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KRAS mutation analysis by droplet digital PCR of duodenal juice from patients with MODY8 and other pancreatic diseases

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ABSTRACT

Background: Maturity-onset diabetes of the young type 8 (MODY8 or CEL-MODY) is an inherited pancreatic disease characterized by chronic inflammation of the pancreas and diabetes. It is not known whether MODY8 patients have increased risk for developing pancreatic cancer. We investigated *KRAS* mutation load in duodenal juice from MODY8 patients, comparing with other groups of pancreatic disease.

Methods: Droplet digital PCR (ddPCR) was used to detect *KRAS* codon 12/13/61 mutations in duodenal juice sampled from 11 MODY8 patients, nine healthy subjects and 100 patients clinically investigated due to suspected pancreatic disease.

Results: *KRAS* mutations were detected in 4/11 patients with MODY8 (36%), 1/9 healthy subjects (11%), 15/44 patients with chronic pancreatitis (CP, 34%), 3/5 patients with pancreatic ductal adenocarcinoma (PDAC, 60%), 3/20 patients with acute pancreatitis (15%), 0/13 patients with other pancreatic disorders and 2/18 patients with nonpancreatic gastrointestinal disease (11%). Of the 28 positive juice samples, 25 (89%) had low-abundance mutations in codons 12/13, with a variant allele frequency (VAF) less than 1%. *KRAS*-positive patients with MODY8 or CP had significantly lower VAFs than patients with PDAC (Mann-Whitney *U* test; $p = 0.041$). Although the overall mutation detection rate was higher for subjects ≥50 years old (26%) than for younger subjects (15%), the difference was not statistically significant.

Conclusions: *KRAS* mutations were detectable in duodenal juice from MODY8 patients, but with low abundance and at the same frequency as in CP patients. The discriminative value of the analysis with regard to other pancreatic disease was limited.

1. Introduction

Among monogenic diabetes forms, maturity-onset diabetes of the young, type 8 (MODY8) is unique as the primary defect arises in the exocrine tissue and not in the islets of Langerhans [1]. The disease is caused by mutations in the *CEL* gene which encodes carboxyl ester lipase, a digestive enzyme specifically produced by the pancreatic acinar cells [2,3]. The parenchyma of the MODY8 pancreas is characterized by lipomatosis, atrophy, fibrosis and cystic lesions [2,4,5], and the patients exhibit pancreatic exocrine insufficiency early in life with onset of diabetes in their 30ies or 40ies [2]. Thus, MODY8 may also be regarded as a form of hereditary chronic pancreatitis (CP) in which secondary diabetes frequently develops.

Patients with CP, especially inherited forms, have an increased risk for developing pancreatic cancer [6]. After the publication of the first report on MODY8 [2], one family member developed *KRAS*-positive pancreatic ductal adenocarcinoma (PDAC) at age 78 [5]. We

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therefore asked the question whether the long-standing pathogenic process of MODY8, which also includes cyst formation in the pancreas, predisposes for development of pancreatic malignancy later in life.

This problem might be addressed by searching for circulating tumor DNA (ctDNA) in pancreatic secretions from the patients. Sampling of pancreatic juice from the duodenum upon secretin stimulation (hereafter termed "duodenal juice") is clinically feasible and relatively safe, and can be integrated into other diagnostic procedures for patients with suspected pancreatic disease. Moreover, oncogenic *KRAS* mutations are early somatic events in the development of PDAC, and several studies have shown that *KRAS* mutations can be frequently detected in pancreatic secretions from PDAC patients [7–10]. The detection of somatic *KRAS* mutations in pancreatic secretions therefore represents a potential screening approach to identify the emerging cancer at a curable stage [11].

In this study, we used the highly sensitive droplet digital PCR technique (ddPCR) [12] to detect and quantify *KRAS* mutations in duodenal juice obtained from MODY8 patients. For comparison, we included juice samples collected as part of the diagnostic workup in various other patient groups. We found that *KRAS* mutations were detectable in some of the MODY8 duodenal juice samples, but not with a higher abundance than in CP patients. In addition, our study highlights challenges of using duodenal juice for early cancer detection.

2. Methods

2.1. Collection of duodenal juice samples

We analyzed duodenal juice samples obtained from 11 MODY8 patients, all being mutation carriers that belong to the largest Norwegian family described in Ræder et al. [2]. Duodenal juice was also collected from 100 other patients as part of their clinical workup at Haukeland University Hospital, Bergen, Norway between 2010 and 2012 (Table 1). *KRAS*-positive MODY8 patients and patients with high mutational load had their medical records checked in 2020 for development of pancreatic neoplastic disease. Moreover, duodenal juice was sampled from nine healthy controls for the purpose of this and other research projects. Secretion of duodenal juice was stimulated by administration of secretin, and the juice was collected endoscopically as described [13]. The juice samples were immediately aliquoted and stored in liquid nitrogen until further use. This study was performed in accordance with the Helsinki II Declaration and was approved by the Research Ethics Committee of Western Norway (REK Vest 2010/198). Written informed consent was obtained from all study participants.

2.2. DNA isolation and quantification

DNA was extracted from duodenal juice using the QIAamp DNA Investigator kit (Qiagen) according to the manufacturer's instructions, but with some minor modifications as follows: The juice sample was mixed with an equal volume of Buffer AL, with the proteinase K solution of the kit (20 mg/ml) added at one-tenth of the starting juice volume. This solution was incubated at 56 °C with shaking for 10 min. After addition of ethanol (50 μl per 100 μl of starting juice volume), the solution was incubated at room temperature for 3 min and then applied to the QIAamp MinElute column supplied by the kit. The column was centrifuged, and washed separately with Buffer AW1, Buffer AW2 and ethanol. When the column had dried, DNA was eluted in Buffer ATE and stored at −20 °C until use. DNA concentration was measured on the Qubit V 3.0 fluorometer using the Qubit dsDNA BR Assay kit (Thermo Fisher Scientific).

2.3. Protein quantification

The protein concentration of each duodenal juice was determined on the Qubit V 3.0 fluorometer using the Qubit Protein Assay kit (Thermo Fisher Scientific) according to the manufacturer's protocol.

2.4. ddPCR assay

KRAS mutations in duodenal juice DNA were detected using the ddPCR *KRAS* G12/G13 Screening kit (Bio-Rad, #1863506), which detects seven different nonsynonymous mutations in codons 12 and 13 (p.G12A, p.G12C, p.G12D, p.G12R, p.G12S, p.G12V and p.G13D) and the wildtype allele. For *KRAS* codon 61 mutations, specific ddPCR mutation/wildtype assays (Bio-Rad, #dHsaMDV2010131 and #dHsaMDV2010133) were used for detection of the two p.Q61H mutations c.183A>T and c.183A>C. All assays were run according to the manufacturer's instructions. In general, each PCR reaction was set up in a total volume of 22 μl with up to 20 ng input DNA, 11 μl 2x ddPCR Supermix for Probes (no dUTP), 1.1 μl 20x primers/probes (FAM + HEX) and nuclease-free water. Droplets were generated using the QX200 Droplet Generator (Bio-Rad), and the PCR reaction was then run immediately afterwards with the following program: 95 °C for 10 min; 40 cycles of 94 °C for 30 s and 55 °C for 1 min; ending with 98 °C for 10 min. A ramp rate of 2 °C/s was included in each PCR amplification step. Droplets were read using the QX200 Droplet Reader (Bio-Rad). For each run, a no-template control and assay-specific positive and negative controls were included.

All samples were run in duplicate or more to achieve ≥3000 wildtype *KRAS*-positive droplets. Sample handling was performed blinded to patient diagnoses, which were disclosed after all *KRAS* VAFs had been determined. Data were analyzed by the program QuantaSoft Analysis Pro 1.0.596 (Bio-Rad) and results were presented as % variant allele frequency (VAF). Samples with ≤12,000 droplets generated were excluded from further analysis. Samples generating a total of ≤2 mutation-positive droplets or having a VAF <0.1% were defined as having no detectable tumor DNA.

Table 1
Overview of the subject groups included in the study.

Subject groups	Number of subjects	Gender (Male/Female)	Mean age at sampling (years ± s.d.)
Maturity-onset diabetes of the young, type 8	11	6/5	50.0 ± 11.7
Chronic pancreatitis	44	26/18	53.9 ± 15.4
Pancreatic ductal adenocarcinoma	5	2/3	55.0 ± 9.1
Acute pancreatitis	20	8/12	54.6 ± 10.9
Other pancreatic disorders[a]	13	3/10	37.8 ± 15.3
Nonpancreatic gastrointestinal disease	18	12/6	59.6 ± 9.3
Healthy controls	9	5/4	48.3 ± 11.0

[a] This group included germline *CEL-HYB* mutation [25,26] without chronic pancreatitis, germline *PRSS1* mutation [27] without chronic pancreatitis, cystic fibrosis, and diabetes mellitus type 1.

2.5. Statistical analyses

Statistical analyses were conducted in R version 3.6.3 using Rstudio version 1.1.463 and GraphPad Prism 8.3.0. Pearson's correlation was used to assess the relationship between the total protein and DNA concentration of duodenal juice, and the concordance between the VAFs of *KRAS*-positive control pancreatic juice samples by ddPCR and deep sequencing from our previous study [10]. Pairwise Mann-Whitney *U* test was used to assess the differences in the VAF of *KRAS* codon 12/13 mutations in relation to disease type. Linear regression was used to assess the relationship between the total DNA concentration of nonmalignant duodenal juice samples positive for *KRAS* codon 12/13 mutations and the detected VAF. This method was also used to evaluate the relationship between patient age at sampling and VAF of *KRAS* codon 12/13 mutations in *KRAS*-positive subjects with nonmalignant disease. The likelihood of detection of *KRAS* mutations in subjects ≥50 years with nonmalignant disease was calculated using the Fisher's exact test. Except for the Mann-Whitney *U* test and the Pearson's correlation test, the PDAC cases were excluded from all analyses in order to avoid a potentially skewed result due to the likely enrichment of *KRAS* mutation in these cases.

3. Results

3.1. Protein and DNA concentrations of the duodenal juice samples

We analyzed duodenal juice specimens from 11 MODY8 patients (6 males, 5 females). Age ranged from 34 to 70 years with a mean value of 50.0 years. For comparison, juice samples were collected from 100 patients as part of their clinical evaluation for suspected pancreatic disease. These patients were diagnosed through a multimodal approach [13] and were subsequently classified into five diagnosis groups as listed in Table 1. At the time of juice sampling, all disease groups had a mean age between 50 and 60 years except for the group denoted 'other pancreatic disorders', in which the mean age was 37.8 years. In addition, duodenal juice was collected from nine healthy individuals (mean age 48.3 years) and included in the study as a control group.

We first determined the total protein and DNA concentration of the juice samples. The concentrations observed for the MODY8 subjects were in the same range as for the other patients (Fig. 1A and B). The mean total protein concentration varied from 1.4 to 2.0 μg/μl for all groups except for other pancreatic disorders where the concentration was 4.2 μg/μl. The mean concentration of DNA in the juice samples showed somewhat larger variation between groups, ranging from 1.0 to 7.2 ng/μl. Notably, no correlation was observed between the concentration of protein and DNA in the samples (Pearson's correlation; $r = 0.08$, $p = 0.38$; Fig. 1C).

3.2. Detection of KRAS codon 12/13 mutations

KRAS mutation status in codons 12/13 was determined using a multiplex ddPCR assay, which detects any of seven different mutations in a single reaction. For quality control, we first tested four pancreatic juice samples obtained from patients with PDAC during surgery. These samples had been analyzed and identified with *KRAS* codon 12 mutations using deep sequencing in a previous study from our group [10]. We found a high degree of concordance between the VAF determined by ddPCR and the value previously estimated by deep sequencing (Pearson's correlation; $r = 0.96$, $p = 0.04$; data not shown).

A *KRAS* codon 12/13 mutation was then identified in 4/11 (36%) of the MODY8 samples (Fig. 2A). All *KRAS*-positive MODY8 patients were diabetic and had pancreatic exocrine insufficiency. Three of them were older than 50 years. The VAFs ranged from 0.15% to 0.35%. No clinical signs of pancreatic cancer were reported in the *KRAS*-positive MODY8 patients on subsequent follow-up.

For the other patient groups, the observed frequencies of *KRAS* positivity were as follows: CP – 14/44 (32%), PDAC – 3/5 (60%), AP – 1/20 (5%), other pancreatic disorders – 0/13 (0%), NPGD – 2/18 (11%), healthy controls – 1/9 (11%) (Fig. 2A). Thus, looking collectively at the 120 subjects who had their duodenal juice analyzed by ddPCR, *KRAS* codon 12/13 mutations were detected in 25 samples (21%).

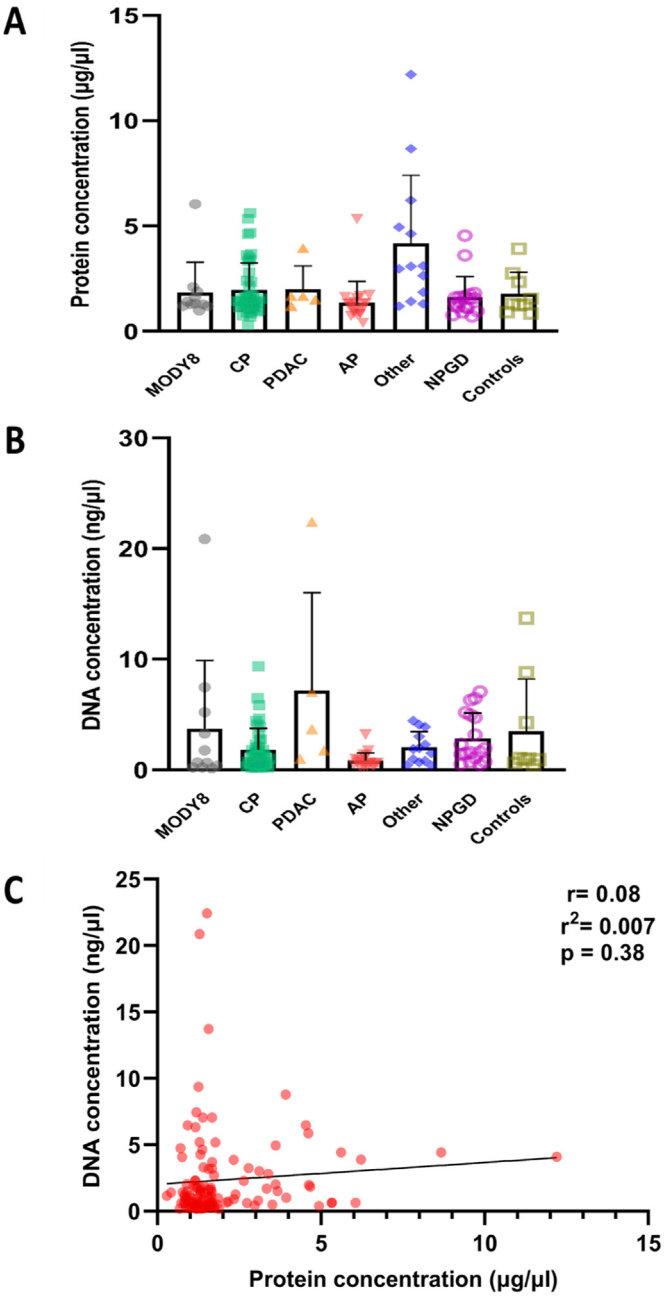

Fig. 1. Protein and DNA concentration of the 120 duodenal juice samples analyzed for *KRAS* mutations. (A) Total protein concentration and (B) total DNA concentration per μl duodenal juice across different subject groups. Error bars are standard deviation. (C) Pearson's correlation analysis between total protein and DNA concentration of the 120 samples. MODY8, maturity-onset diabetes of the young, type 8; CP, chronic pancreatitis; PDAC, pancreatic ductal adenocarcinoma; AP, acute pancreatitis; Other, other pancreatic disorders; NPGD, nonpancreatic gastrointestinal disease. Controls are healthy individuals.

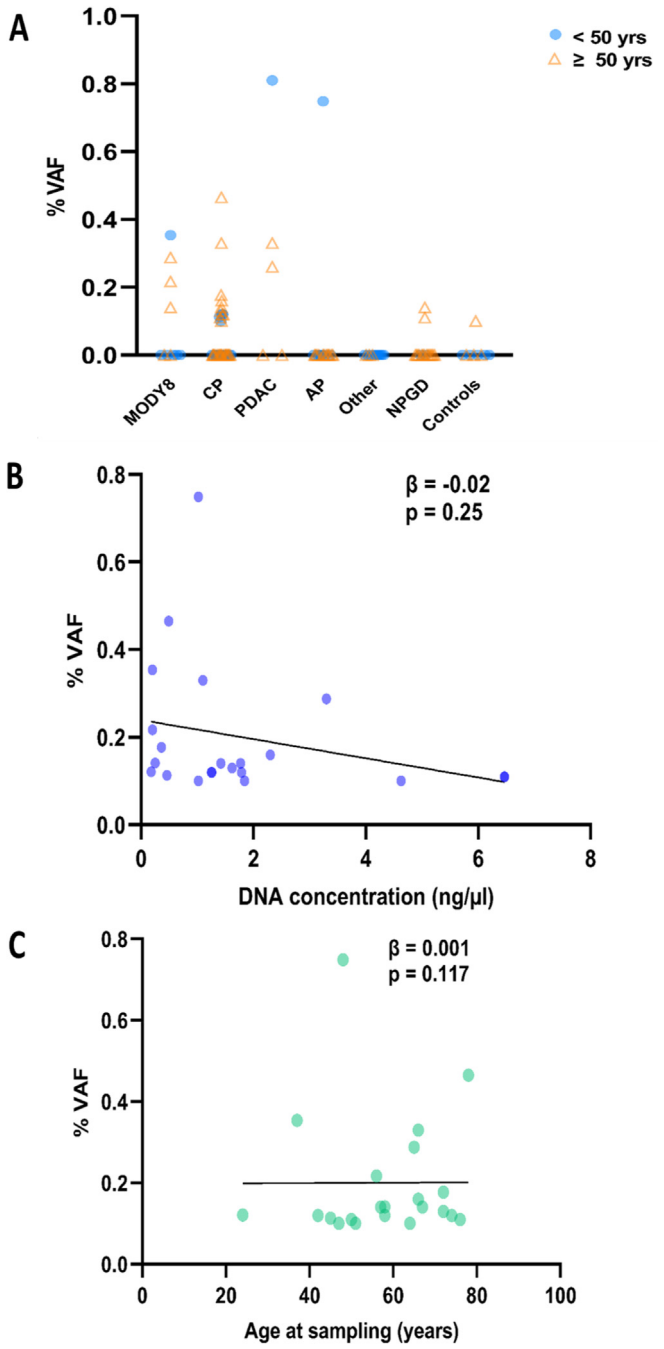

Fig. 2. Detection of *KRAS* codon 12/13 mutations in 120 duodenal juice samples. (A) Observed variant allele frequency (VAF) according to disease group and age at sampling. Disease group abbreviations are explained in Fig. 1. (B) Relationship between total DNA concentration of the duodenal juice samples and *KRAS* codon 12/13 VAF modeled by linear regression. (C) Relationship between the age at sampling and *KRAS* codons 12/13 VAF modeled by linear regression. Only nonmalignant *KRAS*-positive cases (n = 22) were included in (B) and (C).

These mutations were present at low VAFs that varied from 0.10% to 0.81%. Linear regression indicated a slightly negative relationship between the DNA concentration of the duodenal juice and the VAF of mutant *KRAS*, although this relationship was not statistically significant ($\beta = -0.02$, $p = 0.25$; Fig. 2B). When setting VAFs below 0.1% to zero and including all patients in the statistical analysis, patients with MODY8 or CP had a significantly lower VAF compared to patients with PDAC (0.06% vs 0.28%; Mann-Whitney *U* test; $p = 0.041$). Of note, the only positive case with AP was below 50 years of age and had a relatively high VAF of 0.75%. This patient had not developed PDAC on subsequent follow-up.

Finally, for the positive cases we analyzed by linear regression whether there was an association between higher VAF of *KRAS* codon 12/13 mutations and higher patient age at sampling (Fig. 2C). This was not the case ($\beta = 0.001$, $p = 0.117$).

3.3. Detection of KRAS codon 61 mutations

Although codon 12/13 alterations represent the by far most common *KRAS* point mutations in pancreatic cancer [14], also codon 61 can be affected. We therefore used two independent ddPCR assays to evaluate the nonsynonymous mutations c.183A>C and c.183A>T. These nucleotide substitutions lead to the same amino acid change in the KRAS protein (p.Q61H) and are the two most frequent mutations in *KRAS* codon 61 that are associated with PDAC [15].

We did not observe the c.183A>C mutation in any of the 120 analyzed specimens although the assay consistently detected this alteration in two pancreatic juice samples obtained from PDAC patients during surgery (Fig. 3A). These samples were known from our previous study to be positive for c.183A>C [10], thereby confirming the absence of c.183A>C mutations in the current duodenal juice material.

For the c.183A>T mutation, one case with MODY8 was positive with a relatively low VAF of 0.14%. This was, in fact, the only duodenal juice sample in our material that had a detectable mutation both in codons 12/13 (VAF = 0.35%) and in codon 61. The double-positive patient was below 50 years at sampling, and the clinical picture was similar to that of the other MODY8 patients.

Three additional juice samples were positive for c.183A>T: two stemming from AP patients and one from a CP patient (Fig. 3B). Two of the positive subjects were more than 50 years old at the time of sampling. Unlike the *KRAS* mutations in codons 12/13, the codon 61-positive cases had VAFs that were above 1% (AP cases: 1.6% and 27.0%; CP case: 21.9%). None of the three subjects with these high VAFs had developed PDAC at subsequent follow-up.

When the findings of codon 61 mutations were added to the data for codon 12/13, the *KRAS* mutation frequencies increased to 15% (3/20) for the AP group and to 34% (15/44) among CP patients. Taken together, a total of 23% (28/120) *KRAS*-positive cases were observed in our material, of which the dominating fraction (89%, 25 subjects) exhibited mutations in codons 12/13.

Excluding the five PDAC cases, we noted that a *KRAS* codon 12/13/61 mutation was detected in 26% (18/68) of the individuals with a sampling age ≥50 years and in 15% (7/47) of individuals with a sampling age <50 years. Although this observation could indicate that older individuals have a higher detection rate of *KRAS* mutations in duodenal juice, the difference was not statistically different (Fisher's exact test, two sided; odds ratio = 3.08, $p = 0.171$).

4. Discussion

In recent years, there has been a growing interest in the potential clinical utility of detecting ctDNA in pancreatic secretions for surveillance of subjects at high risk of developing PDAC [7–9]. Duodenal juice collected after secretin stimulation originates from the pancreatic ductal tree where most precancerous lesions of PDAC arise [16], and it contains DNA and other macromolecules that probably are released as a result of apoptosis and other cellular turnover processes [17].

Here we report the first mutation analysis of duodenal juice from subjects with the MODY8 syndrome [2]. As the disease progresses, the patients develop pancreatic lipomatosis, atrophy,

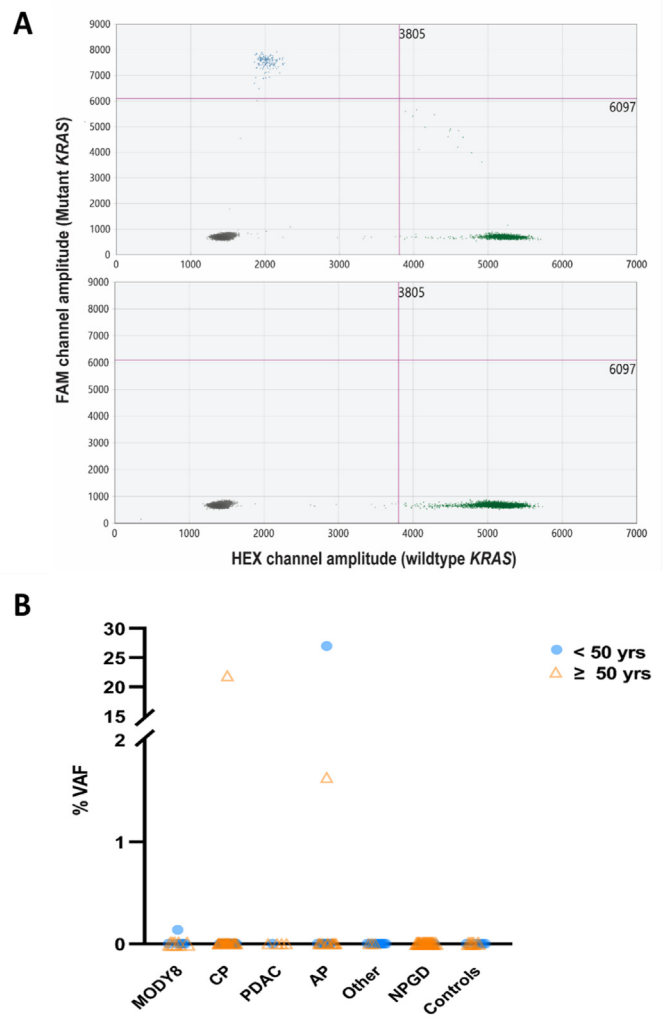

Fig. 3. Detection of the *KRAS* codon 61 mutation Q61H in the duodenal juice samples. (A) Fluorescence (HEX and FAM) signals of the droplets and examples of gating different droplet populations in the detection of wildtype *KRAS* and the p.Q61H (c.183A>C) mutation using QuantaSoft Analysis Pro (Bio-Rad). Upper panel shows the gating profile of a pancreatic juice sample from a PDAC patient as positive control, whereas the lower panel shows the result in a random duodenal juice sample. The bottom grey population represents droplets without DNA, the green population is droplets with wildtype *KRAS* and the blue population is droplets with mutant *KRAS*. (B) Detection of the *KRAS* p.Q61H (c.183A>T) mutation according to disease group and age at sampling. Disease group abbreviations are explained in Fig. 1. VAF = variant allele frequency.

fibrosis and multiple cysts [4,5]. We have previously reported an upregulation of target proteins and cytokines of mitogen-activated protein kinase (MAPK) signaling and an increase in kinase activity in duodenal juice sampled from subjects with MODY8 [5]. MAPK acts downstream of the RAS proteins. Moreover, one MODY8 subject developed PDAC at age 78, before the initiation of the present study. These observations might indicate a link between MODY8 and pancreatic cancer.

To evaluate a potential association between the two diseases, we analyzed duodenal juice samples from 11 MODY8 patients. We used the highly sensitive ddPCR technique to determine the abundance of oncogenic *KRAS* codon 12/13/61 mutations. For comparison, we took advantage of juice samples collected from 100 other patients clinically evaluated for suspected pancreatic disease, as well as from nine healthy controls. The data were analyzed in relation to disease type and patient age at sampling. Notably, the detection rate of *KRAS* mutations was nearly identical between subjects with MODY8 (36%) and CP (34%), and the observed VAFs in *KRAS*-positive cases were in the same range (Fig. 2A). Our mutation analysis therefore did not find evidence for MODY8 subjects having a greater risk of PDAC development than what is generally observed in CP. Although subjects with CP have increased PDAC risk, the effect is modest after long-term follow up, and the large majority of CP patients do not develop pancreatic cancer [6,18].

In persons without malignant disease, low-frequent *KRAS* mutations in duodenal juice may originate from and reflect the presence of benign, low-grade lesions (PanINs) in the pancreatic parenchyma. These lesions are commonly detected in healthy, aged individuals, with low potential to progress further into invasive cancer [19]. It has been reported that *KRAS* mutations are present at a significantly higher frequency in older than in younger subjects without pancreatic cancer [7]. In the latter study, among 194 asymptomatic persons with an inherited predisposition to PDAC, a substantially higher fraction of subjects positive for *KRAS* mutations (50%) was reported than what we observed in the present work. The majority of our samples were collected from cases with relatively low risk of PDAC and among the 115 subjects without malignancy, 22%, (n = 25) were *KRAS*-positive. We did not observe a positive relationship between VAF and sampling age (Fig. 2C), contrasting observations in [7] where screened subjects above 50 years of age had higher mutation concentrations than the younger subjects.

Notably, in three of our subjects with nonmalignant pancreatic disorders, unexpected high frequencies of *KRAS* mutation were identified. These were two cases with AP and a single case with CP, all positive for the codon 61 c.183A>T mutation and with no clinically detectable signs of PDAC on subsequent follow-up. Nevertheless, their duodenal juice samples harbored up to 100-fold more *KRAS*-mutated DNA than the average VAF (0.28%) observed in our patients who received a diagnosis of PDAC. We have no explanation for the high frequency of *KRAS* mutations observed in these three cases and can only speculate that inflammatory and/or necrotic processes somehow have damaged the DNA and made it particularly prone to the codon 61 mutation.

We have previously used deep sequencing to analyze surgically sampled pancreatic juice from the distal dilated pancreatic duct and the matched pancreatic head tumor of resectable PDAC patients [10]. When comparing with the current data, we note a striking difference between pancreatic and duodenal fluids with regard to the abundance of *KRAS* mutations. These alterations were generally present at much lower VAFs (<1%) in the duodenal juice than in the pancreatic juice sampled during surgery. This was even true for the patients with PDAC in the present cohort, and the difference was in the range of 10 to 100-fold (compare with Table 3 of ref. [10]). This discrepancy was unlikely to be caused by different analytical methods as samples from the previous study tested with ddPCR yielded comparable VAFs (data not shown). Instead, the low VAFs in the present study may result from mixing with DNA, both human and bacterial, originating from the duodenal lumen and diluting any *KRAS* mutation present [20]. In fact, there was a nonsignificant trend towards a negative association between VAF value and DNA concentration in the duodenal juice samples (Fig. 2B). A larger number of *KRAS*-positive samples will be needed to confirm whether the dilution effect of duodenal juice obtained after secretin stimulation is a major limiting factor for the detection of somatic mutations.

Another limitation could be the effect of the collection timing after secretin stimulation, as this may influence mutation detection in pancreatic fluids [21]. We used the total protein concentration in the duodenal juice as a surrogate measure to control for any differences in secretion potentially related to the collection timing. We

correlated the protein concentration with the DNA concentration in the juice but did not find statistical evidence supporting an association (Fig. 1C).

An important difference of our previous report [10] is that we here used a multiplex assay that detects and reports the sum of seven common *KRAS* codon 12/13 mutations. The assay therefore does not discriminate between individual mutations. It was chosen due to the limited availability of DNA from each sample which prevented the detection of each individual mutation in separate reactions. Our study is also limited by a low number of PDAC cases with duodenal juice samples available. Hence, we were not able to investigate any potential link between clinical variables and *KRAS* mutations in terms of detection and concentration.

5. Conclusions

We conclude that MODY8 patients do not seem to have a larger *KRAS* mutational load in their duodenal juice samples than patients suffering from chronic pancreatitis of other etiologies. Nevertheless, our analyses highlight the technical challenges of somatic mutation detection in duodenal juice: *KRAS* mutations can be found in subjects with various nonmalignant diseases or even in healthy individuals, although mostly at a low VAF [7–9,20].On the contrary, such alterations are undetectable in pancreatic secretions of around 20–40% of verified PDAC patients [7,8].

Thus, using *KRAS* mutational load as a single marker in pancreatic secretions is currently of very limited clinical utility. Sensitive methods for detecting multiple somatic mutations [9,22] in combination with protein markers [23] and epigenetic signatures [24] are probably necessary to potentiate duodenal juice as a clinically valuable specimen when testing for early pancreatic cancer.

Author contributions

MHC performed experiments, analyzed all data, wrote the manuscript and prepared the figures. ET, TE and HR recruited patients and controls, and evaluated the clinical data. RBF supervised the ddPCR experiments. AM conceived and directed the study with the help of RH. All authors contributed to the revision of the manuscript and approved the final version.

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