Author’s response to reviews

Title: Genetic mutations in SPINK1, CFTR, CTRC in acute pancreatitis

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Author’s response to reviews: see over
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Reviewer's report

Reviewer's report
Title: Genetic mutations in acute pancreatitis
Version: 4 Date: 30 October 2014

Reviewer: Tony G Jacob

Reviewer's report:

Title: consider revising because the authors have not looked at all possible mutations in AP as the title suggests. The title of the article has been changed.

Genetic mutations in SPINK1, CFTR, CTRC in acute pancreatitis

Abstract:
All readers may not know abbreviations of the methods. Please use expanded forms as far as possible. The methods are written in odd English. Needs rephrasing. 6.3% of 221 patients is 14. That means that 14 patients had SPINK1 mutations. If that were the case, then how is it that if 5 of these had alcohol as a causative factor, the percentage is 7.1%. 5 of 14 is 35.7%. Please review the results. The conclusion could have been curtailed to a single sentence regarding the positive results. The methodological part was corrected, the sentence concerning the results and relationship between SPINK1 mutations and etiology of AP was reformatted, and conclusions shortened.

The abbreviations of the methods applied have been expanded according to the reviewer's comment. This part of the manuscript has been corrected as follows:

Methods
In the study included 221 patients treated for acute pancreatitis and 345 healthy subjects as a control group. Peripheral blood samples were collected from each study participant and genomic DNA was isolated. Genotyping of common mutations in the SPINK1 (p.N34S and p.P55S) and CTRC (p.I259V, p.V235I, p.K247_R254del, p.E225A) was performed by using high resolution melting (HRM) method. However, mutations in the CFTR p.F508del (delF508_CTT) were genotyped using allele specific amplification polymerase chain reaction (ASA-PCR). All detected mutations were confirmed with direct capillary DNA sequencing. .

Results – the last sentence has been changed as follows:
The SPINK1 mutation was significantly more frequent in 8 (10.4%) severe than in mild course of the disease - 6 (4.2%) (p<0.05), and was observed in 5/70 (7.1%) patients with alcohol-related AP, 5/81 (6.2%) - with biliary AP, and 4/63 (6.3%) – in those without any established cause of the disease.
Conclusions - shortened

Mutation p.N34S SPINK1 may predispose patients for acute pancreatitis, especially in patients abusing alcohol, and may promote a more severe course of the disease.

**Background:**

The second sentence should not use the word ‘patients’. If they did not develop the disease, why should they be called patients?

Changed as follows:

However, in a considerable number of individuals even many attacks of gallstones and multiple episodes of alcohol abuse do not lead to acute pancreatitis [2,3].

Third sentence needs rephrasing.

The third sentence has been deleted.

Para 2: Which is the ‘presented epidemiological study’? Where is the reference for this study?

The reference for this study was placed in the subsequent sentence. It has been repeated at the reviewer’s suggestion.

The already presented epidemiological investigations have confirmed high morbidity due to acute pancreatitis among adult inhabitants of the Kielce Region in Poland [5].

Most of the mutations mentioned in Para 5 are related to chronic pancreatitis.

Mutations in the PRSS1, SPINK1, CFTR, CTRC and polymorphisms in other genes are the major genetic contributors to the development of AP and CP.

Para 6 goes on to say that SPINK1 mutation are a susceptibility gene for CP. So why bother to study it? ‘Since SPINK1 encodes a protein that guards the pancreas from the effects of recurrent or persistent trypsin activation, it is not a typical susceptibility gene for acute pancreatitis.

The discussion of the role of SPINK1 in pancreatitis has been changed as follows:

*SPINK1* is a specific trypsin inhibitor and an acute phase protein which is secreted by the acinar cells. *SPINK1* protein plays a role in the prevention of premature activation of zymogen that is catalyzed by trypsin within the pancreatic duct system or the acinar tissue. A reactive site in the protein serves as a specific target substrate for trypsin. *SPINK1* polymorphisms are common in the general population (approximately 2%) but are shown to be significantly associated with pancreatitis [15].

There is no citation for the statement made for CFTR gene mutation. Please check recent publications and view the role of CFTR mutations and chronic ER stress in the pancreatic acinar cells.
The role of CFTR and chronic ER stress in pancreatic acinar cells has been discussed. The following reports have been quoted:


This paragraph is as follows:

Recent studies suggest an important role of CFTR in the development of pancreatitis, particularly through its role in intraluminal pH regulation from bicarbonate secretion and the flushing of ductal proteins. Secretory granules of pancreatic acinar cells co-release protons (H+) with digestive enzymes during normal pancreatic secretion. Diminished ductal bicarbonate secretion and consequent reduced alkalinisation of the acinar lumen may promote the development of pancreatitis since acidification of the pancreatic lumen can lead to a loss of tight junction integrity, allowing the leakage of digestive enzymes into the pancreatic duct lumen and interstitial space [16]. Thus, as expected, a wide array of CFTR genotypes was described to associate with pancreatitis. Stressors such as oxidative damage, overloading the protein folding capacity of the ER stress and trigger the unfolded protein response [17].

The reason why a mutated Claudin2 protein should lead to pancreatitis is not given. It is an interesting point.

The problem has been more comprehensively discussed in the paragraph below:

Studies conducted in recent years confirmed that CLDN2 mutation may increase the risk of CP, by interaction with alcohol consumption. These studies partly explain the increased incidence of alcohol-related pancreatitis is higher in males than females. The c.592A>C mutation in CDS CLDN2 causes the change of amino acid from Met to Leu. Physiologically, Claudin-2 protein encoded by the CLDN2 gene is expressed at low levels, co-forming tight junctions (a type of sealed cellular connections) between pancreatic duct cells. When subjected to stress, acinar pancreatic cells abnormally produce high quantities of Claudin-2 protein which may lead to pancreatitis by means of aberrant distribution of ion transport between acinar cells and lumen [18].

Materials and Methods:

Please mention the Ethical clearance at the beginning of the methods section.

Information concerning the expression of consent by the Bioethical Commission were transferred from the section: Statistical analysis
Where is the questionnaire or formulaire that was used to determine risk factors?

What quantity of alcohol consumed warranted inclusion into the alcohol induced pancreatitis?

Alcohol consumption was evaluated with Short Alcohol Dependence Data Questionnaire (SADD) and self-estimated alcohol consumption via interview. Acute pancreatitis diagnosis was made by if a patient gained 10 or more points of SADD Questionnaire or if a period of alcohol abuse was at least one-year-long and the daily dose was 40g of pure ethanol.

**Results:**

Were the number and characteristics of the groups matched? There is a disproportionate number of females as controls and males as patients. Was this a confounder in the search for significant mutations? Their ages seem to be matched, though no statistical test has been shown to determine this.

Principles of selection of the control group were discussed: age, gender. This part of the manuscript has been transferred to the section *Materials and Methods*

Simple random sampling was a method for control group to include adult volunteers without any apparent accompanying diseases which would potentially affect both structure and expression of genes that were to be tested in the study.

The control group consisted of 345 healthy inhabitants of the Kielce Region, 223 females and 122 males; mean age 45.1 (females 44 and males 47), in a general good state of health, with Body Mass Index (BMI) within 18.5-30. The study group covered 221 patients who had undergone acute pancreatitis (88 females and 133 males); mean age 55.4 (females 56.2 and males 51.4). In 65.2% of patients in the study the course of AP was mild, in 17.6% - moderately severe, and in 17.2% - severe. The percentage of females in the study group was significantly lower, compared to the control group, while the mean age was significantly higher in the study than control group.

A large part of the results have been summarized in the tables; they do not need to be repeated in the text form.

The results repeated in tables were omitted in the text.

Why are the legends of the tables included in the results section? Page 12.

Table legends were removed from this part of the manuscript.

**Discussion:**

The first, second and a large part of the third paragraph of discussion is redundant. It is a repetition of the introductory comments. The authors may start with a short summary of the results. Many of the comparisons of the results of other workers could be summarized in a
table with the details of the publication, the number of patients and controls and the findings with respect to the genes. This would reduce the discussion to a more manageable and focused piece. The inferences of the comparisons of the findings and the limitations or advantages of this study should have been highlighted in the discussion.

The sentences indicated by the reviewer have been deleted and the entire text shortened. At the same time, we would like to explain that the results by other researchers quoted in this part of the manuscript always constitute a comparison for the results of our studies.

**Conclusion:**
This could be made more relevant by only mentioning the positive result. The generalized statement borders on hyperbole.

Conclusions have been shortened.

**References:**
I have not checked the reference style. But 33 may be too many for the findings that have been presented.

References No. 33 have been deleted.

**Tables:**
Why do some tables have separating lines and some don't? In table 3, in the column of ‘Concomitant Diseases’- please write ‘None’ instead of ‘Lack’.

Changed according to the reviewer’s comment.
Reviewer's report
Title: Genetic mutations in acute pancreatitis
Version: 4 Date: 28 October 2014
Reviewer: RUPJYOTI TALUKDAR

Reviewer's report:

INTRODUCTION:
1. Introduction is too long. It should be shortened substantially and authors need to be more focused.
Shortened according to the reviewer’s comment.

MATERIAL AND METHODS:
1. It has been mentioned that biliary AP was confirmed based on USG, CT, NMR and/or ERCP. Was ALT levels and EUS not used in making the diagnosis of biliary AP? ERCP is currently not recommended for making a diagnosis biliary AP; but is restricted only to therapeutic ductal clearance for impacted CBD stones with cholangitis in the setting of AP.
The principles of the qualification of patients into the study were explained according to the cause of the disease (alcohol-related, biliary).
Biliary etiology was confirmed based on USG, CT, NMR, and/or ERCP if only there were indications for performance of this procedure e.g. icterus or triple elevation of ALT level.
Endoscopic ultrasonography (EUS) was performed in part of patients to confirm biliary AP
2. Who were the healthy controls and how were they selected and enrolled for the study? Were they age and sex matched?
Principles of selection of the control group were discussed: age, gender. This part of the manuscript has been transferred to the section Materials and Methods
Simple random sampling was a method for control group to include adult volunteers without any apparent accompanying diseases which would potentially affect both structure and expression of genes that were to be tested in the study.
The control group consisted of 345 healthy inhabitants of the Kielce Region, 223 females and 122 males; mean age 45.1 (females 44 and males 47), in a general good state of health, with Body Mass Index (BMI) within 18.5-30. The study group covered 221 patients who had undergone acute pancreatitis (88 females and 133 males); mean age 55.4 (females 56.2 and males 51.4). In 65.2% of patients in the study the course of AP was mild, in 17.6% - moderately severe, and in 17.2% - severe. The percentage of females in the study group was
significantly lower, compared to the control group, while the mean age was significantly higher in the study than control group.

3. Why was PRSS1 mutations not checked? This is one of the most important gene that has been found to be associated with pancreatitis in different cohort worldwide. Authors should also study mutations in the PRSS1 gene.

In all participants exons 2 and 3 of PRSS1 gene were analysed by HRM and Capillary Sequencing. We did not find any mutations in all but one control subject (pI94L). Analysis of patient samples were always accompanied by the analysis of control DNA (exon 2 [p.N29I, p.A16V] i 3 [p.R116C, p.R122D, p.R122C]. Positive control DNA were kindly provided by dr Katarzyna Wertheim - Tysarowska, Institute of Mother and Child, Department of Medical Genetics, Laboratory of Hereditary Diseases Research Warsaw.

STATISTICAL ANALYSIS:

1. Results should also be reported in terms of odd's ratio with 95% confidence intervals.

Table 2 was supplemented by calculations of odds ratio with 95% confidence intervals.

RESULTS:

1. Were all the 221 patients with AP were consecutively admitted? How many patients had first episode and how many had recurrent AP? Many of these patients are likely to have underlying chronic pancreatitis. Did the authors evaluate for the presence of features of underlying chronic pancreatitis?

Into the study were enrolled all the consecutive patients treated in one centre due to AP, who expressed their consent about participating in the genetic tests.

Table 2 contains the number of patients with recurrent AP (82 patients). In this group there were 5 SPINK1 mutations, 1 CFTR mutation, and 2 CTRC mutations. No significant differences in the presence of mutations were observed between patients with and without recurrent AP.

2. How was alcohol related AP defined, in terms of duration and amount of alcohol intake?

Alcohol consumption was evaluated with Short Alcohol Dependence Data Questionnaire (SADD) and self- estimated alcohol consumption via interview. Acute pancreatitis diagnosis was made by if a patient gained 10 or more points of SADD Questionnaire or if a period of alcohol abuse was at least one- year-long and the daily dose was 40g of pure ethanol.

3. How many patients had pancreas divisum and what was the relationship of the genetic mutations with pancreas divisum and other aetiologies?
In the examined material we did not encounter pancreas divisum; therefore, such a situation was not described.

4. Authors should also report the frequency of acute pancreatitis in the presence of each mutations that were studied. They should also mention if there was multiple mutations in patients and if any, what was the relationship with the development of acute pancreatitis and its severity.

In the manuscript it was previously stated: **In one patient with a moderately severe course of AP of alcohol etiology with recurrence of the disease, the presence of p.N34S mutation in SPINKI and p.V235I mutation in CTRC was observed.**

5. Headings of Tables 2, 3 and 4 appears to be incorporated in the main text. This needs to be removed.

The results repeated in tables have been omitted in the text.

6. Figures 1-7 may be omitted since they doesn't seem to contribute to the manuscript substantially.

Figures 1-7 have been deleted.

7. There are several language and grammatical errors all throughout the manuscript.

The language has been revised from the editorial aspect.