The Role of Genetic Factors in Primary Hyperhidrosis

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The Role of Genetic Factors in Primary Hyperhidrosis

A Thesis Presented

by

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Abstract

Primary Hyperhidrosis (HH) is a disorder characterized by excessive sweating in focal areas, such as the palms, soles, craniofacial and axillary region. Over 2% of the world’s population is affected by some form of HH and often report low quality of life scores (QOLs) due to its physical and psychological effects. Current treatments, burdened with side effects and varying success rates, call for a more effective approach. This paper proposes a genome-wide analysis study (GWAS) to identify any genes and loci that are significantly associated with primary HH. Following the results of the GWAS, a subsequent gene knockout experiment is presented in order to delve into the functions of the identified genes. These proposed experiments are necessary for the further understanding of primary HH and ultimately for the development of an improved and efficient treatment.
Introduction

Hyperhidrosis (HH) is a disorder characterized by an abnormal, excess amount of sweating. While sweat is necessary to maintain thermoregulation, the sweat production seen in a person with HH far surpasses the normal amount. Globally, more than 2% (~176 million) of the world’s population is affected by hyperhidrosis (Wadhawa, 2019). There are two main types of HH, primary and secondary. While both present similar symptoms, there are differences in areas of sweat concentration and response triggers (Colvin, 2018). For example, primary HH only affects areas that contain eccrine sweat glands while secondary HH involves generalized sweating (Colvin, 2018). Additionally, primary HH has no underlying medical cause unlike secondary HH, in which its symptoms are brought on by an underlying disorder or by medication use (Colvin, 2018). This paper will focus solely on primary hyperhidrosis. The percentage of those affected by primary HH varies widely depending on each country. For example, a hospital-based report showed the prevalence of primary hyperhidrosis to be ~3% in the USA, ~6% in Sweden, ~16% in Germany, ~13% in Japan, ~15% and ~12% in Canada (Wadhawa, 2019). Primary HH affects many of the world’s population, but there is still limited information surrounding it.

According to research, primary hyperhidrosis has no underlying medical cause (Cole, 2023). While heat, physical exertion, and psychological emotions can induce sweating, many with primary hyperhidrosis experience excessive sweating regardless of the presence of a trigger (Harker, 2013). The breakdown of focal areas include: the palms of the hands (palmar hyperhidrosis), soles of the feet (plantar hyperhidrosis), the forehead and cheeks (craniofacial hyperhidrosis), and the armpits (axillary hyperhidrosis). Sweat comes out of these areas the most due to the concentration of eccrine sweat glands. Primary hyperhidrosis is typically diagnosed
through patient history and physical examination (Colvin, 2018). In a 2016 study, over 60% of individuals affected by primary hyperhidrosis experienced childhood onset symptoms before the age of 12 years. Additionally, 88% of the participants reported that the symptoms got worse with age (Lai, 1997). Due to the nature of primary HH, it can significantly affect a person’s life at any time.

Primary hyperhidrosis can present serious effects on an individual’s physical and psychological well-being. Excessive sweating has an effect on normal daily routines and activities. Primary hyperhidrosis can cause complications, such as skin infections, skin discoloration, skin maceration, etc (Cleveland Clinic, 2023). A compilation study by Krishan Parashar revealed 48% of hyperhidrosis patients reported a high level of psychological issues with an increased association of both anxiety and depression. In the study, patients reported feelings of stigma, stress, shame, discomfort, anxiety, depression, etc. Furthermore, the study revealed that activities, such as choosing clothes, occupation, and daily activities, were seen as daunting by those with primary hyperhidrosis (Parashar, 2023). Excessive sweating has the ability to affect a person’s self-esteem as it is an obstacle to an individual’s lifestyle and can alter a person’s whole life routine. Many of these qualities that come from primary HH leave those affected with a deep burden.

Furthermore, there is a stigma surrounding primary hyperhidrosis. There are various factors behind this, such as lack of understanding and awareness, societal norms, media representation, etc. Sweating is often linked to a negative image, due to associated stereotypes that dictate perceptions of what excessive sweating signifies. For example, stereotypes include problems with hygiene, social etiquette, nerves, appearance, etc. Stereotypes like these can contribute to societal attitudes that view excess sweating as an unfavorable trait. There are many
individuals with primary hyperhidrosis that go undiagnosed due to the lack of representation and understanding of it.

While primary HH places a significant effect on those affected, there is still no direct cure for primary hyperhidrosis, but there are treatments to lessen the effects of excessive sweat. Existing treatments for primary hyperhidrosis exhibit varying success rates and side effects. Current treatment options are not enough to balance the damaging effects caused by primary HH. Therefore, a new, effective, and efficient treatment is needed to combat the negative effects of primary hyperhidrosis on an individual’s life. While a treatment is needed to combat the physical and psychological effects of primary HH, the genetics and underlying mechanism of primary HH is not yet fully understood, making it difficult to create one.

Because there is limited information surrounding the genes responsible for primary HH, the first step would be to develop a study that is capable of identifying these genes. A way this can be done is through a genome-wide analysis study (GWAS). A GWAS is a research approach that looks for significant associations between specific genetic variations and observable traits in many different people. While primary hyperhidrosis has been prevalent since the late 19th century, there is still uncertainty surrounding the exact genetic cause and heritability of excess sweating (Lee, 2014). The use of a GWAS will help indicate which genetic markers are linked to primary hyperhidrosis and which genetic variations should be studied more. However, these associations do not automatically mean that these genes are actually involved in primary HH. Therefore, the next step would be to isolate and analyze the functions of these identified genes. This can be done through experiments using model mice, which will further isolate and test each gene’s role in primary HH. Model mice
The development of a study capable of uncovering information surrounding primary HH is essential. With more information, a path to a new and improved treatment will open up. Therefore, this proposal seeks to define a way towards a solution.

**Background**

**Cellular Mechanism of Sweat:**

There are two types of sweat glands, eccrine and apocrine. Eccrine sweat glands are well distributed across the body, with higher concentrations on the palms and soles. Eccrine sweat glands are primarily involved in thermoregulation. Apocrine sweat glands are specific to regions with hair follicles, such as the scalp, axillary region, and inguinal region. Apocrine sweat glands secrete a thicker liquid that contains proteins. Additionally, apocrine sweat glands are associated with pheromones and scent glands. While both are responsible for sweat production, primary hyperhidrosis is heavily associated with eccrine glands while secondary hyperhidrosis can affect both eccrine and apocrine sweat glands (Henning, 2019).

Acetylcholine (ACh) is a neurotransmitter that plays an important role in regulating sweat production in the autonomic nervous system. The sympathetic nervous system is responsible for signaling cholinergic nerve fibers, which then release ACh (Shibasaki, 2010). As ACh is released by nerve endings, it binds to the muscarinic receptors on the sweat gland (Fig. 1). This causes an immediate sweat response. People with hyperhidrosis experience an overstimulation of the ACh signaling pathway. The hyperactivity of the ACh causes immensely responsive sweat glands, leading to excessive sweating. Acetylcholinesterase (AChE), a cholinergic enzyme found in the synapse between nerve cells and muscle cells, is responsible for hydrolyzing or breaking
down ACh. However, due to the over-signalling of ACh, AChE is not able to properly maintain the rate of ACh breakdown, leading to an increase in sweat production (Shibasaki, 2010).

**Figure 1.** Cellular mechanism of sweat release involving acetylcholine (Wiki, 2014).

Criteria for Treatments:

Treatments for Hyperhidrosis can be divided into categories. There are topical treatments, medicinal treatments, cosmetic treatments, and surgical treatments. With each treatment, there are advantages and complications. Doctors strongly recommend starting off with less invasive treatments and then progressing as needed (Figure 1). The decision of choosing which treatment is the most suitable for an individual depends on a list of criteria, such as the following:

1. **Extent of Symptoms:** The intensity and areas of sweat production needs to be taken into account to assess the level of treatment needed. The severity of
hyperhidrosis’ effects on a patient’s daily life should also be determined to gauge the extent of physiological and psychological distress of the patient.

2. Treatment Results: Each patient responds differently to types of treatments as hyperhidrosis is highly individualized. Additionally, the medical history of a patient needs to be noted as each treatment can lead to side effects and possible changes in a patient’s lifestyle.

3. Cost-effectiveness and Accessibility: Treatments vary in price and in availability, with some being more affordable and accessible than others. The factor of treatment duration should also be considered when determining which treatment type is most cost-effective and accessible.

4. Personal Preference: Ultimately, it is important for the patient to select the treatment that they are most comfortable with and they believe benefits them the most.

Figure 2. Doctor-recommended algorithm for treating primary hyperhidrosis (Colvin, 2018).
Treatments:

*Antiperspirants*

Antiperspirants are a common treatment for people who have axillary hyperhidrosis, which is localized under the arm. Antiperspirants for hyperhidrosis usually consist of aluminum salt solutions. Since 1916, aluminum salts were discovered to be effective in preventing excessive sweat (Lee, 2014). As the individual sweats, the antiperspirant is able to be absorbed into the skin. When the antiperspirant enters the body, its components react with mucopolysaccharides, which then damage epithelial cells. As seen in figure 3, this build of precipitate and cells block the sweat gland and prevent sweat from secreting (Campanati, 2015). For best results, aluminum salt antiperspirants should be used daily until desired effect is achieved and then usage can be scaled down to once or twice a week for maintenance.

The limitations to using antiperspirants are duration, side-effects, etc. Antiperspirants must be used regularly and weekly in order to have the best results. The consistent usage of the aluminum salt based antiperspirants can lead to skin and eye irritation.

**Figure 3.** Schematic of “Gel Plug Theory” in which aluminum salts interact with BSA protein. (Yuan, 2015).
Anticholinergic Drugs

The use of anticholinergic drugs to treat primary hyperhidrosis was first seen in the 1950s (Lee, 2014). Anticholinergic drugs work by competitively inhibiting ACh at muscarinic receptors. Due to the decrease in ACh, there is less signaling for sweat production. At the beginning of treatment, anticholinergic drugs must be taken at small dosages and then dosage may increase (Cleveland Clinic, 2023).

Anticholinergic drugs inhibit all ACh function, not just sweat production. Therefore, anticholinergic drugs present many side effects, such as dry mouth, blurred vision, bladder issues, etc. Anticholinergic drugs prevent generalized sweating and can stop sweating in places that are not excessive in sweat, potentially leading to heat related illnesses. Additionally, anticholinergic drugs are not recommended for anyone over the age of 65 years old due to the possible causes of confusion, memory loss, and inhibitions in mental function (Wong, 2022).

Iontophoresis

Iontophoresis uses electrical currents in water to reduce sweat. Iontophoresis treatment for primary hyperhidrosis was developed in the 1950s (Lee, 2014). Iontophoresis is primarily for people who suffer from palmoplantar hyperhidrosis or those who excessively sweat in their hands or feet. The mechanism of action of Iontophoresis is still unknown yet there are many theories on how it works. However, one theory suggests that charged particles physically block sweat glands (Cole, 2015). Another theory suggests the ionization process affects the threshold of the sweat production in the sympathetic nervous (Cole, 2015). The set up for iontophoresis includes a mat containing electrodes, a shallow plastic tray, and tap water. The mat with the
electrodes is placed on the bottom of the tray and then the tray is filled with water. The hands or feet are submerged in the tray for usually 20-40 minutes at a time (Walia, 2000). This process has to be repeated several times a week, consistently before results show. After the effects are apparent, the rate of treatment can decrease, but maintenance treatments, usually once per week, are necessary (Walia, 2000).

In terms of cost, a single iontophoresis session can cost up to $250 while an approved personal machine could cost between $500-$700. Users of iontophoresis have to establish a routine for maximum results. Iontophoresis can present discomfort due to a tingling sensation due to the electrical stimulation (Walia, 2000).

*Botulinum Toxin*

Botulinum Toxin, commonly known as botox, falls under the category of invasive treatment. Botox is a neurotoxin that paralyzes the muscle. While botox is often used for cosmetic purposes, it is also administered to treat medical conditions, such as neuromuscular conditions and hyperhidrosis. Botox’s earliest use of treating primary hyperhidrosis was in 1994 (Lee, 2014). Since 2004, the Food and Drug Administration (FDA) has allowed the use of botox to treat axillary hyperhidrosis, which is localized in the region of the armpits (Osborn, 2022). Essentially, botox works by decreasing the production of sweat by blocking the nerve signals involved. Botox cleaves SNARE proteins that are necessary for the release of ACh into the neuromuscular junction as seen in Figure 3 (Al-Ghamdi, 2014). Without the release of ACh, sweat production signaling is reduced.
Botox is a relied-on treatment due to some of its beneficial features. For example, botox is FDA approved and a high success rate is seen (Al-Ghamdi, 2014). It is a very quick non-invasive procedure, lasting no longer than 30 minutes.

While botox has beneficial qualities, it presents several negative qualities. A single treatment of botox can cost anywhere between $1000-$1500. While botox offers a longer duration than other treatment types, it is not a permanent solution and requires maintenance every 3-6 months (Al-Ghamdi, 2014). Botox is not suitable for every body part and for people with certain medical conditions, such as neurological diseases. Furthermore, botox has been associated with side effects like muscle weakness, vision problems, nausea, vomiting, pain at the injection site, etc.

Figure 4. Process of Botulinum Toxin (Al-Ghamdi, 2014).

Endoscopic Thoracic Sympathectomy
Endoscopic thoracic sympathectomy is a minimally invasive surgery that involves cutting out the nerves linked to sweat production. While sympathectomies existed since 1889, it wasn’t until 1919 when sympathectomies were used to treat primary hyperhidrosis (Lee, 2014). The thoracic sympathetic chain is fundamental to the sympathetic nervous system and it plays a role in the neural control of sweating of the upper body (Cinà, 2007). The thoracic sympathetic chain is made up of interconnected ganglia of the sympathetic nerve that is next to the spine. At the level of the thorax, the ganglia of the sympathetic chain are surgically cut out.

ETS is often left as the last resort due to its results being mainly irreversible. After receiving ETS, it is highly unlikely a second surgery would be needed (Cinà, 2007). This surgery can lead to side effects and complications. The largest complication seen from ETS is compensatory sweating. While troubled areas might sweat less, patients see an increase in sweat in different areas (Cinà, 2007). Furthermore, ETS is an expensive procedure, ranging from $8000-$10000 (Cinà, 2007).

**Drawbacks**

Overall, the current treatment options for primary HH have varying levels of success with some being significantly more effective. Furthermore, there is a lack of efficiency in many of the treatments, leading to frequent maintenance as seen with antiperspirants, iontophoresis, and botox. The risk of side-effects, such as skin irritation and uncomfortableness, and complications like compensatory sweating, leave the public with limited choices in treatments. It is truly important that patients follow the criteria of treatments (Fig.2) and make but also have the best choice for them to deal with persistent symptoms of primary HH. This is why it is worth investigating a new method to treat the condition of excessive sweating.
Methods

The proposed methods in this paper offer a pathway to uncovering more genetic information surrounding Primary HH. In this section, a genome-wide association study (GWAS) will be performed to identify any genes of interest. This section will break down each individual step of the GWAS design. In a subsequent methods section, an experiment using model mice will be conducted, targeting the functions and roles of these genes of interest. The second methods section will lay out the creation process of the model mice and will also lay out the gene function testing process, which will be done using an iodine-starch assay. Both experimental methods will offer insight into the genomic variants and genetic functions surrounding primary HH.

Experiment 1: Genome-Wide Analysis Study (GWAS)
A GWAS has a complex setup design therefore it is important to break down the individual steps involved (Fig. 5). Each step is essential in ensuring that the GWAS’s result are valid and generalizable. Therefore, the following section below will outline the proposed setup for a GWAS on Primary HH.

Selecting a Study Cohort

The first and foundational step of the GWAS is to select a study population. Typically, researchers choose between a population-based study and a family-based study, in which both cohorts have their advantages and disadvantages.

Population-based studies involve unrelated individuals and tend to have higher statistical power than family-based studies. GWAS often require a large sample size in order to identify significant associations with a high statistical power as well as limit the false positives (Uffelmann, 2021). Because population-based studies involve strangers, it is easier to obtain a larger sample size unlike family-based studies that often lead to a lower quantity of participants. Another difference is that population-based studies have a bigger variety of genetic backgrounds, which increases the likelihood of identifying common genetic variants. Furthermore, population-based studies avoid confounding variables that could be involved in family-based studies such as shared environmental factors (Uffelmann, 2021).

Family-based studies involve related individuals. Because a large amount of genetic material is shared between the participants, it is easier to avoid population stratification. Additionally, rare genetic variants are more likely to be identified within a family and cues of the genetic inheritance of the disorder might be observed.
Because both cohorts offer favorable qualities, this proposed GWAS will have a mixed-model cohort. A mixed-model cohort will still allow there to be genetic variety with the potential to see genetically linked components involved. This cohort improves the accuracy of identifying true genetic associations by lowering the risks of false positives associated with population structure. Furthermore, it allows researchers to simplify the effects of genetic variants from the effects of different genetic backgrounds, providing a more reliable analysis. The mixed model also ensures that the population size can be obtained more strategically and therefore there will be high statistical power present (Uffelmann, 2021).

Overall recruitment of participants will be done through an extensive questionnaire. The questionnaire will cover points on important topics, such as symptoms, intensity of symptoms, family background, etc. For recruiting familial linked participants, participants, who qualify from the questionnaire, will receive the invitation to invite family members.

**Genotyping**

Genomic data will be collected through either DNA or saliva. Using a spin column method, DNA will be extracted from ethylenediaminetetraacetic acid (EDTA) blood due to its ability to prevent blood clotting and preserve the DNA. Saliva will be collected using a saliva collection kit, such as the OrageneDNA OG-500 kit. A microarray (Infinium Global Screening Array with Cytogenetics-24 or Illumina HumanCore-24) will then be performed on the genomic DNA. The microarray will identify patterns of gene expression, genetic variations, and the presence of specific sequences (Uffelmann, 2021).

**Quality Control**
Quality control is necessary to avoid the effects of population stratification. Population stratification refers to the presence of systematic differences in allele frequencies within a population. This is often caused by the variety in the participants’ genetic backgrounds. PLINK is a software genomic toolset that uses genetic data to identify patterns of variation and creates plots to visualize these patterns. By applying Principal Component Analysis (PCA), it corrects for population differences in subsequent analyses, ensuring that study results aren't skewed by variations in genetic ancestry. PLINK is necessary to ensure that the associations detected are not confounded by differences in genetic ancestry. Additionally, PLINK will minimize the occurrence of false positives and false negatives (Uffelmann, 2021).

**Imputation**

Imputation refers to the process of predicting and replacing any missing data. IMPUTE2, a software tool, will be used for imputation. IMPUTE2 compares a reference panel of broad genomic data to the experimental data in order to fill in any missing genotypes into the dataset. Imputation will allow the dataset to have a more extensive set of genetic variants and overall increase the density of genotypic data (Uffelmann, 2021).

**Association Testing**

Due to the cohort being a mixed-model cohort, the variables of both population structure and relatedness need to be taken into account. A Wald Test will be performed to evaluate the significance of individual Single Nucleotide Polymorphism (SNP) associations with primary HH. A Wald Test will work by assessing the association between an individual SNP and primary HH by comparing the estimated effect size to its standard error. Significant associations will be
identified using the common GWAS p-value of $5 \times 10^{-8}$ but will be adjusted as needed to minimize false positives and false negatives (Uffelmann, 2021).

**Meta-Analysis**

A meta-analysis is needed to increase the overall statistical power of the experiment. A Genome-Wide Association Meta-Analysis (GWAMA), which is a statistical technique that pools data to enhance sample size and data points using multiple independent GWAS studies, will be performed. This will be done to make sure findings are significant in a broader population size (Uffelmann, 2021).

**Replication**

Replication is a necessary step to validate any genetic associations found in one study by conducting additional independent studies. Replication ensures that the associations found are not due to chance or are unique to a specific population. The same GWAS design will be repeated however on a different, independent population in order to generalize findings and minimize any false associations (Uffelmann, 2021).

**Post-GWAS Analyses**

Post-GWAS Analyses are important for identifying any discoveries that are derived from the statistical analysis results. From the results, a Manhattan plot will be made to visualize the associations between the genetic variants and primary HH (Fig. 5e). A post-GWAS analysis can also uncover the biological processes involved in primary HH. This will allow us to dive into the possible genetic inheritance of primary HH (Uffelmann, 2021).
**GWAS Expected Results**

From the GWAS, the expected results should show significant associations between certain genomic variants and primary HH. A post-GWAS analysis will reveal p-values, which will show us what genes and associations are significant. A Manhattan plot and quantile-quantile (QQ) plot are both visual methods that will help show the significance of association between SNPs. In this experiment, a Manhattan plot contains the variants that are organized by their chromosomal location on the x-axis while the y-axis shows the strength of association between the SNP and trait of interest, primary hyperhidrosis (Uffelmann, 2021). Another important aspect of the results would be a table of Loci. This table summarizes the specific genomic regions where significant associations were seen. The table further contains the specific locations and genes that contain relevant relationships. These visual aspects of the results are crucial in understanding the relationship between the SNPs and primary HH.

**Potential Genes of Interest**

From previous studies, there are identified genes of interest that researchers have concluded could play a part in primary HH. In this study, the expected genes of interest could overlap with previous findings. One study concluded that the butyrylcholinesterase (BCHE) gene might be involved in primary HH (Simes, 2018). Additionally, the researchers concluded that the cholinergic system, influenced by the –116A and K-variants on the BCHE gene, may be associated with primary HH (Simes, 2018). The BCHE gene encodes for the butyrylcholinesterase enzyme that plays a role in the breakdown of acetylcholine, which triggers sweat response, therefore it is possible that the GWAS would support this finding (Simes, 2018).
In a family-based GWAS, researchers concluded that 4 loci were significantly identified throughout familial generations. These loci included, PFH: 1q41-1q42.3, 2p14-2p13.3, 2q21.2-2q23.3, and 15q26.3-15q26.3. Notably, three pedigrees shared a locus at 2q21.2-2q23.3, reinforcing its significance with a genome-wide significant LOD score of 3.45 (Schote, 2020). These results weren’t able to solidify a specific gene responsible for primary HH but were able to conclude that there is genetic heterogeneity involved in primary HH. The proposed GWAS could uncover cues of genetic heritability, such as found in the Schote study.

Overall, a GWAS is a helpful first step in identifying genes associated with primary HH however it is not entirely reliable. Even though a gene’s association is significant in a GWAS, it does not necessarily mean that it is actually related to the trait of interest. Therefore, it is important to execute follow up experiments to uncover the gene’s actual role.
Methods

Experiment 2: Gene Knockout Using Model Mice

Given the results of the GWAS, the next step would be to further isolate the functions of the targeted genes to test its role in primary hyperhidrosis. A way to test the effects of the targeted gene would be to create model mice. There are two types of model mice, knockout (KO) and knock-in (KI). Knockout mice are genetically modified mice in which a specific gene is disrupted or affected while knock-in mice are genetically modified so that a specific DNA sequence is altered or replaced (Gurumurthy, 2021). The decision of which model mice to employ is entirely dependent on the results of GWAS. If the significantly associated gene is a full loss of function mutation, then KO mice will be used to uncover the specific functions of the deleted gene (Gurumurthy, 2021). However, if partial loss of function and/or gain of function mutations are found, KI mice will need to be created. This is because KI mice will maintain the natural regulation of the targeted gene whilst allowing us to decipher what specific modifications to the genome actually do (Gurumurthy, 2021). While both might be necessary given the results of the GWAS, only a KO model mice experiment will be proposed in this paper. Overall, mice have similar genetics to humans and can provide insight to the underlying mechanism of primary hyperhidrosis.

*Cr*ispr-*Cas*9

Crispr-Cas9 is a genome editing tool that allows for specific DNA sequences to be altered, added, or excised (Fig. 5). Crispr-Cas9 is made up of two key elements, which include Cas9, an enzyme, and single-guide RNA (sgRNA). A sgRNA is complementary to and binds to the DNA target sequence. The Cas9 enzyme then binds to the sgRNA and cuts both DNA
strands. The cut is then repaired and the deletion/mutation is now introduced. According to Gurumurthy, knockout mice are more efficiently made nowadays by injecting pronuclei with gRNA in addition to Cas9 protein, which will create double-stranded break at the targeted location (2021).

Figure 6. Mechanism of Crispr-Cas9 (Gurumurthy, 2021).

To set up the Crispr-Cas9 knockout experiment, a single-guide RNA (sgRNA) is first needed. Now that a targeted gene is identified, a targeted sequence within the gene must be selected. It is important for the selected sequence to be somewhat unique and specific in order to avoid off-target effects. The sgRNA, typically 17-24 nucleotides long, is created from the DNA targeted sequence and is its complementary base pair. Both shorter or longer nucleotide lengths for a sgRNA result in the possibility of off-target results. Next, a protospacer adjacent motif (PAM) next to the targeted sequence must be identified. Typically, a PAM sequence is
5’-NGG-3’, where N can be any nucleotide base. PAM is necessary because the Cas9 protein will be able to recognize it, allowing for the Cas9 protein to bind to the targeted sequence. If PAM is absent, Cas9’s ability to bind correctly is very limited and the introduction of a double-strand break is less likely. Therefore, if PAM is not compatible with the selected targeted sequence then a new sequence will be needed.

Next, mice zygotes will be injected with the sgRNA and the Cas9 mRNA, in which the Crispr-Cas9 system will proceed and make cuts to the targeted sequence. The fertilized eggs will then be transferred to the oviduct of a pseudopregnant female mouse that underwent progesterone therapy. The mice that are born will be screened by pcr amplification to assess whether or not gene knockout has been achieved. The KO mice are then ready for the next step of the experiment, which would be testing sweat rates.

**Iodine-Starch Assay**

After having identified the gene of interest from the GWAS, the next step would be to test the effects of the genomic alteration by observing differences in sweat production rates. Two groups of mice (n=50) will be employed, in which one group is the control (n=25) and the other will be the experimental group (n=25). In this experiment, variables, such as type and strain of mice, sex, age, environment, diet, and overall health will be controlled as much as possible. These variables should not fluctuate drastically to ensure that any results are not influenced by confounding variables.

To measure sweat production rates, an iodine-starch assay will be performed. In mice, it is important to note that eccrine sweat glands only exist in their paw pads and hence will be the only area measured for sweat secretion (Chee, 2017). Pilocarpine, a cholinergic agonist or a
sweat-inducing substance, will first be injected into the mice’s hind paws. This will subsequently activate the mice’s sweat glands and then an iodine solution will be applied to the paws along with a starch solution. The iodine is then able to react with the starch to form a visible dark color. Each dark dot observed corresponds to an individual sweat gland, making the number and size of these dots indicative of sweat production rates. The increase in the number of dark dots and the intensity of the color will help determine sweat production rates (Klar, 2014).

After measuring all of the sweat production rates, all of the results from the control and KO mice are able to be analyzed. The percentage difference of sweat production rates will be obtained and any significant results will be highlighted.
Expected KO Model Mice Results

**Figure 7.** Iodine-starch assay of an anhidrosis gene knockout mice experiment (Klar, 2014)

From the iodine-starch assay, the expected results will show the difference in sweat production rates between control mice and KO mice. Figure 6 shows the expected data visually in each part. However, it is important to note that figure 6 shows a gene knockout experiment on anhidrosis, which is a condition in which the sweat glands don’t function properly. Therefore, we would expect to see completely flipped results, in which a gene knockout for primary HH genes would produce a higher volume of responded sweat glands. This would mean that the gene KO mice would have a higher percentage of black dots on the paws and therefore a higher sweat gland response than control mice (Fig. 6A). Additionally, the KO mice would overall have bigger dot diameters (Fig. 6B). Finally, figure 6C would be very similar because they would both show the lack of fluorescence, indicating the knocked-out gene. Overall, the expected results of
this experiment will help confirm or deny the relationship between the genes of interest and primary HH.

**Discussion**

Due to the lack of efficient treatments for primary HH, a new approach is needed in order to counteract the mental and physical effects that come along with it. To start the journey to a solution, a better understanding of primary HH is first needed. With the combination of a GWAS and model mice experiment, it is possible to identify genes of interest and uncover the genetic relationship of primary HH. The expected GWAS results will identify any significant associations among a mixed population, potentially supporting previously identified associations. Additionally, KO mice expected results will confirm any suspicions of a gene’s involvement in excessive sweating. Both experiments’ results work to build more information on primary HH.

While the proposed experiments offer a structured setup design, there are still limitations and possible sources of error that may arise. For example, a possible source of limitation that may occur in the GWAS are false positives. A false positive is when an association is shown to be significant even when there is no biological meaning behind it (Ehret, 2010). The presence of false positives can be combated through a Bonferroni correction. A Bonferroni correction modifies the significance threshold, in which the original p-value is divided by the number of tests performed. By doing this, the standard of significance increases, decreasing the risk of false positives. Furthermore, a small sample size could lead to chance associations or random relationships. In terms of the gene knockout model mice experiment, it is very possible that a knock-in model mice experiment may be needed. If partial loss or gain of function is observed, then a new experiment design will be needed. This can still be done using a Crispr-Cas9
approach, but it will involve more steps, such as adding a reporter gene or tag. Additionally, a repair template, which includes an extra DNA sequence, is needed to precisely introduce the genetic modification of interest. Limitations and sources of error may affect the results however they will be addressed as the experiment progresses.

**Conclusion**

Primary hyperhidrosis is a disorder that negatively affects many aspects of an individual’s life. Primary hyperhidrosis not only inhibits daily physical activities, but it also interferes with a person’s mental well-being and overall lifestyle. While there are developed ways to treat primary hyperhidrosis currently, the current treatments require maintenance and upkeep. Understanding the genetics of primary hyperhidrosis is truly crucial for the advancement of medical research and for the development of more personalized, targeted, and effective treatments. Through experimental designs using GWAS and gene knockout mice, it is possible to uncover the genetics and mechanism behind primary hyperhidrosis. Additionally, more studies on primary hyperhidrosis can be used to end the stigma and lack of awareness surrounding excessive sweating.
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