinguished by both the sensations they evoke and the reverse reflex pattern. Pain triggers the withdrawal reflex leading to retraction, and therefore protection. Itch induces the scratching reflex to localize the affected skin site and draw attention to it.

Scratching also represents a potentially damaging noxious stimulus. The inhibition of itch by painful stimuli has been shown experimentally using different types of painful stimuli, for example, heat, electrical current and mustard oil (Ward et al., 1996). Histamine-induced itch was significantly reduced by painful electrical stimulation at a distance of up to 10 cm outside the stimulated site, suggesting a central mode of action (Nilsson et al., 1997). Consistent with these results, histamine-induced itch including alloknesis and hyperknesis was suppressed inside the zone of capsaicin-induced allodynia (Brull et al., 1999). This antagonism between pain and itch finds its ground in genetics: sensitivity in pain was inversely correlated to sensitivity in itch in mice strains (Green et al., 2006).

2.8 Instruments to Measure Itch

Several instruments have been designed to measure itch. Verbal rating scale (VRS) and visual analogue scale (VAS) have been the most used in the clinical setting. VRS and

VAS measure the absolute intensity of itch, whereas, the general version of the Labeled Magnitude Scale (gLMS) measures the comparative magnitude of the sensation.

Specifically, VAS has been used for more than half a century to evaluate subjective symptoms. Subjects are presented with a 100mm horizontal line, with one end labeled as complete absence of the symptom, and the other end labeled as the worst symptom imaginable. Compared with VRS, VAS more closely assessed what a patient actually experienced with regard to change in sensory intensities (Ohnhaus et al., 1975).

General version of the Labeled Magnitude Scale (gLMS) enables comparison of the perceived intensities of itch and nociceptive sensations on a common scale (LaMotte et al., 2009). The gLMS was an invention that combined the magnitude estimation and labeled scale estimation (Bartoshuk et al., 2004). Like the labeled scale, gLMS consisted of intensity descriptors, such as barely detectable at the lower end, weak, moderate, strong and very strong in the middle, with the strongest imaginable sensation of any kind at the other end. The labeled scale enables comparison between difference groups of sensation. Magnitude estimation provided a valid comparison independent of the measured sensation. The gLMS has been used to measure different nociceptive sensations (Green and Schoen, 2007).

Different itch questionnaires were created to measure the quality of life that is affected by itch. Questionnaires with respect to specific skin diseases have been created, such as

psoriasis (Yosipovitch et al., 2000), chronic urticaria (Yosipovitch et al., 2002a), atopic dermatitis (Yosipovitch et al., 2002b) and uremic pruritus (Yosipovitch et al., 2001). Itchy QoL was a new questionnaire specifically targeting quality of life in patients with pruritus from various origins (Desai et al., 2008). Itchy QoL has two versions: bother and frequency versions. The itchy QoL questionnaire encompasses three sections based on the psychological conception of "quality of life": emotions, functioning and symptoms. The content of itchy QoL questionnaire derived from in-depth patient interviews. This questionnaire was further validated in the clinical setting, confirming its reliability and sensitivity to changes in trial patients (Desai et al., 2008). The frequency version of itchy QoL questionnaire was used in our study.

2.9 Uremic Pruritus

2.9.1 Characteristics and Proposed Causes of Uremic Pruritus

Pruritus is a prevalent symptom of individuals with end-stage renal disease (ESRD) on hemodialysis. Termed uremic pruritus, this symptom affects 22–90% of patients with ESRD and can severely affect quality of life, including sleep alteration and induction of both depression and anxiety (Feramisco et al., 2010; Zucker et al., 2003; Wang and Yosipovitch, 2010).

Despite numerous proposed etiologic factors (Table 2-1), including inappropriate dialysis, xerosis, secondary hyperparathyroidism, derangement of divalent ions, such as phosphorus, defective sweating, and mast cell proliferation and degranulation, the

pathogenesis of uremic pruritus remains poorly understood (Keithi-Reddy et al., 2007; Szepietowski and Schwartz, 1998; Kumagai et al., 2012). Among all proposed causes of uremic pruritus, uremia, xerosis, anemia, hyperparathyroidism and high phosphorus and calcium levels are currently clinically proven itch causes. Despite clinical efforts to provide appropriate dialysis and correct the above abnormalities, some patients still have recalcitrant pruritus. In our study, we chose to study specifically this recalcitrant pruritus.

Table 2-1. Proposed Causes of Uremic Pruritus

Uremia
Xerosis
Anemia (iron, or Vitamin B12)
Secondary Hyperparathyroidism
Ion Derangement (Calcium, Phosphorus, magnesium, and aluminum)
Defective Sweating
Mast Cell Degranulation
Retention of Poorly Dialyzable Molecules (e.g. morphine)
Pro-Inflammatory Cytokines in The Skin

2.9.1.1 Opioid as A Cause of Uremic Pruritus

The role of systemic circulating opioids in uremic pruritus remains controversial. The dramatic resolution of itch after renal transplantation led to the theory that a poorly dialyzable pruritogenic molecule could be the culprit causing uremic itch. Systemic, circulating opioids, acting in the central nervous system (CNS), are possible candidates.

Various pain-relieving opioids were found to accumulate in renal failure, and no consensus exists if the parent opioid compounds and their metabolites can be adequately dialyzed by hemodialysis (Dean 2004). The most studied drug has been morphine. Morphine and its metabolites are effectively removed from the blood during hemodialysis, but their levels rebound as they re-equilibrate between the CNS and plasma. Specifically, morphine metabolites accumulate in the blood stream between dialysis sessions.

Morphine activates the mu-opioid receptor (Ballantyne et al., 1988). Dynorphin activates the kappa-opioid receptor (Chavkin et al., 1982). In general, activation of mu-opioid receptors cause itch, while activation of kappa-opioid receptors has anti-pruritic effects. Studies correlating beta-endorphin (a mu-agonist) levels with itch intensity in uremic pruritus have been mixed with one study showing a positive correlation and the other, no correlation (Odou et al., 2001; Mettang et al., 1998). The efficacy of mu-opioid antagonists in treating uremic pruritus has been mixed as well (Andersen et al., 1984; Peer et al., 1996; Pauli-Magnus et al., 2000). Kappa-opioid agonists have shown mild-to-moderate efficacy in treating uremic pruritus. The itch reduction in kappa-opioid agonist nalfurafine 5mg group was 32–37% compared to a 20% decrease in the placebo group (Wikstrom et al., 2005; Kumagai et al., 2009). Thus, further work is required to elucidate the contribution of opioids to the pathophysiology of uremic pruritus. In this study, subjects taking opioid drugs were excluded in both the case and control groups.

2.9.1.2 Immunological Alteration as A Cause of Uremic Pruritus

Although there is no sign of inflammation in the skin of uremic pruritus. Immunological alteration, characterized by a pro-inflammatory pattern, is another theory proposed to explain uremic pruritus. In this theory, pro-inflammatory mediators, such as interlukin-2 (that enhance the Th1 response), have been suggested to mediate uremic pruritus (Mettang et al., 2002). However, experimental results have been mixed. For example, tacrolimus ointment, which suppresses TH1 lymphocyte differentiation, showed dramatic relief of itch in a pilot study of 3 patients (Pauli- Magnus et al., 2000) but no efficacy in another controlled study (Duque et al., 2005).

2.9.1.3 Other Postulated Causes of Uremic Pruritus

Other less-well characterized mechanisms of uremic pruritus include antigen sensitization from dialysis, elevated levels of serum magnesium, plasma essential fatty acid abnormalities, aluminum overload, erythropoietin deficiency, and the serotonin theory (Kurban et al., 2007).

2.9.2 ESRD Related Small Fiber Neuropathy

The sensory symptoms in ESRD subjects follow a pattern of length-dependent, small-fiber sensory neuropathy. Specifically, skin biopsies at the distal leg showed that IENF density is largely diminished in ESRD patients and negatively correlated with duration of

kidney disease (Lauria et al., 2010; Chao et al., 2011). There have not been any published articles to date reporting the relationship between IENF density and intensity of pruritus in either length-dependent or non-dependent areas, although one article observed a correlation between uremic pruritus and secondary neuropathy in ESRD subjects (Jedras et al., 1998). However, I could not evaluate the significance of these findings because only the abstract was available in English, and the abstract did not state how the relationship between uremic pruritus and secondary neuropathy was measured.

2.10 Hypotheses

2.10.1 Hypothesis 1: Uremic Pruritus is Partially Caused by Augmented Signaling Through the Histamine Independent, Protease-Activated Itch Pathway

As described in the Literature Review chapter, (2.4.4), clinical experience with topical capsaicin and narrow band UVB phototherapy indicate that peripheral sensitization of itch-signaling cutaneous nerves contributes significantly to uremic pruritus. The lack of efficacy of systemic anti-histamines to treat uremic pruritus further suggests that the histamine-dependent pathway does not contribute to uremic pruritus.

Whether peripheral sensitization of histamine-independent signaling cutaneous nerves contributes to uremic pruritus is unknown. We propose that histamine-indepndent protease-activated itch pathway is augmented in uremic pruritus. Cowhage-induced itch is a marker of histamine-independent itch pathway, but it has not been reported to date if alterations exist in cowhage responsiveness in the skin of uremic pruritus patients. To test

this hypothesis, we measured the time course and intensity of itch sensation from cowhage application in uremic pruritus and compared them with those in control subjects.

2.10.2 Hypothesis 2: Uremic Pruritus is Partially Caused by Persistent Cutaneous Itch Fiber Signaling in the Setting of Decreased Itch-Inhibiting Cutaneous Pain Fiber Signaling

As described in the Introduction chapter (2.4.2), evidence suggests that central sensitization also contributes to uremic pruritus Central sensitization to itch could arise by loss of itch-inhibiting spinal cord dorsal horn input from the selective loss of intraepidermal pain nerves with preservation of DEJ itch signaling fibers (Timmes et al., 2013). However, it is not been well-established if neuroanatomical changes occur in itch and/or pain-sensing epidermal and papillary dermal nerves in uremic pruritus.

To answer this question and test this hypothesis, we quantified epidermal nerves and papillary dermal nerves with different peptidergic markers for itch and/or pain in uremic pruritus subjects and compared them with those in controls.

CHAPTER 3. AIMS OF THE STUDY

Hypothesis 1: Uremic Pruritus is partially caused by augmented signaling through the histamine independent, protease-activated itch pathway.

(a) **Specific Aim 1:** Determine if cowhage-induced itch is augmented in uremic pruritus patients on hemodialysis compared to age- and sex-matched ESRD patients on hemodialysis without pruritus.

(b) **Specific Aim 2:** Determine if itch induced by central sensitization tests (brush-stroke, pin-prick and thermal tests) is present in uremic pruritus patients on hemodialysis compared to age- and sex-matched ESRD patients on hemodialysis without pruritus.

Hypothesis 2: Uremic pruritus is partially caused by persistent cutaneous itch fiber signaling in the presence of loss of itch-inhibitory cutaneous pain fiber signaling.

(a) **Specific Aim 1:** Determine the percentage of BNP-expressing or SP-expressing or CGRP-expressing nerve fibers at the dermoepidermal junction (DEJ) in the skin of ESRD patients on hemodialysis with and without uremic pruritus.

(b) **Specific Aim 2:** Determine the intraepidermal nerve fiber density (IENF) in the skin of ESRD patients on hemodialysis with and without uremic pruritus.

(c) **Specific Aim 3:** Determine if there is a difference in the percentage of BNP-expressing or SP-expressing or CGRP-expressing nerve fibers at the DEJ in the skin of ESRD patients on hemodialysis with and without uremic pruritus.

(d) **Specific Aim 4:** Determine if there is a difference in the IENF density in the skin of

ESRD patients on hemodialysis with and without uremic pruritus.

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CHAPTER 4. METHODOLOGY

4.1 Study Design

We performed a pilot study using a case-control design. All protocols were approved by Boston University Institutional Review Board (IRB) under the protocol number H-32330, and by DaVita Dialysis Center.

4.2 Time Frame

Subjects had a) one-time study visit lasting about 2.5 hrs and b) one optional suture removal visit.

4.3 Subject Recruitment

All English-speaking patients undergoing hemodialysis at DaVita Boston were informed individually about this study with the help of Dr. Jasvinder Bhatia. A flyer describing the study was also given to them. All staff and physicians working at DaVita Boston were informed about this study. Flyers (Appendix VII) describing the study were also placed in the waiting room of the Boston Medical Center (BMC) Dermatology outpatient clinic.

All subjects having end-stage renal disease were undergoing hemodialysis at DaVita Boston. All patients undergoing hemodialysis at DaVita Boston were asked individually about their itch status and their willingness to participate in the study. Only those who expressed willingness to join the study were screened with the screening questionnaire

(Appendix I). Inclusion and exclusion criteria (Appendix II) were applied to all subjects that passed the screening. Only subjects that met the criteria were recruited. In addition, we excluded subjects complaining of itch only from their lower legs or distal arms. One subject initially presented with generalized pruritus and so was first scheduled to be a case subject. During screening, his source of pruritus was identified as xerosis and not uremic pruritus. This subject's pruritus completely resolved after adequate moisturization. Subsequently, this subject was recruited into the control group. Recruitment was divided into two phases. Phase one was case-recruitment. Cases were defined as individuals with generalized uremic pruritus without other known causes and with a self-reported VAS score of average itch in the preceding week for itch of 30 or greater (out of 100), and concurrently on non-dialysis days. Phase two involved recruiting controls without pruritus who were age-and sex-matched with cases. Each individual control was selected to have an age range within 5 years of the matched case's age (Lauria et al., 2010).

4.4 Study Visit

Written consent took place in the outpatient Dermatology clinic at BMC. An IRB-approved Informed Consent Form was presented to the subject, and the details of the procedures, risks, benefits, alternatives, costs, etc., were outlined. The subject was given the opportunity to ask any and all questions regarding the study. Each subject was given a photocopy of the signed written consent.

After subjects were consented to the study, all subjects 1) reported duration of hemodialysis. 2) completed the frequency version Itchy Quality of Life (QoL) questionnaire (appendix III) (Desai et al., 2008), and completed three Visual Analogue Scale (VAS) scores (Appendix IV) to assess pruritus: average itch in the preceding week, worst itch in the preceding week, and itch on the day of the visit; 3) underwent 4 non-invasive tests of sensitization (described below); 4) underwent one 6 mm punch biopsy from itchy, normal-looking, non-scratched, non-eroded, non-ulcerated, lesion-free pruritic skin obtained from the lower back of all subjects to avoid length-dependent small fiber neuropathy (Chao et al., 2011).

4.4.1 Methods to Test Sensitization

Sensitization tests were performed on clinically normal-appearing pruritic skin located on the lower back close to the midline. Before the testing, subjects were taught the study definitions to describe cutaneous sensations, including itching, pain, burning, stinging, pricking, tingling, warm, and hot (Appendix V). Central sensitization tests included brush-stroke test, pin-rick test and thermal test.

4.4.1.1 Assessment of Brush-Stroke Induced Sensation

Test sites were stroked smoothly and lightly by a cotton swab attached to the end of a flexible coping saw blade at a rate of 1 Hz (Sikand et al., 2009). The participants were asked to report the evoked sensation (including presence of pain and/or itch) in five

separate trials. To be scored as present, the sensation has to be experienced in more than 1 out of the 5 trials.

4.4.1.2 Assessment of Pin-Prick Induced Sensation

Pinprick stimuli were applied to the skin with a series of probes consisting of the following: 2 mL or 10 mL syringe barrels containing a free-floating sterile 27 gauge cannula above which rested one of the following weights: 1.0, 2.3, 3.7, 8.6, or 14.8 g. Each probe was laid down slowly and perpendicularly onto the skin surface in a manner that prevents insertion into the skin, and then held for 2 seconds. The subjects were asked to describe the quality and intensity of the evoked sensation. The intensities of itch and pain were reported separately by drawing a line on a 100mm length Visual Analogue Scale (VAS) with one end of this scale anchored as no sensation and the other end anchored as maximal sensation imaginable (Appendix VI). This procedure was repeated 5 times with each load in the area of the skin. For reasons of hygiene and to eliminate cross-infection, fresh sterile needles and syringe barrels were used for each subject.

4.4.1.3 Assessment of Thermal Induced Sensation (sensation of mildly painful and non-damaging heat as itch)

A single thin metal rod (diameter of ~10mm) was preheated to 49 °C in a temperature-controlled water bath and then sequentially applied perpendicularly to the skin and held for 10 seconds. This temperature with this short length of application was not enough to cause thermal damage to the skin. The subjects were asked to rate the intensities of the

evoked itch and pain on the VAS as described in the "pin-prick induced sensation". This procedure was performed once in the same area of skin for all other sensory tests.

4.4.1.4 Assessment of Cowhage-Spicule Induced Sensation

The spicules were inserted by rubbing in a circular fashion limited to an area of ~1.5 cm^2 for 40 seconds (Papoiu et al., 2011) and approximately 40 spicules were placed on the skin at each trial. Given the tiny size of the spicule, subjects could not feel the actual act of the insertion. Inserting the spicule did not cut the patient or cause any damage to the skin. After inserting the spicule and every 30 seconds afterwards, the subjects were asked to describe the sensation they felt and record its maximum intensity on a labeled magnitude scale using a computer software program specifically written and adapted to this purpose. This computer program, created for the laboratory of Dr. LaMotte, was graciously shared with us as a courtesy.

This computer program uses a labeled magnitude scale (LaMotte et al., 2009; Sikand et al., 2009). The subjects rate the perceived intensity of itch, burning and pricking/tingling using the general version of the Labeled Magnitude Scale (gLMS). This scale presents subjects with intensity markers including "no sensation", "barely detectable", "weak", "moderate", "strong", "very strong" positioned at appropriate locations along the scale in relation to the "strongest imaginable sensation of any kind", that was placed at the top. Ratings of each of the three sensory qualities were obtained every 30 s from the moment of spicule insertion until 20 min elapsed or until each quality was judged as "barely

detectable" 3 times in a row. No subjects in either group exceeded the 20-minute mark. The subjects judged the magnitude of each quality by using a computer mouse to move the cursor along the scale as presented on a video screen. Each subject received prior training during which they used the gLMS to judge the magnitude of familiar sensory experiences such as the "sting of a bee" and the "itch from a mosquito bite." Any sensation rated below barely detectable on the scale was considered as "zero". These instructions on how to use the computer to assess the itch are included in the text for the description of different sensations (Appendix V). The generalized scale on the computer appears as a vertical line 230 mm high. Considering the scale to be 100 units, the labels were placed no sensation, 0; at barely detectable, 1.4; weak, 6; moderate, 17; strong, 34.7; very strong, 52.5; strongest imaginable sensation of any kind, 100.

This computer program only stores responses entered by the user with no other associated information. The files containing these data were named with the subject's unique study ID and the date of acquisition.

4.4.2 Biopsies

The principal investigator (Deon Wolpowitz, MD, Ph.D.) performed a single 6mm punch biopsy procedure at the area where the sensitization tests were performed, according to standard of care practices. Biopsy sites were closed using non-absorbing sutures. Sutures were removed 2 weeks after the biopsy either at the outpatient clinic of the Dermatology Department or at DaVita Dialysis Center. Wound care instructions were discussed with

the subject, including daily application of a Band-Aid to the dry wound covered with plain petroleum jelly until it healed. Specimens were labeled with the date of visit and subjects/control's unique identifying code only. Left over tissue was saved in a repository with informed, written consent, otherwise the left over tissue was discarded after the study.

4.5 Immunohistochemistry

Skin tissue were placed directly into Periodate-lysine-paraformaldehyde (PLP) fixative for 24 hrs, then cryoprotected with 20% sucrose in 0.1M Sorrenson's buffer, frozen with dry ice, then cut into 50μm sections with a cryostat. Skin tissue sections were then processed for multi-labeled immunofluorescence localization of nerves with the following combinations of primary antibodies applied in the sequential order listed: rabbit anti-NPPB, guinea pig anti-Protein Gene Product (PGP)9.5, then mouse anti-CGRP; rabbit anti-NPPB, mouse anti-PGP9.5, then guinea pig anti-SP. Rabbit anti-TRPV-1 was used for single-label immunofluorescence. Sequential fluorophore-conjugated tyramide detection (Perkin Elmer, Waltham, MA/Invitrogen, Grand Island, NY) was accomplished by horse-radish peroxidase (hrp) inactivation with hydrogen peroxide incubation. For each antibody combination, appropriate negative controls omitting each primary antibody were carried out, confirming complete inactivation of the first hrp-conjugate as well as absence of cross-reactivity of the secondary antibodies.

4.5.1 Primary Antibodies

Primary antibodies were as follows: guinea pig anti-substance P (SP, 1:6000; Neuromics, Edina, MN), mouse anti-calcitonin-gene-related peptide (CGRP, 1**:**250; Enzo Life Sciences), mouse anti-protein gene product 9.5 (PGP9.5, 1:1500; Abcam, Cambridge, MA), guinea pig anti-protein gene product 9.5 (PGP9.5, 1:750, Neuromics), rabbit anti-NPPB antibody (NPPB, 1:750; Proteintech, Chicago, IL), and rabbit anti-TRPV-1 (TRPV-1, 1:175, Pierce, Rockford, IL). Secondary antibodies conjugated to hrp, were donkey anti-mouse, donkey anti-guinea pig, which were obtained from Jackson Immunoresearch (West Grove, PA) and vector rabbit from ImmPRESS reagents (Vector Labs, Burlingame, CA).

4.6 Nerve Length Quantitation

All skin specimens derived from the 6mm punch biopsy. Three consecutive sections from each specimen, corresponding to the central, largest portion of the biopsy, were used to represent each subject in nerve length quantitation. For each 50 µm section, fluorescence images were acquired at 4 µm intervals throughout the depth of the specimen using a 20x lens (Z-series), separately for each fluorescent signal, using the Nikon deconvolution wide-field epifluorescence system. To quantify nerve lengths in these sections, Nikon software was used to image two pieces of each section in consecutive, non-overlapping 20x fields. In the triple-labeled specimens, each piece was constructed by tiling 3X2 Z-stacks into one composite Z-stack. Each 3X2 tiling Z-stack gave an average linear epidermal length of 1.3mm, so two 3X2 tiling Z-stacks with average 2.7mm linear

epidermal length were chosen to represent the whole section, which was comparable to a 3mm biopsy. Each 3X2 tiling field was chosen where the most NPPB-staining nerves were visually seen in the triple-label staining. In the single-label immunofluorescence with rabbit anti-TRPV-1, each piece was constructed with 4X2 tiling Z-stack. Each 4X2 tiling Z-stack gave an average linear epidermal length of at least 1.6mm. Two 4X2 Z-stacks with linear epidermal length 3.2mm were chosen to represent the section. Location of the two pieces in one section applies to the other two sections from the same specimen. In all cases, the imaging field had at its most rostral edge, the stratum corneum. Thus, images contained the entire epidermis, papillary dermis, at least the superficial reticular dermis (including a depth of at least 300 μm beneath the dermal-epidermal junction). Three-dimensional reconstruction of each Z-series was produced using Image J software (NIH) with the micro-manager plug-in. Each Z-series was then projected into a single in-focus image (Z-projection), and nerve fibers within the papillary dermis or epidermis, corresponding to single, double, or triple-labeled fibers were identified by manual comparison of the respective fluorescent Z-series. Neuron J software was used to manually trace nerve fibers in each Z-projection as well as to quantitate the resulting nerve length (Hirai et al., 2000; Meijering et al., 2004 ; Timmes et al., 2013).

4.7 Intraepidermal Nerve Fiber (IENF) Density

For IENF quantitation, 3 consecutive sections from each specimen, corresponding to the central, largest portion of the biopsy, were stained with TRPV-1. Intraepidermal nerve

fiber densities were determined under epifluorescence microscope (Nikon Eclipse E400, TRITC channel) using standard procedures, counting only branches that cross the dermoepidermal junction (DEJ) at 40x (objective) and 10x (eyepiece) (Lauria et al., 2005). Only the pieces that were imaged for nerve length quantitation were used for IENF counting. Image J was used to measure the linear length of epidermis.

4.8 Data Analysis

1) Duration of Hemodialysis: mean and standard deviation (SD) were used to represent the cases/controls group. Student's t-test with equal variances was used to compare cases and controls, to compare female subjects in cases and controls and to compare male subjects in cases and controls.

2) Three VAS scores: median and 25–75% quantiles were used to represent the cases/controls group. Non-parametric Wilcoxon rank-sum test was used to compare cases and controls, to compare female subjects in cases and controls, to compare male subjects in cases and controls, to compare female vs. male subjects within cases, and to compare female vs. male subjects within controls.

3) QoL questionnaire: scores were enumerated as indicated in the QoL questionnaire (1 = never, 2 = rarely, 3 = sometimes, 4 = often, 5 = all the time), then it was categorized into total symptoms score, total functioning score and total emotions score as follows. Median

and 25–75% quantiles were used to represent the cases/controls group. Non-parametric Wilcoxon rank-sum test was used to compare cases and controls, to compare female subjects in cases and controls, to compare male subjects in cases and controls, to compare female vs. male subjects within cases, and to compare female vs. male subjects within controls.

Total symptoms score = average of responses (Question: 1–6)

Total functioning score = average of responses (Question: 7–16)

Total emotions score = average of responses (Question: 17–22)

4) Assessment of brush-stroke induced sensation: more than once rating positive itch or pain out of five rounds was required to qualify that subject as having itch or pain induced from cotton swab rubbing, and was scored as 1. Zero or a single rating positive for itch or pain was deemed as a negative sensation for itch or pain, and was scored as 0. Median and 25–75% quantiles were used to represent the cases/controls group. Non-parametric Wilcoxon rank-sum test was used to compare cases and controls.

5) Pin-prick induced sensation: for each subject the mean of five ratings of either itch or pain was used to represent the individual. Median and 25–75% quantiles were used to represent cases/controls group. Non-parametric Wilcoxon rank-sum test was used to compare cases and controls.

6) Thermal-induced sensation: Median and 25–75% quantiles were used to represent cases/controls group. Non-parametric Wilcoxon rank-sum test was used to compare cases and controls.

7) Assessment of cowhage-spicule induced sensation: From the ratings of each sensory quality for each subject, the values of the following parameters were obtained: the peak magnitude of the ratings, the time from the onset of sensation to the peak, the duration of sensation (time between onset of sensation and the first of the three consecutive ratings of zero) and the area under the curve (AUC). Median and 25–75% quantiles were used to represent case/control groups. Non-parametric Wilcoxon rank-sum test was used to compare cases and controls. Cowhage data from subject 1 was excluded for analysis because this subject could not be adequately trained either to understand the different cutaneous sensations or how to use the computer software to rate them. Accordingly, subject 1 was excluded from correlation analysis involving cowhage spicule-induced sensations. Because subject 1 could adequately understand how to rate itch using the VAS scales, quantitation of nerves from subject 1 was included for comparison of nerve measurements between cases and controls and correlation studies with a VAS score.

8) IENF density: IENF number was determined in three consecutive sections and the sum divided by the total linear length of epidermis in these three sections. Mean and standard deviation (SD) were used to represent case and control groups. Two-tailed Student's t-test was used to compare cases and controls.

9) Nerve Length Quantitation: Two areas of each tissue section were selected depending on the antibodies used. In triple immunohistochemical stains that included NPPB, the areas visually bearing the most NPPB nerves were selected. For the TRPV-1 immunohistochemical stain, bilateral edges of each piece were selected. The values from each area were combined to obtain a single representative value for that entire tissue section. Each section was treated as one data point for the individual subject. For each tissue section, epidermis length was measured using Image J software. Epidermis to total epidermal and papillary dermal ratio of TRPV-1 nerve length (%Epi) was calculated as the total nerve length of TRPV-1 in epidermis divided by the sum of the total nerve length of TRPV-1 in both the epidermis and papillary dermis. Papillary dermis to total epidermal and papillary dermal ratio of TRPV-1 nerve length (%PD) was calculated as the total nerve length of TRPV-1 in the papillary dermis divided by the total nerve length of TRPV-1 in both the epidermis and papillary dermis. Intraepidermal TRPV-1 nerve length (IENL)/mm epidermis was calculated as the total nerve length of TRPV-1 in epidermis divided by the linear length of epidermis as determined by the linear length of the stratum corneum. Papillary dermal TRPV-1 nerve length (PDNL)/mm epidermis was calculated as the total nerve length of TRPV-1 in papillary dermis divided by the linear length of epidermis as determined by the linear length of the stratum corneum. Mean and standard deviation (SD) were used to represent case and control groups. Student's t-test with unequal variances was used to compare cases and controls in %Epi, %PD, IENL/mm epidermis and PDNL/mm epidermis.

For calculation of ratio of CGRP nerve length to total papillary dermal (pd)-PGP9.5 nerve length (%PD-CGRP(+)), ratio of SP nerve length to total pd-PGP9.5 nerve length (%PD-SP(+)), ratio of NPPB nerve length to total pd-PGP9.5 nerve length (%PD-NPPB(+)), ratio of CGRP(+)/NPPB(-) nerve length to total pd-PGP9.5 nerve length (%PD-CGRP(+)/NPPB(-)), ratio of CGRP(+)/NPPB(+) nerve length to total pd-PGP9.5 nerve length(%PD-CGRP(+)/NPPB(+)), ratio of CGRP(-)/NPPB(+) nerve length to total pd-PGP9.5 nerve length(%PD-CGRP(-)/NPPB(+)), ratio of SP(+)/NPPB(-) nerve length to total pd-PGP9.5 nerve length(%PD-SP(+)/NPPB(-)), ratio of SP(+)/NPPB(+) nerve length to total pd-PGP9.5 nerve length(%PD-SP(+)/NPPB(+)), and ratio of SP(-)/NPPB(+) nerve length to total pd-PGP9.5 nerve length(%PD-SP(-)/NPPB(+)), individual nerve length with each specific marker was divided by total PGP9.5 nerve length in the papillary dermis. Median and 25–75% quantiles were used to represent case and control groups. Non-parametric Wilcoxon rank-sum test was used to compare cases and controls.

In subgroup quantification: For each tissue section from each tissue specimen, the "% of (marker A) nerve length that is also positive for (marker B)" was calculated as the total amount of a nerve length in that entire tissue section that co-expressed (markers A and B) divided by the total amount of nerve length in the papillary dermis in that entire tissue section expressing (marker A). %CGRP that is NPPB(+), %CGRP that is NPPB(-), %NPPB that is CGRP(+), %NPPB that is CGRP(-), %NPPB that is SP(+), %NPPB that is SP(-), %SP that is NPPB(+) and %SP that is NPPB(-) were measured. Median and 25–

75% quantiles were used to represent case and control groups. Non-parametric Wilcoxon rank-sum test was used to compare cases and controls.

10) Correlation analysis: the distribution of the Spearman rank correlation coefficient under the null hypothesis was computed through permutation test (10,000 permutations per variable) using R statistical software. Correlation analysis was used to test relationship between two variables with the outcome defined as the correlation coefficient(CC). CC was a value between -1 to +1 and shows if changes in one item will result in changes in the other item. For correlations involving nerve measurements, the total respective nerve lengths, obtained from all tissue sections from a given biopsy specimen, were aggregated and then used to generate a single cumulative respective nerve length percentage for the entire tissue specimen. There were four sets of correlation analyses performed: 1) nerve measurements and cowhage-induced itch data; 2) nerve measurements and VAS score-average itch preceding week; 3) duration of hemodialysis and VAS- average itch preceding week; and 4) duration of hemodialysis and cowhage-induced itch data.

11) Software: JMPPro software (JMP, Cary, NC) was used to identify medians and quantiles; mean and standard deviation; and to perform non-parametric Wilcoxon rank-sum test or Student's t-test.

12) P value: P value <0.05 for Wilcoxon rank-sum test, two-tailed Student's t-test and correlation was defined as significant.

CHAPTER 5. FINDINGS

5.1 Demographics of Subjects (Table 5-1)

12 cases and 13 controls were recruited. There were 6 females and 6 males in the case group. There were 7 females and 6 males in the control group. 9 out of 12 cases were African American/Hispanic and 3 out of 12 cases were Caucasian. 10 out of 13 controls were African-American/Hispanic, 3 out of 13 controls were Caucasian. The ratio of African-American/Hispanic to Caucasian subjects in the cases and controls were balanced (p=1.0). The predominantly African-American population in our study resulted from the skewed African-American population at DaVita Dialysis Center Boston. No significant differences existed in age between cases and controls, including when subgrouped by sex (Table 4-1). The median age of cases vs. controls was 55 years (25–75% quantiles: 45.75–70.75) vs. 59 years (51–72). In the group of males, the median age of cases vs. controls was 55 years (41.5–71.25) vs. 55 years (45–73.5). In the group of females, the median age of cases vs. controls was 59 years (47.25–72.25) vs. 67 years (51.00–71.00). There was no difference in hemodialysis duration between cases and controls, even when sub-grouped by sex. Moreover, within cases or controls group, there was also no difference of hemodialysis duration between female and male (cases: p=0.52; controls: p=0.60), consistent with the literature that uremic pruritus is independent of dialysis duration (Bernhard, 1994).

Table 5-1. Demographics of Subjects

	Cases	**Controls**	**P value**
Total	12	13	1.00
Males	6	6	1.00
Females	6	7	1.00
Black/Hispanics	9	10	1.00
White	3	3	1.00
Age, all subject (year) (median, 25%–75%)	55.00 (45.75–70.75)	59.00 (51.00–72.00)	0.67
Age, males	55.00 (41.50–71.25)	55.00 (45.00–73.50)	0.86
Age, females	59.00 (47.25–72.25)	67.00 (51.00–71.00)	0.71
Hemodialysis Duration (year) (mean±SD*)	3.73 ± 2.18	2.73 ± 2.23	0.28
Hemodialysis Duration, males (year)	4.15 ± 1.85	3.09 ± 2.59	0.44
Hemodialysis Duration, females (year)	3.23 ± 2.65	2.41 ± 2.03	0.55

*SD: Standard Deviation

5.2 Itch Characterization (Table 5-2, 5-3)

Three VAS scores were obtained to initiate the study visit: average itch for the preceding week, worst itch for the preceding week, and itch on the day of the study visit. There were no differences in VAS scores between females and males. VAS scores of cases, for all three measures, were significantly higher than those of controls. These data confirm that we have recruited two distinct populations of end-stage renal disease (ESRD) subjects: one with and one without pruritus. All cases reported itch involving the trunk area, especially the back, while some reported itch also involving the face and shoulders. Other areas with pruritus in individual subjects included, but were not limited to, the arms and legs.

Table 5-2. Itch Characterization (median, 25%–75% quantiles)

	Cases	Controls	P value
VAS, day of visit	41.50 (33.75–69.00)	0 (0–0.50)	<0.0001
VAS, average itch preceding week	78.00 (64.25–85.75)	0 (0–5.00)	<0.0001
VAS, worst itch preceding week	85.50 (73.00–93.75)	0 (0–3.00)	<0.0001
VAS, day of visit—female	66.00 (34.50–72.75)	0 (0–0)	0.002
VAS, average itch preceding week—female	80.00 (63.25–96.25)	0 (0–0)	0.002
VAS, worst itch preceding week— female	87.00 (67.50–91.25)	0 (0–0)	0.002
VAS, day of visit—male	40.50 (31.25–54.00)	0 (0–5.00)	0.004
VAS, average itch preceding week—male	78.00 (60.75–85.25)	3.50 (0–15.25)	0.005
VAS, worst itch preceding week—male	84.00 (75.00–98.50)	2.00 (0–22.25)	0.005

VAS: Visual Analogue Scale

Table 5-3. Itch Characterization by Sex (median, 25%–75% quantiles)

	Female	**Male**	**P value**
VAS, day of visit—controls	0 (0–0)	0 (0–5.00)	0.50
VAS, average itch preceding week—controls	0 (0–0)	3.50 (0–15.25)	0.06
VAS, worse itch preceding week—controls	0 (0–0)	2.00 (0–22.25)	0.07
VAS, day of visit— cases	66.00 (34.50–72.75)	40.50 (31.25–54.00)	0.47
VAS, average itch preceding week—cases	80.00 (63.25–96.25)	78.00 (60.75–85.25)	0.75
VAS, worse itch preceding week—cases	87.00 (67.25–91.25)	84.00 (75.00–98.50)	0.81

VAS: Visual Analogue Scale

5.3 Itchy QoL Frequency Questionnaire (Table 5-4, 5-5)

Cases exhibited higher scores compared to controls in total symptoms score (3.00 vs. 1.00), total functioning score (3.40 vs. 1.00) and total emotions score (2.59 vs. 1.00), regardless of sex. These data confirm that the itchy subjects experienced clinically meaningful pruritus that adversely affected their quality of life. In the cases, the ratings for all three were comparable to ratings for patients with pruritus from dermatitis, urticaria, and idiopathic causes (Desai et al., 2008)

Table 5-4. Itchy QoL Frequency Questionnaire (median, 25%–75% quantiles)

	Cases	**Controls**	**P value**
Itchy QoL total symptoms score	3.00 (2.54–3.42)	1.00 (1.00–1.00)	<0.0001
Itchy QoL total functioning score	3.40 (2.88–3.88)	1.00 (1.00–1.00)	<0.0001
Itchy QoL total emotions score	2.59 (1.87–3.67)	1.00 (1.00–1.00)	<0.0001
Itchy QoL total symptoms score—female	3.00 (2.83–3.75)	1.00 (1.00–1.17)	0.002
Itchy QoL total functioning score—female	3.45 (3.33–4.03)	1.00 (1.00–1.00)	0.002
Itchy QoL total emotions score—female	3.50 (1.96–4.29)	1.00 (1.00–1.00)	0.002
Itchy QoL total symptoms score—male	2.75 (2.46–3.54)	1.00 (1.00–1.00)	0.003
Itchy QoL total functioning score—male	3.00 (2.25–3.60)	1.00 (1.00–1.00)	0.003
Itchy QoL total emotions score—male	2.42 (1.67–2.92)	1.00 (1.00–1.00)	0.003

QoL: Quality of Life

Table 5-5. Itchy QoL Frequency Questionnaire by Sex (median, 25%–75% quantiles)

	Female	Male	P value
Itchy QoL total symptoms score—controls	1.00 (1.00–1.17)	1.00 (1.00–1.00)	0.21
Itchy QoL total functioning score—controls	1.00 (1.00–1.00)	1.00 (1.00–1.00)	0.44
Itchy QoL total emotions score—controls	1.00 (1.00–1.00)	1.00 (1.00–1.00)	1
Itchy QoL total symptoms score—cases	3.00 (2.83–3.75)	2.75 (2.46–3.54)	0.52
Itchy QoL total functioning score—cases	3.45(3.33–4.03)	3.00 (2.25–3.60)	0.17
Itchy QoL total emotions score—cases	3.50 (1.96–4.29)	2.42 (1.67–2.92)	0.20

QoL: Quality of Life

5.4 Tests for Sensory Sensitization

5.4.1 Brush-Stroke Induced Sensation (Table 5-6)

Brush-stroke sensation was induced by gently stroking with a cotton swab. Alloknesis (the sensation of itch induced by normally non-itchy mechanical stimuli) from this brush stroke was absent in 75% (9 out of 12) of cases and was absent in 92.3% (12 out of 13) of controls. Allodynia is the inappropriate perception of pain from non-painful stimuli. Brush-stroke induced allodynia was absent in cases and controls. There was no significant difference between cases and controls in regard to the presence or absence of either alloknesis or allodynia.

Table 5-6. Brush-Stroke Induced Sensation (median, 25%–75% quantiles)

	Cases	**Controls**	**P value**
cotton swab rubbing—itch (alloknesis)	0 (0–0.75)	0 (0–0)	0.27
cotton swab rubbing—pain (allodynia)	0 (0–0)	0 (0–0)	1.00

5.4.2 Pin-Prick Induced Sensation

Tests for pain and/or itch induced by a pin-prick carrying a load of 1.0 gm, 2.3 gm, 8.6 gm and 14.8 gm were performed.

5.4.2.1 Pin-Prick Induced Pain (Punctate Hyperalgesia)

Cases reported more pin-prick induced pain than controls. Pin-prick carrying a load of 1.0 gm induced pain in 4/12 cases, 0/13 controls. Pin-prick carrying a load of 2.3 gm induced pain in 4/12 cases, 1/13 controls. Pin-prick carrying a load of 8.6 gm induced pain in 4/12 cases, 1/13 controls. Pin-prick carrying a load of 14.8 gm induced pain in 5/12 cases, 2/13 controls. Nevertheless, for all tested weights, only the 1.0 gm load showed a statistically significant difference in the amount of pinprick-induced pain between cases and controls (Table 5-7). Also, the low intensity of pain experienced by cases (median, 2.5 out of 100, Table 5-7) was of questionable biologic relevance. Thus, punctate hyperalgesia (enhanced pin-prick induced pain) was absent in cases. For either cases or controls, there were no statistically significant differences in pain sensation with increasing weight loads (Table 5-8). These findings indicate cases and controls exhibit hypoalgesia to pin-prick pain. This interpretation is not favored given that subjects experienced pain from the local anesthetic injection. Instead, these results are interpreted as experimental error secondary to the weights selected not being heavy enough to induce meaningful pin-prick pain. The weights may have been insufficient either because of the increased thickness of back skin and/or because these subjects have an unusually high pain tolerance from being regularly subjected to more intense mechanical noxious stimuli (i.e., dialysis with wide-bore needles).

Table 5-7. Pin-Prick Induced Pain (Punctate Hyperalgesia) (median, 25%–75% quantiles)

Weights (gm)	Cases–-pain	Controls–-pain	P value
1.00	2.50 (0–10.30)	0 (0–0.10)	0.03
2.30	0 (0–17.10)	0 (0–0.50)	0.31
8.60	0 (0–21.10)	0 (0–1.00)	0.34
14.80	3.50 (0–29.90)	0 (0–2.90)	0.17

Table 5-8. Pin-Prick Induced Pain Comparison of Different Weight Loads

Pain		Cases	Controls
Level	Level	P value	P value
weight 1.00	weight 14.80	0.42	0.18
weight 2.30	weight 8.40	0.90	0.88
weight 1.00	weight 8.40	0.90	0.60
weight 1.00	weight 2.30	0.67	0.67
weight 14.80	weight 8.40	0.41	0.33
weight 14.80	weight 2.30	0.26	0.29

5.4.2.2 Pin-Prick Induced Itch (Punctate Hyperknesis)

Cases experienced significantly more pin-prick induced itch versus controls (Table 5-9).

However, the biological significance of this difference is uncertain give the relatively low

level of itch induced (VAS range, 1.4 to 3.1 out of 100) and absence of significant differences in itch-induction for different weights within cases or controls (Table 5-10).

Table 5-9. Pin-Prick Induced Itch (Punctate Hyperknesis) (median, 25%–75% quantiles)

Weights (gm)	Cases–-itch	Controls–-itch	P value
1.00	1.40 (0–17.15)	0 (0–0)	0.03
2.30	1.40 (0–34.40)	0 (0–0)	0.02
8.40	3.10 (0–39.35)	0 (0–0)	0.04
14.80	1.90 (0–40.30)	0 (0–0)	0.005

Table 5-10. Pin-Prick Induced Itch Comparison of Different Weight Loads

Itch		Cases	Controls
Level	Level	P value	P value
weight 1.00	weight 14.80	0.78	0.36
weight 2.30	weight 8.40	0.88	0.64
weight 1.00	weight 8.40	0.64	0.64
weight 1.00	weight 2.30	0.85	1.00
weight 14.80	weight 8.40	0.93	0.17
weight 14.80	weight 2.30	1.00	0.36

Punctate hyperalgesia and punctate hyperknesis are mediated by Aδ-fibers (Schmelz 2006). Immunohistochemical staining with an antibody to neurofilament (NF)-200 to identify Aδ-fibers was not performed.

5.4.3 Thermal Induced Sensation

Thermal-induced sensations of heat pain and/or itch were not technically possible in all subjects. A metal rod was preheated to 49 °C in a water bath then applied to the skin for 10 sec with one cross-section end touching the skin. Pain was induced in only 4 subjects in total, 2 cases and 2 controls. Itch was induced in 3 cases and 1 control. There were no statistically significant differences between cases and controls for itch or pain sensation (Table 5-11). We attribute these results to rapid dissipation of heat from the metal rod, and its subsequent inability to preserve the desired temperature for 10 seconds outside of the water bath. Thus, heat pain or heat-induced itch could not be meaningfully assessed in cases or controls.

Table 5-11. Thermal Induced Sensation (median, 25%–75% quantile)

	Cases	**Controls**	**P value**
Heat--itch	0 (0–1.50)	0 (0–0)	0.29
Heat--pain	0 (0–1)	0 (0–0)	0.74

5.4.4 Cowhage-Spicule Induced Sensation (Table 5-12)

Cowhage spicules induce cutaneous sensations via the histamine-independent, protease-dependent pathway (Steinhoff et al., 2003). Cowhage produces predominantly itch with lesser components of pricking/tingling and burning (LaMotte et al., 2009). The dimensions and intensities of cowhage-spicule induced itch were measured to evaluate for sensitization of this histamine-independent itch pathway. Our data show that area under the curve (AUC) for itching (-itching) and peak perceived intensity of itch (peak itch) were significantly higher in cases compared to controls (Table 5-12). There was no statistical difference in prickling/tingling or burning between cases or controls.

Table 5-12. Cowhage-Spicule Induced Sensation (median, 25%–75% quantile)

	Cases	Controls	P value
peak itch	53.60 (53.30–78.80)	34.20 (20.90–55.60)	0.02
AUC* itching	175.40 (101.00–252.20)	42.40 (24.00–160.70)	0.04
time to peak itch	2.50 (1.00–3.00)	1.00 (0.50–1.50)	0.06
total time itch	7.00 (5.50–9.50)	4.00 (3.00–7.25)	0.11
peak burning	70.20 (53.10–91.00)	52.80 (11.50–76.70)	0.17
AUC burning	183.80 (80.30–266.40)	38.80 (3.30–156.90)	0.16
time to peak burning	1.00 (0.50–4.00)	0.50 (0.50–0.80)	0.05
total time burning	5.50 (2.00–8.00)	2.50 (1.80–3.50)	0.15
peak pricking/tingling	52.90 (18.20–59.10)	17.00 (0.30–66.40)	0.26
AUC pricking/tingling	105.80 (27.40–201.00)	12.70 (0.30–165.00)	0.27
time to peak pricking/tingling	1.50 (1.00–2.50)	1.00 (0.30–1.50)	0.10
total time pricking/tingling	5.50 (2.50–6.50)	2.50 (0.80–4.50)	0.10

*AUC, Area Under the Curve

Table 5-13. Comparison of Cowhage-Spicule Induced Sensation within Cases or Controls

		Cases	Controls
		Cases	**Controls**
Level	Level	**P value**	**P value**
AUC* itching	AUC burning	0.69	0.63
AUC pricking/tingling	AUC burning	0.32	0.49
AUC pricking/ting	AUC itching	0.15	0.27

*AUC, Area Under the Curve

5.5 Analysis of Epidermal and Papillary Dermal Innervations (Table 5-13)

5.5.1 Intraepidermal Nerve Fiber (IENF) Density

Cases showed an IENF density of 13.3±7.9/mm, and controls showed an IENF density of 17.7±3.8/mm. IENF density was not statistically significantly different between cases and controls (p=0.10).

5.5.2 Nerve Length Quantitation

5.5.2.1 Description of Nerve Innervation in Subjects' Skin

In all subjects, CGRP, NPPB and SP positive nerves reside in the dermis, predominantly the papillary dermis. None of the nerves staining with these three peptidergic markers crossed the dermal-epidermal junction and into the epidermis.

In controls, of the three peptidergic nerve markers, calcitonin gene related peptide (CGRP) comprised the highest percentage of nerves in the papillary dermis (median, 28%). Substance P (SP) comprised ~3% and natriuetic polypeptide precursor B (NPPB), ~1.8% of papillary dermal nerves. In cases, CGRP still comprised the highest percentage in the papillary dermis (median, 8%). SP comprised about 3% and NPPB comprised about 0.8% of papillary dermal nerves.

In controls, more than 80% of NPPB(+) nerves were CGRP(+). In cases, NPPB(+) nerves rarely co-stained with CGRP(+) nerves (% PD-NPPB(+)/CGRP(+): median (25%–75%

quantiles), 0% (0–0.8%)). In all subjects, NPPB(+) nerves only rarely co-expressed SP(+) (% PD-NPPB(+)/SP(+): median (25%–75% quantiles), cases 0% (0–0), controls 0% (0–0.1%)). In controls, the majority of CGRP(+) nerves were NPPB(-) (%CGRP that is NPPB(-): median, 87.9%). In cases, almost all CGRP(+) nerves were NPPB(-) (median, 100%). In all subjects, almost all SP(+) nerves were also NPPB(-) (median, 100%).

5.5.2.2 Comparison of Epidermal Innervation in Cases and Controls

In both cases and controls, there was no statistical difference in %Epi between cases (24.80%) and controls (25.30%). In controls, IENL/mm was 963.0 ± 294.8/mm; in cases, IENL/mm was 854.1 ± 572.5/mm. There was no statistical difference in IENL/mm between cases and controls. Taken together, there was no difference in epidermal innervation in cases and controls.

5.5.2.3 Comparison of Dermal Innervation in Cases and Controls

Consistent with no difference in %Epi in cases and controls, there was no difference in %PD in cases and controls, with about 75% of total nerves residing in the papillary dermis. However, PDNL/mm was 20% lower in cases compared to controls (p=0.0009). In controls, PDNL/mm was 2952.60 ± 855.90/mm; in cases, PDNL/mm was 2241.60 ± 917.14/mm. Consistent with the loss of papillary dermal nerves, %PD-CGRP(+) was also reduced in cases versus controls (p<0.0001), from 28% in controls to 7% in cases (corresponding to an absolute reduction of 20% of the entire nerve population and a relative 75% reduction in the value of %PD-CGRP(+) nerves). We therefore attribute the

reduction in PDNL/mm in cases to a decrease in number of CGRP nerves in the papillary dermis. The reason behind the discrepancy in %PD and PDNL/mm was the epidermal nerve length was taken into account in calculating %PD. Given the variability in epidermal nerve length, we cannot exclude that with a larger sample size, %PD could become statistically significant.

The population of CGRP(+) nerves in the papillary dermis was further divided into CGRP(+)/NPPB(+) nerves and CGRP(+)/NPPB(-) nerves. When each one is normalized to the total papillary dermal PGP9.5 nerve length, both percentages were significantly reduced in cases when compared to ($p = 0.0003$, $p < 0.0001$, respectively). Furthermore, the ratio of CGRP(+)/NPPB(+) nerves to the total CGRP(+) nerve population, expressed as %CGRP that is NPPB(+), was statistically significantly reduced (cases vs. controls (median): 0% vs. 12.10%; $p=0.03$).

In cases, %PD-NPPB(+) was also reduced, (cases vs. controls: 0.8% (0%–2.9%) vs. 1.8% (0.2%–5.2%); $p=0.01$). NPPB(+) nerves in the papillary dermis were further divided into NPPB(+)/CGRP(+) and NPPB(+)/CGRP(-) nerves. There was a statistically significant reduction of NPPB(+)/CGRP(+) nerves, with preservation of NPPB(+)/CGRP(-) nerves. The reduction of NPPB(+)/CGRP(+) nerves can be expressed in two ways. The first was by comparing NPPB(+)/CGRP(+) nerves to total papillary dermal nerves, expressed as %PD- NPPB(+)/CGRP(+) nerves, that was statistically significantly reduced in cases versus controls ($p= 0.0003$). The second was by comparing NPPB(+)/CGRP(+) nerves to

total papillary dermal NPPB(+) nerves, expressed as %PD-NPPB(+) that is CGRP(+), that was also significantly decreased in cases versus controls (p= 0.0002). Thus, we conclude that loss of papillary dermal NPPB nerves was attributable to the loss of papillary dermal CGRP nerves.

There was no significant difference between cases and controls in regard to %PD-SP(+), %PD-NPPB(-)/SP(+) and % PD-NPPB(+)/SP(+). Virtually no NPPB and SP co-staining nerves were identified in the papillary dermis in both cases and controls. Thus, we conclude that there was no change in SP expression in the papillary dermal nerves in cases compared to controls. In accordance with the above results, %NPPB that was SP(+), %NPPB that was SP(-), %SP that was NPPB(+), and %SP that was NPPB(-), were unchanged in cases compared to controls. In conclusion, changes in the neuroanatomy of SP-expressing cutaneous nerves were of little importance in the pathophysiology of uremic pruritus.

Table 5-14. Analysis of Epidermal and Papillary Dermal Innervation (median, 25%–75% quantiles)

	Cases	Controls	P value
IENF*§	13.30 ± 7.90	17.70 ± 3.80	0.10
IENL/mm*§	854.10 ± 572.50	963.00 ± 294.80	0.31
%Epi §	24.80 ± 11.30	25.30 ± 7.00	0.85
PDNL*/mm§	2241.60 ± 917.14	2952.60 ± 855.90	0.0009
%PD*§	75.20 ± 11.30	74.70 ± 7.00	0.85
%PD-CGRP* (+)	7.20 (1.90–17.90)	28.10 (19.70–36.20)	<0.0001
%PD-SP* (+)	2.70 (0.80–4.70)	3.60 (2.00–5.90)	0.10
%PD-NPPB* (+)	0.80 (0–2.90)	1.80 (0.20–5.20)	0.01
%PD-NPPB(+)/CGRP(-)	0.60 (0–1.20)	0.40 (0–0.90)	0.30
%PD-NPPB(-)/CGRP(+)	6.80 (1.70–16.60)	22.80 (12.50–31.40)	<0.0001
%PD-NPPB(+)/CGRP(+)	0 (0–0.80)	3.00 (0–8.20)	0.0003
%PD-NPPB(+)/SP(-)	0.80 (0–1.60)	1.20 (0–3.40)	0.32
%PD-NPPB(-)/SP(+)	2.10 (0.60–4.70)	3.30 (1.40–5.60)	0.15
%PD-NPPB(+)/SP(+)	0 (0–0)	0 (0–0.10)	0.37
%NPPB that is CGRP(-)	44.10 (0–100)	7.90 (0–24.90)	0.06
%NPPB that is CGRP(+)	0 (0–50.70)	84.20 (0–97.80)	0.0002
%CGRP that is NPPB(-)	100.00 (68.30–100.00)	87.90 (70.00–100.00)	0.16
%CGRP that is NPPB(+)	0 (0–15.90)	12.10 (0–30.00)	0.03
%NPPB that is SP(-)	81.50 (0–100.00)	89.90 (0–100.00)	0.88
%NPPB that is SP(+)	0 (0–0)	0 (0–1.50)	0.44

%SP that is NPPB(-)	100.00 (69.60–100)	100.00 (83.20–100)	0.80
%SP that is NPPB(+)	0 (0–0)	0 (0–2.50)	0.27

§: mean±SD

*IENF: intraepidermal nerve fiber

IENL/mm: Intraepidermal nerve length/mm epidermis

PDNL/mm: Papillary dermal nerve length/mm epidermis

PD: Papillary Dermis

CGRP: Calcitonin Gene Related Peptide

SP: Substance P

NPPB: Natriuretic Polypeptide Precursor B

5.6 Correlation Analysis

5.6.1 Correlation of Nerve Measurements and Cowhage-Spicule Induced Itch

In cases, AUC itching correlated negatively with %PD-CGRP(+) (CC= -0.40, p=0.02) and %PD-NPPB(-)/CGRP(+) (CC= -0.43, p= 0.009) (Table 5-15). Peak itch was not significantly correlated with any nerve measurements statistically. These data suggest that loss of papillary dermal CGRP nerves may contribute to augmented cowhage itch by loss of an itch-inhibition signal.

Table 5-15. Correlation of Nerve Measurements and Cowhage-Spicule Induced Itch in Cases

Cases	CC*-AUC* itching	P value-AUC itching	CC-peak itch	P value- peak itch
IENF*	-0.04	0.41	-0.08	0.35
IENL/mm*	-0.18	0.20	-0.13	0.27
%Epi	-0.23	0.14	-0.24	0.13
PDNL/mm*	0.32	0.06	0.31	0.07
%PD*	0.23	0.14	0.24	0.13
%PD-CGRP* (+)	-0.40	0.02	-0.26	0.10
%PD-SP* (+)	0.05	0.40	-0.29	0.08
%PD-NPPB* (+)	-0.12	0.30	-0.14	0.25
%PD-NPPB(+)/CGRP(-)	0.003	0.46	-0.11	0.29
%PD-NPPB(-)/CGRP(+)	-0.43	0.0086	-0.28	0.09
%PD-NPPB(+)/CGRP(+)	-0.14	0.27	-0.09	0.33
%PD-NPPB(+)/SP(-)	-0.05	0.43	-0.22	0.15
%PD-NPPB(-)/SP(+)	0.06	0.39	-0.22	0.15
%PD-NPPB(+)/SP(+)	-0.04	0.49	-0.34	0.05[§]
%NPPB that is CGRP(-)	0.15	0.23	0.05	0.41
%NPPB that is CGRP(+)	0.11	0.29	0.13	0.27
%CGRP that is NPPB(-)	-0.10	0.30	0.19	0.18
%CGRP that is NPPB(+)	0.10	0.30	-0.19	0.18
%NPPB that is SP(-)	0.11	0.32	0.20	0.17

%NPPB that is SP(+)	0.34	0.06	0.11	0.30
%SP that is NPPB(-)	-0.09	0.31	0.14	0.26
%SP that is NPPB(+)	0.09	0.31	-0.14	0.26

§: the original P value is larger than 0.05 but is expressed as 0.05 due to rounding off to two decimal places.

*CC: Correlation Coefficient

AUC: Area Under the Curve

IENF: intraepidermal nerve fiber

IENL/mm: Intraepidermal nerve length/mm epidermis

PDNL/mm: Papillary dermal nerve length/mm epidermis

PD: Papillary Dermis

CGRP: Calcitonin Gene Related Peptide

SP: Substance P

NPPB: Natriuretic Polypeptide Precursor B

In controls, %PD-NPPB(+) (CC=0.42, p=0.017) and %PD-NPPB(+)/CGRP(+) (CC=0.45, P=0.01) both positively correlated with peak itch. These data suggest that in controls, PD-NPPB(+) nerves may mediate cowhage-induced itch. We observed a statistically significant correlation between the nerve measurement of SP and peak itch. Peak itch significantly negatively correlated with %PD-SP that is NPPB(-) but not with %PD-SP(+). As there are virtually no SP(+)/NPPB(+) nerves, these two pieces of data contradict each other. We cannot exclude with a larger sample size, %PD-SP would have reached statistical significance. Nevertheless, with our current sample size, these data are inconclusive.

Table 5-16. Correlation of Nerve Measurements and Cowhage-Spicule Induced Itch in Controls

Controls	CC*-AUC* itching	P value-AUC itching	CC-peak itch	P value-peak itch
IENF*	-0.0005	0.48	0.28	0.09
IENL*/mm	0.20	0.18	0.08	0.35
%Epi	0.15	0.25	-0.05	0.40
PDNL*/mm	-0.04	0.43	0.15	0.23
%PD*	-0.15	0.25	0.05	0.40
%PD-CGRP* (+)	0.07	0.36	-0.11	0.30
%PD-SP* (+)	-0.09	0.34	-0.28	0.09
%PD-NPPB* (+)	0.28	0.10	0.42	0.02
%PD-NPPB(+)/CGRP(-)	-0.11	0.34	-0.27	0.10
%PD-NPPB(-)/CGRP(+)	-0.05	0.43	-0.30	0.07
%PD-NPPB(+)/CGRP(+)	0.30	0.09	0.45	0.01
%PD-NPPB(+)/SP(-)	-0.22	0.14	0.01	0.48
%PD-NPPB(-)/SP(+)	-0.24	0.12	-0.50	0.007
%PD-NPPB(+)/SP(+)	0.35	0.06	0.49	0.003
%NPPB that is CGRP(-)	0.13	0.27	0.03	0.44
%NPPB that is CGRP(+)	-0.13	0.27	-0.03	0.44
%CGRP that is NPPB(-)	-0.20	0.18	-0.38	0.03
%CGRP that is NPPB(+)	0.20	0.18	0.38	0.03
%NPPB that is SP(-)	-0.57	0.003	-0.39	0.03
%NPPB that is SP(+)	0.34	0.06	0.49	0.01
%SP that is NPPB(-)	-0.30	0.08	-0.49	0.005
%SP that is NPPB(+)	0.30	0.08	0.49	0.005

*CC: Correlation Coefficient

AUC: Area Under the Curve

IENF: intraepidermal nerve fiber

IENL/mm: Intraepidermal nerve length/mm epidermis

PDNL/mm: Papillary dermal nerve length/mm epidermis

PD: Papillary Dermis

CGRP: Calcitonin Gene Related Peptide

SP: Substance P

NPPB: Natriuretic Polypeptide Precursor B

5.6.2 Correlation of Nerve Measurements and VAS-Average Itch Preceding Week

Controls had no pruritus, as evidenced by their VAS itch scores (Table 5-1). Therefore, correlating VAS itch scores and nerve measurements has no biological significance in controls and so was not performed. In cases, VAS itch score for the average itch in the preceding week of the study visit represents the most accurate reflection of the true itch state of the subjects. This is because itch in uremic pruritus can be paroxysmal and show day-to-day variation from our clinical experience and reports in the literature (Yosipovitch et al., 2001). Therefore, permutation correlation analysis was performed between nerve measurements and VAS score for the average itch in the preceding week.

PDNL/mm, %PD-CGRP(+) and %PD-NPPB(-)/CGRP(+) all were negatively correlated with VAS-average itch preceding week (Table 5-17). These findings are consistent with the significant decrease of PDNL/mm, %PD-CGRP(+) and %PD-NPPB(-)/CGRP(+) in cases compared to controls. Consistent with the above, %CGRP that was NPPB(-) also

correlated negatively with VAS-average itch preceding week. Taken together, these data suggest that loss of papillary dermal CGRP nerves may contribute to augmented itch in uremic pruritus patients by loss of an itch-inhibition signal. In accordance with the negative correlation between VAS-average itch preceding week and %PD-CGRP(+)/NPPB(-), VAS- average itch preceding week positively correlated with %PD-CGRP(+)/NPPB(+); which is of uncertain biological significance, given the negligible number of CGRP(+)/NPPB(+) nerves in cases.

The contribution of NPPB-expressing nerves to uremic itch is ambiguous. %PD-NPPB(+)/SP(-) was significantly positively correlated with VAS-average itch preceding week (CC= 0.38, p= 0.04), whereas %PD-NPPB(+) was positively correlated with VAS-average itch preceding week without statistical significance (CC= 0.24, p= 0.14). Given the virtual absence of NPPB(+)/SP(+) nerves in cases, these data are inconclusive and suggest a larger sample size may be needed to obtain statistical significance.

IENF, IENL/mm and %Epi negatively correlated with VAS-average itch preceding week. However, IENF, IENL/mm and %Epi did not show significant differences in cases compared to controls (Table 5-14). These data raise the possibility that epidermal nerve fibers may normally inhibit itch and loss of these nerves contributes to the severity of uremic pruritus. IENF, IENL/mm and %Epi did not correlate with cowhage-induced itch sensations in either cases or controls. Thus, if epidermal nerve fibers inhibit itch sensation, our data suggest that uremic pruritus itch signaling pathways are multifactorial,

and epidermal itch inhibition is silencing a non-protease-dependent itch signaling pathway contributing to uremic pruritus.

Table 5-17 Correlation of Nerve Measurements and VAS-Average Itch Preceding Week in Cases

Cases	CC*-VAS* (average itch preceding week)	P value-VAS (average itch preceding week)
IENF*	-0.46	0.01
IENL*/mm	-0.61	0.0004
%Epi	-0.46	0.01
PDNL*/mm	-0.53	0.003
%PD*	0.46	0.01
%PD-CGRP* (+)	-0.37	0.03
%PD-SP* (+)	-0.19	0.17
%PD-NPPB* (+)	0.24	0.14
%PD-NPPB(+)/CGRP(-)	0.23	0.16
%PD-NPPB(-)/CGRP(+)	-0.48	0.004
%PD-NPPB(+)/CGRP(+)	0.14	0.27
%PD-NPPB(+)/SP(-)	0.38	0.04
%PD-NPPB(-)/SP(+)	-0.27	0.1
%PD-NPPB(+)/SP(+)	0.31	0.07
%NPPB that is CGRP(-)	-0.17	0.20
%NPPB that is CGRP(+)	-0.32	0.07
%CGRP that is NPPB(-)	0.36	0.04
%CGRP that is NPPB(+)	-0.36	0.04
%NPPB that is SP(-)	-0.25	0.11

%NPPB that is SP(+)	0.06	0.39
%SP that is NPPB(-)	0.25	0.12
%SP that is NPPB(+)	-0.25	0.12

*CC: Correlation Coefficient

VAS: Visual Analogue Scale

IENF: intraepidermal nerve fiber

IENL/mm: Intraepidermal nerve length/mm epidermis

PDNL/mm: Papillary dermal nerve length/mm epidermis

PD: Papillary Dermis

CGRP: Calcitonin Gene Related Peptide

SP: Substance P

NPPB: Natriuretic Polypeptide Precursor B

5.6.3 Correlation of Hemodialysis Duration and VAS-Average Itch Preceding Week

There was no significant correlation between hemodialysis duration and VAS itch scores
in all subject or in case-only groups. This was consistent with the result that there was no
difference in hemodialysis duration between cases and controls.

Table 5-18. Correlation of Hemodialysis Duration and VAS-Average Itch Preceding Week in All Subjects

All subjects	CC-Hemodialysis Duration	P value-Hemodialysis Duration
VAS- average itch preceding week	0.22	0.14

Table 5-19. Correlation of Hemodialysis Duration and VAS-Average Itch Preceding Week in Cases

Cases	CC-Hemodialysis Duration	P value-Hemodialysis Duration
VAS- average itch preceding week	0.16	0.22

5.6.4 Correlation of Hemodialysis Duration and Cowhage-Spicule Induced Itch in All Subjects

There was no correlation between cowhage-induced itch and hemodialysis duration in all subjects (Table 5-20).

Table 5-20. Correlation of Hemodialysis Duration and Cowhage-Spicule Induced Itch in All Subjects

| All subjects	**CC-Hemodialysis Duration**	**P value-Hemodialysis Duration**
AUC itching	0.19	0.19
peak itch	0.26	0.12
total time itch	0.14	0.26
time to peak itch	-0.008	0.49

AUC: Area Under the Curve

5.7 Images of Multi-Label Immunohistochemistry Staining

5.7.1 Specificity of Immunohistochemical Staining of a Primary Antibody Raised to Pre-proNPPB$_{1-134}$ in Human Ventricle and Human Skin

In equine atrium, immunohistochemistry detected NPPB-like expression within secretory granules of cardiomyocytes (Figure 5-1, Figure 5-2; Mifune and Richter, 1995). In our study, we obtained an as-of-yet commercially unavailable rabbit antibody raised to the full-length human NPPB protein (pre-proNPPB$_{1-134}$). Using human cardiac tissue, we identified NPPB/NPPB precursor expression in secretory granules (Figure 5-3). This expression was not present when (a) the primary antibody was omitted (data not shown) or (b) the primary antibody was pre-incubated with the full-length immunizing peptide (pre-proNPPB$_{1-134}$) (Figure 5-3). This expression persisted when the primary antibody was pre-incubated with the active peptide, NPPB$_{1-32}$. We then utilized this antibody in human skin from subjects without pruritus and identified a population of primary

cutaneous nerves (as identified by PGP9.5 antibody staining, a pan-neuronal marker) that expressed NPPB/NPPB precursor (Figure 5-5). Again, this human skin staining was lost with either omission of the primary antibody or pre-incubation with the full-length immunizing peptide (pre-proNPPB$_{1-134}$) (Figure 5-4). Importantly, the human skin staining persisted when the primary antibody was pre-incubated either with a non-specific protein (GRP) (data not shown) or the active peptide, NPPB$_{1-32}$ (Figure 5-4). Together, these observations indicate this primary antibody specifically identifies in human skin the N-terminal component of the full-length NPPB peptide precursor.

Figure 5-1. Secretory Granules of NPPB-like Expression in Cardiomyocytes (Mifune and Richter, 1995).
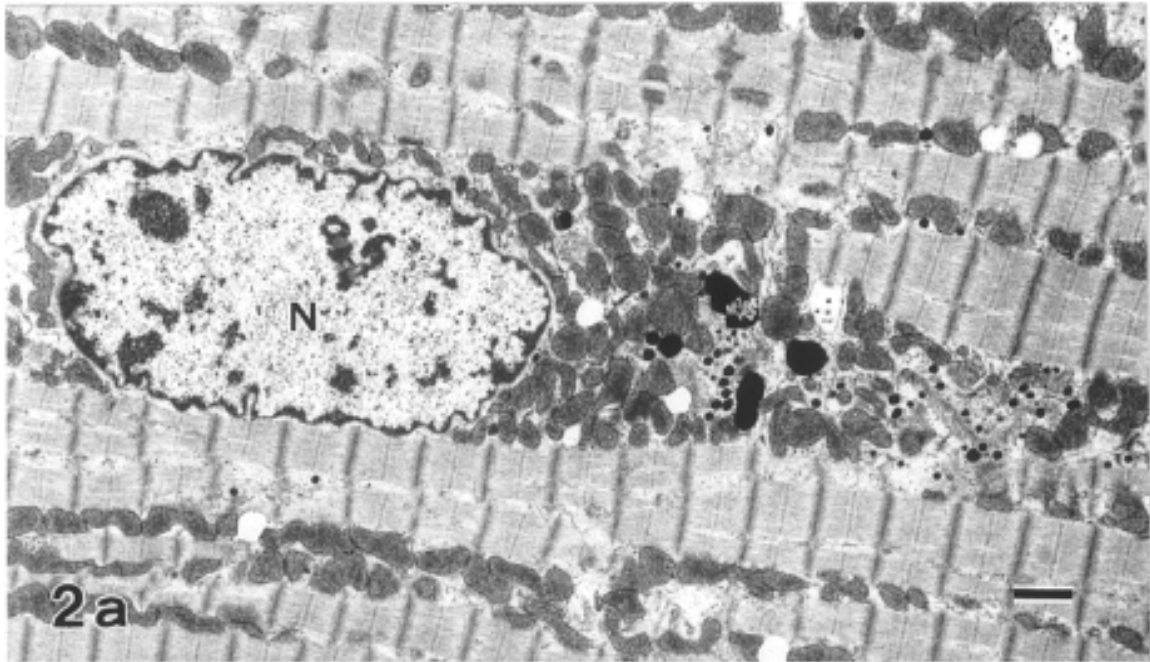

In this electron microscope image, secretory granules are the dense circles of various sizes and are found in the perinuclear region predominantly at the poles of the elongated nucleus, the. (N:Nucleus). Scale bar: 1μm.(Figure from Mifune and Richter, 1995; legend adapted from Mifune and Richter, 1995)

Figure 5-2. NPPB-like Expression Within Secretory Granules of Cardiomyocytes

(Mifune and Richter, 1995).
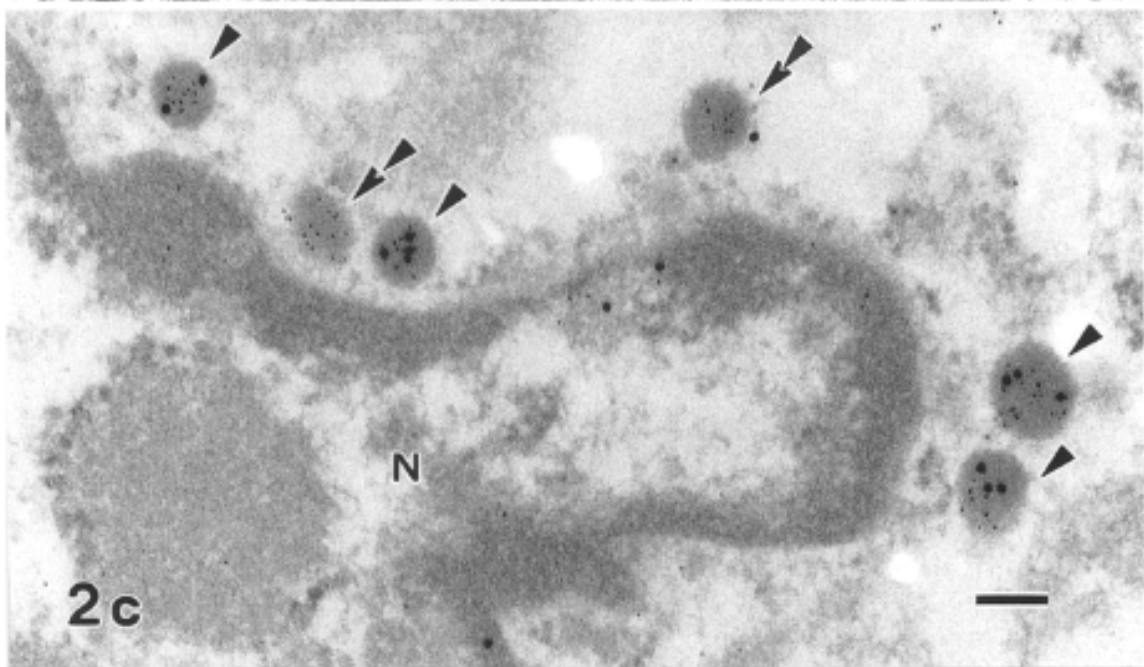
Electron microscope image of double immuno-gold labeling of equine atrium. Large gold particles (15 nm in diameter) demonstrate IR- hCDD/ANP and small gold particles (5 nm in diameter) demonstrate IR-pBNR. Most of the secretory granules show both large and small gold particles (single arrow-head). Few show small gold particles only (double arrow-head). (N: nucleus, G: Golgi apparatus, IR: immunoreactive, hCDD/ANP: human cardiodilatin/Atrial Natriuretic Peptide, pBNP: porcine Brain Natriuretic Peptide). Scale bar, 100nm. (Figure from Mifune and Richter, 1995; legend adapted from Mifune and Richter, 1995)

Figure 5-3. Specificity of NPPB/NPPB Precursor Antibody Staining in Human

Cardiomyocytes.
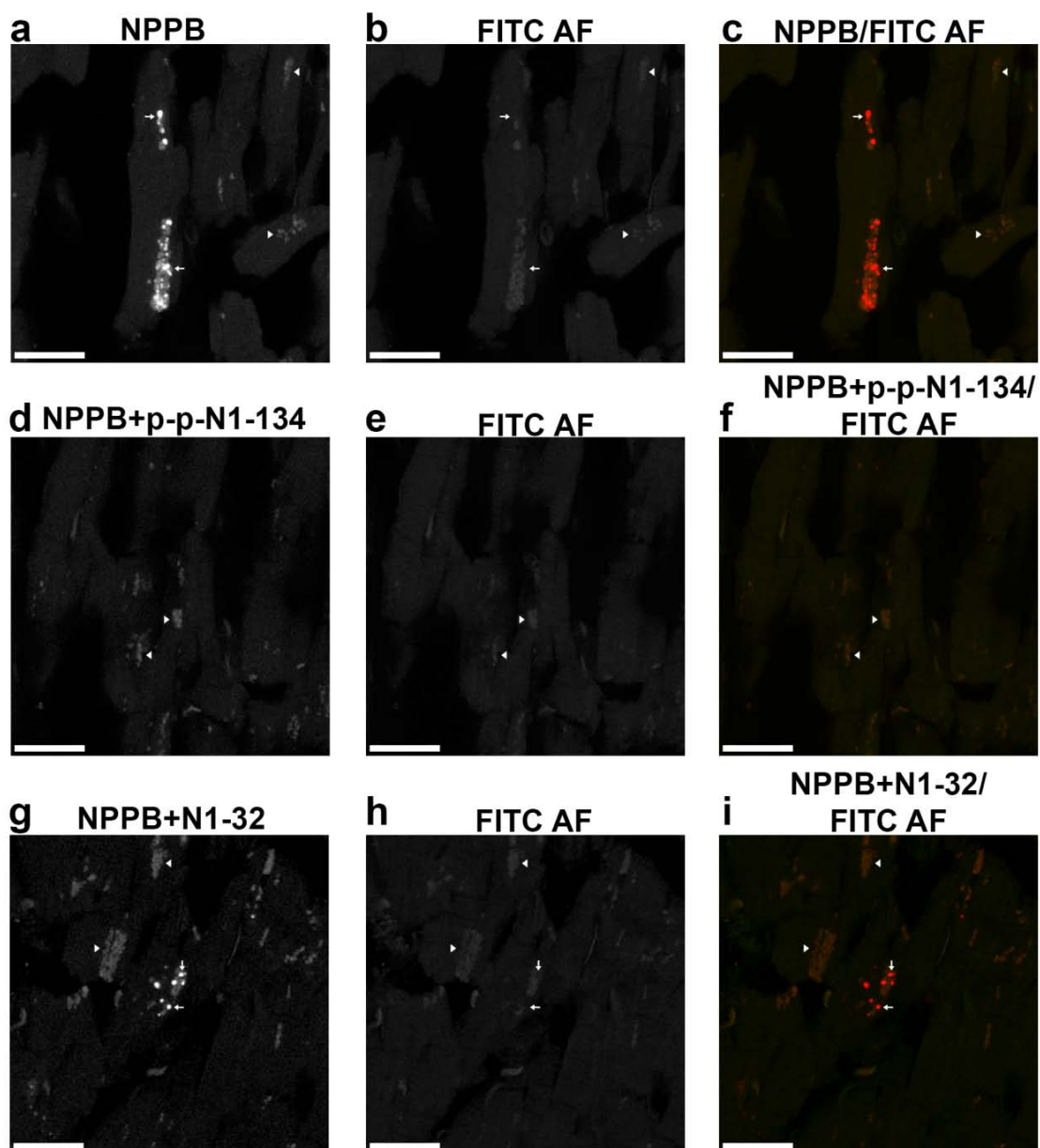
(a-c) NPPB/NPPB precursor antibody alone, (d-f) NPPB/NPPB precursor antibody pre-incubated with immunizing peptide (pre-proNPPB$_{1\text{-}134}$) or (g-i) pre-incubated with NPPB$_{1\text{-}32}$. NPPB/NPPB precursor antibody detection used Alexa568 (generates a fluorescence signal only in the TRITC channel). (a,d,g) TRITC channel. (b,e,g) FITC

channel images of sections in (a,d,g respectively). (c,f,i) Composite of Alexa 568 and FITC channels. (a-i) Arrows show secretory granule staining visible only in the TRITC channel (a and g) that is abolished by pre-incubation with immunizing peptide (d) but not with $NPPB_{1-32}$ (g). Arrowheads show endogenous autofluorescence from lipofuscin, visible in both the TRITC and FITC channels with (d-f; g-i) and without (a-c) immunizing peptides. p-pN1-134, pre-proNPPB$_{1-134}$; FITC AF, FITC autofluorescence; N1-32,NPPB$_{1-32}$. Scale bars, 100μm.

Figure 5-4. Specificity of NPPB/NPPB Precursor Antibody Staining for Papillary

Dermal Nerves in Human Skin.
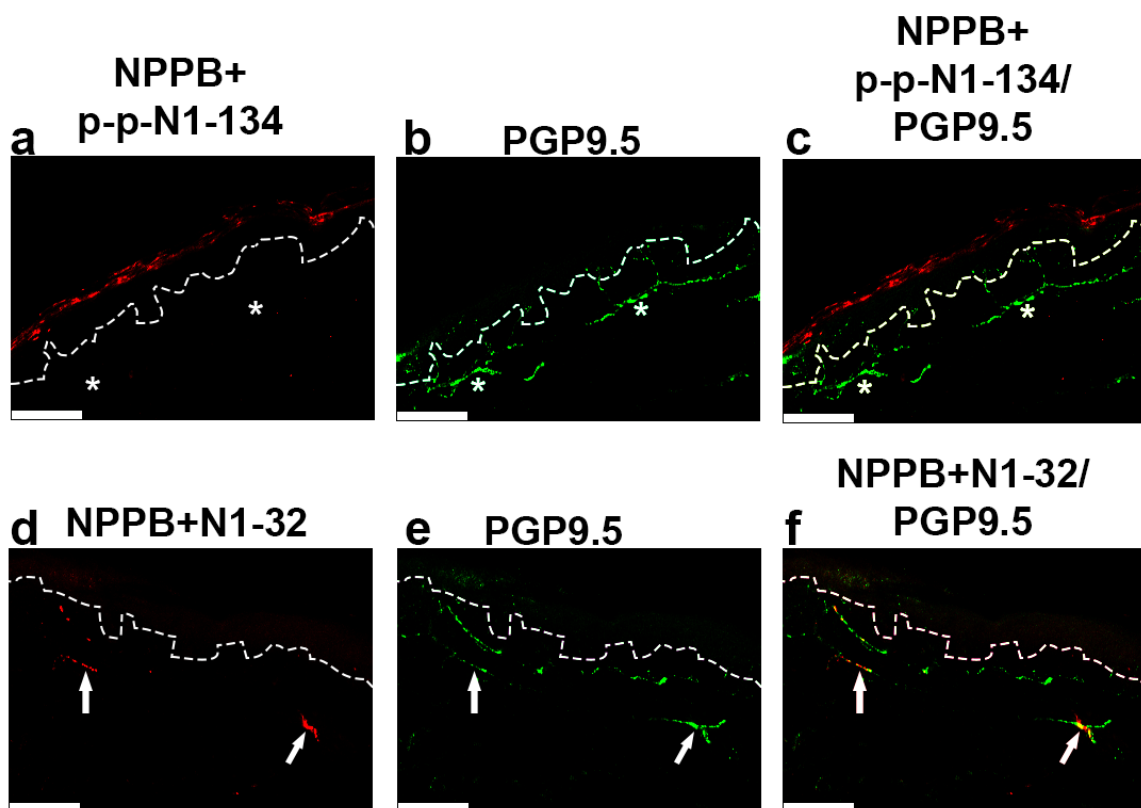
Double immunohistochemistry of PGP9.5 antibody with (a–c) NPPB/NPPB precursor antibody pre-incubated with immunizing peptide (pre-proNPPB$_{1-134}$); or (d–f) NPPB/NPPB precursor antibody pre-incubated with NPPB$_{1-32}$. (a–c) following pre-incubation with immunizing peptide, asterisks highlight (a) the absence of NPPB/NPPB precursor antibody staining of (b) PGP9.5(+) nerves. (c,d) Arrows show co-localization of (d) NPPB/NPPB precursor staining with (e) PGP9.5-positive cutaneous nerves that terminate near the DEJ. (c,f) Composite images of (a&b; d&e, respectively). p-pN1-1-134, pre-proNPPB$_{1-134}$; N1-32, NPPB$_{1-32}$. (a–f) Dashed lines indicate the dermoepidermal junction. Scale bars, 100μm.

5.7.2 Characterization of NPPB Positive Cutaneous Nerves in Subjects' Skin

Mishra and Hoon demonstrated that in mice, only a minority of NPPB-positive neurons co-stained with substance P, that ~25% co-stained with calcitonin gene related peptide (CGRP), and that 50% of them co-stain with Neuromedin B (Mishra and Hoon, 2013). We reported the percentage of such nerves that co-express peptidergic markers (SP and CGRP), that are C- and/or Aδ-fibers in section 5.5.2.1. Here we provide examples of triple immunohistochemical experiments with NPPB, PGP9.5, and either SP or CGRP in uremic pruritus and control skin, respectively. NPPB commonly co-stained with CGRP, whereas, NPPB rarely co-stained with SP.

Figure 5-5. Multi-label (NPPB, CGRP, and PGP9.5) Immunohistochemical

Localization of Peptidergic Nerves in Control Skin.
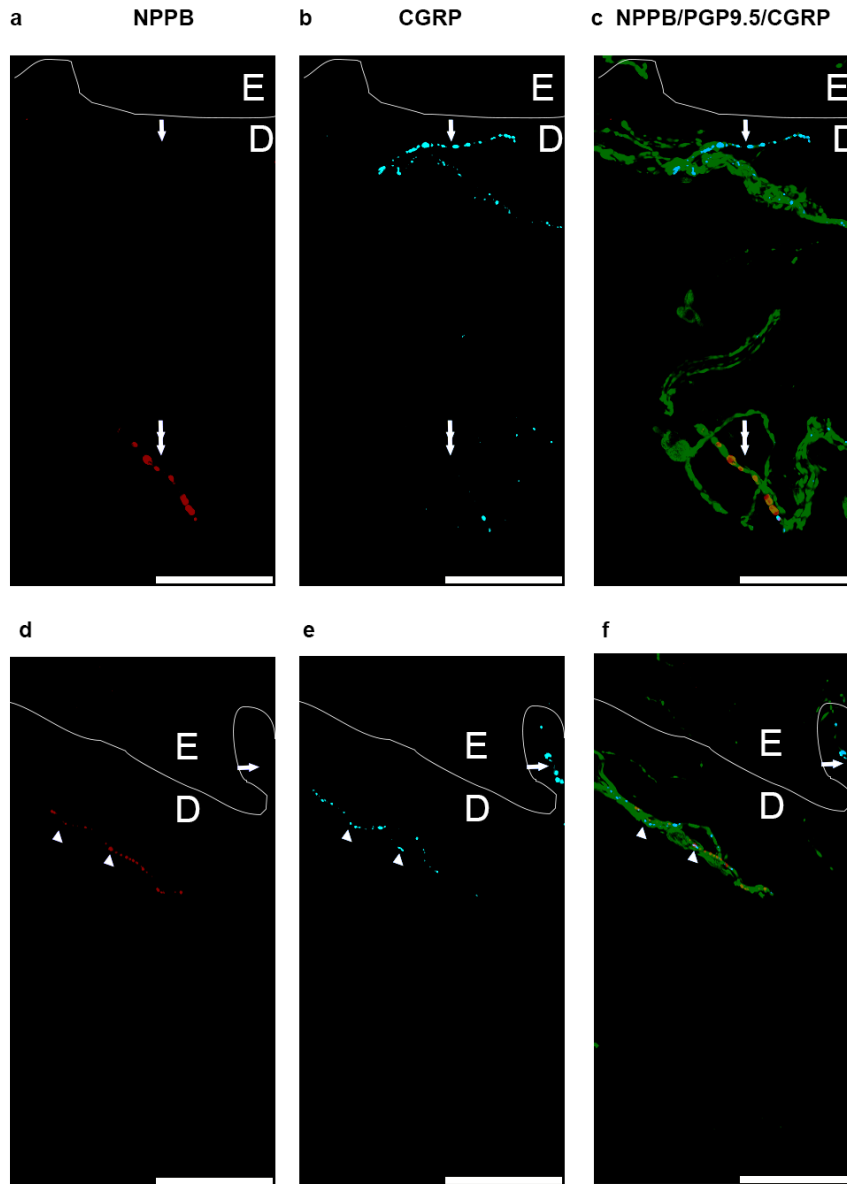
(a,d) NPPB staining (red); (b,e) CGRP staining (blue); (c,f) composite of NPPB, CGRP and PGP9.5 (green) triple staining. Arrows show papillary dermal nerves positive for CGRP. Double arrows show papillary dermal nerves positive for NPPB. Arrowhead shows nerves positive for both NPPB and CGRP. E: epidermis; D: dermis; white line, dermoepidermal junction. Similar results were observed in uremic pruritus skin (data not shown). Scale bar: 50μm.

Figure 5-6. Multi-label (NPPB, SP, and PGP9.5) Immunohistochemical Localization of Peptidergic Nerves in Uremic Pruritus Skin.
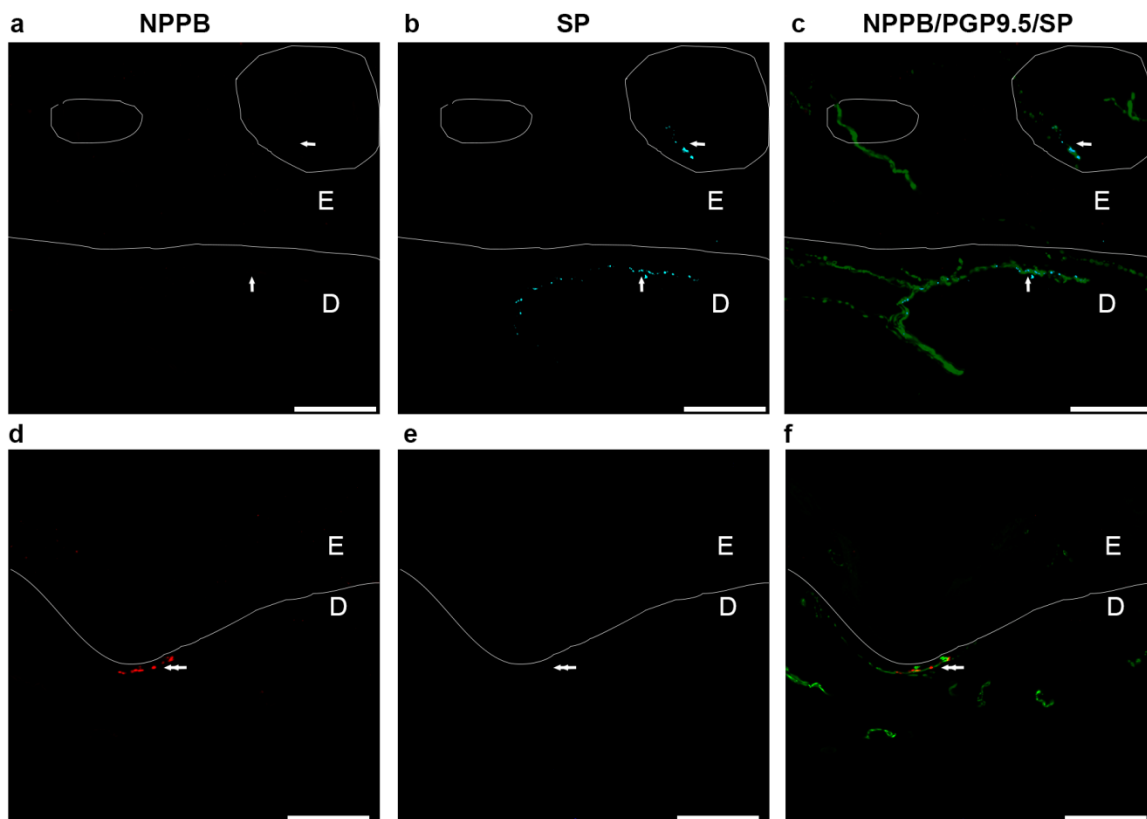
(a,d) NPPB staining (red); (b,e) SP staining (blue); (c,f) composite of NPPB, SP and PGP9.5 (green) triple staining. Arrows show nerves positive for SP. Double arrows show nerves positive for NPPB. Similar results were observed in control skin (data not shown). E: epidermis; D: dermis; white line, dermoepidermal junction. Scale bar: 50μm.

5.7.3 Characterization of TRPV-1 Positive Nerves in the Skin of Uremic Pruritus and Controls.

As described in section 5.5.2.2 and 5.5.2.3, there was no difference in TRPV-1 positive nerves in the epidermis of cases with uremic pruritus compared to controls. TRPV-1 positive nerves were decreased in the dermis of cases with uremic pruritus compared to controls.

Figure 5-7. Immunohistochemical Localization of TRPV-1 in Peptidergic Nerves in

Uremic Pruritus and Control Skin.
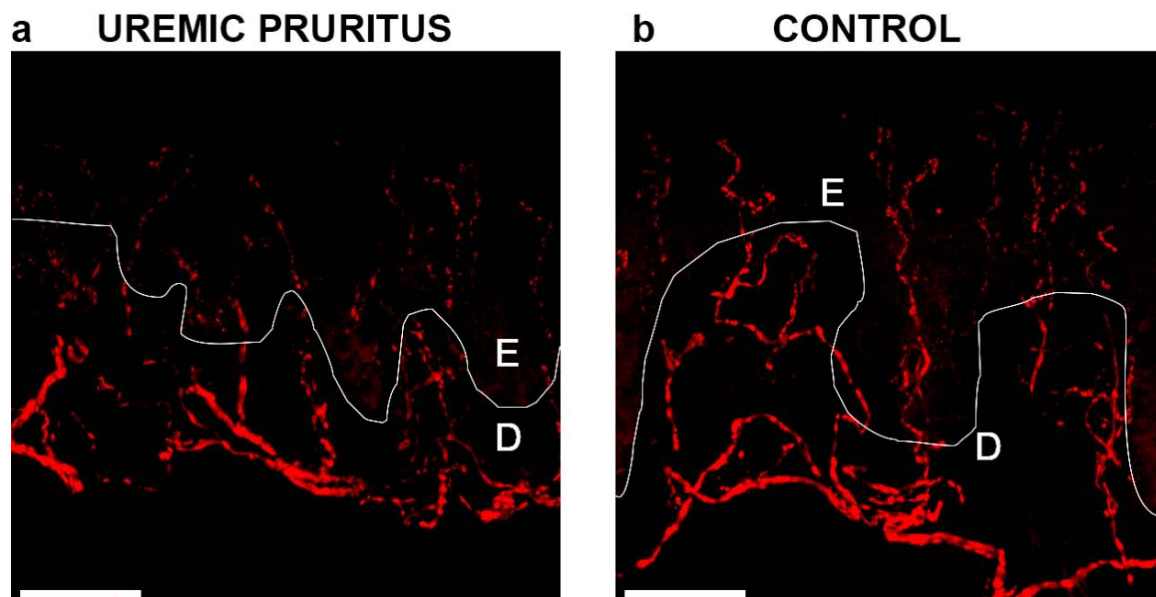
(a) Uremic Pruritus; (b) Control. E: epidermis; D: dermis; white line, dermoepidermal junction. Scale bar: 50μm.

CHAPTER 6. DISCUSSION

Uremic pruritus is a paroxysmal symptom that occurs in the setting of chronic renal failure. Uremic pruritus is a misnomer as the pruritus is not secondary to elevated serum urea levels. In fact, the clinical entity of uremic pruritus is multifactorial, and its causes and treatments have evolved over time as a consequence of advances in dialysis technique. Lower than normal iron, vitamin B12, and/or hemoglobin levels are common in end-stage renal disease, and their correction relieves itch in a subset of these patients. Similarly, elevated serum parathyroid hormone (PTH), calcium and phosphorus are also causes of intractable pruritus, relieved either by parathyroidectomy and/or oral medicine to lower phosphorus, and/or PTH levels (Chou et al., 2000). Finally, xerosis (i.e., dry skin) is very common in ESRD patients, and untreated could cause pruritus. Optimal skin care regimen based on diligent moisturizing dramatically relieves this itch.

Given this diversity of causes of pruritus that can occur in the setting of end-stage renal disease and so be encompassed by the generic clinical term "uremic pruritus," we sought to define more specifically this term for this study. Subjects with uremic pruritus had itch as follows: 1) arising from end-stage renal disease; 2) persistent despite proper dialysis (on dialysis for at least 6 months with a Kt/V>1.20 (Kt/V is defined as the dialyzer clearance of urea (**K**) multiplied by the duration of the dialysis treatment (**t**, in minutes) divided by the volume of distribution of urea in the body (**V**, in mL), which is approximately equal to the total body water, corrected for volume lost during ultrafiltration. We used the KDOQI guidelines of > 1.2 being considered "adequate" to

rule out a patient having pruritis due to being underdialyzed.)); 3) persistent despite serum chemistries within the acceptable range (average for the last 6 months) according to National Kidney Foundation Kidney Disease Outcomes Quality Initiative (KDOQI) guidelines; and 4) not attributable to other systemic or skin conditions.

In our study, 12 subjects with uremic pruritus, as defined above, were recruited according to our inclusion and exclusion criteria (see Appendix II), which made up about 10% of the hemodialysis population in this single dialysis facility. From the recent literature, the prevalence of uremic pruritus ranged from 40% to 60% in the population on adequate hemodialysis (Pisoni et al., 2006; Duque et al., 2006). In these studies, uremic pruritus was more broadly defined, only requiring an index for proper dialysis but not excluding subjects with abnormal serum chemistries or dry skin. For example, in the international Dialysis Outcomes and Practice Patterns Study (DOPPS), serum phosphorus level was higher than the level allowed in our study based on the KDOQI guideline. Conversely, in our study, the stricter and narrower definition of uremic pruritus and high quality of the dialysis regimen resulted the lower 10% prevalence rate in the single utilized dialysis facility. This 10% prevalence rate still fell within the 5%–75% inter-facility range reported in multi-facility DOPPS study (Pisoni et al., 2006).

The medical literature indicates uremic pruritus is independent of gender, ethnicity, age, and duration of hemodialysis. Our data are consistent with literature in that uremic pruritus was not related to duration of hemodialysis. The intensity of uremic itch ranges

from sporadic to restless. The frequency of uremic pruritus ranges from intermittent to persistent. This vacillating quality was reflected by the three VAS itch scores obtained in this study: VAS-average itch preceding week, VAS-worst itch preceding week, and VAS-itch day of visit. In our study subjects, VAS-worst itch preceding week was the same or higher than VAS-average itch preceding week, while VAS-itch day of visit was either lower than, higher than, or similar to VAS-average itch preceding week.

Uremic pruritus may be generalized or localized, and predominantly affects the back (70%), followed by abdomen (46%), head (44%), and arms (43%) (Gilchrest et al., 1982). This was also consistent with our clinical observation that uremic pruritus was present on the back of all subjects with itch. In our study, we purposely excluded subjects with itch only involving the lower legs to exclude the confounding factors of dry skin and ESRD-related length-dependent small fiber neuropathy (Lauria et al., 2010).

Uremic pruritus adversely affects quality of life. The DOPPS study found that HD patients with moderate-to-extreme pruritus were more likely to feel drained and have poor sleep quality, depression, and lower mental and physical composite scores of quality of life than subjects with no or mild pruritus (Pisoni et al., 2006). In accordance with the result from this large cohort study, the quality of life in our case group was statistically significantly worse than that of control group using the frequency version of itchy QoL questionnaire (Desai et al., 2008). Our results confirm that our subjects experienced uremic pruritus that was very symptomatic and adversely affected both emotions and

social functioning.

Uremic pruritus is a type of chronic pruritus. Two pathways can cause chronic itch: central sensitization and peripheral sensitization. We sought evidence for three types of central sensitization in the uremic pruritus population: mechanical and non-noxious touch-to-itch, pin-prick type pain-to-itch, and heat pain-to-itch. Our data definitively excluded mechanical and non-noxious touch-to-itch central sensitization. Technical issues rendered our data inadequate to assess for either pin-prick pain-to-itch or heat pain-to itch. Our study utilized a set of weighted needles shown to efficacious to induce incrementally increase pain scores in the forearms and scalps of healthy subjects without ESRD or dialysis. In both the uremic pruritus and control groups, pin-prick-induced pain did not increase as the weight load of the needles increased. This failure of the weight set to induce incrementally increasing pain may have resulted from the weights being too light for the thick back skin as compared to forearm/scalp skin and/or from the abnormally high mechanical pain threshold of this unique study population, accustomed to needle pricks from much larger and wider needles used routinely in dialysis. Future studies would determine the appropriate weight set reference range for back skin in healthy, age- and sex-matched subjects without ESRD and without dialysis.

Non-sedative histamine receptor antagonists are rarely efficacious for relieving uremic pruritus. Therefore, uremic pruritus may be mediated through a neuroanatomic pathway that is specific for non-histamine dependent itch. Mucunain, a cysteine protease

concentrated in the tips of cowhage plant spicules, was utilized to test the protease-mediated, histamine- independent itch pathway. In our study, we have demonstrated that cowhage-induced total itch, expressed as area under the curve (AUC)-itching, and peak itch were significantly higher in cases than controls. No differences were found in prickling/tingling or burning sensation induced by cowhage spicules between cases and controls. This novel finding implies that augmented activity via a protease-dependent, histamine-independent neuroanatomic pathway is involved in the pathophysiology of uremic pruritus.

Few studies have investigated changes in cutaneous innervations in subjects with uremic pruritus. We sought to investigate if uremic pruritus, as we narrowly defined it (see Discussion Chapter in thesis), arose in the setting of alterations in epidermal and papillary dermal cutaneous innervation. We utilized the pan-neuronal marker PGP9.5 and TRPV-1, both of which are expressed in histamine-dependent and histamine-independent itch nerve pathways. Histamine-independent itch nerves in the skin are mechanical and heat sensitive (CMH) C- and Aδ- fibers that terminate in the papillary dermis and in the epidermis. The neuroanatomical site of termination of histamine-dependent, mechanically insensitive (CMiHis+) C-fibers are unknown. In our study, the length of papillary dermal nerves per mm epidermis staining positively for TRPV1 antibodies was statistically significantly lower in uremic pruritus group than that in controls. Specifically, nerves expressing the CGRP peptide were statistically significantly lost in uremic pruritus group compared to the control group and were lost in an amount sufficient enough to account

for the lost population of papillary dermal TRPV-1 positive nerves.

CGRP-expressing cutaneous nerves have been shown to mediate nociceptive heat and pain in animal models (Mogil et al., 2005; Sun et al., 2004). Experiments in different animal and human studies have shown that cutaneous pain stimuli inhibit coincident cutaneous itch (Ward et al., 1996; Green et al., 2006). We postulate that loss of cutaneous-derived itch inhibition from the loss of CGRP-expressing pain nerves contributes to itch in uremic pruritus. Correlation studies further supported this hypothesis. The ratio of CGRP positive nerves in the papillary dermis is negatively correlated with VAS-average itch last week in uremic pruritus subjects. CGRP positive nerves that did not co-stain with NPPB (more than 80% of all CGRP positive nerves) were decreased in the uremic pruritus, and also correlated negatively with VAS-average itch last week in uremic pruritus subjects. This implies that loss of CGRP-positive nerves, specifically the subset that does not co-express NPPB, contributes to uremic pruritus and itch intensity in uremic pruritus. Furthermore, in uremic pruritus subjects, the ratio of all CGRP-positive nerves in the papillary dermis and those that did not express NPPB, were both negatively correlated with AUC-itching from cowhage stimulation. This implies that loss of CGRP nerves contributed to augmented cowhage-induced itch. In conclusion, we propose that CGRP(+)/NPPB(-) nerves in the papillary dermis signal pain and may inhibit histamine-independent, protease-mediated itch in normal skin. Loss of these nerves in uremic pruritus results in a loss of this itch inhibition, contributing to augmented cowhage signaling and itch in uremic pruritus.

NPPB was proposed to be involved exclusively in the cutaneous itch pathway (Mishra and Hoon, 2013). In our lab, for the first time, NPPB was found to be expressed in the peripheral endings of primary afferent nerves in human skin. The majority of NPPB nerves co-stained with CGRP peptide. In our study, NPPB-positive nerves were decreased, specifically those that co-stained with CGRP, with the preservation of NPPB-positive nerves that did not co-stain with CGRP. Thus, we conclude that loss of NPPB nerves in the papillary dermis resulted from loss of the CGRP nerve population. NPPB nerves comprised a very small percentage of papillary dermal nerves, 1.8% in controls, 0.8% in cases. This is consistent with recent RNA-sequencing data from human trigeminal ganglia (Goswami et al., 2014). Moreover, almost all NPPB nerves that co-stained with CGRP were lost in cases. Thus, the correlation between NPPB ratio and other measurements in the uremic pruritus population was not plausible. In controls, %PD-NPPB and %PD-NPPB(+)/CGRP(+) were both positively correlated with peak itch induced by cowhage spicules. In conclusion, NPPB nerves were involved in histamine-independent, protease-mediated itch sensation.

The contribution of epidermal nerve innervation to uremic pruritus is not well-understood. Two articles published in the 1980s reported conflicting results: one found that epidermal sprouting was seen in all patients on hemodialysis independent of itch status (Stahle-Backdahl, 1989) and the other found that there was a reduction in epidermal innervation in ESRD patients compared to healthy controls (Fantini et al., 1992). Both of these studies had serious enough methodological flaws to call into

question the significance of their findings. Both studies utilized 14μm sections, which is a thickness that is suboptimal for evaluating nerve fiber density. In the Stahle-Backdahl study, biopsies were taken from finger tips and lower legs and only visually qualitative assessment of nerve number was performed. In the Fantini et al. study, biopsies were taken from volar aspect of forearms, and assessment of nerve amount used a "semi-quantitative evaluation" where the number of neuron-specific enolase (NSE)-immunoreactive (IR) nerve fibers was subjectively classified into absent/moderate/numerous in epidermis or dermis. This evaluation relied only upon visual inspection and statistical analysis was not performed when comparing the values for ESRD subjects on hemodialysis and healthy controls.

We investigated epidermal nerve innervation using TRPV-1 and found it was not statistically significantly decreased in uremic pruritus subjects. However, in the correlation analysis, intraepidermal nerve fiber density and intraepidermal nerve fiber length/mm epidermis, were both negatively correlated with VAS-average itch last week in the uremic pruritus population. This implies that epidermal innervation contributes to the pathophysiology of uremic pruritus and the intensity of itch in uremic pruritus. Loss of epidermal innervation paralleled hypoalgesia in normal skin, indicating these nerves signal at least pain (Nolano et al., 1999). Taken together, we postulate that in uremic pruritus, epidermal nociceptor-mediated itch inhibition is one mechanism to compensate for the loss of papillary dermal CGRP-mediated itch inhibition.

Our study was observational and while could show correlations, could not prove causal relationships between the above described neuroanatomic changes and uremic pruritus. Rodent models are one class of future studies that could be used to prove causation. However, rodent skin has inherent differences compared to human skin that may impede their use for such studies. For example, in rodents, CGRP positive nerves comprise a much larger subset of all papillary dermal nerves than in human skin, and unlike humans, CGRP(+) nerves are present in the epidermis of rodents. Accordingly, existing rodent studies implicate CGRP-expressing nerves in both pain and itch pathways, and this may be a consequence of the expanded expression pattern in rodents versus humans. Future studies could examine the skin of other animals to identify a model whose cutaneous CGRP population mirrors that of humans. In humans, technical methodology does not currently exist to selectively ablate CGRP-expressing cutaneous nerves. Alternatives in humans include administration of systemic acting CGRP and/or synthetic molecules that activate the CGRP receptor, as our theory predicts such molecules should reduce uremic pruritus. Interestingly, amylin, a peptide co-secreted with insulin from pancreas, is also an agonist of CGRP receptor (Poyner et al., 2002). The synthetic analog (Pramlintide) was used to treat diabetes (Ratner et al., 2004), and an oral form has been investigated (Andreassen et al., 2014). Anti-pruritic effects of these synthetic CGRP receptor agonists have not been investigated. Conversely, administration in humans of CGRP receptor antagonists should enhance experimental and possibly clinical pruritus. Of the above mentioned alternatives, CGRP receptor antagonists are under investigation in humans to treat migraines. However, these antagonists do not cross blood brain barrier, precluding

them from reaching receptors in the dorsal horn of spinal cord where presumably the CGRP is acting to inhibit itch. Intrathecal injection of CGRP receptor antagonists could overcome the blood brain barrier impermeability but is not an ethical experimental design in humans. While the causes of uremic pruritus are multi-factorial, our data raise the possibility of a novel therapeutic approach to treating uremic pruritus: stimulation of CGRP-mediated input into the dorsal horn of the spinal cord. Therefore, these data warrant additional experiments in appropriately chosen animal models and in humans with existing and novel CGRP receptor agonists to investigate and extend further our findings.

CHAPTER 7. CONCLUSION

This study was motivated by the clinical observation that in some patients with end-stage renal disease on dialysis, uremic pruritus persists despite correction of all known metabolic and dermatologic causes. Although placebo effects cannot be entirely excluded, the efficacy of narrow-band UVB to relieve uremic pruritus suggested that uremic pruritus may arise from a cutaneous source. In this regard, uremic pruritus occurs in the absence of skin inflammation, suggesting an alteration in cutaneous nerve signaling pathways. Unlike other studies published to date, we investigated the innervation of the epidermis and papillary dermis in subjects with uremic pruritus compared to age- and sex-matched controls, with both study groups having ESRD on effective hemodialysis, including without serum or metabolic abnormalities. Our study generated several novel findings: We found evidence for sensitization of a protease-mediated, histamine-independent cutaneous itch pathway in uremic pruritus. We propose that this augmented protease-mediated itch and uremic pruritus occur at least in part from central sensitization. Specifically, we found that both augmented protease-mediated itch and uremic pruritus occurred in the setting of the partial loss of CGRP(+)/NPPB(-) nerves in the papillary dermis. We hypothesize that these CGRP(+)/NPPB(-) nerves mediate pain in normal human skin, that this cutaneous pain input inhibits coincident cutaneous itch in the dorsal horn of the spinal cord, and that loss of these nerves contributes to augmented protease-dependent itch and uremic pruritus in our study population.

This study is a first step in investigating the role of peripheral nerves in the pathophysiology of uremic pruritus and more broadly, chronic pruritus. Causal studies are required to prove that the loss of CGRP-expressing papillary dermal nerves mechanistically explains uremic pruritus. Additional research is required to determine the factors causing the selective loss of this nerve population. Future research is also needed to examine if loss of papillary dermal CGRP nerves occurs in other forms of chronic itch without skin inflammation.

In conclusion, for the first time, we found anatomic and functional changes in the skin of subjects with uremic pruritus: loss of papillary dermal CGRP nerves and sensitization to cowhage-induced histamine-independent itch. We believe that this knowledge gained will contribute to future efforts to generate more effective treatments for this debilitating symptom.

APPENDICES

Appendix I. Screening Questionnaire

Phone Date: _____

Thank you for your interest in our study about why the skin itches in subjects with end-stage renal disease and on hemodialysis. We would like to let you know this study involves 1 clinical visit to Boston Medical Center, Dept. of Dermatology. At this visit, you will fill out questionnaires about if your skin itches, undergo testing to see how sensitive your skin is to warm heat, touch, prick, and itch, and then have a single small piece of skin from either your abdomen or back cut out which is called a skin biopsy for which you will be paid. If these procedures are OK with you, I would like to ask you a few questions to see if this study is a good fit for you and if you are a good fit for this study.

 Section I: Inclusion/Exclusion Criteria Script of anonymous screening questions without collection of personal health information

Inclusion criteria:

1. Age 18 years or older: yes   OR   no

2. Do you have end-stage renal disease: yes   OR   no?

3. Are you on hemodialysis? Yes   OR no?

4. Does your skin itch? If so, how much in a given week on a scale of 0–100 with 0 being no itch and 30 being just at that amount of itch that makes you want to scratch and 100 being worst imaginable itch?

4. Are you willing to come to the Dermatology building for the 1 time study visit? YES or NO?

Exclusion Criteria:

(1) Do you have a history of any other type of skin disease now or in the past (per verbal report of subject/control):   yes   OR   no

(2) Are you able to read and then answer written questions about your itch? Yes   OR no

 (3) Do you have a history of allergy and/or history of adverse reaction to lidocaine used for the skin biopsy: yes   OR   no

 (4) If female: are you pregnant or do you think you might be pregnant?

Appendix II. Inclusion and Exclusion Criteria

Inclusion Criteria

- Individuals 18yrs old or older.

- Individuals with chronic (6 weeks or greater) of generalized pruritus, rated on a VAS scale as 30 or more (out of 100), that started after diagnosis of chronic kidney disease (CKD)(this is only for cases group, not for controls).

- Individuals with underlying ESRD on hemodialysis for at least 6 months with Kt/V ratio of >1.2.

Exclusion criteria

- Individuals with known psychiatric, neurologic, or substance abuse disorders that would interfere with cooperation with the requirements of the trial.

- Individuals with an allergy and/or history of adverse reaction to lidocaine used for the biopsy.

- Women who are pregnant and/or breast-feeding.

- Individuals who are taking opioid derivatives, such as Buprenorphine, Codeine, Oxycodone, Dextropropoxyohene, Methadone, Hydromorphone, Tramadol, Butorphanol, Fetanyl, Alfentanil, Sufentanil, Remifentanil, Morphine, Meperidine, Nalmefene, Naltrexone, Naloxone and Nalfurafine.

- Individuals with psychologic cause(s) of pruritus.

- Individuals with a primary skin disease (e.g. atopic dermatitis, cutaneous T-cell lymphoma) and/or other systemic disease that can cause pruritus.

- Individuals with pruritus where the pruritus is clinically attributed to an existing disease, such as abnormal PTH, Calcium, Phosphorus levels (per KDOQI guideline), VitB12 deficiency, Iron deficiency, Hgb<9g/dl, or active thyroid disease.

Appendix III. Itchy Quality of Life Questionnaire

Itchy Quality of Life

Item no.	Description of the item **In the past 1 week, how frequently have you experienced the following…..**	Scoring
1	My itchy skin condition bleeds	[ ] Never [ ] Rarely [ ] Sometimes [ ] Often [ ] All the time
2	My skin hurts because of my itchy skin condition	[ ] Never [ ] Rarely [ ] Sometimes [ ] Often [ ] All the time
3	My itchy skin condition burns or stings	[ ] Never [ ] Rarely [ ] Sometimes [ ] Often [ ] All the time
4	I get scars from my itchy skin condition	[ ] Never [ ] Rarely [ ] Sometimes [ ] Often [ ] All the time
5	I need to scratch my itchy skin condition	[ ] Never [ ] Rarely [ ] Sometimes [ ] Often [ ] All the time
6	Temperature/seasonal changes aggravate my itchy skin condition	[ ] Never [ ] Rarely [ ] Sometimes [ ] Often [ ] All the time
7	I spend a lot of money treating my itchy skin condition	[ ] Never [ ] Rarely [ ] Sometimes [ ] Often [ ] All the time
8	My itchy skin condition makes it hard to work or do what I enjoy	[ ] Never [ ] Rarely [ ] Sometimes [ ] Often [ ] All the time
9	My itchy skin affects my interaction with others	[ ] Never [ ] Rarely [ ] Sometimes [ ] Often [ ] All the time
10	My itchy skin condition affects how well I sleep	[ ] Never [ ] Rarely [ ] Sometimes [ ] Often [ ] All the time
11	My itchy skin condition often makes it difficult to concentrate	[ ] Never [ ] Rarely [ ] Sometimes [ ] Often [ ] All the time
12	My itchy skin condition limits the types of clothes I can wear	[ ] Never [ ] Rarely [ ] Sometimes [ ] Often [ ] All the time
13	My itchy skin condition forces me to buy special soaps, detergents, and lotions	[ ] Never [ ] Rarely [ ] Sometimes [ ] Often [ ] All the time

14	I am frustrated by my itchy skin condition	[ ] Never [ ] Rarely [ ] Sometimes [ ] Often [ ] All the time
15	I am embarrassed by my itchy skin condition	[ ] Never [ ] Rarely [ ] Sometimes [ ] Often [ ] All the time
16	My itchy skin condition drives me crazy/nuts	[ ] Never [ ] Rarely [ ] Sometimes [ ] Often [ ] All the time
17	My itchy skin condition makes me feel angry or irritable	[ ] Never [ ] Rarely [ ] Sometimes [ ] Often [ ] All the time
18	My itchy skin condition makes me feel depressed or sad	[ ] Never [ ] Rarely [ ] Sometimes [ ] Often [ ] All the time
19	I worry about what other people think about me because of my itchy skin condition	[ ] Never [ ] Rarely [ ] Sometimes [ ] Often [ ] All the time
20	I worry that the itching will last forever	[ ] Never [ ] Rarely [ ] Sometimes [ ] Often [ ] All the time
21	I feel self-conscious because of my itchy skin condition	[ ] Never [ ] Rarely [ ] Sometimes [ ] Often [ ] All the time
22	My personality has changed because of my itchy skin condition	[ ] Never [ ] Rarely [ ] Sometimes [ ] Often [ ] All the time
		1 = never, 2 = rarely, 3 = sometimes, 4 = often, 5 = all the time Total *symptoms* score = avg of responses (1–6) Total *functioning* score = avg of responses (7–16) Total *emotions* score (17–22) = avg of responses (17–22)

Appendix IV. Three VAS Itch Questionnaires

Pt ID:_____

Date:_____

How severe has your skin itch been today?

Place a vertical mark on the line below to indicate how bad you feel your itch in your skin is today.

No itch | ████████████████████ | Worst itch imaginable

Examples:

No itch | ████████████████████ | Worst itch imaginable

Weak

No itch | ████████████████████ | Worst itch imaginable

Moderate

No itch | ████████████████████ | Worst itch imaginable

Severe

Pt ID:_____

Date:_____

Overall, how severe has your skin itch been over this past week?

Place a vertical mark on the line below to indicate how bad you feel your itch in your skin is today.

No itch |————————————————————————| **Worst itch imaginable**

Examples:

No itch |——|————————————————| **Worst itch imaginable**

Weak

No itch |——————|——————————————| **Worst itch imaginable**

Moderate

No itch |——————————————|————————| **Worst itch imaginable**

Severe

Pt ID:_____

Date:_____

In the past week, describe the worst itch you felt at any time.

Place a vertical mark on the line below to indicate how bad you feel your itch in your skin is today.

No itch |━━━━━━━━━━━━━━━━| Worst itch imaginable

Examples:

No itch |━┃━━|━━━━━━━━━━| Worst itch imaginable

Weak

No itch |━━|━━┃━━━━━━━| Worst itch imaginable

Moderate

No itch |━━|━━━━━━┃━━| Worst itch imaginable

Severe

Appendix V. Description of Sensations and Instructions on How to Use a Computer to Assess Cowhage-Induced Itch

Date:

Name of Experimenter:

Patient ID #:

Subject is given the description of cutaneous sensations

Cutaneous Sensation Qualities

BURNING: A sensation most often associated with thermal burns and sun burns, but can also result from other stimuli, such as skin abrasions, strong cold, and chemical irritants. Can be either painful or non-painful, and may or may not be accompanied by a temperature sensation.

STINGING: A sharp, well-localized sensation (e.g., as from an insect bite) that can be painful or non-painful, and may or may not be accompanied by a temperature sensation.

PRICKING: A sharp, well-localized sensation similar to stinging, but brief or intermittent (e.g., as from a needle "prick"), which may or may not be painful.

ITCHING: A generally persistent sensation that when sufficiently strong provokes the desire to scratch.

TINGLING: A lively "pins-and-needles" sensation

WARM: The sensation of mild heating.

HOT: The sensation associated with temperatures that are more than warm but is not necessarily painful.

NUMBNESS: The diffuse (i.e., "fuzzy") sensation produced during the onset or offset of an anesthetic (i.e., Novocaine). *NOT* the absence of sensation.

ICY: A sensation like that produced by touching ice to the skin.

ACHE: A dull, uncomfortable sensation that fluctuates in strength, appears to come from below the skin's surface, and is usually difficult to localize.

TICKLE: A light, attention-grabbing sensation of contact or movement; different from itch but may also provoke the desire to rub or scratch.

PAIN: Any sensation that 'hurts' and to which you might respond by saying "ouch!"

Alloknesis: Enhanced itch evoked by light touch or enhancement of an ongoing itch by light touch. If skin is lightly stroked with a cotton swab it does not produce sensation of itch. It may be ticklish but does not evoke itch. If the light stroking with cotton swab induces itch or exacerbates an ongoing itch then it is called alloknesis.

Hyperalgesia: Brief pricking of the skin by a 50µm tip von Frey filament that I am going to show you produces two sensations. The first sensation is a prickle or pricking pain this is followed about 1 second later by a second sensation of itch. If the pricking pain is enhanced (more than usual) that is called Hyperalgesia and if there is enhanced itch to the stimulus it is called **Hyperknesis.**

D

Subject is given instructions for gLMS

Instructions for the generalized LABELED MAGNITUDE SCALE:

You will be asked to rate the intensity of a variety of real and remembered sensations by indicating where they lie on a scale of all possible sensations. The scale contains commonly used terms like weak and strong, and the top of the scale is the strongest sensation of any kind that you can imagine experiencing.

When you make your ratings you should use the terms just as you would in daily life. But do not limit your ratings to the terms themselves. A good strategy is to first decide which term most closely describes the strength of a sensation, then fine-tune your rating by moving the cursor between that descriptor and the next most appropriate one. For example, if you think a sensation is about moderate, but a little bit stronger, you should move the cursor to the appropriate place just above moderate. If you think another sensation is more than just barely detectable but less than weak, you should move the cursor to the appropriate place between barely detectable and weak, and so on.

It is important to emphasize that the top of the scale is "strongest imaginable", which represents the most intense--and therefore most painful--sensation <u>that you can ever imagine experiencing</u>.

Do you have any questions about how to use the scale?

****

<u>Training Task</u>

To acquaint you with the scale, I would like you to move the cursor to the place on the scale that best describes the intensity of the following remembered or imagined sensations (read aloud to subject):

1. Washing your hands with cold water.

2. A friendly tap on the shoulder.

3. The itch of a mosquito bite.

4. The warmth of a cat sitting on your lap.

5. Stubbing your toe.

6. A warm breeze on your face.

7. Briefly touching a hot light bulb.

8. Getting into a hot bath.

9. An itch on your scalp.

10. Dipping your hand in scalding hot water.

11. A cotton swab touched to your cheek.

12. Pricking your finger with a needle.

13. Putting a hat on your head.

14. Knocking on a wooden door.

15. Briefly touching an ice cube with a fingertip.

16. Slamming a door on your finger.

17. The summer sun on your face.

18. The winter sun on your face.

19. A bee sting.

20. A fly landing on you arm.

E

The purpose of the study and method of the experiment is explained to the subject

Instruction for the experiment

Instructions to subjects

Sensation produced by cowhage

Introduction: The purpose of this study is to measure the magnitude and duration of different kinds of sensations on your skin.

The first and second tests will be combined and use a cotton swab and a series of 5 weighted needles. The swab will be gently rubbed on your skin. After each trial of rubbing you will answer a yes or no question. Each weighted needle will be gently applied to your skin in such a way that does not cut your skin. Each needle will touch your skin for 2 seconds. We will test your back or abdomen. You will NOT be allowed to look at the test site during this procedure. Each weight will be applied for a total of 5 times. After each application, mark on the scale bar what you felt. For both the cotton swab and the needles, we are NOT asking you to record the sensations of touch or pressure. We want you to tell us about either pain or itch. Pain is different than touch or pressure because pain should feel like a sharp or slightly pricking or burning sensation. Itch is different than touch or pressure because itch should feel like a desire to scratch.

Remember this is not a test. There are no wrong or right answers. All trials will have

touch or pressure but only some will have pain and/or itch. It is OK to feel no pain and/or no itch. Please describe what you feel as best as you can.

The third will be the application of a heated metal rod to your skin. It is not hot enough to burn your skin. It will be applied to your skin for 10 seconds ONE time. It will be applied to your back or abdomen. You will may or may not feel the sensation of temperature/heat when it is placed on your skin. If you do feel temperature or heat, we are NOT asking you to record the ability to feel the temperature or the heat. We are asking you to mark on the scale bar the intensity of itch, pain, both, or none, that you felt with the heating sensation.

Again, remember this is not a test. There are no wrong or right answers. It is OK to feel no pain and/or no itch. Please describe what you feel as best as you can.

The fourth will be produced by a very superficial insertion of a cowhage spicule into the skin of your back or abdomen. Cowhage is a naturally occurring plant and the spicules are neither harmful nor is the insertion of spicule painful.

You will use the labeled magnitude scale to rate the intensity of the sensations you feel. You will be prompted to rate three sensations in a sequence one after the other in 30 sec. The scale is going to repeat itself in the same sequence after every 30 seconds. You may or may not feel all the sensations you are prompted for so just rate 0 for any sensation

you do not feel. The first screen will ask you to rate the intensity of itch, so you will move the cursor to a place on the labeled magnitude scale which corresponds to the maximum intensity of Itch you have felt during the last 30 seconds and give your rating by clicking on that place. A window will appear after your click, asking you to confirm your rating. You can select "Yes" to confirm your rating or "No" to go back and change your rating. The second screen will ask you to rate pricking/stinging. Again you will move the cursor on the labeled magnitude scale to a point that corresponds to the highest intensity of pricking/stinging you have felt during the last 30 seconds and give your rating. The third screen will ask you to rate "burning" sensation in the same manner as you have rated itch and pricking/stinging.

If you take more than 30 seconds to rate these sensations the scale will repeat itself and will come back to the first sensation, i.e. itch. You will also hear a beep just before every prompt. Please keep in mind that this is not a test, so there is no right or wrong answer. We are relying on your careful reports to discover what kinds of sensations are evoked by cowhage spicules.

Do you have any questions?

Appendix VI. Recording Sheets for Pin-Prick Test

Pt ID:_____

Round: 1 2 3 4 5   Date:_____

Describe the sensation you are feeling.

Place a vertical mark on the line below to indicate what
you are feeling in the treated skin.

1

No pain |████████████████████████████| Worst pain
imaginable

2

No pain |████████████████████████████| Worst pain
imaginable

3

No pain |████████████████████████████| Worst pain
imaginable

4

No pain |████████████████████████████| Worst pain
imaginable

5

No pain |████████████████████████████| Worst pain
imaginable

Pt ID:_____

Date:_____

Round: 1 2 3 4 5

Describe the sensation you are feeling.

Place a vertical mark on the line below to indicate what you are feeling in the treated skin.

1

No itch | ▬▬▬▬▬▬▬▬▬▬▬▬▬▬ | Worst itch imaginable

2

No itch | ▬▬▬▬▬▬▬▬▬▬▬▬▬▬ | Worst itch imaginable

3

No itch | ▬▬▬▬▬▬▬▬▬▬▬▬▬▬ | Worst itch imaginable

4

No itch | ▬▬▬▬▬▬▬▬▬▬▬▬▬▬ | Worst itch imaginable

5

No itch | ▬▬▬▬▬▬▬▬▬▬▬▬▬▬ | Worst itch imaginable

Appendix VII. Flyers

Flyers for Cases

Study of Cause of Itch that Happens with Hemodialysis

Are you a male or female age 18 or older?

Do you speak English?

Do you have end-stage kidney disease?

Are you on hemodialysis?

Does your skin itch?

Boston University School of Medicine, Department of Dermatology is currently enrolling people with end-stage kidney disease that are on hemodialysis and have itch, in a research study to determine what causes the itch in this setting.

• We will ask you to answer some questions over the phone.

• You may be asked to fill out a few surveys about your skin.

• We will test your skin to see how sensitive it is to warm heat, pressure, and itch.

• You will undergo 10 weeks of 3 times per week treatment in the Shapiro Center at Boston Medical Center, with light.

• Lastly, we will take 1 small (6 mm wide; narrower than your smallest finger nail) sample of the skin either from your belly or your back.

You can earn $50 for completing all study tasks and undergoing the skin biopsy.

To find out more, contact our research team at katedu@bu.edu or 617-638-5574.

Flyers for Controls

Study of Causes of Itch that Happens with Hemodialysis

Are you a male or female age 18 or older?

Do you speak English?

Do you have end-stage kidney disease?

Are you on hemodialysis?

Does your skin NOT itch?

Boston University School of Medicine, Department of Dermatology is currently enrolling people with end-stage kidney disease that are on hemodialysis but do NOT have itch, to be controls in a research study to determine what causes the itch in this setting.

• We will ask you to answer some questions over the phone.

• You may be asked to fill out a few surveys about your skin.

• We will test your skin to see how sensitive it is to warm heat, pressure, and itch.

• Lastly, we will take 1 small (6 mm wide; narrower than your smallest finger nail) sample of the skin either from your belly or your back.

You can earn $50 for completing all study tasks and undergoing the skin biopsy.

To find out more, contact our research team at katedu@bu.edu or 617-638-5574.

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