Atherosclerosis

Glycerol Kinase Deficiency in adults: description of 4 novel cases, systematic review and development of a clinical diagnostic score

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Corresponding Author: Itziar Lamiquiz-moneo, PhD
Hospital Universitario Miguel Servet
Zaragoza, SPAIN

First Author: Itziar Lamiquiz-moneo, PhD

Order of Authors:
Itziar Lamiquiz-moneo, PhD
Rocio Mateo-Gallego, PhD
Jacinto Fernández-Pardo, PhD, MD
Chuan López-Ariño
Victoria Marco-Benedí
Ana M Bea
Lia Ferraro
Estibaliz Jarauta, PhD, MD
Ana Cenarro, PhD
Fernando Civeira, PhD, MD

Abstract:

Background
Glycerol kinase deficiency (GKD) is a rare genetic disorder characterized by hyperglycerolemia and glyceroluria, which could be misdiagnosed as a moderate to severe hypertriglyceridemia (HTG). We aimed to describe four novel cases of GKD, to complete a systematic review of all cases of isolated GKD published so far and to develop a suspicion clinical diagnostic score for GKD.

Material and Methods
We reported four cases with suspicion of GKD and compared their phenotype with 584 males with triglycerides (TG) > 300 mg/dL, selected as control group (HTG non-GKD). The GK gene was sequenced in all cases. Lipoprotein particle concentrations were measured in all the cases with GKD. The systematic review involved a Pubmed, Cochrane and Scopus databases search to identify anthropometric and biochemical characteristics of all described cases with GKD.

Results
The systematic review retrieved a total of 15 articles involving 39 subjects with GKD. GKD cases reported a history of high TG levels resistant to lipid-lowering therapy. Compared to GKD subjects (n=43), HTG non-GKD subjects (n=584) showed significantly higher BMI, total cholesterol, non-HDL cholesterol and gamma-glutamyltransferase; significantly lower HDL cholesterol and TG; and higher prevalence of diabetes than subjects with GKD. The proposed diagnostic score was significantly higher in GKD than in HTG non-GKD subjects.

Conclusions
This is the first systematic review that compiles all GKD cases reported to date including 4 novel cases, and examine the differential GKD phenotype compared to other types of HTG. The proposed score would have a broad utility in clinical practice to avoid unwarranted lipid lowering treatment in GKD patients.
Highlights

- Glycerol Kinase Deficiency (GKD) is a rare genetic disorder often misdiagnosed as hypertriglyceridemia (HTG)
- We describe 4 novel GKD cases and complete the first systematic review that compiles all previously published subjects with GKD
- The distinctive clinical features between GKD and HTG patients are described
- This article proposes the first pseudo-HTG diagnostic score
Glycerol kinase deficiency (GKD) is a rare genetic disorder characterized by hyperglycerolemia and glyceroluria, which could be misdiagnosed as a moderate to severe hypertriglyceridemia (HTG).

1º Describe four novel cases of GKD
2º Systematic review of isolated GKD
3º Develop the first Pseudo-HTG diagnostic score

Systematic review involved Pubmed, Cochrane and Scopus databases identifying 39 subjects with isolated GKD.

584 males with TG > 300 mg/dL selected as control group (HTG non-GKD)

### Pseudo-HTG diagnostic score criterion

<table>
<thead>
<tr>
<th>Points*</th>
<th>Age of HTG diagnosis</th>
<th>Therapy-resistant high TG levels</th>
<th>Pancreatitis history</th>
<th>BMI, kg/m²</th>
<th>Glucose, mg/dL</th>
<th>HDL cholesterol, mg/dL</th>
<th>Non-HDL cholesterol, mg/dL</th>
<th>GGT, UI/L</th>
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<tbody>
<tr>
<td>2</td>
<td>&lt; 40 years</td>
<td>No</td>
<td>No</td>
<td>&lt;25</td>
<td>&lt;100</td>
<td>&gt;60</td>
<td>&lt;150</td>
<td>&lt;20</td>
</tr>
<tr>
<td>1</td>
<td>40-60 years</td>
<td>No</td>
<td>Yes</td>
<td>25-30</td>
<td>100-125</td>
<td>40-60</td>
<td>150-200</td>
<td>20-50</td>
</tr>
<tr>
<td>0</td>
<td>&gt; 60 years</td>
<td>Yes</td>
<td>Yes</td>
<td>&gt;30</td>
<td>&gt;125</td>
<td>0</td>
<td>&gt;200</td>
<td>&gt;50</td>
</tr>
</tbody>
</table>

*Pseudo hypertriglyceridemia diagnosis, if the sum of points is:

- < 8 points: no pseudo hypertriglyceridemia
- 8-10 points: unlikely pseudo hypertriglyceridemia
- >10-14 points: possible pseudo hypertriglyceridemia

Subjects with isolated GKD showed significantly lower BMI, total cholesterol, non-HDL cholesterol and GGT than HTG non-GKD subjects.

Subjects with isolated GKD had significantly higher HDL cholesterol and TG and lower prevalence of diabetes than HTG non-GKD subjects.

Distribution of the percentage of subjects according to the diagnostic index in patients with non-GKD HTG or subjects with isolated GKD.
Glycerol Kinase Deficiency in adults: description of 4 novel cases, systematic review and development of a clinical diagnostic score

Authors

Lamiquiz-Moneo I\textsuperscript{1}, Mateo-Gallego R\textsuperscript{1,2}, Fernández-Pardo J\textsuperscript{3}, López-Ariño C\textsuperscript{1}, Marco-Benedí V\textsuperscript{1}, Bea AM\textsuperscript{1}, Ferraro L\textsuperscript{3}, Jarauta E\textsuperscript{1,4}, Cenarro A\textsuperscript{1,5}, Civeira F\textsuperscript{1,4}

Affiliation

\textsuperscript{1}Hospital Universitario Miguel Servet, Instituto de Investigación Sanitaria Aragón (IIS Aragón), CIBERCV, Zaragoza, Spain.

\textsuperscript{2}Departamento de Fisiatría y Enfermería, Facultad de Ciencias de la Salud y del Deporte, Universidad de Zaragoza, Huesca, Spain.

\textsuperscript{3}Servicio de Medicina Interna (Unidad de Lípidos), Hospital General Universitario Reina Sofía, Murcia, Spain.

\textsuperscript{4}Departamento de Medicina, Psiquiatría y Dermatología, Facultad de Medicina, Universidad de Zaragoza, Zaragoza, Spain.

\textsuperscript{5}Instituto Aragonés de Ciencias de la Salud (IACS), Zaragoza, Spain

Corresponding author

Itziar Lamiquiz-Moneo, PhD

Address: Hospital Universitario Miguel Servet, Instituto de Investigación Sanitaria Aragón (IIS Aragón), CIBERCV, Avenida Isabel La Católica, 1-3, 50009, Zaragoza, Spain

Telephone number: (34) 976765500 (EXT 142895)

Email address: itziarlamiquiz@gmail.com
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Keywords

Glycerol Kinase Deficiency, pseudo-HTG, lipid-lowering therapy resistant, triglycerides

Number of tables: 5

Number of figures: 3
1. INTRODUCTION

Glycerol kinase deficiency (GKD) is a rare genetic disorder with a prevalence of 1:10⁶. Glycerol kinase (GK) induces the phosphorylation of glycerol to glycerol-3-phosphate, necessary in the formation of triglycerides (TG) in the liver and other tissues. GKD produces hyperglycerolemia and glyceroluria [1]. GKD is a X-linked genetic disorder caused by pathogenic mutations in the GK gene. GK is located at the locus Xp21.3 of the X chromosome, and expands 21 exons. Three distinct clinical phenotypes of this enzymopathy have been described: the complex infantile form, the juvenile form, and the benign or adult form [2]. The complex infantile form is an Xp21 contiguous gene syndrome involving not only the GK locus but also the contiguous congenital adrenal hypoplasia locus or the Duchenne muscular dystrophy locus, or both [3]. This is because infantile GKD is due to large genetic deletions involving not only the GK gene, but also the continuous genes that encodes congenital adrenal hypoplasia or Duchenne muscular dystrophy. The clinical features of patients with complex GKD depend on the loci that are involved, and may be associated with intellectual disability and dysmorphic features [4]. In contrast, the juvenile and adult forms result from either point mutations or small rearrangements within the GK gene, not resulting in congenita adrenal hypoplasia or Duchenne muscular dystrophy [5]. The juvenile form is the least studied form, it can involve severe episodes of vomiting, metabolic acidosis, stupor and coma in the first years of life [6]. The adult form of isolated GKD has been poorly studied, usually it is described as benign and frequently is diagnosed in the context of the study of a primary hypertriglyceridemia (HTG) [5]. The most common biochemical methods to determine triglycerides are based on quantifying the plasmatic glycerol levels. Therefore, when GKD is present, the associated high amount of glycerol would be quantified as high amount of TG, diagnosing these patients with a false HTG. Other reports have associated an increased risk of diabetes [7,8]: however, the knowledge we have on the clinical features of patients with GKD derives from sporadic cases, lacking recent reviews. For this reason, it is important to carry out the first systematic review of this disease to identify the clinical characteristics of the adult form of GKD and differentiate it from common HTG.
HTG is a common complex metabolic trait resulting in increased plasma TG levels and frequently associated with premature cardiovascular disease, atherosclerosis risk, metabolic syndrome and in severe cases, high risk of acute pancreatitis [9]. The treatment of HTG includes dietary restrictions and lipid-lowering drugs for long periods. However, patients with pseudo-HTG due to isolated GKD do not benefit from any lipid-lowering treatment, hence a correct GKD diagnosis is extremely important to avoid unnecessary medical interventions. Recently, we have had the opportunity to study four cases of isolated GKD, three of them belonging to the same family, with falsely elevated TG concentration that did not decrease with lipid-lowering therapy. Taking advantage of this chance, we have elaborated the first systematic review to identify anthropometric, clinical and biochemical characteristics of previously published descriptive cases of isolated GKD.

The objectives of the current study were to describe four novel cases of GKD; to review the clinical information published so far on patients with isolated GKD; to characterize the clinical consequences of the disease; to identify clinical and biochemical differences with HTG non-GKD; and finally, to develop a suspicion clinical diagnostic score, that would help in the diagnosis of GKD in clinical practice.

2. MATERIAL AND METHODS

2.1 Cases and control study subjects

Cases. We present four cases, three of them belonging to the same family, attended in the Lipid Unit at the Hospital Universitario Reina Sofía, Murcia, Spain, who were referred in 2018 because moderate HTG resistant to lipid-lowering therapy, defined as less than 20% decrease of TG levels using fibrates during at least 6 weeks of treatment. In these patients, morning urine samples, after 10-12 hours of fasting, were collected to determine TG levels in urine after ruling out kidney disease in all of them.

Controls. All males with TG levels higher than 300 mg/dL without lipid-lowering treatment and good lipid-lowering therapy response that attended to the Lipid Unit at the Hospital
Universitario Miguel Servet, Zaragoza, Spain, from January 2012 to December 2019, were selected to use them as HTG non-GKD, considering the low prevalence of GKD disease in the general population. All subjects signed an informed consent following a protocol previously approved by the local ethics committee (Comité Ético de Investigación Clínica de Aragón, Zaragoza, and Comité Ético del Servicio Murciano de Salud, Murcia).

2.1 Biochemical analysis

Lipid and lipoprotein analyses in cases and controls were performed using blood samples collected after at least 10 hours overnight fasting and no use of lipid lowering drugs for at least 6 weeks. Total cholesterol and TG levels were determined by standard enzymatic methods. High-density lipoprotein (HDL) cholesterol was measured directly by an enzymatic reaction using cholesterol oxidase (UniCel DxC 800; Beckman Coulter, Inc, Brea, CA). Gamma-Glutamyl Transferase (GGT) was determined by an automated technique in a Beckmann equipment (AU 5800).

2.1.2 Genetic analysis

Whole blood genomic DNA was isolated using the commercial product of Flexigene® DNA (Qiagen). Promoter, coding regions, and intron-exon boundaries of GK (NM_000167.5) were amplified by polymerase chain reaction and purified by ExoSap-IT (USB) using the temperatures and primers described in Supplemental Table 1. Amplified fragments were sequenced by Sanger method using the BigDye 3.1 sequencing kit (Applied Biosystems) in an automated ABI 3500xL sequencer (Applied Biosystems). DNA sequences were analyzed using Variant Reporter software (Applied Biosystems).

2.1.3 Lipoprotein particle analysis

Lipoprotein particle concentrations were measured by 2D Nuclear Magnetic Resonance (NMR)-based on LipoScale® test in the GKD cases from the Hospital Universitario Reina Sofía. The lipid concentration and mean size of very low density lipoproteins (VLDL) were determined as previously reported [10]. Briefly, particle concentration and diffusion coefficients were obtained
from the NMR measurements of the lipid methyl group. The methyl signal was surface fitted with 9 Lorentzian functions associated with each lipoprotein subclasses: large, medium and small. The area of each Lorentzian function was related to the lipid concentration of each lipoprotein subclass, and the size was calculated from their diffusion coefficient.

### 2.2 Systematic review

The systematic review was conducted according to the Preferred Reporting Items for Systematic Reviews [11].

#### 2.2.1 Search strategy and study selection

A systematic search of the relevant literature was performed by using PubMed, Scopus and Cochrane databases in order to identify anthropometric, clinical and biochemical characteristics of all described cases of GKD. References of studies and reviews included were manually checked for additional studies. The structured search strategies used the combination of adult form of GKD: [Glycerol Kinase Deficiency OR GKD OR Pseudo-HTG OR Pseudo hypertriglyceridemia]. Articles retrieved were then included or excluded based on the following criteria. Inclusion criteria were the following: a) articles published in a peer-reviewed journal; b) descriptive studies; c) studies conducted in adults; d) case studies that reported clinical, anthropometric and biochemical characteristics of the adult form of isolated GKD. Exclusion criteria were the following: a) review articles of GKD; b) case studies of juvenile form of isolated GKD <14 years; c) case studies of infantile complex form of GKD; d) studies not conducted in humans. The search was limited to bibliography written in English, French or Spanish.

#### 2.2.2 Outcome measures

Outcomes of interest were body mass index (BMI), blood levels of TG, total cholesterol, HDL cholesterol, non-HDL cholesterol and glucose. Besides, age, family history of HTG, mutation in the GK gene, pharmacotherapy, prevalence of pancreatitis, diabetes mellitus (DM) and all other diseases reported were registered too.
2.2.3 Quality measures

The quality of each included trial was assessed based on the previously validated methodology developed by Kmet et al [12]. The Prisma checklist is available in Supplemental Table 2. The methodology was derived from a checklist for assessing the quality of quantitative studies, which included the following criteria: 1) Question, objective sufficiently described?; 2) Study design evident and appropriate?; 3) Method of subject, comparison group selection or source of information, input variables described and appropriate?; 4) Subject and comparison group (if applicable) characteristics sufficiently described?; 5) If interventional and random allocation was possible, was it reported?; 6) If interventional and blinding of investigators was possible, was it reported?; 7) If interventional and blinding of subjects was possible, was it reported?; 8) Outcome and (if applicable) exposure measure(s) well defined and robust to measurement, misclassification bias? Means of assessment reported?; 9) Sample size appropriate?; 10) Analytic methods described, justified and appropriate?; 11) Some estimate of variance is reported for the main results?; 12) Controlling for confounding?; 13) Results reported in sufficient detail?; 14) Conclusion supported by the results. Each question can be answered with “yes”, “partial”, “no” or “not applicable”. Scoring process was done according to the following formula: (number of “yes” x 2) + (number of “partial” x 1) / (total possible sum (28) – (number of “not applicable” x 2)). The score ranged from 0 to 1; thus, the closer the value is to 1, the higher is the quality of the trial. Quality assessment of each trial was performed by two different researches (ILM and RMG). Two researchers performed the quality checklist of each trial; If a discordance was found (difference mean score more than 0.1 points), a third review by an independent researcher (AMB) was performed.

2.3 Statistical analysis

Analyses were performed using statistical computing software R version 3.5.0 [13]. The distribution of the variables was analyzed with the Shapiro test. Quantitative variables with a normal distribution were expressed as mean ± standard deviation and they were compared by the T-Student test. Variables with a skewed distribution were expressed as median and
interquartile ranges and they were compared with the U Mann Whitney test. Qualitative
variables comparison was expressed as percentages and it was conducted with the Chi-squared
test. The level of significance was set at $p<0.05$. Logistic binary regression was used to
determine which clinical parameter could be used as a diagnostic marker of the isolated GKD
phenotype. We found a difference of 78 mg/dL of mean TG levels between GKD and HTG non-
GKD. A total sample size of at least 349 subjects should be included in the control group
considering 90% power ($Z_{\beta}$ unilateral=1.28) to detect differences in clinical characteristics
between both groups with a confidence interval ($1-\alpha$) of 95% ($Z_{\alpha}$ unilateral= 1.645).

3. RESULTS

3.1 Study subjects with the adult form of isolated GKD

Table 1 shows clinical, anthropometric and biochemical characteristics from the four studied
patients with the adult form of isolated GKD. These patients presented a history of TG levels
between 352 to 578 mg/dL in previous routine blood analysis. None of these patients reported
history of pancreatitis, DM nor any other chronic relevant disease. All of them showed a
medical history of therapy-resistant HTG for several years, despite the combination of a lipid-
lowering diet and fibrates. Interestingly, they showed normal-high levels of HDL cholesterol,
with a range from 52 to 73 mg/dL and normal levels of GGT, lower than 20 UI/L. Urine TG
determination revealed high levels, and since none of these subjects had renal disease, this fact
made their clinician suspect false HTG, as these high levels were actually due to glycerol in
urine. The LipoScale® test allowed to identify the cause of the false HTG in these patients,
showing normal levels of plasma TG, with a range from 54 to 104 mg/dL, and high levels of
plasma free glycerol (>0.2 mmol/L). Genetic analysis showed one pathogenic mutation in the
GK gene, all 4 subjects carrying the same mutation: c.1213C>T, which produces a stop codon,
p.(Arg405*). Nevertheless, out of the four patients GKD analyzed, only three of them belonged
to the same family, apparently (Figure 1).

3.2 Systematic review
3.2.1 Study selection

The systematic search retrieved a total of 764 studies, of which 289 were identified in Pubmed, 408 in Scopus and 67 in Cochrane. After removing 221 duplicated articles, we screened 543 manuscripts, of which 238 were excluded because they did not meet the eligibility criteria. We reviewed 160 full-text studies, excluded 145 articles for different reasons: most articles reported cases of complex GKD (n=93) or infantile and juvenile cases of isolated GKD (n=11), were in vitro studies or developed in animal models (n=22), were published in German, Japanese or Swedish language (n=6), lacked a HTG history (n=2), had no access to full-text manuscript (n=9) or reporting high TG levels due to the exogenous administration of glycerol or severe renal disease (n=1). Finally, 15 articles fulfilled the eligibility criteria and were included in qualitative synthesis (Figure 2).

3.2.2 Participants and main study characteristics

A detailed description of the included studies can be found in Table 2. The 15 studies included a total of 39 adult participants (aged 23-70 years). All articles reported severe HTG: thirteen articles showed a range of TG from 484 to 1992 mg/dL [5,7,8,14–23], however two of them did not report concrete values of TG [24,25]. Twelve articles reported high levels of plasma glycerol [5,7,8,14,15,17–24], with a range from 0.195 to 210 mmol/L, and eight of them showed the presence of glycerol in urine. Regarding to the incidence of other diseases associated with isolated GKD, none of the 39 adults included showed any case of pancreatitis, only two subjects had DM, four had cardiovascular disease, seven presented hypertension, two had overweight and two of them had renal disease. Nine articles reported the causative mutation in the GK gene; a) two of them showed a frameshift mutation: c.730-¿833+? del, p.(Lys244Valfs*10) [14] and IVS6 G-1--C; which produces the deletion of AG at nucleotides 553-554 [18]; b) four articles reported six missense variants responsible of GKD disease: p.(Asn288Asp), p.(Ala305Val), p.(Gln438Arg), p.(Thr96Ile) p.(Cys358Tyr), p.(Gly280Ala) [5,20,21,24]; c) three articles [20,22,25] reported four causative splice site mutation: IVS3+1G>A, IVS10+1G>T, IVS4-52ins316alu and IVS9A–1G>A, causing a premature stop
codon; d) one article showed the deletion of a serine in the amino acid 355 [8]. Interestingly, one of most determining characteristic of isolated GKD was that HTG persisted in spite of lipid-lowering therapy and lifestyle change, and this fact was indicated by eight articles [8,14–18,20,21].

3.2.3 Quality of the studies

The overall quality score of the included studies is summarized in Table 2, and ranged from 0.70 to 1.00, with a mean score of 0.85. Detailed description of each issue assessment for each study is included in Supplemental Table 2. Most articles have obtained a very high-quality score. However, the our greatest concern was absence of correct description of analytic methods, observed in four articles [14,16,17,25], and the poor description of subjects included in seven articles [5,7,19,20,23–25]. Among 15 trials, only in 1 of them, the sample size should have been calculated, although they did not report having done so [5].

3.3 Anthropometric, clinical and biochemical differences between patients with GKD and with HTG non-GKD: a diagnostic score

3.3.1 Clinical characteristics of subjects with HTG non-GKD versus subjects with GKD

Those subjects with isolated GKD registered within the systematic review and those studied by our group were mixed, and they were compared with the HTG non-GKD group. Subjects with HTG non-GKD showed significantly higher levels of BMI, total cholesterol, non-HDL cholesterol and GGT levels compared to than subjects with GKD (\(p<0.001\) for all of them). Besides, subjects with isolated GKD had significantly higher values of HDL cholesterol and TG than subjects with HTG non-GKD (\(p=0.002\) and \(p<0.001\), respectively). Interestingly, subjects with HTG non-GKD showed significantly higher prevalence of DM (17.6%), than subjects with isolated GKD (4.65%, \(p=0.021\)). However, both groups showed similar percentage of cardiovascular disease (11.7 vs 11.3%, \(p=0.965\)) (Table 3).

3.3.2 Suspicion diagnostic score
A suspicion diagnostic score was constructed by comparing subjects with isolated GKD and HTG non-GKD subjects by binary logistic regression (Table 4). From a total of 43 subjects with isolated GKD, including 39 subjects previously reported in the literature and our 4 patients, 33 subjects reported enough data to calculate the total score, all of them being diagnosed as possible pseudo-HTG (all of them had more than 10 points). According to the calculated diagnostic score of pseudo-HTG, of the 584 subjects with HTG non-GKD, only one would have been diagnosed as possible pseudo-HTG, 71 subjects would be diagnosed as unlikely pseudo-HTG and 512 subjects would have been diagnosed as no affected, as they had less than 8 points. Among 71 subjects who had between 8 to 10 points, categorized as unlikely diagnosis, only three of them had 10 points. The GK gene was sequenced in subjects with HTG non-GKD who had 10 or 11 points in the diagnostic score, not finding any pathogenic mutation in these two patients (Figure 3). Besides, the value of the diagnostic score was significantly higher in isolated GKD subjects than in subjects with other forms of HTG (11.7 ± 1.36 vs 4.89 ± 1.82, p < 0.001).

The distribution of each criteria proposed in the diagnostic score showed significantly different distribution between isolated GKD and HTG non-GKD, except for the presence of pancreatitis, probably due to the low incidence of this condition (Table 5).

4. DISCUSSION

GKD is a rare disease, possibly underdiagnosed, which benefits from an early diagnosis, avoiding unnecessary explorations and treatments. The diagnosis is usually delayed for years due to the lack of clinical criteria of suspicion, and also because an accurate diagnosis requires determinations not usually available in most clinical laboratories. In the present work, we report 4 novel cases with the disease, compile all the available information reported on its clinical repercussion and propose some simple suspicion criteria for its diagnosis with high specificity. To our knowledge, this is the first systematic review aiming to explore the differential clinical characteristics in patients with pseudo-HTG due to GKD. The analysis of the clinical
characteristics of 39 adult males with isolated GKD previously reported together with 4 novel cases and the comparison with a large cohort of subjects with HTG non-GKD, have allowed us to create the first diagnostic score for pseudo-HTG. This diagnostic score would have a wide practical utility allowing to discern between subjects with isolated GKD and subjects with HTG non-GKD, reaching a rapid diagnosis of these individuals and avoiding unjustified lipid lowering treatment. More than 37% of subjects with isolated GKD had taken a prolonged lipid-lowering therapy [8,14–18,20,21]. This diagnostic score should apply in males with HTG moderate-severe, with TG levels above 350-400 mg/dL, relatively young, normal values of BMI and GGT, without DM and whose TG levels do not decrease with lipid-lowering treatment. Of the 584 with non-GKD HTG subjects used as control group, only three of them (0.6%) had 10 or more score points. Hence our score selects a very small group of males within the large number of HTG patients. In these subjects would be advisable to perform blood glycerol determination or even urine TG determination, if these patients do not have severe renal disease, to obtain the correct diagnosis and avoid unjustified lipid-lowering treatment, and with this diagnostic score would be possible to identify them.

GKD is characterized by the absence of GK activity, which is often misdiagnosed as a HTG because excess serum glycerol, caused by this disorder, interferes with the enzymatic assay for TG determination used in most clinical laboratories. It is interesting to highlight that all subjects with isolated GKD presented HTG, with a mean of almost 700 mg/dL TG, without presenting any of the risk factors associated with HTG [26]. As expected, none of the 43 subjects with isolated GKD described in the current study suffering from acute pancreatitis, despite it is widely accepted that this process is related with severe HTG [27]. The incidence of DM is another differential clinical characteristic of subjects with isolated GKD: only two subjects (4.65%) had a previous diagnosis of diabetes, while the 17.6% of subjects with HTG non-GKD had the diagnosis of DM. In this sense, several studies have demonstrated the close relationship between high TG levels and a higher incidence of DM [28–30]. Another distinguishing clinical feature of subjects with isolated GKD is a normal BMI versus overweight
and obesity commonly observed in subjects with true HTG. It is interesting to highlight that none of the subjects with isolated GKD had obesity, while 13.3% of subjects with HTG non-GKD were obese, very similar percentage to that previously described in a Spanish working population with moderate HTG [31]. The decrease of TG levels under lipid-lowering therapy was not observed in none of the subjects with isolated GKD, although it is well known that the use of fibrates achieves reductions of TG between 30 to 50% [32]. This lack of decrease is expected, since the subjects do not actually have high TG, but high glycerol levels. The age at diagnosis of HTG is lower in subjects with isolated GKD and usually is diagnosed in the first routine blood test. Besides, TG levels will remain constant despite the age, therapy diet and drug treatment, whose relationship is clearly confirmed [28]. Finally, another distinguishing clinical characteristic would be GammaGT levels, that were significantly lower in subjects with isolated GKD than in subjects with HTG non-GKD. The positive linear relationship between TG and the GGT has previously been described [31], while this relation has not been observed in subjects with isolated GKD.

In addition to GKD, there are other rare diseases that combined could explained the falsely elevated TG levels. For example, a severe renal and hepatic disease combined with a high consumption of white wine or beer, drinks especially rich in glycerol, would produce a high consume of glycerol that would not be removed from the blood due to kidney failure [33]. Besides, high glycerol plasma concentrations could be explained by exogenous glycerol administration in the differential diagnosis of hearing loss at low frequencies [34]. In such cases, medical records of the patient should be carefully evaluated to avoid a false diagnosis of pseudo-HTG by GKD.

It must be pointed out that although the quality assessment of trials was very high, most articles revised were case reports, with limited information about the clinical and anthropometric characteristics of the patients, which could partially limit the interpretation and conclusions obtained.
Our study has some limitations worth mentioning: First, the prevalence of GKD is very low in the general population, only 39 cases of isolated GKD have previously been described in the scientific literature so far, and some of them reported few clinical characteristics. Nevertheless, clinical characteristics of cases and controls are different enough to find statically significant differences. Second, only eight cases reported their values of GGT, although all of them were lower than 20U/L. Third, the age at HTG diagnosis was not significantly lower in subjects with isolated GKD than in subjects with HTG non-GKD. However, it may be due to the fact that not all authors refer to the age at which the patients were first diagnosed as hypertriglyceridemic.

5. CONCLUSIONS

In conclusion, this is the first systematic review that compiles all studies which have reported patients with pseudo-HTG due to GKD to identify their differential clinical characteristics and to create the first pseudo-HTG diagnostic score. We can conclude that GKD is a benign disease without any associated morbidity. A systematic review, together with 4 novel cases be cases not previously described, showed that subjects with isolated GKD present a different phenotype from subjects with true HTG. These differential clinical characteristics have allowed us to create the first diagnostic score to help with the suspicion of pseudo-HTG. The use of this diagnostic score would have a wide utility in our daily clinical practice, distinguishing subjects with isolated GKD from subjects with true HTG, although the results of this study warrant further validation in a prospective clinical setting. This diagnostic score for possible pseudo-HTG identifies subjects in which its advisable to perform blood glycerol determination or even urine TG determination in absence of renal disease to obtain the correct diagnosis and to avoid unjustified lipid-lowering treatment.

Conflict of interest

The authors declare no conflict of interest.
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All authors have read and approved the final manuscript.
REFERENCES


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Table 1. Clinical, anthropometric and biochemical characteristics of 4 novel patients with adult form of isolated GKD

<table>
<thead>
<tr>
<th>Studied patients</th>
<th>Age, years</th>
<th>BMI, kg/m²</th>
<th>Total cholesterol, mg/dL</th>
<th>HDL cholesterol, mg/dL</th>
<th>Non-HDL cholesterol, mg/dL</th>
<th>Plasma triglycerides by routine lab method (mg/dL)</th>
<th>Plasma triglycerides by NMR (mg/dL)</th>
<th>VLDL particles by NMR (nmol/L)</th>
<th>Plasma free glycerol by NMR (nmol/L)</th>
<th>GGT, U/L</th>
<th>Glucose, mg/dL</th>
<th>CVD</th>
<th>DM</th>
<th>Pancreatitis</th>
<th>Mutation in GK gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient 1</td>
<td>25</td>
<td>18.2</td>
<td>163</td>
<td>73</td>
<td>90</td>
<td>352</td>
<td>63.0</td>
<td>26.0</td>
<td>3.04</td>
<td>11</td>
<td>96</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>c.1213C&gt;T; p.(Arg405*)</td>
</tr>
<tr>
<td>Patient 2</td>
<td>25</td>
<td>21.3</td>
<td>121</td>
<td>53</td>
<td>68</td>
<td>471</td>
<td>54.0</td>
<td>19.0</td>
<td>4.22</td>
<td>11</td>
<td>89</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>c.1213C&gt;T; p.(Arg405*)</td>
</tr>
<tr>
<td>Patient 3</td>
<td>58</td>
<td>25.7</td>
<td>302</td>
<td>52</td>
<td>250</td>
<td>578</td>
<td>104</td>
<td>46.0</td>
<td>4.14</td>
<td>19</td>
<td>92</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>c.1213C&gt;T; p.(Arg405*)</td>
</tr>
<tr>
<td>Patient 4</td>
<td>36</td>
<td>22.0</td>
<td>172</td>
<td>67</td>
<td>105</td>
<td>460</td>
<td>66.0</td>
<td>32.0</td>
<td>1.64</td>
<td>19</td>
<td>96</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>c.1213C&gt;T; p.(Arg405*)</td>
</tr>
</tbody>
</table>

GKD: Glycerol kinase deficiency; BMI: Body Mass Index; HDL: High density lipoprotein; VLDL: Very low-density lipoprotein; NMR: nuclear magnetic resonance; GGT: Gamma-Glutamyl Transferase; DM: Diabetes Mellitus
<table>
<thead>
<tr>
<th>Author, year</th>
<th>N</th>
<th>Age, years</th>
<th>TG, mg/dL</th>
<th>Plasma glycerol, mmol/l</th>
<th>Urine glycerol</th>
<th>Pancreatitis, (%)</th>
<th>DM, (%)</th>
<th>Other co-morbidities</th>
<th>Family history of HTG</th>
<th>Mutation in GK gene</th>
<th>Pharmacotherapy</th>
<th>Lifestyle change</th>
<th>Quality score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arrieta F, 2018</td>
<td>1 subject</td>
<td>58</td>
<td>585 to 1242</td>
<td>9.95</td>
<td>4.99 mmol/l</td>
<td>No, (0%)</td>
<td>No, (0%)</td>
<td>CVD, HTA and overweight</td>
<td>NR</td>
<td>c.730?833+? del, p.(Lys244Valfs*10)</td>
<td>Omega 3 (3g/day) + Fenofibrate 250 mg</td>
<td>Yes</td>
<td>0.875</td>
</tr>
<tr>
<td>Arrobas-Velilla T, 2013</td>
<td>1 subject</td>
<td>23</td>
<td>497 to 638</td>
<td>4.17</td>
<td>NR</td>
<td>No, (0%)</td>
<td>No, (0%)</td>
<td>No other associated pathology</td>
<td>NR</td>
<td>NR</td>
<td>Gemfibrozil 600 mg/12h</td>
<td>Sportsman</td>
<td>1.000</td>
</tr>
<tr>
<td>Backes, 2012</td>
<td>2 unrelated subjects</td>
<td>34.5 ± 10.9</td>
<td>484 ± 64.5</td>
<td>NR</td>
<td>NR</td>
<td>No, (0%)</td>
<td>No, (0%)</td>
<td>HTA</td>
<td>NR</td>
<td>NR</td>
<td>Fenofibrate 160 mg + Omega 3 + Fibrates + omega 3 low-carbohydrate diet</td>
<td>Low-carbohydrate diet</td>
<td>0.750</td>
</tr>
<tr>
<td>Backes, 2015</td>
<td>1 subject</td>
<td>37</td>
<td>552 to 695</td>
<td>0.195</td>
<td>NR</td>
<td>No, (0%)</td>
<td>No, (0%)</td>
<td>HTA and stroke</td>
<td>NR</td>
<td>NR</td>
<td>Rosuvastatin 20 + Fenofibrate 160</td>
<td>NR</td>
<td>0.750</td>
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<tr>
<td>Delanghe, 1994</td>
<td>1 subject</td>
<td>61</td>
<td>764</td>
<td>8.19</td>
<td>210 mmol/l</td>
<td>No, (0%)</td>
<td>No, (0%)</td>
<td>No other associated pathology</td>
<td>Sister and daughter non affected</td>
<td>c. del553_554AG, frameshift, reduced levels of GK</td>
<td>Gemfibrozil</td>
<td>Therapy diet</td>
<td>0.938</td>
</tr>
<tr>
<td>Dipple, 2001</td>
<td>3 unrelated subjects</td>
<td>39.6 ± 4.57</td>
<td>Elevated</td>
<td>Elevated</td>
<td>Elevated</td>
<td>No, (0%)</td>
<td>No, (0%)</td>
<td>S1: CVD with 38 years</td>
<td>S1: NR S2: HTA S3: Borderline high DBP</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>0.722</td>
</tr>
<tr>
<td>Gaudet D, 2000</td>
<td>18 subjects from three different families</td>
<td>46.4 ± 14.2</td>
<td>547 ± 98.8</td>
<td>3.99 ± 0.71</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>c.862A&gt;G; p.(Asn288Asp)</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>0.700</td>
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<tr>
<td>Goussault Y, 1982</td>
<td>1 subject</td>
<td>74</td>
<td>892</td>
<td>4.30</td>
<td>290 mmol/24 h</td>
<td>No, (0%)</td>
<td>No, (0%)</td>
<td>No other associated pathology</td>
<td>One sister and daughter non affected</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>0.938</td>
</tr>
<tr>
<td>Hellerud C, 2003</td>
<td>4 subjects from three</td>
<td>61 ± 3.87</td>
<td>688 ± 103</td>
<td>5.43 ± 0.72</td>
<td>4499 ± 2001</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
<td>Two subjects</td>
<td>S1: c.287C&gt;T; p.(Tyr96Ile)</td>
<td>NR</td>
<td>Hypocaloric diet</td>
<td>0.813</td>
</tr>
<tr>
<td></td>
<td>different families</td>
<td>mmol/mmol creatinine</td>
<td>were brothers, no more siblings affected</td>
<td>S2: c.1073G&gt;A; p.(Cys358Tyr)</td>
<td>S3 and S4: splice acceptor site mutation, IVS9A–1G&gt;A, which produces a premature stop codon</td>
<td>&lt;20% of calories</td>
<td></td>
<td></td>
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<tr>
<td>Rose, 1978</td>
<td>1 subject</td>
<td>55</td>
<td>966</td>
<td>7.6</td>
<td>13g/24h</td>
<td>NR</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>No, (0%)</td>
<td>Yes, (100%)</td>
<td>DM and CVD</td>
<td>NR</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Strong family history of DM (his mother and 4 of 11 siblings)</td>
<td></td>
<td>NR</td>
<td></td>
<td></td>
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<tr>
<td>Sanson-Raffin ML, 1989</td>
<td>1 subject</td>
<td>54</td>
<td>800</td>
<td>NR</td>
<td>No, (0%)</td>
<td>NR</td>
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<td></td>
<td></td>
<td></td>
<td>No, (0%)</td>
<td>duodenal ulcer</td>
<td>NR</td>
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<tr>
<td>Walmsley TA, 2008</td>
<td>1 subject</td>
<td>43</td>
<td>840</td>
<td>9.9</td>
<td>71mmol/l</td>
<td>NR</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>No, (0%)</td>
<td>Yes, (100%)</td>
<td>HTA and obesity</td>
<td>NR</td>
<td></td>
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<td></td>
<td>NR</td>
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<tr>
<td>Wibmer T, 2005</td>
<td>1 subject</td>
<td>60</td>
<td>552</td>
<td>10</td>
<td>No, (0%)</td>
<td>NR</td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td>No, (0%)</td>
<td>unexpected death for CVD</td>
<td>NR</td>
<td></td>
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<td></td>
<td></td>
<td>c.1064_1066delCT T, p.(Ser355del)</td>
<td>NR</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Zhang Y, 2000</td>
<td>1 subject</td>
<td>36</td>
<td>1992</td>
<td>210.1</td>
<td>No, (0%)</td>
<td>NR</td>
<td></td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td>No, (0%)</td>
<td>HTA</td>
<td>(IVS4-52ins316alu)</td>
<td>NR</td>
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<td></td>
<td>NR</td>
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<td></td>
</tr>
<tr>
<td>Zhang YH, 2006</td>
<td>2 subjects</td>
<td>70</td>
<td>HTG</td>
<td>NR</td>
<td>No, (0%)</td>
<td>NR</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>No, (0%)</td>
<td>S1: nephrolithiasis and glaucoma</td>
<td>NR</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>NR</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

Zhang YH, 2006 | 2 subjects | 70 | HTG | NR | No, (0%) | S1: nephrolithiasis and glaucoma | NR | NR | 0.750 |
|   |   |   |   | S2: No other associated pathology |

GK: Glycerol Kinase; CVD: Cardiovascular disease; HTG: Hypertriglyceridemia; NR: Not reported; S1: subject 1; S2: subject 2; HTA: Hypertension; DM: Diabetes mellitus; DBP: Diastolic blood pressure.
Table 3. Clinical and biochemical characteristics of all patients included in the present study according to the presence or absence of mutation in *GK* gene

<table>
<thead>
<tr>
<th></th>
<th>Subjects with isolated GKD (N=43)</th>
<th>Subjects with non-GKD HTG (N= 584)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>44.9 ± 16.3</td>
<td>46.8 ± 11.2</td>
<td>0.586</td>
</tr>
<tr>
<td>Men, n (%)</td>
<td>43 (100%)</td>
<td>584 (100%)</td>
<td>NA</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>23.6 ± 2.91</td>
<td>29.1 ± 3.77</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>625 (547 - 814)</td>
<td>475 (358- 714)</td>
<td>0.005</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>178 ± 50.2</td>
<td>284 ± 83.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>61.2 ± 22.8</td>
<td>38.0 ± 11.5</td>
<td>0.001</td>
</tr>
<tr>
<td>Non-HDL cholesterol, mg/dL</td>
<td>117 ± 51.4</td>
<td>244 ± 75.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>GGT, UI/L</td>
<td>14.0 (12.0 - 20.0)</td>
<td>39.0 (28.0- 61.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Glucose, mg/dL</td>
<td>92.9 (90.6 – 96.0)</td>
<td>97.0 (97.0- 112)</td>
<td>0.441</td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td>2 (4.65%)</td>
<td>103 (17.6%)</td>
<td>0.021</td>
</tr>
<tr>
<td>Acute pancreatitis</td>
<td>0 (0.00%)</td>
<td>7 (1.20%)</td>
<td>0.213</td>
</tr>
<tr>
<td>Cardiovascular disease</td>
<td>5 (11.7%)</td>
<td>66 (11.3%)</td>
<td>0.965</td>
</tr>
</tbody>
</table>

*GK*: Glycerol Kinase; *GKD*: Glycerol Kinase Deficiency; *BMI*: Body Mass Index; *HDL*: High density lipoprotein; *GGT*: Gamma-Glutamyl Transferase; *DM*: Diabetes mellitus; NA: Not applied. Quantitative variables are expressed as means ± standard deviations, except for variables not following normal distribution that are expressed as medians (interquartile ranges). Qualitative variables are expressed as percentage. The p value was calculated by T-Student test or U Mann Whitney and Chi-square, as appropriate.
Table 4. Pseudo-HTG diagnostic score

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Points*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical history</strong></td>
<td></td>
</tr>
<tr>
<td>Age at HTG diagnosis</td>
<td></td>
</tr>
<tr>
<td>&lt; 40 years</td>
<td>2</td>
</tr>
<tr>
<td>40-60 years</td>
<td>1</td>
</tr>
<tr>
<td>&gt; 60 years</td>
<td>0</td>
</tr>
<tr>
<td>Therapy-resistant high TG levels</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>0</td>
</tr>
<tr>
<td>Yes</td>
<td>2</td>
</tr>
<tr>
<td>Pancreatitis history</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>0</td>
</tr>
<tr>
<td>Yes</td>
<td>-2</td>
</tr>
<tr>
<td><strong>Physical examination</strong></td>
<td></td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td></td>
</tr>
<tr>
<td>&lt;25</td>
<td>2</td>
</tr>
<tr>
<td>25-30</td>
<td>1</td>
</tr>
<tr>
<td>&gt;30</td>
<td>0</td>
</tr>
<tr>
<td><strong>Biochemical data</strong></td>
<td></td>
</tr>
<tr>
<td>Glucose, mg/dL</td>
<td></td>
</tr>
<tr>
<td>&lt;100</td>
<td>2</td>
</tr>
<tr>
<td>100-125</td>
<td>1</td>
</tr>
<tr>
<td>&gt;125</td>
<td>0</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td></td>
</tr>
<tr>
<td>&gt;60</td>
<td>2</td>
</tr>
<tr>
<td>40-60</td>
<td>1</td>
</tr>
<tr>
<td>&lt;40</td>
<td>0</td>
</tr>
<tr>
<td>Non-HDL cholesterol, mg/dL</td>
<td></td>
</tr>
<tr>
<td>&lt;150</td>
<td>2</td>
</tr>
<tr>
<td>150-200</td>
<td>1</td>
</tr>
<tr>
<td>&gt;200</td>
<td>0</td>
</tr>
<tr>
<td>Gamma-Glutamyl Transferase, UI/L</td>
<td></td>
</tr>
<tr>
<td>&lt;20</td>
<td>2</td>
</tr>
<tr>
<td>20-50</td>
<td>1</td>
</tr>
<tr>
<td>&gt;50</td>
<td>0</td>
</tr>
</tbody>
</table>

HTG: Hypertriglyceridemia; HDL: High-density lipoprotein; BMI: Body Mass Index.

*Pseudo hypertriglyceridemia diagnosis, if the sum of points is:

- **< 8 points**: no pseudo hypertriglyceridemia
- **8-10 points**: unlikely pseudo hypertriglyceridemia
- **>10-14 points**: possible pseudo hypertriglyceridemia
Table 5. Number of subjects based on the criteria they met according to the phenotype they had

<table>
<thead>
<tr>
<th>CRITERION</th>
<th>Subjects with isolated GKD (N=43)</th>
<th>Subjects with HTG non-GKD (N=584)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRITERION 1: Age of HTG diagnosis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;40 years</td>
<td>15 (34.1%)</td>
<td>70 (12.0%)</td>
<td>0.011</td>
</tr>
<tr>
<td>40-60 years</td>
<td>23 (52.3%)</td>
<td>360 (61.6%)</td>
<td></td>
</tr>
<tr>
<td>&gt;60 years</td>
<td>5 (11.4%)</td>
<td>152 (26.0%)</td>
<td></td>
</tr>
<tr>
<td>CRITERION 2: High TG levels therapy-resistant</td>
<td>No</td>
<td>0 (0%)</td>
<td>584 (100%)</td>
</tr>
<tr>
<td>Yes</td>
<td>43 (100%)</td>
<td>0 (0%)</td>
<td></td>
</tr>
<tr>
<td>CRITERION 3: Pancreatitis history</td>
<td>No</td>
<td>43 (100%)</td>
<td>577 (98.8%)</td>
</tr>
<tr>
<td>Yes</td>
<td>0 (0%)</td>
<td>7 (1.20%)</td>
<td></td>
</tr>
<tr>
<td>CRITERION 4: Body mass index, kg/m²</td>
<td>&lt;25</td>
<td>15 (41.6%)</td>
<td>60 (10.3%)</td>
</tr>
<tr>
<td>25-30</td>
<td>21 (58.3%)</td>
<td>285 (48.8%)</td>
<td></td>
</tr>
<tr>
<td>&gt;30</td>
<td>0 (0%)</td>
<td>208 (35.6%)</td>
<td></td>
</tr>
<tr>
<td>CRITERION 5: Glucose levels, mg/dL</td>
<td>&lt;100</td>
<td>28 (90.3%)</td>
<td>313 (53.6%)</td>
</tr>
<tr>
<td>100-125</td>
<td>2 (6.45%)</td>
<td>141 (24.1%)</td>
<td></td>
</tr>
<tr>
<td>&gt;125</td>
<td>1 (3.23%)</td>
<td>97 (16.6%)</td>
<td></td>
</tr>
<tr>
<td>CRITERION 6: HDL cholesterol, mg/dL</td>
<td>&gt;60</td>
<td>5 (31.3%)</td>
<td>5 (0.85%)</td>
</tr>
<tr>
<td>40-60</td>
<td>11 (68.8%)</td>
<td>188 (32.2%)</td>
<td></td>
</tr>
<tr>
<td>&lt;40</td>
<td>0 (0%)</td>
<td>369 (63.2%)</td>
<td></td>
</tr>
<tr>
<td>CRITERION 7: non-HDL cholesterol, mg/dL</td>
<td>&lt;150</td>
<td>12 (75%)</td>
<td>27 (4.62%)</td>
</tr>
<tr>
<td>150-200</td>
<td>3 (18.8%)</td>
<td>129 (22.1%)</td>
<td></td>
</tr>
<tr>
<td>&gt;200</td>
<td>1 (6.25%)</td>
<td>420 (71.9%)</td>
<td></td>
</tr>
<tr>
<td>CRITERION 8: Gamma-Glutamyl Transferase, U/L</td>
<td>&lt;20</td>
<td>8 (100%)</td>
<td>52 (8.90%)</td>
</tr>
<tr>
<td>20-50</td>
<td>0 (0%)</td>
<td>301 (51.5%)</td>
<td></td>
</tr>
<tr>
<td>&gt;50</td>
<td>0 (0%)</td>
<td>191 (32.7%)</td>
<td></td>
</tr>
</tbody>
</table>

GKD: Glycerol Kinase Deficiency; HTG: Hypertriglyceridemia; TG: Triglycerides; HDL: High-density lipoprotein. The p value was calculated by Chi-square.
Figure legends

**Figure 1.** Pedigree of patients carrying the pathogenic mutation p.(Arg405*) in the GK gene.

**Figure 2.** Inclusion and exclusion criteria for the systematic review. Study Flow.

**Figure 3.** Distribution of the percentage of subjects according to the score classification in patients with HTG non-GKD or subjects with isolated GKD.
Reviewer 1:

I carefully read the manuscript by Lamiquiz-Moneo which is of outstanding interest and really very well written.

My only comment regards the definition of "therapy-resistant HTG" (i.e. were patients resistant to PUFAs / fenofibrate / gemfibrozil ?). In my opinion, authors should specify this information in the manuscript.

Answer

We thank the Reviewer for his/her comment about our study. According with the Reviewer`s suggestion, we have included the next sentence in the Material and Methods section to better define "therapy-resistant HTG":

“Cases. We present four cases, three of them belonging to the same family, attended in the Lipid Unit at the Hospital Universitario Reina Sofia, Murcia, Spain, who were referred in 2018 because moderate HTG resistant to lipid-lowering therapy, defined as less than 20% decrease of TG levels using fibrates during at least 6 weeks of treatment. In these patients, morning urine samples, after 10-12 hours of fasting, were collected to determine TG levels in urine after ruling out kidney disease in all of them.”

Reviewer 2:

I read the case series entitled 'Glycerol Kinase Deficiency in adults: Description of 4 new cases, systematic review of the phenotype and creation of a new diagnostic score'. As it defines a new score of pseudohyperTGs, I think the manuscript should be published.

I have 2 major comments

1. As a rule systematic reviews should cover at least 3 different databases however this has involved only Pubmed and Scopus databases

Answer

We thank the Reviewer for his/her comment. As suggested, we have included a third database, Cochrane, in the systematic review. New papers have been reviewed, without new GKD cases. This new information has been included in the “Material and Methods” and “Results” sections.

2. The evaluation of the cases and including the family should also cover the presence of atherosclerotic events.

Answer
According to Reviewer’s suggestion, we have included the evaluation of the presence of atherosclerosis events in table 1 and table 3. We have found that do not exist significant differences in the presence of cardiovascular disease between subjects with isolated GKD and subjects with non-GKD hypertriglyceridemia.

We have included the next sentence in the results section:

“However, both groups showed similar percentage of cardiovascular disease (11.7 vs 11.3%, p=0.965).”

**Reviewer 3:**

In this work the authors propose a pseudo-HTG diagnosis score, describe 4 novel cases of GKD and review the literature. The conclusions suggest that the application of the score is able to identify patients with pseudo-HTG.

Please comment that the results of this study warrant further validation in a prospective clinical setting.

**Answer**

According to the Reviewer’s suggestion, we have included the next sentence in the conclusion paragraph. In the current version, it reads:

“In conclusion, this is the first systematic review that compiles all studies which have reported patients with pseudo-HTG due to GKD to identify their differential clinical characteristics and to create the first pseudo-HTG diagnostic score. We can conclude that GKD is a benign disease without any associated morbidity. A systematic review, together with 4 novel cases be cases not previously described, showed that subjects with isolated GKD present a different phenotype from subjects with true HTG. These differential clinical characteristics have allowed us to create the first diagnostic score to help with the suspicion of pseudo-HTG. The use of this diagnostic score would have a wide utility in our daily clinical practice, distinguishing subjects with isolated GKD from subjects with true HTG, although the results of this study warrant further validation in a prospective clinical setting. This diagnostic score for possible pseudo-HTG identifies subjects in which it's advisable to perform blood glycerol determination or even...
urine TG determination in absence of renal disease to obtain the correct diagnosis and to avoid unjustified lipid-lowering treatment.”

Reviewer 4:
- More suitable Highlights should be presented.

Answer

According to the Reviewer’s suggestion, we have rewritten the highlights. Now, they read as follows:

- “Glycerol Kinase Deficiency (GKD) is a rare genetic disorder often misdiagnosed as hypertriglyceridemia (HTG)
- We describe 4 novel GKD cases and complete the first systematic review that compiles all previously published subjects with GKD
- The distinctive clinical features between GKD and HTG patients are described
- This article proposes the first pseudo-HTG diagnostic score”

- More suitable title should be selected for the article.

Answer

We have rewritten the title:

“Glycerol Kinase Deficiency in adults: description of 4 novel cases, systematic review and development of a clinical diagnostic score”.

- The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone.

Answer
We had included the purpose of the research in the background section:

“We aimed to describe four novel cases of GKD, to complete a systematic review of all cases of isolated GKD published so far and to develop a suspicion clinical diagnostic score for GKD”.

Besides, we had included the principal results, discussing that

“Compared to GKD subjects (n=43), HTG non-GKD subjects (n=584) showed significantly higher BMI, total cholesterol, non-HDL cholesterol and gamma-glutamyltransferase; significantly lower HDL cholesterol and TG; and higher prevalence of diabetes than subjects with GKD. The proposed diagnostic score was significantly higher in GKD than in HTG non-GKD subjects”.

- It is suggested to present the structure of the article at the end of the introduction.

Answer

We have modified the final sentence of the introduction. In the current version reads as follows:

“The objectives of the current study were to describe four novel cases of GKD; to review the clinical information published so far on patients with isolated GKD; to characterize the clinical consequences of the disease; to identify clinical and biochemical differences with HTG non-GKD; and finally, to develop a suspicion clinical diagnostic score, that would help in the diagnosis of GKD in clinical practice.”

Furthermore, we have numbered in order sections and subsections, with the goal to structure the article.

- The necessity and innovation of the article should be presented to the introduction.

Answer

According to the Reviewer’s suggestion, we have added the next sentence in the introduction section:
“For this reason, it is important to carry out the first systematic review of this disease to identify the clinical characteristics of the adult form of GKD and differentiate it from common HTG.”

- The major defect of this study is the debate or Argument is not clear stated in the introduction session. Hence, the contribution is weak in this manuscript. I would suggest the author to enhance your theoretical discussion and arriveres your debate or argument.

Answer

We appreciate the reviewer's comment on this important aspect. We believe that with the modifications made and discussed above, we have improved what was commented by the Reviewer.

- Sections and sub-sections should be numbered in order.

Answer

According to the Reviewer's suggestion, we have numbered in order all sections and sub-sections.

- More suitable title should be selected for the figure 1 instead of "Flow-chart".

Answer

We have rewritten the title for the Figure 2. Now, it reads:

“Inclusion and exclusion criteria for the systematic review. Study Flow”.

- Following, you will find some new related references which should be added to literature review:

Swierczynski, A. Pathogenicity of Endocrine Dysregulation in Autism: The Role of the
Melanin-Concentrating Hormone System;

An et al. GRIK3 rs490647 is a Common Genetic Variant between Personality and Subjective Well-being in Chinese Han Population;

Ebrahimipour et al. Isolation and Characterization of Glutaminase-free L-asparaginase Produced by Staphylococcus sp. MGM1.

Answer

We do not believe the references provided are related to our article and we do not see the need for their inclusion.

- Page 7: the following paragraph is unclear, so please reorganize that:

"Briefly, particle concentration and diffusion coefficients were obtained from the measured amplitudes and attenuation of their spectroscopically distinct lipid methyl group NMR signals using the 2D diffusion-ordered 1H NMR spectrometry (DSTE) pulse. The methyl signal was surface fitted with 9 lorentzian functions associated with each lipoprotein subclasses: large, medium and small of the main lipoprotein classes. The area of each lorentzian function was related to the lipid concentration of each lipoprotein subclass, and the size was calculated from their diffusion coefficient."

Answer

According with the Reviewer’s suggestion, we have rewritten the next paragraph to try to simplify it. Now, it reads:

“Briefly, particle concentration and diffusion coefficients were obtained from the NMR measurements of the lipid methyl group. The methyl signal was surface fitted with 9 Lorentzian functions associated with each lipoprotein subclasses: large, medium and small. The area of each Lorentzian function was related to the lipid concentration of each lipoprotein subclass, and the size was calculated from their diffusion coefficient.”
- Much more explanations and interpretations must be added for the results, which are not enough.

**Answer**

In the results section, we have included the following sections:

3.1 Complete description of table 1, which includes the clinical, anthropometric and biochemical characteristics of the four studied patients with the adult form of isolated GKD.

3.2 Results of the systematic review, including the study selection, participants characteristics, studies types and quality of the studies.

3.3 Anthropometric, clinical and biochemical differences between patients with GKD and with non-GKD HTG, which compares the clinical characteristics of subjects with non-GKD HTG versus GKD subjects and the creation of suspicion diagnostic score.

According with the reviewer suggestion, we have analyzed the prevalence of cardiovascular disease in both diseases. Therefore, we have added the next sentences in the results section:

“*However, both groups showed similar percentage of cardiovascular disease (11.7 vs 11.3%, p=0.965).*”

- It is suggested to compare the results of the present research with some similar studies which is done before.

**Answer**

Although we agree with the Reviewer that it would be interesting to compare the results of the present research with some similar studies done before. However, it cannot be done because this is the first systematic review of isolated GKD and the first comparing the anthropometric, clinical and biochemical characteristics between GKD and non-GKD HTG patients. However, in the discussion section, we compared the clinical characteristics of isolated GKD, commenting
that none of these subjects reported associated disease related with HTG, like pancreatitis, high BMI, high levels of GGT, lower prevalence of DM, etc.

- Please make sure your conclusions’ section underscore the scientific value added of your paper, and/or the applicability of your findings/results, as indicated previously. Please revise your conclusion part into more details. Basically, you should enhance your contributions, limitations, underscore the scientific value added of your paper, and/or the applicability of your findings/results and future study in this session.

Answer

According with the Reviewer’s suggestion, we have rewritten the conclusion section. Now, it reads:

“In conclusion, this is the first systematic review that compiles all studies which have reported patients with pseudo-HTG due to GKD to identify their differential clinical characteristics and to create the first pseudo-HTG diagnostic score. We can conclude that GKD is a benign disease without any associated morbidity. A systematic review, together with 4 novel cases be cases not previously described, showed that subjects with isolated GKD present a different phenotype from subjects with true HTG. These differential clinical characteristics have allowed us to create the first diagnostic score to help with the suspicion of pseudo-HTG. The use of this diagnostic score would have a wide utility in our daily clinical practice, distinguishing subjects with isolated GKD from subjects with true HTG, although the results of this study warrant further validation in a prospective clinical setting. This diagnostic score for possible pseudo-HTG identifies subjects in which its advisable to perform blood glycerol determination or even urine TG determination in absence of renal disease to obtain the correct diagnosis and to avoid unjustified lipid-lowering treatment.”

- "Notation" should be added to the article.
We have checked the units of all data, like mg/dL, U/L, mmol/L, etc, and they are correctly indicated.

- DOI of the references must be added (you can use "https://crossref.org/").

We added all available DOI of the references.

Reviewer 5:

The manuscript of Lamiquiz-Moneo et al is a report of novel pathologic GK variants and a systematic review of the reported patients in order to establish a pseudohypertriglyceridemia score. In sum the systematic review provides interesting and novel characterization of adult GKD patients and raises important awareness for pseudohypertriglyceridemia.

Major points:

1) The authors discuss that their diagnostic score has "enormous practical utility". They should clarify who should use this score in which setting and estimate the number of patients needed to find a misdiagnosed patient and based on this number, discuss the practical benefit. I am not sure about the benefit of this score in clinical practice for such a rare disease as pointed out in the introduction of the manuscript.

According with the Reviewer’s suggestion, we have reduced the forcefulness of the sentence about the enormous practical utility of our diagnostic score. Now, it reads:

“This diagnostic score could have a wide practical utility”.

Besides, in the same paragraph, we have discussed the use of this score and we have indicated the number of misdiagnosed patients based on this score. In the current version, it reads:
“This diagnostic score should apply in males with HTG moderate-severe, with TG levels above 350-400 mg/dL, relatively young, normal values of BMI and gamma-glutamyltransferase, without DM and whose TG levels do not decrease with lipid-lowering treatment. Of the 584 with non-GKD HTG subjects used as control group, only three of them (0.6%) had 10 or more score points. Hence our score selects a very small group of males within the large number of HTG patients.”

2) The problem with the diagnostic score used is that it cannot be validated in a second validation cohort as all reported patients are included in the design of the score. The authors discuss that also non-GKD cohorts of hyperglycerolemic patients exist ("severe renal and hepatic disease combined with a high consumption of white wine or beer, drinks especially rich in glycerol" [...]; "glycerol exogeneous administration in the differential diagnosis of hearing loss at low frequencies"). The authors should try to assess the novel score on these patient groups as well to see the specificity for GKD patients versus non-GKD pseudo-HTG (also sometimes called pseudo-pseudo-HTG, see PMID: 7720256) to see if this score is just useable for adult GKD or if it is a score of all pseudo-HTG forms. Please also try to use this score for juvenile GKD. The authors should provide evidence that this score might be specific and sensitive enough to be useable for more than the 39 patients which are included to design the score.

Answer

Although we agree with the reviewer that the diagnostic score cannot be validated in a second validation cohort, because all 43 GKD patients (39 previously reported in the literature and four new patients) are included to develop the score.

We appreciate your suggestion to apply the score to populations with pseudo-pseudo HTG, but these types of patients tend to have other clinical characteristics that the clinical history identifies, and we sincerely believe that they are outside the scope of this study.
It is a suggestion that we take into consideration and we will try to explore in the future with different inclusion criteria that could include this population.

Respect to GKD juvenile, although would be interesting to try to apply the diagnostic score, we considered that practical application is not utility, because these patients do not present adult HTG, but vomiting and acidemia to stupor and unconsciousness in the first years of life. Therefore, the diagnostic of juvenile GKD should happen before these individuals reach maturity and they show a very different phenotype from HTG.

3) Which benefit has this score compared to a non-glycerol dependent measurement of TG in HTG patients? Maybe the outcome of the review could also be to run lipoprotein electrophoresis or non-glycerol assays after primary assessment of high TG in routine lab measurements?

Answer

We agree and we appreciate the comment. The aim of the article is to point out the importance of GKD and identify potential patients for a definite diagnosis of the disease. Whether it is necessary in the suspicion patients to carry out TG determination in urine, genetic analysis, lipoprotein electrophoresis or blood glycerol determination is outside the scope of this work. We suggest the determination of TG in urine, since it is a simple method and it is available to all clinical laboratories.

This idea has been included in the discussion section:

“This diagnostic score for possible pseudo-HTG identifies subjects in which its advisable to perform blood glycerol determination or even urine TG determination in absence of renal disease to obtain the correct diagnosis and to avoid unjustified lipid-lowering treatment.”
Minor points:

1) Please provide all used NM numbers for description of the genetic variants mentioned in the manuscript. It will be easier then to see whether the reported novel variant is already reported e.g. in gnomAD or 1000 genomes. In gnomAD there is already a Arg413Ter variant (reported in PMID: 9719371) which might be the same as the reported Arg411Ter depending on the genetic reference codes used?

Answer

We agree with the reviewer that depending on the RefSeq used, the mutation nomenclature can vary, and therefore, it can be some confusing for comparison. We have checked the RefSeq used in the mentioned paper PMID: 9719371, being GenBank X78211.1. With this RefSeq we can conclude that the mutation identified in our study is different from that of the paper of D.R. Sjarif et al. 1998, p.(Arg413*), as with that RefSeq, the identified mutation in our study would read c.1213C>T, p.(Arg405*). According with that, and to avoid confusions and clarify this issue, we have used the NM_000167.5 for the mutation reported in our manuscript, as it renders the same numbering as GenBank X78211.1. Therefore, we have used the RefSeq NM_000167.5, indicated in Material and Methods section, and we have changed the nucleotide and amino acid numbering of our mutation throughout all manuscript. In this new version, it reads as c.1213C>T, p.(Arg405*), because with this way of numbering it is easier to see that they are two different mutations, and there is no doubt about if our mutation could be the same as that reported by PMID: 9719371. We think that with this change, the issue addressed by the reviewer is clarified.

2) "The complex infantile form is an Xp21 contiguous gene syndrome involving not only the GK locus but also the contiguous congenital adrenal hypoplasia locus or the Duchenne muscular dystrophy locus, or both". Please provide more insight into the genetic connection of the three mentioned disease that the reader can understand why there are connected cases and why there are cases which are not connected to

Answer
According with the reviewer suggestion, we have added the next sentence in the introduction section. Now, it reads:

“This is because infantile GKD is due to large genetic deletions involving not only the GK gene, but also the continuous genes that encodes congenital adrenal hypoplasia or Duchenne muscular dystrophy.”

3) "[…] and the poor description of subjects included in seven articles [5,7,19,19,20,24,25]." Two of the seven articles seem to be the same (19, 19).

Answer

We agree with the Reviewer. We have checked the seven articles with poor description. Now, it reads: “and the poor description of subjects included in seven articles [5,7,19,20,23–25].”

Reviewer 6:

The authors have made a decent attempt to address one of the rare genetic disorder and to develop a diagnostic score which would help to identify GKD patients from HTG non-GKD. However, there are certain queries that require clarification before the manuscript can be considered for publication

Queries

Introduction

1. Line 38-46. The statement regarding the association between elevated glycerol and elevated TG leading to pseudo-HTG is unclear. Kindly reframe it.

Answer

According with the reviewer suggestion, we have rewritten these lines. In the current manuscript version, it reads:

“The most common biochemical methods to determine triglycerides are based on quantifying the plasmatic glycerol levels. Therefore, when GKD is present, the associated high amount of
glycerol would be quantified as high amount of TG, diagnosing these patients with a false HTG.”

Material and methods

1. Mention the sample size in the controls section

According to the reviewer suggestion, we have commented the sample size in the controls section in the statistical analysis section. In the current version, it reads:

“We found a difference of 78 mg/dL of mean TG levels between GKD and HTG non-GKD. A total sample size of at least 349 subjects should be included in the control group considering 90% power (Zβ unilateral=1.28) to detect differences in clinical characteristics between both groups with a confidence interval (1-α) of 95% (Zα unilateral= 1.645).”

2. There is no mention of TG estimation being done from the urine sample. Need to provide the details of urine sample collection

According with the Reviewer’s suggestion, we have included the next paragraph in the material and methods section:

“In these patients, morning urine samples, after 10-12 hours of fasting, were collected to determine TG levels in urine after ruling out kidney disease in all of them.”

3. Mention the recruitment period for both cases and controls

The recruitment period is included in the current version for cases and controls.

4. Information regarding the genetic analysis is greatly inadequate. There is no information on the method was used for DNA extraction and primer sequences. Provide all the details including the references

Answer
According to the reviewer suggestion, we have included the method used for DNA extraction and the primer sequences in the supplemental table 1. In the current version, it reads:

“Whole blood genomic DNA was isolated using the commercial product of Flexigene® DNA (Qiagen). Promoter, coding regions, and intron-exon boundaries of GK (NM_000167.5) were amplified by polymerase chain reaction and purified by ExoSap-IT (USB) using the temperatures and primers described in the Supplemental Table 1.”

Results and Discussion

1. **Provide additional details about the suspicion diagnostic score**

   **Answer**

   Additional details are described. The new information is included in the previous comments to the Reviewers.

2. **Page 13, line 54-55, rectify number of subjects with diagnostic score less than 8..instead of 512 it mentions 956**

   **Answer**

   We agree with the Reviewer. We have corrected the number 956, writing the correct 512 subjects.

3. **Page 13, line 59-60 – reframe the statement as it appears incorrect / incomplete**

   **Answer**

   According to the Reviewer’s suggestion, we have rewritten the next sentence:

   “Besides, the value of the diagnostic score was significantly higher in isolated GKD subjects than in subjects with other forms of HTG”.

4. **Page 14, line 47-49- provide a reference for the statement “…More than 37% of subjects with isolated GKD had reported a prolonged lipid-lowering therapy”**

   **Answer**
This percentage was obtained according to the lipid-lowering combined lifestyle change reported in all subjects, 12, included in the present systematic review, together to our new four cases (16/43 = 37.2%). We have added the references of these articles.

5. Page 15, 8-9 – Rectify the percent of subject with HTG non-GKD and DM. It states 14% whereas in table 3, it mentions 17.6%

Answer

We thank the Reviewer. We have corrected the percentage of subjects with HTG non-GKD with DM. The correct percentage is 17.6%.

6. Figure 1 shows three patients with age as 24, 36 and 58 years however table 1 shows patient 2 age is 25 years. Kindly rectify the error.

Answer

We have rectified the age of the patient. The correct age is 25 years, this age has been corrected in the Figure 1. We have checked that the rest ages and they are correct.

Editorial Office comments:

Answer

All comments have been reviewed. Besides, English edition has been revised by Ferraro L, who has an English-Spanish mother language.

- A graphical abstract is required at revision.

Answer

Included.
Statement of originality

With this document all authors confirm that the paper entitled “Glycerol Kinase Deficiency in adults: Description of 4 new cases, systematic review of the phenotype and creation of a new diagnostic score” is an original work and it has not been previously published in any journal.

All authors have made a substantial contribution to the study and all of them have approved the final draft. None of the authors has any conflicts of interest to declare.

Itziar Lamiquiz-Moneo

Unidad Clínica y de Investigación en Lípidos y Arteriosclerosis

Hospital Universitario Miguel Servet, Zaragoza, Spain
Editor-in-Chief

Dear Professor Arnold von Eckardstein,

Please, find enclosed the manuscript entitled: “Glycerol Kinase Deficiency in adults: description of 4 novel cases, systematic review and development of a clinical diagnostic score”, that we resubmit as Original Research for *Atherosclerosis Journal*. This is the revised version of the manuscript with the same title with the ID ATH-D-20-00800.

In this revised version, we have answered all the questions addressed by the Reviewers. We have highlighted in yellow the changes that we have made according to the Editor’s and Reviewer’s suggestions.

On behalf of all authors, I state that all authors have approved the submitted version of the manuscript, that the manuscript, including related data, figures and tables, has not been previously published and that the manuscript is not under consideration elsewhere.

Thank you for taking the time to consider our work.

Looking forward to hearing from you.

Itziar Lamiquiz-Moneo, PhD

Unidad Clínica y de Investigación en Lípidos y Arteriosclerosis,

Hospital Universitario Miguel Servet, Instituto de Investigación Sanitaria de Aragón (IIS Aragón), Zaragoza, CIBERCV, Spain
Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:
**Figure 3.** Distribution of the percentage of subjects according to the diagnostic index in patients with HTG non-GKD or subjects with isolated GKD

$p<0.001$
Figure 1. Pedigree of patients carrying the pathogenic mutation p.(Arg405*) in the GK gene
Figure 2. Flow chart of study selection

Records identified through database searching:
PubMed (n =289) Scopus (n = 408) and Cochrane (n=67)

Records after duplicates removed (n =543)

Records screened (n=398)

Full-text articles assessed for eligibility (n =160)

Studies included in quantitative synthesis (review) (n =15)

Full-text articles excluded for the review (n = 145)
- n = 93 studies reported cases of complex glycerol kinase deficiency
- n = 11 studies reported infantile and juvenile cases of isolated glycerol kinase deficiency
- n = 12 studies in mice
- n = 2 studies did not report triglyceride values or hypertriglyceridemia history
- n = 10 studies in vitro
- n = 6 studies reported in German, Japanese and Swedish language
- n= 1 study reported high levels of TG due to the inoculation of glycerol in blood
- n = 1 study reported high levels of TG due to hepatic and renal deficiency combined with a high consume of drinks rich in glycerol
- n = 9 unable to access to full-text manuscript

Records excluded (n =145)

Full-text articles excluded (n =238)
- n = 232 not meeting systematic review inclusion criteria
- n = 6 unable to access to manuscript
Click here to access/download
Supplementary Material for online publication only
Supplementary table_review_ACL.docx
Atherosclerosis style guide checklist

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Manuscript structure and style

Does your manuscript contain all the below essential elements, in this order? (please stick to the headers as indicated below)

- Title
- Authors, Affiliations, Contact Information
- Abstract in the Atherosclerosis format (Background and aims, Methods, Results, Conclusions)
- Introduction
- Materials and methods (or Patients and methods)
- Results
- Discussion
- Conflict of interest (mandatory)
- Financial support (if applicable)
- Author contributions (mandatory)
- Acknowledgements (if applicable)
- References
- Figures and Tables (with legends in the suitable style)

Abstract style

Is the Abstract structured in the below sections?

- Background and aims
- Methods
- Results
- Conclusions

Figure and table legends

Are figure and table legends formatted as described below?

Each figure and table legend should have a brief overarching title that describes the entire figure without citing specific panels, followed by a description of each panel, and all symbols used.

If a figure or table contains multiple panels, the letter describing each panel should be capitalized and surrounded by parenthesis: i.e. (A)(B)(C)(D).

Please make sure to apply the formatting requirements to figures and tables where necessary (e.g. style of p values, gene and protein nomenclature).

Footnotes to tables

Are footnotes to tables formatted as described below?

Footnotes to tables should be listed with superscript lowercase letters, beginning with “a.” Footnotes must not be listed with numbers or symbols.

Abbreviations

Are abbreviations defined when first used in the text?

Use of abbreviations should be kept at a minimum.
**Units**
Are units expressed following the international system of units (SI)?

If other units are mentioned, please provide conversion factors into SI units.

**DNA and protein sequences**
Are gene names italicized?

Gene names should be italicized; protein products of the loci are not italicized.

For murine models, the gene and protein names are lowercase except for the first letter.
(e.g., gene: *Abcb4*; protein: Abcb4)

For humans, the whole gene name is capitalized.
(e.g., gene: *ABCB4*; protein ABCB4)

**Mouse strains and cell lines**
Are knock-out or transgenic mouse strains and cell lines italicized and the symbol superscripted? Yes  No

(e.g. *ob/ob*, *p53+/−*, *p53−/−*)

**p values**
Are p values consistently formatted according to the below style throughout the manuscript
(including figures and tables)? Yes  No

p <X
p >X
p=X

**Language**
Is your manuscript written in good English? Yes  No

Please make sure that you consistently use either American or British English, but not a mixture of them.

Please make sure that words are written consistently in the same way throughout the manuscript.

e.g. non-significant or nonsignificant

e.g. down-regulation or downregulation

**Artwork**
Have you submitted high-resolution versions of your original artwork? Yes  No

Please make sure to use uniform lettering and sizing in your original artwork, including letters to indicate panels, consistently throughout all figures.
Author contribution statement

Conceptualization, ILM and FC; Data Curation, ILM, RMG and CLA; Formal Analysis ILM, FC and JFP; Funding Acquisition, FC, AC; Investigation, EJ, LF, VMB; Methodology, ILM and CLA; Project Administration, FC; Resources, FC; Software ILM and CLA; Supervision RMG, ILM and FC; Validation ILM, RMG and AMB; Visualization, AMB, JGG, SPC and LBR; Writing – Original Draft Preparation, ILM and FC; Writing – Review & Editing, RMG, JFP, AC, LF and FC.