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Organization and implementation of a diagnostic care model for rare kidney diseases

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1. Introduction

1.1 Introduction to pediatric CKD

Chronic kidney disease (CKD) is a global health problem and a major contributor to deaths worldwide. Due in part to the rise in its risk factors, such as obesity and diabetes mellitus, the number of patients affected by CKD has also been increasing, affecting an estimated 843.6 million individuals worldwide in 2017 (1). Accordingly, in the same year CKD has been reported as causing 1.2 million deaths globally. (2) CKD is often a silent disease with sometimes slow and indolent evolution. According to kidney function it is classified internationally by five stages, the last of which is the end stage kidney disease (ESKD) (3). Data coming from epidemiological studies in adults provide dramatic evidence that ESKD represents probably only the 'tip of the iceberg' of CKD and suggest that the number of patients with earlier stages of the disease are likely to exceed those reaching ESKD by as much as 50 times (4). The same data indicating CKD as a global health problem are considered valid also for pediatric and young adult patients (5). Recent aggregated numbers from international registries from Europe and other western countries reported prevalence rates varying from 55 to 77 people per million age-related population in 2015 (6). These numbers are moreover thought to increase in the next years due to medical improvements in the management of preterm newborn, a condition associated with CKD due to low nephron endowment (7). Besides this, no precise data on the incidence and prevalence of pre-terminal CKD in the pediatric population are available for the majority of countries (8).

Also, there is substantial difference between the main causes of CKD in pediatric and young adult and adult population. In the pediatric age group, the leading cause of ESKD is congenital anomalies of the kidney and urinary tract (CAKUT), which account for up to 50% of pediatric diagnoses in international registries. These are followed by steroid-resistant nephrotic syndrome, chronic glomerulonephritis (e.g. Alport syndrome) and renal

ciliopathies, which respectively represent 10%, 8%, and 5% of diagnoses (5). Within early-onset CKD cases, specifically those under 25 years of age, there's a variation in the frequency of diagnoses: while CAKUT is the predominant diagnosis in pediatrics, chronic glomerulonephritis becomes more prevalent during puberty. As age progresses, this epidemiological pattern increasingly aligns with CKD in adulthood, where, albeit due to different etiologies, chronic glomerulonephritis stands out as the primary cause of ESKD (5).

From a prognostic standpoint, it's becoming increasingly evident that many patients diagnosed with pediatric CKD progress to ESKD during early adulthood (9). This transition period, from pediatric to adult nephrology, often witnesses a gap in specialized medical care. Population studies have shown that patients with a history of renal disease during childhood, even if demonstrating preserved renal function during adolescence, faced an elevated risk of ESKD later on (9). This realization has, in recent years, catalyzed efforts to establish dedicated transition pathways for pediatric nephropathy patients transitioning into adulthood. However, the preliminary step remains the accurate identification of these patients (10). In routine clinical practice, the International Nephrology Society guidelines (KDIGO) rely on creatinine levels to assess renal function. However, the classification system for CKD staging, as per the KDIGO guidelines, is primarily derived from adult data. This introduces several limitations when applied to the pediatric population (5,11). For instance, it cannot be reliably used for children under two years of age, as their kidney function is not yet fully matured, making it challenging to ascertain true renal function. Additionally, since creatinine production is tied to muscle mass, its levels might be misleadingly low in prepubescent children, given their relatively underdeveloped musculature (11).

Given these intricacies in diagnosing and managing pediatric CKD, it becomes paramount to precisely identify and appropriately manage these patients from an early stage. Early diagnosis not only ensures tailored medical interventions but also aids in prognostic determinations. Furthermore, the early onset and presentation of CKD in these patients underscores a significant clinical consideration: a substantial proportion might have an underlying genetic etiology. This pivotal connection between pediatric CKD and genetic predispositions paves the way for ensuing discussion on genetic kidney diseases, emphasizing the profound implications of genetics in nephrology.

1.2 The genetic landscape

From the 1980s onwards, genetics has progressively infiltrated clinical practice, significantly expanding our understanding of diseases and broadening the diagnostic possibilities for conditions with varying population frequencies. Within the realm of nephrology, the pioneering associations established were those of autosomal dominant polycystic kidney disease (ADPKD) and Alport syndrome. These conditions epitomize the so-called monogenic diseases, exemplifying the “one gene = one disease” correlation (12).

The delineation of monogenic diseases was originally achieved through techniques such as Sanger sequencing. These methods were characterized by their specific approaches and limitations. Sanger sequencing, introduced by Frederick Sanger in the 1970s, was the first method developed to sequence DNA and has since been foundational in the world of genetics. It operates on the principle of chain termination, using dideoxynucleotides to selectively halt DNA synthesis at every possible position. The resulting fragments are then separated by electrophoresis, and the sequence is read based on the length of these fragments. Though revolutionary at its inception, Sanger sequencing has limitations in terms

of throughput, as it is typically used to sequence short DNA stretches, making it labor-intensive and less feasible for sequencing large genomes (13).

Over the past decade, the development of next-generation sequencing (NGS) techniques has provided a significant impetus to the integration and essentiality of genetic diagnostics in clinical research. Contrasted with older generation techniques, these new methods allow for the sequencing of broader DNA regions, thereby enabling the examination of more extensive genetic material. In this case there was the use of gene panels that represent a more targeted approach. They involve the simultaneous testing of multiple genes known to be associated with a particular condition or group of related disorders. Panels utilize a variety of sequencing techniques, often combined with capture methodologies to selectively enrich the regions of interest from the genome (13,14). While they offer a more efficient way to scan multiple genes of interest compared to traditional methods, they are constrained by their predefined set of genes, potentially missing novel or less-known mutations outside these selected regions. The progressive cost reduction associated with these methodologies has facilitated their transition from the research to bedside. The NGS techniques that have made the most significant contributions to clinical nephrology are primarily centered around whole exome sequencing (WES). WES has progressively broadened our understanding of disease phenotypes. This expansion of knowledge is attributed to the capability of WES to sequence all the protein-coding regions of genes, known as exons, which constitute about 1% of the human genome but harbor approximately 85% of known disease-related variants (13,14). By focusing on this crucial subset of the genome, WES provides an effective approach to identify new potential disease-causing mutations. Moreover, since the analysis is conducted *in silico*, it allows for the stored DNA data to be reanalyzed in the future, accommodating advancements in knowledge or when seeking clarity on previously ambiguous results. Indeed, as research progresses and

new disease-associated genes or variants are identified, the exome data can be revisited and reanalyzed, lending it a dynamic edge. This iterative nature of WES has enabled clinicians and researchers to continually refine and expand the genotype-phenotype correlations, revealing previously unrecognized manifestations of genetic kidney diseases and contributing to personalized medicine approaches. Moreover, WES has facilitated the discovery of novel genes associated with renal diseases, uncovering intricate molecular pathways and fostering the development of targeted therapeutic strategies (14).

These advancements have facilitated a transition from the traditional Mendelian disorders to those with more complex heredity patterns. The vast amount of information garnered over the years means that today, we recognize more than 625 Mendelian disorders associated with kidney and urinary tract traits (15). However, as our knowledge base continues to expand, the focus is now shifting towards studying diseases that demonstrate non-Mendelian inheritance patterns. Such diseases often have multifactorial etiologies, where a combination of multiple genes and environmental factors contribute to the disease phenotype. This complexity presents unique challenges in diagnosis and management, as they do not follow the predictable inheritance patterns seen in Mendelian disorders since both genetic predispositions and external factors play roles in disease manifestation (16).

The integration of this vast amount of genetic information into clinical practice, especially when conveying results to patients, has underscored the need for establishing multidisciplinary teams (13,17,18). These teams are essential for evaluating the true pathogenicity of DNA sequence variations encountered when analyzing extensive portions of DNA (13). Among the challenges faced in genetic interpretation are the Variants of Unknown Significance (VUS). These are genetic variants for which the clinical implications are not yet well-defined, often because they are rare or have not been previously reported in the context of a specific disease. The presence of a VUS complicates genetic counseling, as

it neither confirms nor rules out a genetic basis for the disease in question. Given the potential for ambiguous or uncertain findings with VUS, it's crucial to have a holistic assessment that takes into account clinical, laboratory, and genetic data. Collaborative efforts among geneticists, clinicians, and laboratory experts help in interpreting these variants, leveraging databases, functional studies, and patient phenotypes. Only with such comprehensive evaluations can truly informed clinical decisions be made, ensuring that the information is appropriately communicated to patients and guiding them in potential further actions or surveillance. These premises paved the way for the establishment of renal genetic clinics (17).

1.3 Personalized medicine in chronic kidney disease by detection of genetic variants

Over the past fifteen years, NGS studies combined with the use of animal models have enabled the establishment of correlations between specific genetic mutations and clinical syndromes. The initial studies with larger case numbers were performed in the pediatric population with an early onset of CKD and then in the adult population (14,17). These studies revealed that renal diseases in pediatric age have genetic causes in up to about 30% of cases. This percentage appears to be more variable in the adult population, ranging from 5 to 30% (14). These diagnostic rate differences are currently being re-evaluated. In fact, more recent studies have shown that the diagnostic yield would be the same in both populations (19). Indeed, the diagnostic yield is also highly variable depending on the considered clinical group. For instance, the diagnostic yield is particularly high in cystic and tubular diseases (up to 80-100% in some studies), but it drops below 20% in CAKUT (13). The broad variations are related to various factors: the number of mutations discovered in different diseases, different disease transmission and mechanisms among various diagnostic groups, sample selection criteria, and the DNA sequence analysis pipeline.

Regardless of the diagnosis rate, the use of NGS techniques has accelerated the concept of personalized medicine. Traditionally, diagnostics often relied on a mix of clinical features, histological findings, and laboratory markers. With the rise of genetics, especially tools like WES and targeted gene panels, clinicians can now identify the precise molecular foundations of a patient's kidney disease. This precision is especially beneficial in cases with non-standard presentations or when several potential diagnoses are possible. Indeed, some mutations can manifest as phenocopies of other genetic kidney disease (20). A phenocopy can be understood as a clinical scenario where a patient shows symptoms identical to a genetic disease but doesn't have the typical genetic makeup associated with that disease. In the context of nephrotic syndrome, this has been found to be particularly relevant. Sometimes, patients with steroid-resistant nephrotic syndrome are treated by immunosuppressive drugs, when common genetic tests, particularly those identifying genes associated with podocytopathies, come back negative. However, they exhibit all the clinical signs of the genetic disease, making them phenocopies of monogenic podocytopathies. This term implies that these patients' conditions mimic those caused by specific podocytopathies genotypes, although these patients don't carry those genotypes themselves. Utilizing advanced tools like WES and adopting a "reverse phenotyping" approach can help clarify these complex cases. Briefly, reverse phenotyping is an approach in which specific clinical features are interrogated in a subsequent clinical examination once a likely molecular genetic diagnosis has been established (21). Our group recently showed that using these tools, it was possible to increase the diagnostic rate of genetic tests from 30% to 58% when genetic phenocopies are considered in patients affected by nephrotic syndrome (22). In particular, we used WES in 111 patients affected by nephrotic syndrome: according to literature, we found pathogenic variants in 30% of patients; however, a substantial number of variants were identified in other genes, not commonly associated with isolated nephrotic

syndrome. Reverse phenotyping, on the basis of a personalized diagnostic workflow, allowed to identify previously unrecognized clinical signs of an unexpected underlying genetic nephropathy in a further 28% of patients.

Moreover, understanding the genetic roots of a kidney ailment has consequences that go beyond simple diagnostics. It opened up possibilities for tailored treatments. For instance, discerning the specific genetic mutation in a patient with a well-defined form of kidney disease can guide treatment choices, potentially sidestepping treatments that might be ineffective while emphasizing those that directly tackle the underlying genetic cause. A prime example of this is primary hyperoxaluria type 1 caused by a mutation in the *AGXT* gene. The identification of the mutated gene and the understanding of the resulting damage mechanism has allowed researchers to pinpoint a specific pharmacological target and, consequently, develop a drug tailored for this specific condition, blocking the endogenous oxalate production (23). Also, establishing genotype-phenotype correlations has led to improved prognosis insights. Some genetic variants may be associated with a more aggressive disease manifestation or a heightened risk of progressing to end-stage renal disease. Such insights provide clinicians the tools to customize monitoring and management strategies accordingly. In this context, for diseases such as polycystic kidney diseases, Alport syndrome and Bartter and Gitelman Syndromes, it's now possible to tentatively predict the severity of the patient's condition and the likelihood of progressing to ESKD during lifecourse (24–26).

1.4 The cost related to genetic sequencing: a possible trade-off.

Given the epidemiology of pediatric CKD, which encompasses profound clinical, familial and social consequences, the healthcare systems worldwide are exposed to an important economic burden. For instance, in the United States, the total annual direct costs attributable

to CKD are approximately USD 49 billion (27) and, in Italy, approximately 9.6% (5.8 million people) of Italian patients had CKD which resulted in an annual expense of 1.81 billion euros in 2016 (28). Taking these numbers into account, advancing knowledge of etiologic causes of CKD is paramount for understanding the pathogenesis, for adequate patients' classification, prognosis and treatment and to provide a "personalized medicine approach" for patients with CKD.

As previously discussed, the implementation of genetic diagnostics can undoubtedly play a pivotal role in this process. However, it also carries the potential of increasing healthcare costs, paradoxically exacerbating the financial burden that renal diseases exert on the healthcare system. Initial studies in other clinical specialties seem to emphasize that, despite the higher costs of genetic methods compared to standard diagnostic pathways, the benefits might outweigh the costs (29). On the other hand, the prevailing opinion within the scientific community is that the progressive reduction in sequencing costs will further facilitate the widespread adoption of genetic diagnostics (30).

The key to achieving cost-efficiency lies in optimizing the workflow for diagnosing rare kidney diseases using genetic methods. While significant advancements have been made on the research front in the past decade, its application in general clinical practice is still in its infancy. This necessitates the development of efficient service delivery models, which are now beginning to be proposed by various international groups (17). Establishing these models requires addressing existing barriers and in the very last years first guidelines for the implementation of genetic testing in clinical nephrology have been issued by the International Society of Nephrology (18).

Foremost among main barriers is the appropriate selection of patients, aiming to maximize the diagnostic rate by applying NGS techniques to those with a high likelihood of a positive result. Given the intricacies associated with NGS reporting, such diagnostics

will likely need centralization in facilities with diverse expertise that collaborates cohesively. Conversely, it's equally vital to establish networks with peripheral facilities that refer patients, ensuring system-wide optimization.

Given the infancy of renal genetic services, data on cost-effectiveness on the wide adoption of NGS in clinical practice are lacking. Only one study demonstrated that genetic testing conducted early in a patient's diagnostic journey, while increasing diagnostic costs, is cost-effective in diagnosing podocytopathies and collagenopathies in both pediatric and adult populations (29). It's also essential to recognize that the benefits are not solely economic. There are indeed significant clinical advantages, as immediate diagnoses are attained, preventing unnecessary treatments for these conditions. The immediate diagnoses facilitate targeted treatments, reducing the physical and psychological burden on patients subjected to a myriad of tests and treatments, often with little to no improvement in their conditions. In this context, the value of a swift, accurate diagnosis is immeasurable. It heralds a paradigm shift in patient management, anchoring treatment plans in precision medicine, tailored to the unique genetic makeup of each patient.

In the grander scheme, the ripple effects of these immediate, accurate diagnoses permeate the entire healthcare ecosystem. They influence policy-making, guide resource allocation, and inform clinical guidelines, ultimately contributing to enhanced patient outcomes, optimized resource utilization, and improved quality of care. The integration of these findings can catalyze a transformation in renal genetic services, marking a transition from a predominantly clinical and symptomatic approach to a genetically informed, patient-centric model of care.

1.5 Aim of the thesis

CKD is a disease characterized by diagnostic challenges, with its clinical and economic impacts manifesting as early as in pediatric ages. The evolution of genetic diagnostic techniques has highlighted the significant role of genetic diseases, attributing them as the causative factor in up to 50% of pediatric cases (5). Early identification plays a crucial role in not only the clinical management but also the holistic care of the patient. However, as genetic diagnostics has only recently been integrated into clinical practice, there is a lack of substantial evidence on how to optimize its incorporation into service delivery models to maximize clinical benefits while mitigating associated costs.

The core objective of this research is predicated on the hypothesis that we can significantly enhance the diagnostic yield and realize substantial cost savings for the healthcare system. This can be achieved by devising and implementing a streamlined workflow that addresses three pivotal challenges. First, establishing clear and efficient selection criteria to identify patients who would benefit most from genetic testing. Second, enhancing the rate and precision of genetic diagnoses through optimized testing protocols. And third, pinpointing specific kidney diseases where early genetic intervention is not only clinically beneficial but also yields economic advantages for healthcare providers, reducing the costs of genetic diagnosis and thus favoring the spread of genetic sequencing in clinical practice.

2. Methods

2.1 Selection criteria definition

To establish and validate clinical criteria for patient selection and genetic testing prioritization, we retrospectively revised the results of genetic testing with WES in all the patients referred to the laboratory of Medical Genetics of Meyer Children' Hospital IRCCS of Florence (Italy) from 2015 (when WES was first adopted as a sequencing strategy) to 2018. Based on an extensive review of existing literature and recommendations, we evaluated various variables as potential indicators for successful genetic testing via WES. These variables included:

1) Resistance to Treatments: The non-responsiveness to treatments like steroids and immunosuppressive drugs, as defined by current guidelines.

2) Family History and Consanguinity: Having a family history of kidney disease or parental consanguinity, characterized by kidney ailments or CKD in first, second, or third-degree relatives.

3) Extra-Renal Involvement, the presence of symptoms or complications outside the kidneys.

4) Nephrotic Syndrome.

5) Persistent Metabolic Abnormalities, such as acidosis or alkalosis without kidney impairment, or calcium-phosphate metabolism imbalance lasting over three months (confirmed by at least two consecutive blood tests) after ruling out secondary causes.

6) CAKUT with CKD Stage G2 or Higher, as outlined in the KDIGO guidelines(3).

7) CAKUT without CKD.

8) Identification of at least two cysts in each kidney through ultrasound scanning.

9) Hyperechoic Kidneys or Nephrocalcinosis detected via kidney ultrasound scanning.

10) Hypertension.

11) Isolated Complement Abnormalities.

12) Age of the patient at the time of evaluation.

We employed univariable logistic regression analysis, treating these features as independent variables and a positive genetic diagnosis as the dependent variable. To mitigate bias associated with rare events, Firth's method was applied. The features that emerged from this analysis were then utilized as criteria for selecting and prioritizing patients for genetic testing.

2.2 Study population: Referral and Selection Process

In 2018, the Tuscany Region in Italy initiated government-funded projects to enhance the personalized diagnosis of genetic kidney disorders. Patients suspected of having these disorders were referred to our center at Meyer University Hospital IRCCS of Florence for genetic testing via WES. The hospital established in conjunction with other secondary-level hospitals across the region a network for patient referral (31).

Nephrologists across the region referred patients to the tertiary center if they met at least one established selection criteria indicating a potential monogenic cause of kidney disease. These referrals were independent of clinical phenotype and kidney biopsy results, in order to standardize the selection basing it only on the established framework of criteria.

At the tertiary center, patients underwent a thorough evaluation by a specialized nephrologist and a geneticist. Comprehensive demographic, clinical, and laboratory data were collected, including a detailed family history. Patients were excluded from genetic

testing if secondary causes of kidney phenotype were identified, if there was an absence of clinical information, or if a genetic diagnosis was already confirmed.

2.3 Genetic Testing and Classification:

Eligible patients were offered WES, and the collected data, including medical records and familial history, contributed to the establishment of clinically suspected diagnoses. Patients were categorized into eight clinical categories, including podocytopathies, collagenopathies, CKD of unknown origin (CKDu), tubulopathies, ciliopathies, CAKUT, syndromic CKD, and metabolic kidney disorders. Segregation analysis was performed on all probands, and first-degree relatives were either pre-included in the study or invited to participate post-identification of potentially causative variants (31).

Genetic testing was conducted at Meyer University Hospital's accredited laboratory in Florence, Italy. DNA extracted from peripheral blood underwent WES, utilizing standard kits and procedures as previously described (22). The DNA sequences were aligned with the human reference hg19 genome. Variant annotation was conducted using Annovar tool, and the predictions of the variant effect using different software (SIFT, Polyphen2, MutationTaster, MutationAssessor, FATHMM and FATHMM MKL)(22). Quality control of sequencing showed that 96% of the reads were mapped to the reference genome (hg19), and 97% of the targeted regions were covered by ≧ 30× reads with average depth of 100× (31).

Key inclusion criteria for variants included their association with kidney diseases or phenotype as per Online Mendelian Inheritance in Man (OMIM), Human Gene Mutation Database (HGMD), or recent scientific literature. The pipeline considered variants with a minor allele frequency (MAF) ≤ 0.01 for autosomal recessive and MAF ≤ 0.001 for autosomal dominant genes in specific population databases, and those absent in the

gnomAD database of healthy control populations. Additionally, variants had to be listed as “pathogenic”, “likely pathogenic”, or “variant of unknown significance (VUS)” according to the American College of Medical Genetics and Genomics (ACMG) guidelines and using annotation tool Varsome. VUS were only retained if supported by strong evidence of pathogenicity, either through functional studies, prediction software tools or a strong correlation with clinical phenotype (22).

The process included the validation of selected variants via Sanger sequencing or real-time PCR, adhering to the pattern of inheritance and clear genotype-phenotype correlation when parents' DNA samples were unavailable for segregation analysis. This rigorous approach ensured the identification and validation of genetic variants crucial in understanding and managing genetic kidney disorders.

2.4 Assessment of genetic diagnosis and result return

After identifying the genetic variants, a multidisciplinary team evaluated their relevance to the patients' phenotypes. A variant was deemed diagnostic if it aligned with the clinical phenotype and inheritance patterns, or if reverse phenotyping and extended family testing supported the genetic findings. Reverse phenotyping involved a comprehensive review and additional investigations to identify overlooked symptoms by previous clinical evaluation suggested by the genetic results.

Diagnoses were labeled as "confirmed" when genetic findings aligned with initial clinical diagnoses. In cases where genetic results diverged but were validated through reverse phenotyping as pertaining still to the same clinical group, diagnoses were "modified." The term "reclassified" was reserved for patients whose genetic findings fell from a different clinical spectrum (category) than initially suspected. The diagnostic yield

was computed from the number of confirmed and modified diagnoses after applying both WES and reverse phenotyping, relative to the total number of patients tested.

Although certain polymorphic variants like biallelic APOL1 risk genotypes and CFHR1-CFHR3 deletions were noted, they weren't counted towards the diagnostic yield as they aren't considered disease-causing. If a patient received a genetic diagnosis, cascade screening was offered to other family members.

Nephrologists provided specific recommendations and clinical work-ups upon returning WES results to patients, family members, and referring nephrologists. The study did not include the analysis of medically actionable secondary findings due to protocol and consent restrictions. Non-renal genetic diagnoses were only confirmed through *in silico* analysis of WES results if they were previously identified before the study's commencement. All genetic results were directly returned to the participants and/or their families in compliance with the consent protocols.

2.5.1 Modeled cost-analysis

The study included a comprehensive cost-analysis to scrutinize the economic impacts associated with the newly proposed service delivery model for the genetic diagnosis of inherited kidney diseases. The analysis was conducted on the study population selected by the predefined criteria. It comprised two distinct yet complementary approaches: a modeled cost-analysis and a real-life cost-analysis (31).

The modeled cost-analysis offered theoretical insights, drawing from simulated scenarios and assumptions to evaluate the potential cost implications in the considered population. In contrast, the real-life cost-analysis was grounded in actual data, reflecting the practical and tangible costs encountered in the real-world setting. The necessity of a double model, derived from the fact that real-life costs were unretrievable for all the patients often

due to long clinical histories (29). Such a limitation would have reduced the sample available for the cost analysis.

These dual approaches provided a robust assessment of the economic aspects, ensuring both theoretical depth and practical relevance.

2.5.2 Diagnostic Trajectories and Tiers

Two diagnostic trajectories were defined: the Late WES Model and the Early WES Model. The Late WES Model involves a complete diagnostic pathway before the employment of WES, while the Early WES Model introduces WES after initial basic examinations to just confirm the clinical suspicion of genetic kidney disease. The different examinations encountered through the diagnostic process were divided into three tiers of investigations of increasing complexity and cost (Table 1): Tier 1: Basic examinations for initial clinical diagnosis categorization; Tier 2 and 3: Advanced and more expensive evaluations, with Tier 3 featuring first-choice genetic tests for each clinical category, excluding WES, which was considered only if a definitive diagnosis was not established by another genetic test. In particular, patients were modeled to undergo targeted gene panels according to clinical suspicion with the exception of Syndromic CKD, who underwent array comparative genomic hybridization (aCGH) as a first-choice genetic strategy. For Collagenopathies, Tubulopathies and Ciliopathies we also considered multiplex ligation-dependent probe amplification analyses (MLPA) as integrating targeted gene panels to cover genetic mechanisms reported to be frequently responsible for the disease (i.e., copy number variations, CNVs). Single gene Sanger sequencing was considered the standard genetic testing for assessment of variant segregation within the family. Of note, the costs of genetic testing (Sanger sequencing, panel sequencing, MLPA, aCGH) included the cost of sequencing and genetic clinic consultations.

Patients with Chronic Kidney Disease of unknown origin (CKDu) were not included in the analysis, due to the undeterminable origin of CKD and consequent absence of ideal diagnostic trajectory.

Table 1. List of the investigations required for the diagnosis of kidney diseases.

	Tier 1	Tier 2	Tier 3	Total €
Podocytopathies	kidney function, blood count, electrolytes, ABG, liver enzymes, LDH, coagulation tests, albumin, SPEP, 25OH-vit. D, PTH, iron tests, urinalysis, urine culture, urinary p/c ratio (or 24h proteinuria), abdomen US	Complement, autoimmunity tests, ANCA, immunoglobulins, hepatitis B and C serology, HIV, CRP, ESR, cryoglobulins, anti-DNase, SPEP, anti-GMB, anti-PLA2R, Rheumatoid factor, Beta-2-microglobulin, FLC, UPEP, urinary c/c ratio, urinary FLC	Diagnostic immunosuppression trial**, Kidney biopsy***, gene panel	
	221 €	**218 €**	**3 909 €**	**4 348 €**
Collagenopathies	kidney function, blood count, electrolytes, ABG, liver enzymes, LDH, coagulation tests, albumin, SPEP, 25OH-vit. D, PTH, iron tests, urinalysis, urine culture, urinary p/c ratio (or 24h proteinuria), parent's urinalysis	Complement, autoimmunity tests, ANCA, immunoglobulins, hepatitis B and C serology, HIV, CRP, ESR, cryoglobulins, anti-DNase, SPEP, anti-GMB, Rheumatoid factor, UPEP*, urinary c/c ratio, abdomen US*, ophthalmology and audiology evaluations	Kidney biopsy, urology visit§, cystoscopy§, gene panel, MLPA	
	196 €	**361 €**	**4 265 €**	**4 822 €**
Tubulopathies	kidney function, blood count, electrolytes, renin, aldosterone, ABG, liver enzymes, LDH, coagulation tests, albumin, SPEP, uric acid, 25OH-vit. D, PTH, iron tests, urinalysis, urine culture, urinary p/c ratio (or 24h proteinuria), 24h urinary calcium (or c/c ratio), 24h urinary potassium, 24h urinary sodium, chloride fraction excretion, abdomen US	Urinary SPEP, 24h urinary phosphate, magnesium, citrate, oxalate, uric acid	Urine amino acids, stone analysis, ECG, gene panel, MLPA°	
	320 €	**94 €**	**1 775 €**	**2 189 €**
Ciliopathies	kidney function, blood count, electrolytes, ABG, liver enzymes, LDH, coagulation tests, albumin, SPEP, 25OH-vit. D, PTH, iron tests, HbA1c, urinalysis, urine culture, urinary p/c ratio (or 24h proteinuria), abdomen US*	Parent's abdomen US, urinary c/c ratio#	Abdomen MRI, gene panel, MLPA	
	251 €	**141 €**	**2 143 €**	**2 535 €**
CAKUT	kidney function, blood count, electrolytes, ABG, liver enzymes, LDH, coagulation tests, albumin, SPEP, 25OH-vit. D, PTH, iron tests, urinalysis, urine culture, urinary p/c ratio (or 24h proteinuria), abdomen US	Urinary SPEP, Voiding cystourethrogram/kidney sequential scintigraphy^	gene panel	
	240 €	**115 €**	**1 646 €**	**2 001 €**
Syndromic CKD	kidney function, blood count, electrolytes, ABG, liver enzymes, LDH, coagulation tests, albumin, SPEP, 25OH-vit. D, PTH, HbA1c, iron tests, urinalysis, urine culture, urinary p/c ratio (or 24h proteinuria), abdomen US, other specialist visits§§	Other specialists visit and examinations§§	Other major diagnostic imaging testing§§, CGH-array, Sanger sequencing	
	296 €	**120 €**	**2 109 €**	**2 525 €**
Metabolic kidney disorders	kidney function, blood count, electrolytes, ABG, liver enzymes, LDH, coagulation tests, albumin, SPEP, uric acid, 25OH-vit. D, PTH, iron tests, urinalysis, urine culture, urinary p/c ratio (or 24h proteinuria), 24h urinary calcium (or c/c ratio), abdomen US	Urinary SPEP, 24h urinary phosphate, magnesium, citrate, oxalate, uric acid, sodium, potassium, chloride, FGF-23, ALP, 1-25OH-vit.D	Urine amino acids, urology visit, cystoscopy, stone analysis, gene panel	
	254 €	**226 €**	**1 762 €**	**2 242 €**

For each clinical category we report genomic and non-genomic investigations required for diagnosis according to current guidelines, available literature and local clinical practice. Investigations are divided

in Tiers according to increasing complexity and cost. Genomic investigations excluding WES are in Tier 3 for all categories. Prices are reported in Euros.

*In adults; Autoimmunity panel includes: ANA, ENA, Anti-DNA; **Diagnostic immunosuppressive challenge has been calculated differently for adult and pediatric patients (standard adult 70 Kg patient and pediatric 3-8 years old patient), according to KDIGO immunosuppressive treatment guidelines for glomerular diseases; [***]Price based on mean expense at our institution for patient's representative sample from the study population; [§]When macroscopic hematuria is present; [°]Only for clinical suspicion of Bartter syndrome; [#]In case of hyperechoic kidneys at ultrasound not clearly distinguishable from microlithiasis in early stages; [^]According to abdomen ultrasound findings and clinical history; [§§]Price based on mean expense for patient's representative sample from the study population.

US, ultrasound scanning; ABG, arterial blood gas; LDH, lactate dehydrogenase; SPEP, serum protein electrophoresis; PTH, parathyroid hormone; p/c ratio, protein to creatinine ratio; c/c ratio, calcium to creatinine ratio; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; anti-GMB, anti-glomerular basement membrane antibody; anti-PLA2R, phospholipase A2 receptor antibody; FLC, free light chains; UPEP, urinary protein electrophoresis; MLPA, multiplex ligation dependent probe amplification; CGH array, comparative genomic hybridization; CAKUT, congenital anomalies of the kidney and urinary tract; CKD, chronic kidney disease.

2.5.3 Investigations and Costs

Thus, the diagnostic trajectories included in the two models incorporate both genomic and non-genomic investigations, such as blood and urinary exams, imaging studies, kidney biopsies, and specialist consultations. Different genetic testing strategies are modeled according to the specific kidney disease category, including the costs of sequencing and genetic clinic consultations. This detailed analysis of the diagnostic pathway of each clinical category was based on routine guidelines, current clinical practices, and extensive literature,

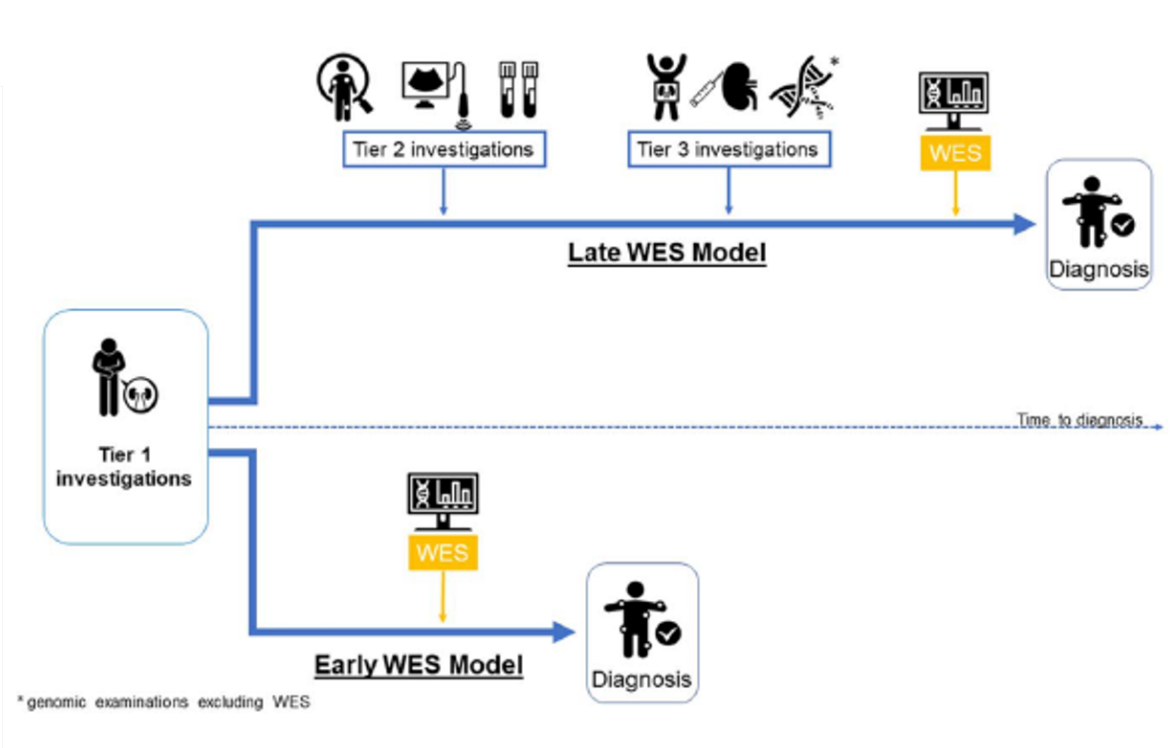
to provide a comprehensive perspective on the economic aspects associated with the diagnostic pathways of inherited kidney diseases (31).

2.5.4 Late vs. Early WES Model

In the late WES Model patients undergo all three tiers of investigations. WES is introduced only if the initial stages do not provide a conclusive diagnosis. The cost per diagnosis is derived by summing all tier costs, adding WES costs where necessary, and dividing by the total number of genetic diagnoses.

In early WES Model WES is introduced post-Tier 1. Moreover, the model accounts for the costs of additional confirmatory tests (reverse phenotyping) for some patients when needed. The cost per diagnosis is obtained by adding Tier 1 and WES costs, including additional evaluations, and dividing by the total number of genetic diagnoses (Figure 1).

Figure 1. Schematic view of Early WES and Late WES Models.



2.5.5 Cost Calculation and Comparison

The costs of the two models were meticulously calculated and compared. The cumulative costs were divided for the number of genetic diagnoses to obtain the cost per diagnosis. In the Early WES Model, costs for Tier 1 were summed with those for WES for all the patients belonging to each clinical category. We also considered costs for reverse phenotyping, as mentioned above. The cumulative costs were divided for the number of genetic diagnoses to obtain the cost per diagnosis. Results of the comparison between the two models are expressed as incremental cost per diagnosis.

2.5.6 Sensitivity analysis

A deterministic sensitivity analysis was performed to evaluate the robustness of the modeled cost-analysis results, focusing on understanding the impact of input uncertainties on the output. This approach consists in varying individual uncertain parameters of the modeled analysis within a specified range (defined by lower and upper values sourced from existing literature) while keeping others constant, to assess the influence of each on the overall model.

Parameters Analyzed in the sensitivity analysis were:

1) Cost Variation of Genomic Investigations and Kidney Biopsy: The fluctuation in the costs associated with genomic tests and kidney biopsies, considering the variability and uncertainty in pricing.

2) Cost Variation of Reverse Phenotyping: Analyzing the impact of different cost scenarios for reverse phenotyping, accounting for variations in expenses related to this process.

3) Proportion of Patients Needing Biopsy and Reverse Phenotyping: Assessing the number of patients requiring these specific procedures and evaluating how variations in this proportion affect the overall cost and effectiveness of the model.

4) Prevalence of Podocytopathy: Given that podocytopathy was the most represented class in the study, its prevalence rate's impact on the model's outcome was specifically analyzed.

This sensitivity analysis aims to offer a comprehensive insight into the potential variability in the modeled cost-analysis outcomes, ensuring a well-rounded and robust evaluation of the economic implications associated with different diagnostic approaches to inherited kidney diseases. Each parameter's influence is meticulously assessed to enhance the reliability and applicability of the model in real-world scenarios.

2.5.7 Definition of costs

The cost analyses in this study strictly focused on diagnostic investigation costs (table 1). Expenditures associated with subsequent clinical management, stemming from the presence or absence of a genetic diagnosis, were not included. The analysis considered only direct medical costs, encompassing expenses for laboratory and radiologic tests, procedures, outpatient consultations, and any prior genetic tests. Each cost, calculated in line with the Regional Health Reimbursement System, included expenses for supplies, staff, results reporting, and overheads, ensuring a comprehensive account of the financial aspects involved in the diagnostic process. Prices are expressed in Euros. Exclusions from the cost analysis included indirect medical costs (such as lost working days and caregiver expenses), costs associated with cascade genetic testing, and expenses tied to disease progression resulting from an absent genetic diagnosis.

The analysis adopted a 12-month time horizon, covering the period from the initial patient presentation to three months post-WES and reverse phenotyping results, offering a detailed overview of the incurred costs within this timeframe. The study was conducted from the perspective of the Regional Healthcare System, ensuring relevance and applicability to the local healthcare context and policies.

2.6 Statistical analysis

Statistical analysis was performed using SPSS software v27.0 (SPSS, Inc., Evanston, IL). Quantitative data are shown as median and interquartile range (IQR). Categorical variables are expressed as numbers or percentages for each item. The chi-square test was used for categorical variables. Univariable logistic regression was used to identify selection criteria for WES. The level of statistical significance was set at a value of $P<0.05$.

3. Results

3.1 Assessment of Clinical Criteria for Patient Selection and Genetic Testing Prioritization

To establish clinical criteria for patient selection, we performed a comprehensive retrospective review of genetic testing outcomes in patients before the set up of the renal genetic service. This involved evaluating data from 392 probands who, between 2015 and 2018, were referred to the Medical Genetics laboratory at our hospital under the suspicion of harboring genetic kidney diseases. WES was the principal diagnostic tool and we registered during this period a diagnostic yield of 35.7% (140 out of 392 cases).

The next step was an assessment of various clinical features to gauge their efficacy in predicting a positive genetic diagnosis via WES. We enlisted a range of possible predictors for this purpose. The univariable analysis spotlighted several clinical features that were statistically significant in predicting a genetic diagnosis. These included: 1) resistance to conventional treatments, 2) a familial history of kidney disease or parental consanguinity, 3) manifestations of extrarenal involvement, 4) evidence of CAKUT and CKD stage ≥G2, 5) the presence of at least two cysts in each kidney as revealed by ultrasound scanning, 6) persistent hyperechoic kidneys or nephrocalcinosis on ultrasound, and 7) persistent metabolic abnormalities. Other variables including age (when considered as a continuous variable), the presence of nephrotic syndrome, hypertension, CAKUT in the absence of kidney function impairment, and isolated complement abnormalities were found to lack predictive power in anticipating a genetic diagnosis (Table 2).

Table 2. Univariable logistic regression analysis of selected inclusion criteria predicting genetic diagnosis after whole exome sequencing.

Variables	Odds ratio	C.I. 95%	p
Familial history	2.67	1.70 - 4.19	**<0.001**
Extra-renal involvement	2.3	1.39 - 4.06	**0.002**
Resistance to treatments	11.47	2.61 - 50.37	**0.001**
Persistent metabolic abnormalities	3.66	2.10 - 6.42	**<0.001**
At least 2 cysts in each kidney at kidney US	4.06	2.05 - 8.04	**<0.001**
Persistent nephrocalcinosis/hyperechoic kidneys at kidney US	3.93	1.89 - 8.18	**<0.001**
CAKUT and CKD ≥G2	6.0	1.08 - 33.32	**0.041**
Age	0.986	0.97 - 1.002	0.079
Isolated complement abnormalities	0.54	0 - 2.50	0.58
Nephrotic syndrome	1.19	0.322 - 4.40	0.794
CAKUT without CKD	0.4	0.063 - 2.52	0.329
Transient metabolic abnormalities	0.03	0.01 - 0.55	**0.018**
Hypertension	1.892	0.598 - 5.984	0.278

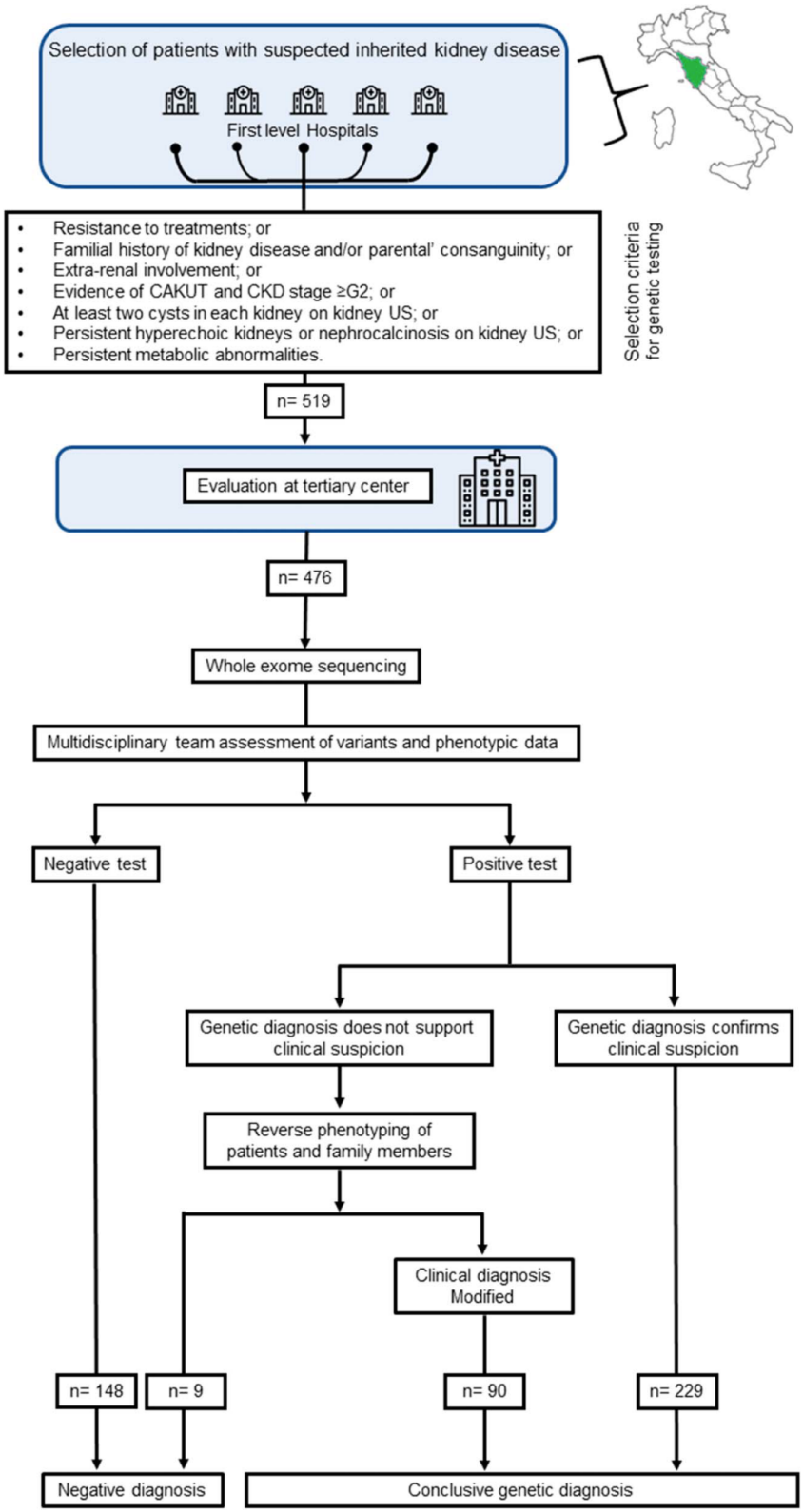
A noteworthy observation was the significant augmentation in diagnostic yield when at least one of the identified clinical criteria was applied to the unselected population. The diagnostic yield increased to 47.2% from 35.7% (p=0.003). In the cohort without any of these specific clinical criteria, the diagnostic rate decreased to 2.5%.

These refined criteria were shared with the hospitals in the regional network involved in referral of patients suspected of having genetic kidney diseases.

3.2 The workflow increases the rate of genetic testing

Following the establishment of the prespecified selection criteria, a total of 519 patients who fit these criteria were either referred to or directly chosen by our tertiary center for further investigation. However, after applying exclusion criteria, 476 probands, originating from 430 distinct families, were ultimately selected for genetic testing. This selection process and the criteria applied are detailed in Figure 2.

Figure 2. Diagnostic workflow for diagnosis of genetic kidney disease.



A breakdown of the demographic data reveals that of the 476 patients, 219 were female, accounting for 46.0% of the cohort, while males constituted 54.0% with 257 individuals. Most of the population was made up of pediatric cases (220 patients, 67.2%), where the presentation of disease was congenital (at birth) in 4.4% of cases. The mean age at presentation was 11 years (IQR 4-23 years), which was consistent with the early onset of CKD in the genetic kidney disease population. A significant majority of patients, 94.1%, were of European ancestry, as per self-reporting, while consanguinity was declared in 18 families, representing 3.9% of the total (Table 3). Mean turnaround time of genetic testing was 7 months.

Table 3. Baseline characteristics of patients included in the study.

	Podocytopathies	Collagenopathies	CKDu	Tubulopathies	Ciliopathies	CAKUT	Syndromic	Metabolic Disorders	Study Population
Congenital, n (%)	0	0	0	12 (11)	5 (7.2)	2 (13.3)	1 (3.1)	1 (2.8)	21 (4.4)
Age <18 yr, n (%)	82 (61.7)	33 (68.8)	16 (47.1)	63 (57.8)	42 (60.9)	11 (73.3)	23 (71.9)	29 (80.6)	299 (62.8)
Age >18 yr, (%)	51 (38.3)	15 (31.3)	18 (52.9)	34 (31.2)	22 (31.9)	2 (13.3)	8 (25.0)	6 (16.7)	156 (32.8)
Age at referral, year, median (IQR)	15 (6-23)	13 (8-34)	21 (11–40)	8 (2–22)	9 (4–24)	1 (0–5)	8 (1–18)	7 (2–15)	11 (4,0–23,0)
Female, n (%)	62 (44.6)	27 (56.3)	11 (32.4)	56 (51.4)	26 (37.7)	5 (33.3)	18 (56.3)	14 (38.9)	219 (46,0)
European, n (%)	119 (89.5)	43 (89.6)	28 (82.4)	98 (88.3)	67 (97.1)	13 (86.7)	26 (81.3)	31 (86.1)	425 (89.2)
Resistance to treatments, n (%)	128	3	0	0	0	0	3	0	134 (28.2)
Family history, n (%)	32	42	28	28	37	8	15	22	212 (44.5)
Extrarenal involvement, n (%)	26	11	6	28	13	4	32	5	125 (26.3)
CAKUT and CKD G2, n (%)	2	0	0	1	2	15	12	4	36 (7.6)
At least two cysts, n (%)	0	0	1	1	60	0	3	8	73 (15.3)
Persistent hyperechoic kidneys/nephrolithiasis, n (%)	1	0	0	32	4	1	5	27	70 (14.7)
Persistent metabolic anomalies, n (%)	0	0	0	103	0	0	2	7	112 (23.5)
Diagnostic yeald, % (n)	54.1 (72/133)	69.2 (29/48)	17.6 (6/34)	86.2 (94/109)	87 (60/69)	46.7 (7/15)	78.1 (25/32)	72.2 (26/36)	67 (319/476)

The patients were classified into eight distinct disease categories, according to their clinical phenotypes. These categories, along with the number of patients in each, were as follows: podocytopathies (133), collagenopathies and other glomerular basement membrane disorders (48), CKDu (34), tubulopathies (109), ciliopathies (69), CAKUT (15), syndromic CKD (32), and metabolic kidney disorders (36). Table 4 reports a detailed breakdown of the clinical diagnoses associated with each category.

Table 4. Clinical diagnosis of patients included in the study.

Clinical category	**Suspected clinical diagnosis**
Podocytopathies (n=133)	SRNS (n=98)
	CNS (n=4)
	FSGS (n=27)
	MC (n=4)
Collagenopathies (n=48)	Alport syndrome/Thin basement membrane nephropathy (n=48)
Chronic kidney disease of unknown origin (n=34)	Unspecified familial nephropathy (n=13)
	Unexplained ESKD/CKD (n=21)
Tubulopathies (n=109)	Bartter syndrome (n=22)
	Gitelman Syndrome (n=26)
	Bartter/Gitelman Syndrome (n=7)
	Dent disease (n=7)
	Pseudohypoaldosteronism Type I (n=7)
	Pseudohypoaldosteronism Type II (n=4)
	dRTA (n=28)
	pRTA (n=2)
	dRTA/pRTA (n=1)
	Fanconi syndrome (n=2)
	Diabetes insipidus, nephrogenic, 1 (n=2)
	Renal hypouricemia (n=1)
Ciliopathies (n=69)	ADPKD (n=34)
	ARPKD (n=8)

	ADPKD veo/ARPKD (n=7)
	Cystic kidney disease (n=7)
	Nephronophthisis (n=13)
CAKUT (n=15)	Hypodysplasia (n=2)
	PUV (n=3)
	Other CAKUT (n=10)
Syndromic CKD (n=32)	RCAD (n=3)
	VACTERL association (n=1)
	Syndromic CAKUT (n=11)
	Nail-patella syndrome (n=1)
	Fibronectin glomerulopathy (n=1)
	Pseudoxanthoma elasticum (n=2)
	Sensenbrenner syndrome (n=1)
	Bartter syndrome and FSGS (n=1)
	CHARGE syndrome (n=1)
	Fraser syndrome (n=1)
	WAGR syndrome (n=1)
	Proteinuria and Wilms tumor (n=1)
	Proteinuria and intellectual disability (n=3)
	Proteinuria and other findings (n=3)
	Hypertension brachydactyly (n=1)
Metabolic kidney disorders (n=36)	Cystinuria (n=4)
	Familial hypocalciuric hypercalcemia (n=3)
	Infantile Idiopathic Hypercalcemia (n=6)
	Familial hypomagnesemia with hypercalciuria (n=1)
	Nephrocalcinosis (n=17)
	Primary hyperoxaluria (n=2)
	Primary hyperoxaluria/APRT deficiency (n=2)
	Recurrent kidney stones (n=1)

SRNS, steroid-resistant nephrotic syndrome; CNS, congenital nephrotic syndrome; FSGS, focal segmental glomerulosclerosis; MC, minimal-changes; dRTA, distal renal tubular acidosis; pRTA, proximal renal tubular acidosis; ESKD, end-stage kidney disease; CKD, chronic kidney disease; ADPKD, autosomal dominant polycystic kidney disease; ARPKD, autosomal recessive polycystic kidney disease; ADPKD veo, very early-onset autosomal dominant polycystic kidney disease; PUV, posterior urethral valves; CAKUT, congenital anomalies of the kidney and urinary tract; CKD, chronic kidney disease; RCAD, Renal cysts and diabetes syndrome; VACTERL, vertebral defects, anal atresia, cardiac defects, tracheo-esophageal fistula, renal anomalies and limb abnormalities; CHARGE, Coloboma, heart anomaly, choanal atresia, retardation, genital and ear anomalies; WAGR, Wilms tumor, anidria, genitourinary anomalies and mental retardation; APRT, adenine phosphoribosultransferase.

The application of WES, coupled with reverse phenotyping and the multidisciplinary workup, led to the identification of a definitive molecular diagnosis in 319 patients out of the 476, a diagnostic rate of 67.0%.

These findings underscore a crucial insight: the application of specific clinical criteria in the pre-selection of patients enhances the efficiency and success rate of WES in yielding a genetic diagnosis. This integrated approach, combining nuanced clinical assessment with advanced genetic testing, proves instrumental in elevating the precision and reliability of diagnoses in the setting of genetic kidney diseases.

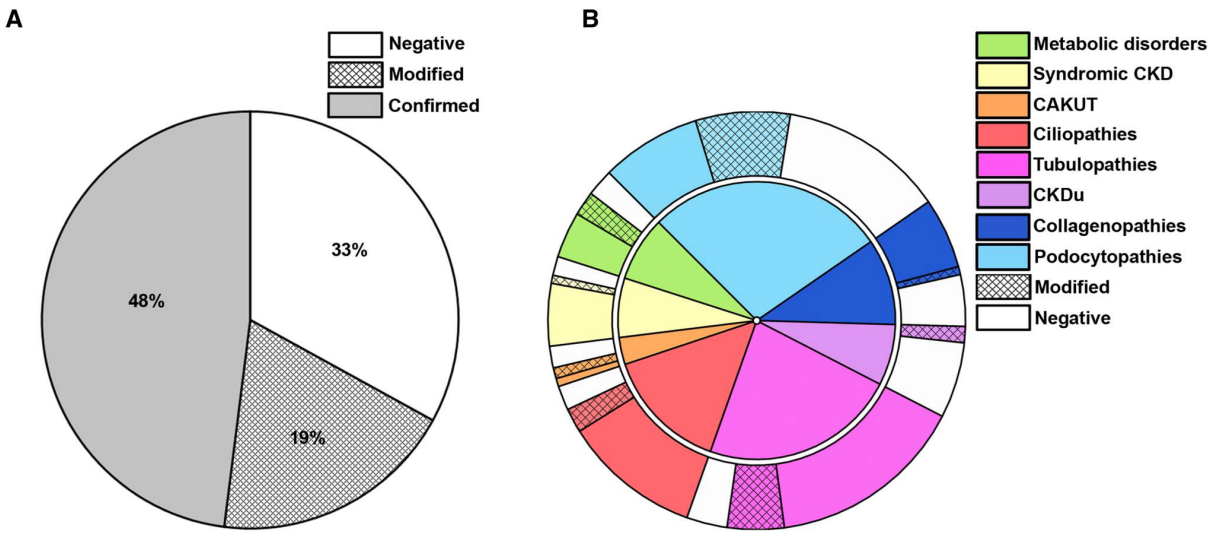
3.3 The workflow increases the accuracy of genetic testing

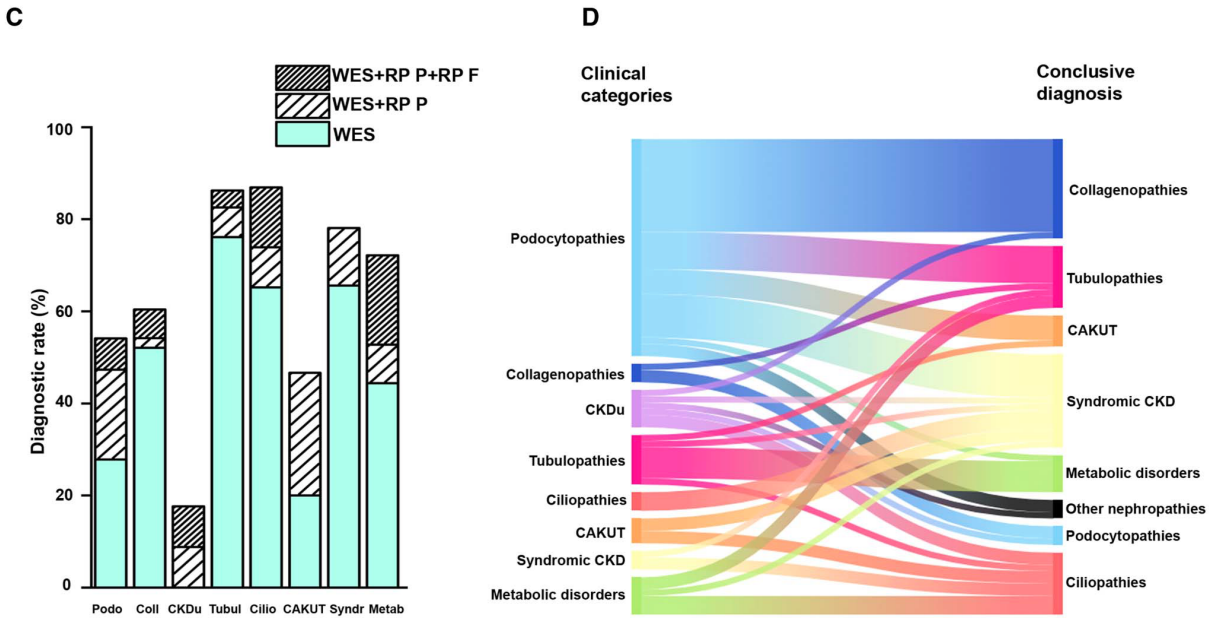
In the subset of 319 patients who received a positive result from genetic testing, the initially suspected clinical diagnosis was confirmed for 229 individuals, representing 48.0% of the total (as illustrated in Figure 3A). Conversely, for 90 patients (19.0%), the initial clinical

diagnosis was revised. Among these, 68 experienced a reclassification of their disease (details provided in Figure 3, A and B).

Breaking down the results by disease category, we found the following diagnosis confirmation rates: podocytopathies at 54.1% (72 out of 133 patients), collagenopathies at 60.4% (29/48), CKDu at 17.6% (6/34), tubulopathies at 86.2% (94/109), ciliopathies at 86.9% (60/69), CAKUT at 46.7% (7/15), syndromic CKD at 78.1% (25/32), and metabolic kidney disorders at 72.2% (26/36), as visualized in Figure 3B.

Figure 3. Diagnostic rate and disease reclassification.





(A) Percentage of diagnosis confirmed (gray), negative (white), and modified (reticulated) by WES and reverse phenotyping. (B) Inner pie chart: distribution of patients according to the eight clinical categories based on pre-WES clinical evaluation (podocytopathies, collagenopathies, CKDu, tubulopathies, ciliopathies, CAKUT, syndromic CKD, metabolic kidney disorders). Outer pie chart: percentage of diagnosis confirmed (solid), modified (reticulated), and negative (white) in patients belonging to the eight clinical categories. (C) Percentage of diagnosis obtained with WES alone (light green), WES coupled with reverse phenotyping in the patient (striped), and WES coupled with reverse phenotyping in the patient and in the family (double striped) of patients belonging to the eight clinical categories. (D) On the left side, the suspected and on the right side, the genetic diagnosis in those patients that underwent disease reclassification after WES. CAKUT, congenital anomalies of the kidney and urinary tract; CKDu, CKD of unknown origin; RPF, reverse phenotyping in the family; RPP, reverse phenotyping in the patient; WES, wholeexome sequencing.

The implementation of reverse phenotyping proved instrumental in the diagnostic process, especially for patients with podocytopathies and CAKUT. It facilitated diagnosis

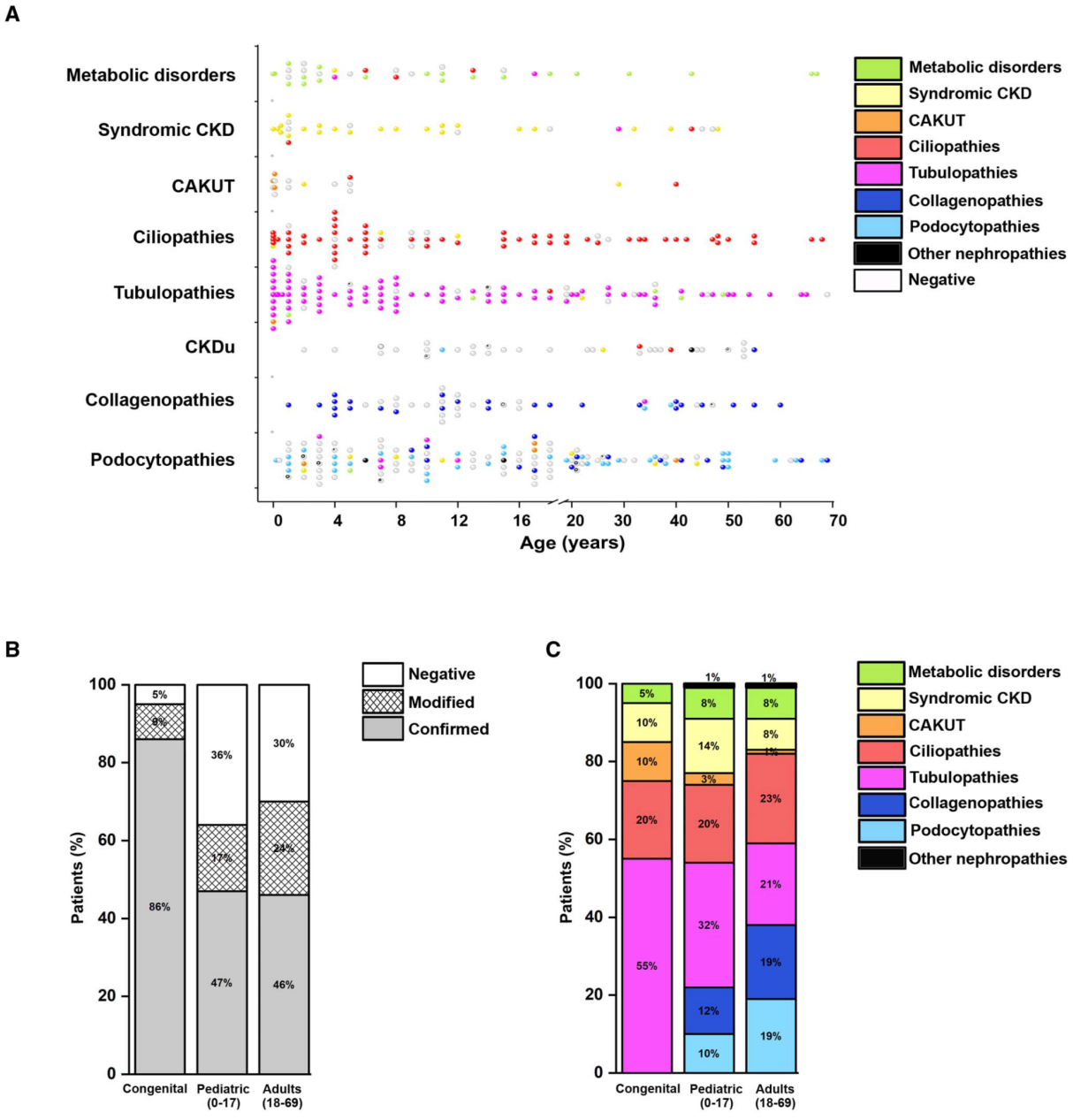
in 57 patients when applied to the probands (Figure 3C). Moreover, extending reverse phenotyping to family members, which included clinical reassessment and segregation analysis, corroborated the genetic diagnosis for an additional 35 patients across the entire cohort. This approach was particularly beneficial in confirming diagnoses of ciliopathies and metabolic kidney disorders (elaborated in Figure 3C).

Remarkably, disease reclassification was observed across all clinical categories, and the subsequent final diagnoses spanned the entire spectrum of genetic groups (depicted in Figure 3D). These findings underscore the efficacy of the diagnostic workflow adopted in this study. It not only amplifies the diagnostic rate but also enhances the precision of identifying and confirming genetic kidney diseases.

3.4 The workflow increases the rate and accuracy of genetic testing in all clinical categories

The efficacy of the diagnostic workflow was assessed separately for different age groups: congenital (disease onset during the fetal period or at birth), pediatric (under 18 years, with an interquartile range (IQR) of 3–12 years), and adult (18 years and older, IQR 23–48 years), as visualized in Figure 4A. The diagnostic success rate was notably high across all groups: 95% in the 21 patients with congenital onset, 64% in the 299 pediatric patients, and 70% in the 156 adult patients, as detailed in Figure 4B.

Figure 4. Diagnostic rate and genetic findings in different age groups.



(A) Distribution of genetic diagnosis according to age in the eight clinical categories (podocytopathies, collagenopathies, CKDu, tubulopathies, ciliopathies, CAKUT, syndromic CKD, metabolic kidney disorders). Each dot represents a patient. Dots are colored according to the genetic diagnosis. Age is reported in years. (B) Diagnostic rate according to age in the study population (476 patients). For each

age group (congenital, pediatric, adults), we show confirmed (gray), modified (reticulated), and negative (white) results. Age is reported in years. (C) Genetic findings according to age in 319 patients with a genetic diagnosis. Age is reported in years. CAKUT, congenital anomalies of the kidney and urinary tract; CKDu, CKD of unknown origin.

A chi-square test revealed no significant difference in the diagnostic rate between patients under and over 18 years of age (chi-square = 0.856, P = 0.355). Age was also not a significant predictor for obtaining a genetic diagnosis, as confirmed by logistic regression (P = 0.22, odds ratio 1.01, with a 95% confidence interval of 0.99 to 1.02). The contribution of reverse phenotyping in enhancing the diagnostic yield was evident across all age groups (Figure 4B).

In the congenital disease category, tubulopathies were the most common genetic diagnosis, followed by ciliopathies, CAKUT, and syndromic CKD (Figure 4C). The genetic diagnosis of CAKUT was particularly prominent in the congenital and pediatric groups. This is likely attributable to the selection criteria that included only patients with CKD stage G2 or above, a group characterized by more severe phenotypes and consequently earlier diagnoses (Figure 4C).

A closer examination revealed a consistent pattern in the distribution of genetic diagnoses between pediatric and adult patients, indicating a uniform prevalence of different genetic kidney diseases across these age groups (Figure 4, A and C). This consistency underscores the conclusion that the diagnostic workflow, as proposed, is highly effective in securing a genetic diagnosis, irrespective of the patient's age.

3.5 The workflow improves clinical management

We proceeded to assess the comprehensive clinical impact of securing a genetic diagnosis on individual patients. Specifically, the insights gained from genetic diagnosis and the reclassification of diseases contributed to several clinical aspects. These included: the refinement of prognosis, particularly concerning the risk of progression to kidney failure, and influenced cascade family member screening, additional examinations and ongoing surveillance.

The diagnostic information also played a pivotal role in: defining treatment plans, leading to the discontinuation of unnecessary treatments or the initiation of more suitable ones, informing decisions related to kidney transplantation, including donor selection and registration on the waiting list, and determining eligibility for clinical trials. Additionally, it facilitated access to disease-specific registries and guided family planning and reproductive counseling.

Our approach led to the reclassification of 90 patients, enhancing the precision of prognosis estimations. Cascade screening was extended to 91 family members across 74 families, uncovering a genetic diagnosis in 79 individuals who were previously undiagnosed or had unspecified kidney disorders. The clinical examination and assessment trajectory were revised for an average of 52.0% of patients. Genetic testing insights were instrumental in shaping kidney transplant decisions for 11.9% of patients and influenced the treatment course for 29.2% of patients.

Beyond these, ten patients received an additional molecular diagnosis. Among these, we identified three patients with the biallelic polymorphic G1/G2 risk alleles in APOL1, which is known to increase the risk of CKD; one patient with the CFHR1-CFHR3 homozygous deletion, associated to the development of anti-factor H complement

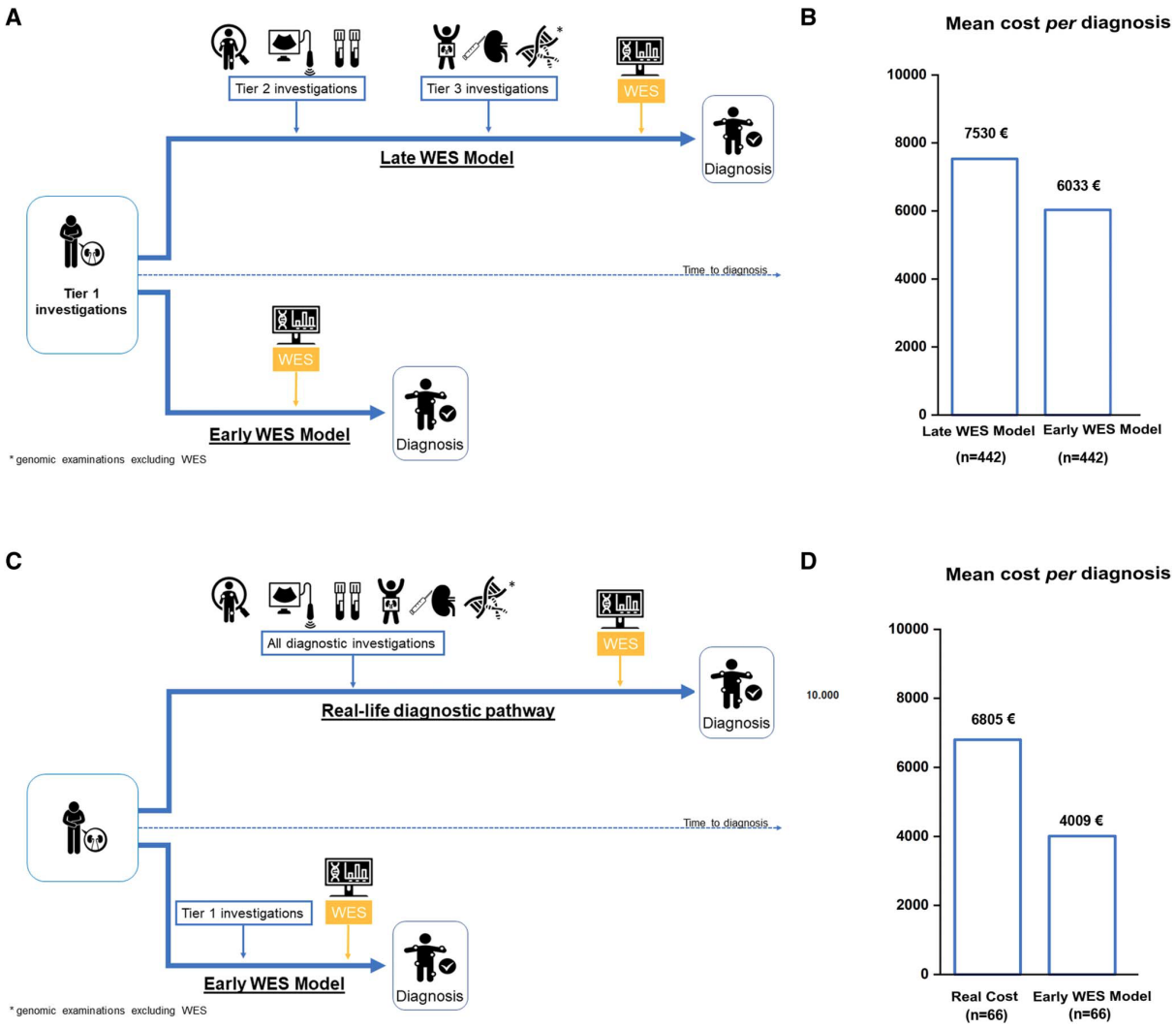
antibodies and hemolytic uremic syndrome; one patient carrying pathogenic variant in GLA gene, associated to Fabry disease; one patient with a VUS in PKD1 gene.

These findings underscore the profound impact of our diagnostic workflow in enhancing patient care, by facilitating tailored clinical examinations, personalized treatment plans, and informed clinical counseling.

3.6 The workflow is cost-saving

To evaluate the financial impact of our diagnostic workflow, we executed a modeled cost analysis first. Our analysis involved 442 patients from the study population, excluding those with CKDu due to the impossibility to model the diagnostic trajectory for this condition.

Figure 5. Cost-analysis.



Our cost analysis revealed that the early WES model resulted in a 20% cost reduction per diagnosis compared to the late WES model (shown in Figure 5A). On average, the cost per diagnosis in the late WES model was estimated at €7530, while it was €6033 in the early WES model. This difference translates to a saving of €1497 per tested patient (Figure 5B). The one-way sensitivity analysis affirmed the base case analysis outcomes (see Table 5), with the early WES model consistently offering cost reductions, except when variations in the costs of genetic investigations were considered (e.g., increased WES costs or decreased costs for other genetic tests).

Table 5. One-way sensitivity analysis.

Parameter tested	Base case	Tested values	Cost-variation in cost *per* diagnosis in the study population
Base case			-1 360€
WES cost	3 670€	466€ - 6 670€	-3 888€ to +743
Gene panel cost	1 646€	350€ - 5 060€	+883€ to -6 553€
Biopsy cost	2 198€	1 480€ - 4 366€	-1 020€ to -2 385€
Reverse phenotyping mean cost *per* patient	115€	90€ – 230€	-1 343€ to - 1 366€
Proportion of patients needing biopsy	1	0.5 - 0.7	-821€ to -1 054€
Proportion of patients needing reverse phenotyping after WES	0.1	0 – 0.20	-1 381€ to -1 339€
Proportion of patients with podocytopathies	0.43	0.33 – 0.53	-1 166€ to -1 545€

The table shows the effect of changing one model parameter estimate on the variation in the cost *per* diagnosis in the study population.

WES, whole-exome sequencing; €, euros.

To validate our model, we compared the outcomes from the early WES model with actual diagnostic pathway costs extracted for a representative subset of 66 patients. These patients were randomly chosen from those with comprehensive clinical information and data recorded in administrative databases (depicted in Figure 5C). The mean turnaround time of

genetic test in this group was 9 months. The comparison revealed that the actual costs incurred by these patients surpassed the estimates derived from the early WES model (€6805 vs. €4009, shown in Figure 5D). This variance underscores a 41% cost reduction, thereby corroborating our modeled analysis across the entire cohort.

In summation, these findings advocate for the cost savings of implementing WES in a diagnostic workflow that's steered by well-defined selection criteria, underscoring its financial viability in diagnosing kidney diseases.

4. Discussion

In this study, we endeavored to optimize the management model for genetic analysis in patients with early-onset CKD. We devised a workflow integrating referral criteria, WES, reverse phenotyping, and multidisciplinary board analysis, achieving a 67% diagnostic rate. Early genetic testing resulted in a 20% cost saving per patient in modeled analyses, while a real cost analysis on 66 patients showed a 41% reduction. Thus, the workflow is proven to be effective and economically efficient for diagnosing genetic kidney diseases in real-world settings (31).

As previously emphasized, early onset CKD is often associated with genetic diseases. However, genetic diseases pose a diagnostic challenge due to their diverse phenotypic presentations (20). For instance, CAKUT leading the charge of pediatric CKD are now known as depending on an evident interaction between genetics and environment (16). The advent of NGS, becoming increasingly accessible in clinical settings, has been instrumental in expanding our understanding of genotype-phenotype correlations (12,21,22,24–26). A quintessential example in nephrology is the concept of phenocopies, where the phenotypic manifestation of a disease can clinically mimic a different nosological entity associated with a specific mutation (20). This can make genetic diagnosis difficult. One notable instance is Alport Syndrome, which typically manifests early in life in cases of severe genetic mutations. If not timely diagnosed, the disease progresses into forms of FSGS and might remain undiagnosed. Accordingly, Alport Syndrome is speculated to be one of the most underdiagnosed genetic diseases (10). Another example is nephrotic syndrome, a common manifestation of many glomerulopathies (22).

Our study aims to propose a dedicated pathway for the genetic diagnosis of kidney disease. In doing so, we have strived to optimize patient selection to ensure a high diagnostic rate, earlier diagnosis compared to traditional methods, and consequently, potential cost

containment. The integration of NGS in the clinical realm is not just a technological advancement but a bridge connecting intricate genetic landscapes to tangible clinical phenotypes, fostering a comprehensive, precise, and cost-effective diagnostic journey.

Through the identification of specific criteria for suspecting and referring a patient for genetic diagnosis, the diagnostic rate can be increased. Indeed, the rate demonstrated in this work is currently elevated compared to existing studies in the literature (13,17,19). This improvement was achieved by different means. First, the use of selecting criteria more closely associated with a genetic diagnosis. These criteria, derived from clinical practice on an historical cohort of patients, somewhat affirm the recommendations in recent guidelines, which are based on expert opinions and initial studies conducted in research settings (18).

Furthermore, providing standardized specific criteria to nephrology centers operating in the peripheral territory offers several advantages. The first is the standardization of the diagnostic process, reducing the subjective clinical judgment of the physician. In cases of limited clinical experience in managing rare diseases, this can prevent timely access to genetic testing. Additionally, the standardization of criteria could ensure a reduction in disparities in access to genetic diagnostics for certain population groups.

Beyond the use of selection criteria, another distinguishing feature of the diagnostic process in our algorithm is the contribution of the multidisciplinary team. This team is composed of individuals with varied expertise, including a genetic trained nephrologist, a geneticist, genetic sequencing laboratory technicians, a psychologist, and access to a basic research laboratory with specialized expertise. The exchange of ideas, discussions, and joint evaluations facilitated a comprehensive clinical assessment of patients, followed by an in-depth genetic evaluation and vice versa. This approach also aligns with the recent guidelines from the International Society of Nephrology, which underscore the necessity for specific training for personnel involved in renal genetic clinics (18). The presence of a

multidisciplinary team is instrumental in effectively managing the plethora of information that can emerge from exome sequencing. Indeed, while this technology allows for the testing of multiple genes simultaneously, it also increases the probability of encountering unexpected variants or those of uncertain significance. In such cases, the utilization of diverse tools and expertise becomes pivotal for characterization.

A crucial aspect of the multidisciplinary team's work is reverse phenotyping, a process that begins with the genotype and traces back to the phenotypic manifestation, in contrast with the so-called “forward genetic” screens of classical genetics (20–22). Specifically, when genetic data suggests the possibility of an unnoticed phenotypic correlation associated with the genetic information derived from the test, it is subject to verification. Thus, in this case there is a shift from genotype to phenotype, diversely from the past when the direction was opposite.

A significant portion of patients experienced a reclassification of their clinical diagnosis. This means that although a patient’s phenotype could be clinically categorized under a specific disease or clinical category, the execution of exome sequencing unveiled variants in different genes that subsequently changed the genetic disease category at final diagnosis in respect to the clinical suspicion. This phenomenon, though reported in other case studies, occurred at a higher rate in our study (17). This aspect is of central importance as a change in diagnosis enables a different clinical management approach, allowing for the application of the most targeted therapies possible. Each diagnosis reclassification is not merely a change in category but represents a step towards a more personalized, precise, and effective treatment plan, tailoring medical interventions to the unique genetic and phenotypic profile of each patient. This personalized approach underscores the potential for enhanced treatment efficacy, improved patient outcomes, and optimized resource utilization in the clinical management of renal genetic diseases.

A novel element of the study is that it is one of the first to challenge the traditional understanding that genetic tests are more frequently positive in the pediatric population. This is presumably linked to the fact that in pediatric patients, there is a greater awareness of genetically related diseases due to their earlier presentation compared to adults, where there is less utilization of genetic methods however (32). Indeed, despite a prevalence of pediatric patients, the frequency of positive diagnoses was not significantly different between patients above and under 18 years of age. However, this data should also be interpreted in light of the low referral age to the center, which was 11 years. This could further emphasize that when the correct criteria are employed, age no longer becomes a determining factor. The diagnostic efficacy becomes consistent across different age groups, underscoring the versatility and comprehensive applicability of the established criteria and diagnostic workflow.

Lastly, our algorithm, owing to the increased diagnostic rate and thus maximized efficacy, allowed us to demonstrate its cost-efficiency as well. In the modeled analysis, there was a reduction in costs up to 20% when genetic test was used promptly at the beginning of the diagnostic pathway; when the real-life costs incurred for a subgroup of patients were considered, the savings were even more substantial. This is a crucial aspect. In the context of rare diseases, genetic diagnostics have traditionally been employed as a last resort due to their costs. Moreover, not every setting is sensitive to the detection of rare diseases, which, by their very nature, are often not clinically suspected. As a result, patients undergo investigations and treatments that are not targeted and thus prove to be fruitless. Therefore, the early use of genetics, despite an increased initial cost compared to non-genetic diagnostics, leads to cost savings by eliminating unnecessary diagnostic steps for the patient, thereby compensating for the initial outlay (32). In the literature, only one other cost analysis study exists, and it pertains solely to two categories of renal diseases, podocytopathies, and

collagenopathies (29). Our study not only confirms these data but also extends them for the first time to a broader cohort of patients. Turnaround times of genetic test results is central in the diagnostic process. The rapid return of WES results can directly influence patient management in several ways. Firstly, it can lead to a quicker initiation of appropriate therapeutic interventions. Early intervention often translates into better patient outcomes and may also prevent the progression of disease, which can be particularly critical in the context of genetic kidney disorders where time is often a deciding factor in the prognosis. Secondly, expedited reporting can reduce the psychological burden on patients and their families (33). The stress and anxiety associated with waiting for a diagnosis can be considerable, and quicker turnaround times can alleviate this emotional strain. This improved patient experience is a qualitative benefit that, while not easily quantifiable, is an essential component of patient-centered care. Thirdly, in the realm of healthcare resource utilization, shorter reporting times can lead to more efficient use of healthcare services (34). By reducing the time spent in diagnostic limbo, healthcare providers can allocate resources more effectively, avoiding unnecessary repeat testing and reducing the likelihood of hospital readmissions or emergency department visits that may arise from delayed diagnosis. Furthermore, in the context of genetic disorders, rapid reporting can facilitate earlier genetic counseling and decision-making regarding family planning, which can have far-reaching implications for the patient's family and the broader healthcare system (35). In the modeled cost analysis, turnaround times for reporting results are treated as a pre-specified fixed parametric variable which, while impacting the overall dynamics of care delivery and patient management, does not directly alter the fixed costs associated with the WES procedure. It is essential to emphasize that while faster reporting times may improve the patient experience and potentially expedite the commencement of treatment, in cost modeling, the focus is on the overall economic efficiency of the diagnostic process over an extended

period. The fixed costs of WES, including materials, equipment, and personnel, remain constant regardless of the speed with which results are generated and delivered. Consequently, cost modeling centers on amortizing these fixed costs over the course of a year, irrespective of individual patient reporting times. This approach reflects a long-term management perspective, focused on how integrating WES into the diagnostic pathway can yield savings by reducing direct costs, such as those associated with repeated diagnostic procedures, ineffective treatments, and management of complications. However, it is crucial to acknowledge that cost analysis is not the sole indicator of value in the diagnostic process. The rapidity of reporting has significant implications for patient well-being and the optimization of clinical resources. The expediency of reporting times transcends mere financial metrics, touching upon various qualitative aspects of patient care that contribute significantly to the overall value proposition of the healthcare service provided.

In summing up, our study highlights the practical improvements in diagnosing early-onset CKD by combining new genetic testing methods, the establishment of a multidisciplinary team, and optimized criteria for choosing patients for testing. We showed that NGS and reverse phenotyping can make a big difference in correctly identifying and treating genetic kidney diseases, and it doesn't have to be limited by the patient's age or financial constraints. The cost savings we identified make this approach a realistic option for more healthcare settings. The findings in this study can help to optimize a service where diagnosing and treating rare kidney diseases is quicker, more accurate, and affordable, leading to better outcomes for patients.

Bibliography

1. Jager KJ, Kovesdy C, Langham R, Rosenberg M, Jha V, Zoccali C. A single number for advocacy and communication-worldwide more than 850 million individuals have kidney diseases. *Kidney international* 2019; 96: 1048–1050.

2. GBD Chronic Kidney Disease Collaboration. Global, regional, and national burden of chronic kidney disease, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. *The Lancet* 2020; 395: 709–733.

3. Andrassy KM. Comments on "KDIGO 2012 Clinical Practice Guideline for the Evaluation and Management of Chronic Kidney Disease." *Kidney international* 2013; 84: 622–623.

4. Coresh J, Astor BC, Greene T, Eknoyan G, Levey AS. Prevalence of chronic kidney disease and decreased kidney function in the adult US population: Third National Health and Nutrition Examination Survey. *American journal of kidney diseases: the official journal of the National Kidney Foundation* 2003; 41: 1–12.

5. Becherucci F, Roperto RM, Materassi M, Romagnani P. Chronic kidney disease in children. *Clinical kidney journal* 2016; 9: 583–591.

6. Harada R, Hamasaki Y, Okuda Y, Hamada R, Ishikura K. Epidemiology of pediatric chronic kidney disease/kidney failure: learning from registries and cohort studies. *Pediatric nephrology* 2022; 37: 1215–1229.

7. Crump C, Sundquist J, Winkleby MA, Sundquist K. Preterm birth and risk of chronic kidney disease from childhood into mid-adulthood: national cohort study. *BMJ* 2019; 365: l1346.

8. Warady BA, Chadha V. Chronic kidney disease in children: the global perspective. *Pediatric nephrology* 2007; 22: 1999–2009.

9. Calderon-Margalit R, Golan E, Twig G et al. History of Childhood Kidney Disease and Risk of Adult End-Stage Renal Disease. *The New England journal of medicine* 2018; 378: 428–438.

10. Torra R, Furlano M, Ortiz A, Ars E. Genetic kidney diseases as an underrecognized cause of chronic kidney disease: the key role of international registry reports. *Clinical kidney journal* 2021; 14: 1879–1885.

11. Cirillo L, De Chiara L, Innocenti S, Errichiello C, Romagnani P, Becherucci F. Chronic kidney disease in children: an update. *Clinical kidney journal* 2023; 16: 1600–1611.

12. Hildebrandt F. Genetic kidney diseases. *The Lancet* 2010; 375: 1287–1295.

13. Cocchi E, Nestor JG, Gharavi AG. Clinical Genetic Screening in Adult Patients with Kidney Disease. *Clinical journal of the American Society of Nephrology: CJASN* 2020; 15: 1497–1510.

14. Connaughton DM, Hildebrandt F. Personalized medicine in chronic kidney disease by detection of monogenic mutations. *Nephrology, dialysis, transplantation: official publication of the European Dialysis and Transplant Association - European Renal Association* 2020; 35: 390–397.

15. Rasouly HM, Groopman EE, Heyman-Kantor R et al. The Burden of Candidate Pathogenic Variants for Kidney and Genitourinary Disorders Emerging From Exome Sequencing. *Annals of internal medicine* 2019; 170: 11–21.

16. Carlassara L, Zanoni F, Gharavi AG. Familial Aggregation of CKD: Gene or Environment? *American journal of kidney diseases: the official journal of the National Kidney*

*Foundation*2021; 77: 861–862.

17. Cirillo L, Becherucci F. Genetic Testing in Nephrology: Show Your Pedigree! *Kidney360* 2022; 3: 2148–2152.

18. KDIGO Conference Participants. Genetics in chronic kidney disease: conclusions from a Kidney Disease: Improving Global Outcomes (KDIGO) Controversies Conference. *Kidney international* 2022; 101: 1126–1141.

19. Connaughton DM, Kennedy C, Shril S et al. Monogenic causes of chronic kidney disease in adults. *Kidney international* 2019; 95: 914–928.

20. Becherucci F, Landini S, Cirillo L, Mazzinghi B, Romagnani P. Look Alike, Sound Alike: Phenocopies in Steroid-Resistant Nephrotic Syndrome. *International journal of environmental research and public health [Internet]* 2020; 17. Available from: http://dx.doi.org/10.3390/ijerph17228363

21. Seltzsam S, Wang C, Zheng B et al. Reverse phenotyping facilitates disease allele calling in exome sequencing of patients with CAKUT. *Genetics in medicine: official journal of the American College of Medical Genetics* 2022; 24: 307–318.

22. Landini S, Mazzinghi B, Becherucci F et al. Reverse Phenotyping after Whole-Exome Sequencing in Steroid-Resistant Nephrotic Syndrome. *Clinical journal of the American Society of Nephrology: CJASN* 2020; 15: 89–100.

23. Hoppe B, Martin-Higueras C. Improving Treatment Options for Primary Hyperoxaluria. *Drugs* 2022; 82: 1077–1094.

24. Yamamura T, Horinouchi T, Nagano C et al. Genotype-phenotype correlations influence the response to angiotensin-targeting drugs in Japanese patients with male X-linked Alport syndrome. *Kidney international* 2020; 98: 1605–1614.

25. Rossetti S, Harris PC. Genotype-phenotype correlations in autosomal dominant and autosomal recessive polycystic kidney disease. *Journal of the American Society of Nephrology: JASN* 2007; 18: 1374–1380.

26. Palazzo V, Raglianti V, Landini S et al. Clinical and Genetic Characterization of Patients with Bartter and Gitelman Syndrome. *International journal of molecular sciences [Internet]* 2022; 23. Available from: http://dx.doi.org/10.3390/ijms23105641

27. Honeycutt AA, Segel JE, Zhuo X, Hoerger TJ, Imai K, Williams D. Medical costs of CKD in the Medicare population. *Journal of the American Society of Nephrology: JASN* 2013; 24: 1478–1483.

28. Brück K, Stel VS, Gambaro G et al. CKD Prevalence Varies across the European General Population. *Journal of the American Society of Nephrology: JASN* 2016; 27: 2135–2147.

29. Jayasinghe K, Wu Y, Stark Z et al. Cost-Effectiveness of Targeted Exome Analysis as a Diagnostic Test in Glomerular Diseases. *Kidney international reports* 2021; 6: 2850–2861.

30. Kris A. Wetterstrand MS. The Cost of Sequencing a Human Genome [Internet]. *Genome.gov*2019; [cited 2023 Oct 18] Available from: https://www.genome.gov/about-genomics/fact-sheets/Sequencing-Human-Genome-cost

31. Becherucci F, Landini S, Palazzo V et al. A Clinical Workflow for Cost-Saving High-Rate Diagnosis of Genetic Kidney Diseases. *Journal of the American Society of Nephrology: JASN* 2023; 34: 706–720.

32. Cirillo L, Becherucci F. The evolving role of first-tier exome sequencing in medical diagnostics. *Nephrology, dialysis, transplantation: official publication of the European Dialysis and Transplant Association - European Renal Association [Internet]* 2023;Available from: http://dx.doi.org/10.1093/ndt/gfad222

33. Oliveri S, Ferrari F, Manfrinati A, Pravettoni G. A systematic review of the psychological implications of genetic testing: A comparative analysis among cardiovascular, neurodegenerative and cancer diseases. *Frontiers in genetics* 2018; 9: 624.

34. Morgan S, Hurley J, Miller F, Giacomini M. Predictive genetic tests and health system costs. *journal de l'Association medicale canadienne [Canadian Medical Association journal]* 2003; 168: 989–991.

35. Resta R, Biesecker BB, Bennett RL et al. A new definition of genetic counseling: National Society of Genetic Counselors' task force report. *Journal of genetic counseling* 2006; 15: 77–83.