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Basic Research Protocol: Exome Sequencing in Adults With Loin Pain Hematuria Syndrome: A Pilot Study

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Abstract

Background: Loin pain hematuria syndrome (LPHS) is a poorly understood clinical syndrome characterized by hematuria and either unilateral or bilateral severe kidney pain in the absence of identifiable urological disease. Loin pain hematuria syndrome imposes a significant health and economic impact with a loss of productivity and quality of life in a young population. Owing to an incomplete understanding of its pathophysiology, treatment has been limited to nonspecific pain management. Nearly 60 years after its initial description, we are no further ahead in understanding the molecular pathways involved in LPHS.
Objective: To outline the study design for exome sequencing in adults with LPHS and their families.
Methods: In this single-center case series, 24 patients with LPHS and 2 additional first-degree family members per participant will be recruited. DNA extracted from venous blood samples will undergo exome sequencing on the Illumina NovaSeq 6000 System at 100× depth and will be assessed for pathogenic variants in genes associated with hematuria (number of genes in: glomerular endothelium [n = 10] and basement membrane [n = 8]), and pain pathways (number of genes in: pain transduction [n = 17], conduction [n = 8], synaptic transmission [n = 37], and modulation [n = 27]). We will further examine identified potentially pathogenic variants that co-segregate with LPHS features among affected families.
Conclusions: This pilot study may identify new directions for an investigation into the molecular mechanisms underlying LPHS.

Abrégé

Contexte: Le syndrome de lombalgie-hématurie est un syndrome clinique encore mal compris qui se caractérise par une hématurie et une forte douleur rénale unilatérale ou bilatérale en l'absence d'une maladie urologique identifiable. Le syndrome de lombalgie-hématurie a une incidence importante sur la santé et l'économie en entraînant une perte de productivité et de qualité de vie dans une population jeune. La compréhension de la physiopathologie de ce syndrome étant incomplète, le traitement a été limité à la gestion non spécifique de la douleur. Près de soixante ans après sa description initiale, nous en sommes au même point dans la compréhension des voies moléculaires impliquées dans le syndrome de lombalgie-hématurie.
Objectif: Décrire le plan de l'étude pour le séquençage de l'exome chez les adultes atteints du syndrome de lombalgie-hématurie et des membres de leur famille.
Méthodologie: Pour cette série de cas menée dans un seul center, nous recruterons 24 patients atteints du syndrome de lombalgie-hématurie et deux membres au premier degré de leur famille. L'ADN extrait d'échantillons de sang veineux sera soumis à un séquençage de l'exome sur le système Ilumina NovaSeq 6000 réglé à 100X de profondeur. Il sera également analysé pour la présence de variants pathogènes dans les gènes associés à l'hématurie (nombre de gènes dans l'endothélium glomérulaire [n = 10] et la membrane basale [n = 8]), et aux voies de transmission de la douleur (nombre de gènes dans la transduction [n = 17], la conduction [n = 8], la transmission synaptique [n = 37] et la modulation [n = 27] de la douleur). Nous poursuivrons l'examen des variants potentiellement pathogènes identifiés qui co-ségrègent avec les caractéristiques du syndrome de lombalgie-hématurie parmi les familles touchées.
Conclusion: Cette étude pilote pourrait révéler de nouveaux axes de recherche sur les mécanismes moléculaires qui sous-tendent le syndrome de lombalgie-hématurie.

Keywords

loin pain hematuria syndrome, exome sequencing, rare disease, eGFR, hematuria, chronic pain

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What was known before

Hematuria in patients with loin pain hematuria syndrome is glomerular in origin, but the source of pain is a mystery.

What this adds

We outline our study protocol to investigate genetic variants in the glomerular endothelium and basement membrane as potential contributors to glomerular hematuria in LPHS. We will also evaluate variants in candidate genes in pain pathways. We anticipate that the results from this study will identify new directions for an investigation into the molecular mechanisms underlying LPHS pathophysiology.

Introduction

Loin pain hematuria syndrome (LPHS) remains a poorly understood chronic pain disorder since its first description in 1967.[1] With a reported prevalence of .012%,[2] it typically affects young women in their late 20s or early 30s. Patients with LPHS experience debilitating flank (loin) pain, which can be unilateral or bilateral, along with gross or microscopic hematuria.[2,3] In 1996, Hebert et al proposed glomerular hematuria as the instigating event, sequentially leading to tubular obstruction, back leak of glomerular filtrate, and local parenchymal edema promoting compression of adjacent tubules and subsequent capsular stretch leading to pain.[4] However, the absence of pain in other clinical conditions that include red blood cell and cellular cast formation including acute tubular necrosis (ATN), pyelonephritis, IgA nephropathy (Henoch Schoenlein Syndrome), vasculitis, and antiglomerular basement membrane disease argues against the validity of this mechanism. We believe LPHS likely represents a heterogeneous collection of cases that present with shared phenotypic features of glomerular hematuria and pain originating from the kidneys but with different pathogenesis and natural history.

Glomerular hematuria in the absence of proteinuria is typically associated with defects in the glomerular filtration barrier, as most forms of podocyte injury lead to some degree of proteinuria. The remaining structural components of the filtration barrier include the glomerular basement membrane (GBM) and the specialized fenestrated glomerular endothelial cells (GEC). Glomerular basement membrane plays a key role in keeping the cellular elements of blood from trespassing into the urinary space. The best technique to evaluate GBM structure is electron microscopy of a kidney biopsy. However, electron microscopy has not revealed structural deficits in the GBM structure of most patient's with LPHS.[4]

There is a real need for an enhanced understanding of LPHS pathogenesis using advanced cellular and molecular technologies to explicate, detect, and diagnose both its presence and probable outcome to treatment. Rapid advances in next-generation sequencing (NGS) technologies over the last 2 decades have enabled the extensive unbiased interrogation of the exome, with patient/parent trio being a successful approach for identifying candidates and *de novo* variants. Specifically, the trio exome analysis has added crucial insights for diseases with complex pathogenesis and a heterogenous rate of progressions, such as IgA nephropathy,[5] steroid-resistant nephrotic syndrome,[6] nephrolithiasis, and nephrocalcinosis.[7] In addition, for diseases like chronic kidney disease (CKD) of unknown cause, where renal ultrasounds and kidney biopsies are uninformative and are unable to distinguish between multiple diseases, exome sequencing has led to the identification of 500 monogenic causes of CKD,[8,9] which in turn led to an increased diagnosis rate.[10] Similarly, a phenotypic spectrum of GBM abnormalities has been reported in patients with rare pathogenic variants in type IV collagen genes (*COL4A3/4/5*), classically associated with Alport syndrome, ranging from severe abnormalities in GBM thickness to no visible abnormalities at all. The identification of more than 1000 mutations in the *COL43/4/5* genes[11,12] that encode for the type IV collagen A3A4A5 heterotrimer (a major component of the GBM), has led to the reclassification of Alport syndrome and benign familial hematuria/thin basement membrane nephropathy (TBMN) as type IV collagen nephropathies,[13] promoting genetic testing as the gold standard in understanding the prognosis of the phenotype. Therefore, we believe that the next logical step will be to interrogate the genes that encode the filtration barrier in patients with LPHS. There are 8 genes that encode GBM and receptors and 10 genes that code for endothelial cells that we know of to date.[14]

While hematuria is believed to be glomerular in origin, the genesis of the pain remains an unsolved issue. The transmission of pain from the viscera to the brain involves a pathway that is controlled by a series of genes that code for transduction, conduction, synaptic transduction, and modulation. Specialized receptors, expressed in the peripheral termini of these neurons, allow noxious stimuli to be transduced into electrical impulses. The local membrane depolarization generated by stimulus transduction is transmitted along the axon by specific channels, some of which are expressed

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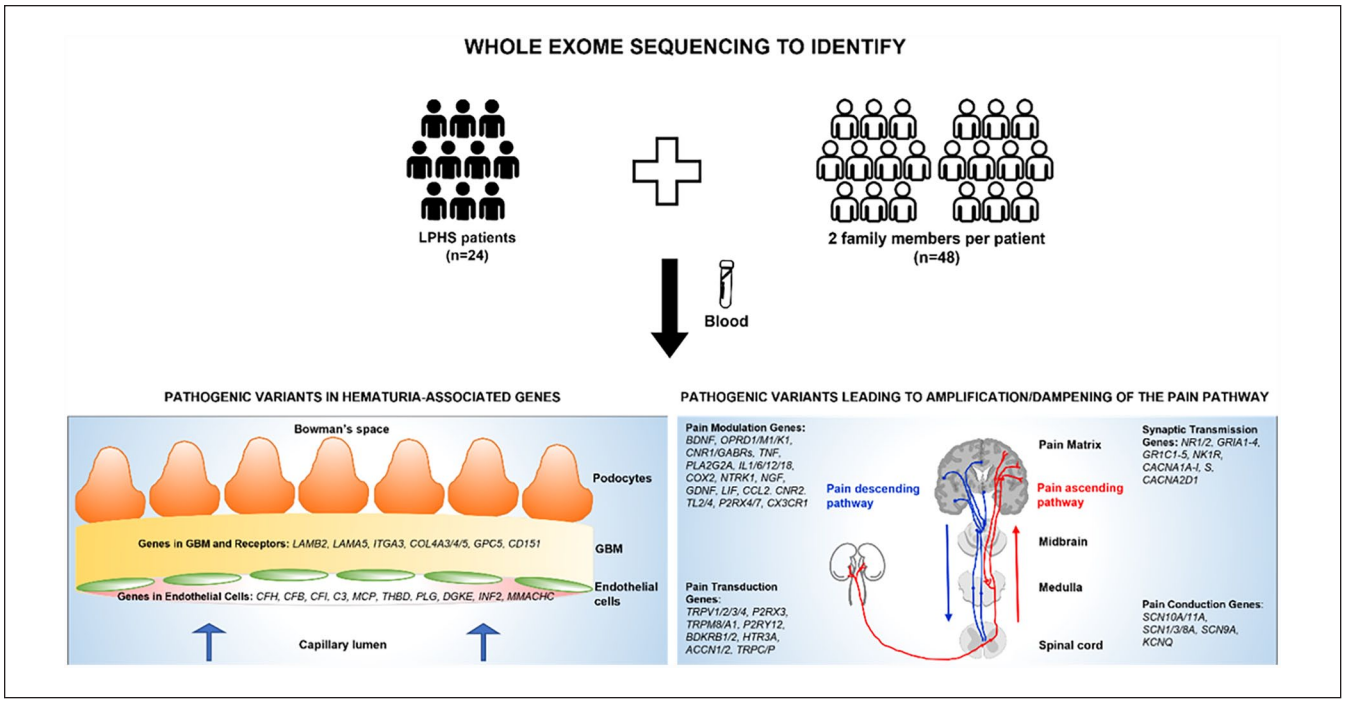

Figure 1. Study design.

specifically in nociceptors. Transmission is modulated by specific channels, which generally act to reduce excitability. Nociceptors terminate in the spinal cord dorsal horn, forming synapses with nociceptive-specific spinal projection neurons. From the spinal cord, information is transmitted to the brain stem and then processed by a pain matrix in multiple brain regions. Descending input from the brain to the spinal cord back to the periphery can both inhibit and facilitate the transmission of information in nociceptive circuits. We believe that LPHS patients have either gain-of-function mutation of the pain pathways and/or a loss-of-function mutation in the pain dampening pathways. We hypothesize that rare variants identified in this study will help delineate disease pathways from a syndrome driven by clinically ascertained phenotype as a diagnosis of exclusion into one based on the molecular basis of disease that leads to hematuria and physically incapacitating pain (Figure 1). To improve the sensitivity and specificity to identify a responsible variant over singleton analysis, we decided to perform a family-based analysis. Exome sequencing will be performed in LPHS-affected probands and participating first-degree family members, to effectively detect *de novo* and compound heterozygous variants. In the absence of parents, analysis will be performed on the other family members such as siblings or cousins.

Systematic Review of Observational Studies

We searched PubMed, Embase, Scopus and Web of Science databases to identify published studies on LPHS (search term: "loin pain hematuria" OR "Loin pain-hematuria" OR "loin pain/haematuria" OR "loin pain haematuria"). The search was generated in September 2021, with no date limits set. A total of 110 articles were identified. Studies with no abstracts or articles were excluded (n = 6). Upon review of 104 LPHS articles included, encompassing 610 LPHS patients, with 68% of the articles and 87% of patients reported from the United States and United Kingdom alone. With all articles published so far focusing on symptomatic pain management using oral narcotic therapy and/or interventional management strategies such as intra-ureteric infusions,[15-17] surgical renal denervation,[18-20] radiofrequency ablation,[21-23] neuromodulation,[24-26] and auto-transplantation,[27-31] we did not identify any studies looking at (A) evidence of familial clustering of LPHS or its components, variability in prevalence across different ancestries, or antecedent exposures or condition, or (B) variants in genes coding for proteins that are involved in GEC, GBM, or the pain pathways.

Research Objectives

The specific objectives of the study are as follows:

Objective 1: Identify potentially pathogenic variants in 18 genes associated with hematuria[14] (Table 1).
Objective 2: Identify potentially pathogenic variants in 89 genes associated with pain syndromes[32] (Table 2).

Methods

We plan to conduct a single-center, pilot study with the aim of decoding the molecular basis of LPHS. The study has

Table 1. Candidate Genes to be Investigated for Contributing to Hematuria in LPHS.

Gene name	Chromosome number	Inheritance[a]	Gene annotation
Glomerular basement membrane and receptors			
LAMB2	chr3	AR	Laminin subunit beta 2
LAMA5	chr20	AR	Laminin subunit alpha 5
ITGA3	chr17	AR	Integrin subunit alpha 3
COL4A3/4/5	chr2/chr2/chrX	AR, AD/AR, AD/XLD	Collagen type IV alpha 3/4/5 chain
GPC5	chr13		Glypican 5
CD151	chr11		CD151 molecule – transmembrane 4 superfamily
Endothelial cells			
CFH	chr1	AR, AD	Complement factor H
CFB	chr6	AR, AD, DD	Complement factor B
CFI	chr4	AR, AD	Complement factor I
C3	chr19	AR, AD	Complement C3
MCP	chr1	AR, AD	Membrane cofactor protein
THBD	chr20	AD	Thrombomodulin
PLG	chr6	AR, AD	Plasminogen
DGKE	chr17	AR	Diacylglycerol kinase epsilon
INF2	chr14	AD	Inverted formin 2
MMACHC	chr1	AR	Metabolism of cobalamin associated C

[a]Inheritance based on OMIM database: AD, autosomal dominant; AR, autosomal recessive; Mu, multifactorial; SMu, somatic mutation; XL, X linked; XLR, X linked recessive; XLD, X linked dominant

Table 2. Candidate Genes to be Investigated for Understanding Pain in LPHS Patients.

Gene name	Chromosome number	Inheritance[a]	Gene annotation
Pain transduction			
TRPV1/2/3/4	chr17/chr17/chr17/chr12	TRPV3/4: AD	Transient receptor potential cation channel subfamily V member 1/2/3/4
P2RX3	chr11		P2X purinergic receptor
TRPM8	chr2		Transient receptor potential cation channel subfamily M member 8
TRPA1	chr8	AD	Transient receptor potential cation channel subfamily A member 1
P2RY12	chr3	AR	Purinergic receptor P2y12
BDKRB1/2	chr14/chr14		Bradykinin receptor B1/2
HTR3a	chr11		5-Hydroxytryptamine receptor 3a
ACCN1/2	chr17/chr12		Acid sensing ion channel subunit 1/2
TRPC/P	chr13		Transient receptor potential canonical
Pain conduction			
SCN10A	chr3	AD	Sodium voltage-gated channel alpha subunit 10
SCN11A	chr3	AD	Sodium voltage-gated channel alpha subunit 11
SCN1,3,8A	chr2/chr2/chr12	AD/AD/AD	Sodium channel protein type 1/3/8A
SCN9A	chr2	AR, AD	Sodium voltage-gated channel alpha subunit 9
KCNQ	chr11	AR, AD	Potassium voltage-gated channel subfamily Q
Pain synaptic transmission			
NR1, 2	chr9/	AR, AD	Nuclear receptor subfamily 1/2
GRIA1-4	chr5/chr4/chrX/chr11	AR, AD/ AD/ XLR/ AD	AMPA receptor 1-4
NK1R	chr2		Tachykinin receptor 1
CACNA1A-I, S	chr19/chr9/chr12/chr3/ chr1/chrX/chr17/chr16/ chr22/chr1	AD/ AR/ AD/ AR, AD/ AD/ XL, XLR/ AD/ AD/ AD/ A R, AD	Calcium voltage-gated channel subunit alpha1 A-S
CACNA2D1	chr7	AR	Calcium voltage-gated channel auxiliary subunit alpha2 delta 1

(continued)

Table 2. (continued)

Gene name	Chromosome number	Inheritance[a]	Gene annotation
Pain modulation			
BDNF	chr11		Brain-derived neurotrophic factor
OPRD1/M1/K1	chr1/ chr6/ chr8		Opioid receptor delta 1/Mu1/kappa 1
CNR1	chr6		Cannabinoid receptor 1
GABRs (GABRD/E/P/Q/A1-A6/BR1-BR3/G1-G3/R1-R3	chr1/chrX/chr5/chrX/chr5/ chr4/chrX/chr4/chr15/ chr5/chr4/chr5/chr15/ chr4/chr5/chr15/chr6/ chr6/chr3	AD/ M/ M/ M/ AD/ AD, Mu/ XL/ M/ AD/ M/ AD/AD/ AD/ M/ AD/ M/ M/ M/ M	Gamma-aminobutyric acid type A receptors
TNF	chr6	AD	Tumor necrosis factor
PLA2G2A	chr1	AD, SMu	Phospholipase A2
IL1/6/12/18	chr2/chr7/chr3/chr11	M/ Mu, SMu, AR, AD/ M/ M	Interleukin 1/6/12/18
COX2	chrMT		Cytochrome C oxidase subunit 2
NTRK1	chr1	AR	High affinity nerve growth factor receptor
NGF	chr1	AR	Beta-nerve growth factor
GDNF	chr5	AD	Glial cell line-derived neurotrophic factor
LIF	chr22	AD	Leukemia inhibitory factor
CCL2	chr17		C-C motif chemokine 2
CNR2	chr1		Cannabinoid receptor 2
TLR2/4	chr4/chr9	AD, SMu/ M	Toll-like receptor 2/4
P2RX4/7	chr12/chr12		P2X purinoceptor 4/7
CX3CR1	chr3		CX3C chemokine receptor 1

[a]Inheritance based on OMIM database: AD, autosomal dominant; AR, autosomal recessive; Mu, multifactorial; SMu, somatic mutation; XL, X linked; XLR, X linked recessive; M, missing or no information in OMIM database.

been approved by the Saskatchewan Health Authority Research Ethics Board (REB-22-66).

Design

Number of Subjects

Twenty-four LPHS patients referred to the nephrology clinic (run by the corresponding author) were approached by the study coordinator, and all 24 agreed to participate in the study. Recruitment of 2 family members of the LPHS patient (parents/siblings/cousins/children) is ongoing. We are the first study to identify and report 2 patients with LPHS (12, 17) with a positive family history of LPHS (Figure 2). In addition, 6 LPHS patients (LPHS: 01, 02, 04, 05, 11, and 21) had a positive family history of intermittent hematuria (Figure 2).

Duration of Study Period

The study period extended for about 3 years (2022-2025).

Participant Selection and Informed Consent

The LPHS patients have been identified by BP (Nephrologist) and FG (Urologist), based on the inclusion and exclusion criteria defined by Spetie et al[4] All patients and family members that meet the eligibility criteria will need to complete the written consent forms followed by baseline data collection (detailed below).

Inclusion criteria for LPHS patient

- ≥18 years of age
- Patients diagnosed with LPHS (by nephrologist and/or urologist).

Exclusion criteria for LPHS patient

- Urological causes of flank pain and hematuria (obstructive uropathy, nephrolithiasis, pyelonephritis, polycystic kidney disease, renal artery embolism, renal artery dissection, renal papillary necrosis, renal vein thrombosis, left renal vein entrapment [nut-cracker syndrome], renal trauma, or renal tumor).
- Additional functional/structural reason for hematuria by kidney biopsy or other interventions.

Inclusion/exclusion criteria for family members

- Only family members (parents/siblings/children/first cousin) of the patient who provide informed consent.

Data Collection, Sample Collection, and Exome Analysis

Demographic Data

Data related to age, sex, gender, self-reported race/ethnicity, occupation, socioeconomic status, weight, height,

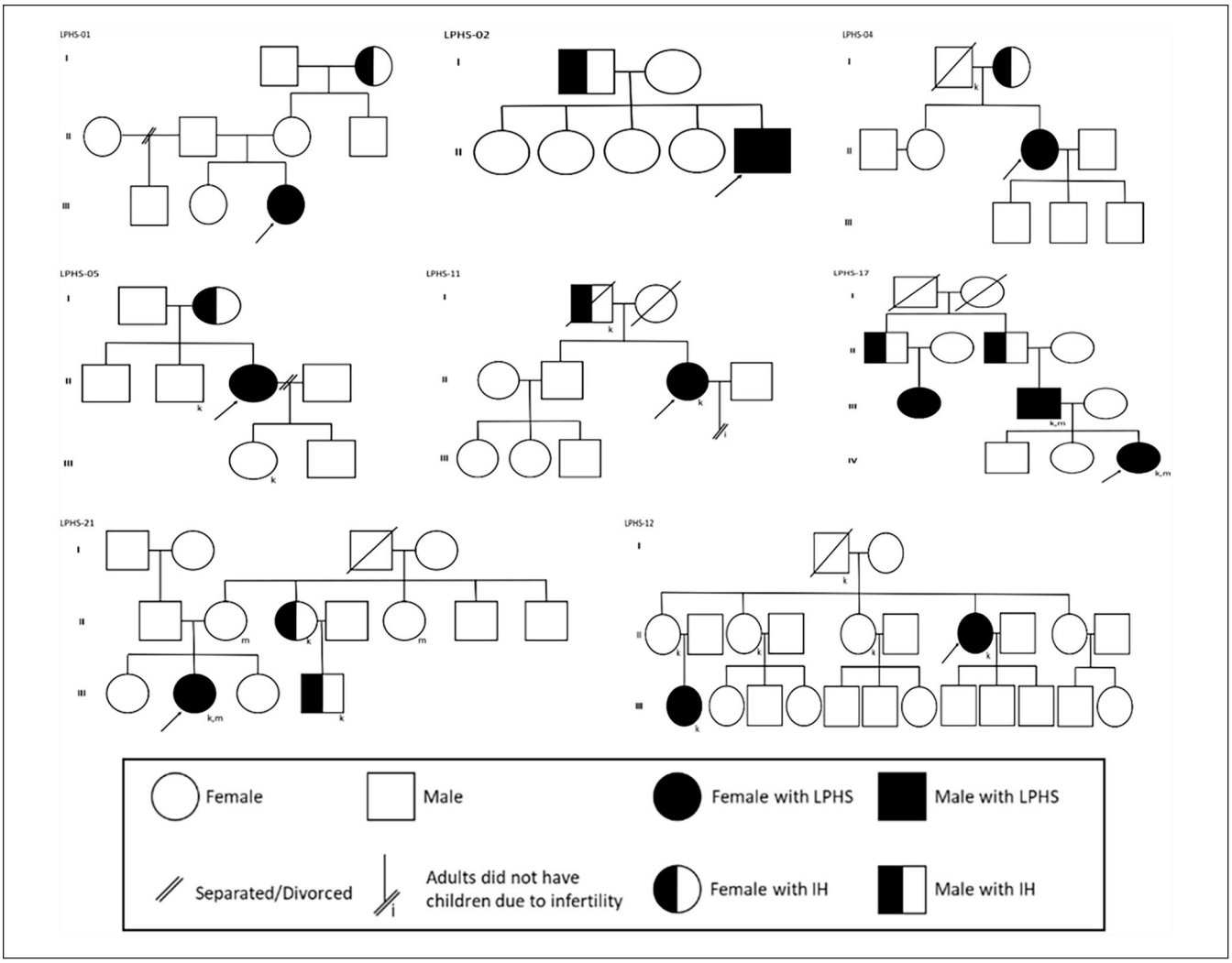

Figure 2. Pedigree of LPHS patients with a family history of LPHS or isolated hematuria (IH). Roman numbers represent the generations of the family.
Note. Proband is indicated by arrow. Abbreviations: k, kidney stones; m, migraine.

comorbidities, family history of diseases (eg, kidney stones, isolated hematuria, LPHS, diabetes, hypertension, chronic fatigue syndrome, mood disorders, asthma, and allergies), the location of pain (bilateral or unilateral), the number and frequency of pain medications, and pain score using the brief pain inventory form and PainDetect questionnaire will be collected for the LPHS patients.

Laboratory Data

Complete blood count, serum electrolytes, serum urea and creatinine, albumin creatinine ratio, and urine analysis.

Genetic Data

Rare variants in genes associated with hematuria (n = 18) and in pain transduction (n = 17), conduction (n = 8), synaptic transmission (n = 37), and pain modulation (n = 27).

Hierarchical Clustering of Patient Phenotype Data

Patients will be grouped based on their demographic, laboratory, and genetic data using unsupervised hierarchical clustering (Ward's method with Euclidean distances).[33] The data will be presented as clustered dendrograms.

Sample Collection, Storage, and Preparation

Venous blood samples will be collected for study probands and participating family members in PAXgene Blood RNA Tubes (Qiagen, Hilden, Germany). DNA will be extracted from PAXgene Blood RNA Tube using the New England Biolabs Monarch Genomic DNA Purification Kit as per the protocol by Kruhøffer et al.[34] DNA libraries will be constructed using the Illumina DNA Prep with Enrichment, Tagmentation kit and IDT xGen Exome Research Panel v2 with xGen Universal Blockers—NXT Mix and dual unique

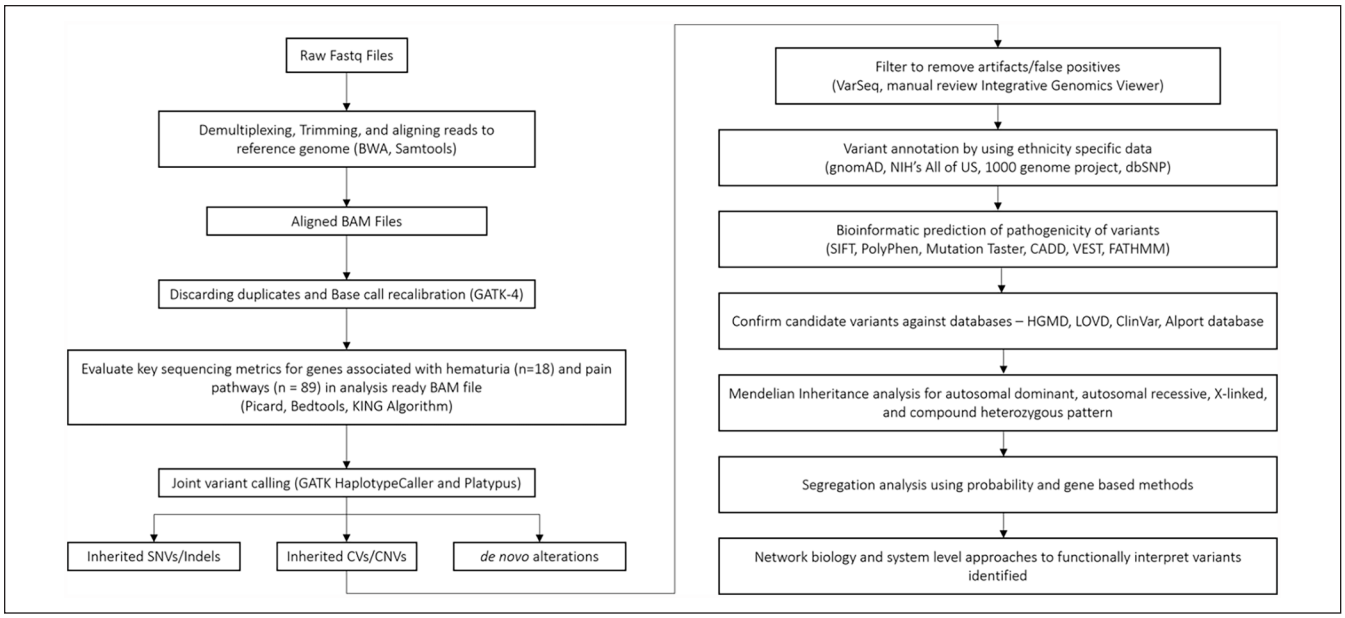

Figure 3. Pipeline for exome data analysis.

barcodes. Paired-end sequencing (2 × 150 bps) on the Illumina NovaSeq 6000 System at 100× depth for exome sequencing. Library preparation and sequencing will be performed for all family members at the same time to minimize potential artifactual differences due to sample preparation. DNA samples will be stored at −80° centigrade to allow for future verification studies.

Exome Analysis

As depicted in Figure 3, raw fastq files will be processed to remove adapter sequences using cutadapt (v1.11).[35] Reads with quality score of ≥30 will be retained for analysis. Contaminating reads will be removed after aligning of the processed reads to the human genome using BWA[36] and Samtools.[37] The reads will be preprocessed using the Genomic Analysis Tool Kit (GATK), as per the recommendations in the Best Practices Workflow by the GATK[38] for all positions with ≥20× coverage, genotype quality ≥20, and minor read ratio ≥0.2 for indel alignment, base quality score recalibration, base alignment quality scoring, and variant calling (single-nucleotide variants [SNVs], indels, short tandem repeats [STRs], structural variant [SV]). Variant allele frequency (VAF) will be calculated as the percentage of sequence reads observed for the alternative allele compared to all coverage of that nucleotide. Exome data will first be evaluated for genes associated with isolated hematuria and pain (as listed in Tables 1 and 2). The variants will be further filtered and prioritized using VarSeq software (Golden Helix, Bozeman, MT, USA) and variant effect predictor. Benign variants with MAF (minor allele frequency) >1% in any ancestry will be eliminated using the 1000 Genomes Project,[39] dbSNP,[40] gnomAD[41] and National Institutes of Health (NIH)'s All of Us.[42] In silico bioinformatic prediction of pathogenicity of variants will be performed using the following prediction algorithms: scale-invariant feature transform (SIFT),[43] PolyPhen2,[44] Mutation Taster,[45] CADD,[46] VEST,[47] and FATHMM.[48] Finally, the candidate variants will be checked against the human gene mutation database (HGMD)[41], LOVD,[12] Clinvar,[49] and Alport database.[50] A multidisciplinary team will then review each variant for evidence of pathogenicity and contribution to the phenotype and classify them according to the American College of Medical Genetics guidelines.[51] Possible pathogenic loci will be screened according to 3 heredity models, namely autosomal recessive (AR) inheritance, autosomal dominant (AD), and X-linked inheritance. Sanger sequencing will be carried out to validate potentially pathogenic variants identified through high-throughput exome sequencing. Special attention will be paid to *de novo* variants, not present in parents, as well as variants with variant allele frequencies suggestive of potential somatic variation. Finally, we will also assess for the presence of copy number variation using CoNIFER,[52] cn.MOPS,[53] and CNVkit.[54] To evaluate the pathogenicity of a rare variant, we will look at the segregation of variants among all sequenced family members using (A) probability-based models by Helbig et al[55] and Jarvik et al,[56] and (B) gene-based segregation methods.[57]

Potential Risks to the Participants

In Canada, people are protected from being required to provide the results of a genetic test by the Genetic Non-Discrimination Act.[58] The genetic results will not be

disclosed to any third party such as employers, insurance companies, or educational institutions. The confidentiality of the participant will be respected. All collected samples will be assigned a unique study number, with no reference to individual identifiers. As this research involves looking at genetic information, it carries the risk of identifying an underlying genetic change(s) which are unrelated to this study and have the potential of affecting the participant. However, this research is being conducted for the scientific purpose of understanding only the cause of pain and hematuria in LPHS patients. In addition, the results of this research project will not be placed in the participant's medical record. All efforts will be made to safeguard participants' privacy.

Author Contributions

BP conceived and designed the study. AS wrote the initial draft. ML and SL assisted with the drafts. ML provided advice regarding genetic study design. BP edited the final manuscript. All authors read and approved the final manuscript.

Declaration of Conflicting Interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: BP has received speaker and advisory fees from Bayer, Otsuka and Astra Zeneca. MBL has received speaker and advisory fees from Otsuka, Reata, Bayer, and Sanofi Genzyme.

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Ethics Approval and Consent to Participate

The study will be conducted in accordance with the second edition of the Tri-Council Policy Statement—Ethical Conduct for Research Involving Humans—TCPS 2. We have received REB certificate of approval for the study (REB-22-66). Written informed consent will be obtained from all the participants in the study.

Consent for Publication

Not applicable as there is no patient identifying information in this article.

Availability of Data and Materials

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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