KRAS and *GNAS* in cell-free DNA and in circulating epithelial cells in patients with intraductal papillary mucinous neoplasms – an observational pilot study

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1 Presentation of the Publication

1.1 Introduction

Intraductal Papillary Mucinous Neoplasms (IPMN) are the most common pancreatic cystic lesions and significant precursors to invasive pancreatic ductal adenocarcinoma (PDAC). Accurate risk assessment and management of IPMN, including surgical resection or active surveillance, are essential to prevent progression to malignancy [1]. IPMN subtypes - main duct, branch duct, and mixed type - vary in malignancy risk, with main and mixed types considered to have the highest risk [2]. Identifying IPMN subtypes and worrisome imaging features such as cyst size or solid components are key to management decisions but can often lead to unnecessary surgeries due to inaccuracies [3, 4]. Emerging diagnostic approaches such as liquid biopsy offer potential, allowing for non-invasive monitoring of biomarkers like *KRAS* and *GNAS* mutations in plasma samples of IPMN patients [5-7]. Additionally, circulating epithelial cells (CEC) have been detected in IPMN plasma samples and their presence may provide valuable information regarding IPMN's malignant transformation [8-12].

The aim of this study was the assessment of the efficacy of *KRAS* and *GNAS* mutation-screening in cell-free DNA (cfDNA) using digital droplet polymerase chain reaction (ddPCR) and CEC detection, as biomarkers for risk stratification in patients with IPMN.

These biomarkers offer potential for guiding clinical decision-making in IPMN cases, aiming in finding a balance between preventing unnecessary surgeries and identifying those IPMNs with a heightened risk of malignant progression necessitating surgical intervention.

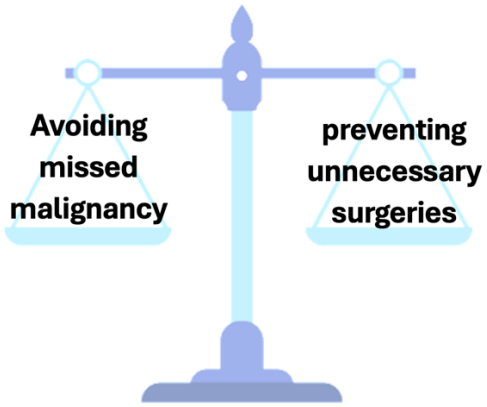

Figure 1: Balance between trying to avoid missing a malignant IPMN and preventing unnecessary surgeries

1.2 Methods

The study focused on 48 IPMN patients at the University Hospital Hamburg-Eppendorf (UKE) between November 2020 and June 2023. These patients were divided into two cohorts: a surgical cohort of 25 patients with main duct, mixed type, or branch duct IPMN with worrisome features; and a conservative cohort of 23 patients with branch duct IPMN and no worrisome features.

Blood samples from each patient were collected before treatment to isolate plasma for analyzing cfDNA and CECs. cfDNA was extracted using the QIAamp Circulating Nucleic Acid Kit, then quantified using a Qubit Fluorometer.
For detecting mutations in the *KRAS* and *GNAS* genes, ddPCR and specific assays targeting the mutations were applied.

CECs detection was done in the first 31 patients using the Parsortix^{TM} device, which enriches cells based on size and deformability. These cells underwent immunofluorescence staining to identify epithelial markers. The markers used were DAPI as a nuclear stain, pan-keratins C11 and AE 1/3 as epithelial markers, and CD45 as a leukocyte marker. However, after interim analysis and negative results, CEC detection was discontinued.

1.3 Results

In the study of IPMN patients, *KRAS* mutations were found in 10.4% and *GNAS* mutations in 18.8% of cases. Within the surgical cohort, 16.0% had *KRAS* mutations and 32.0% had *GNAS* mutations. In contrast, the conservative cohort had one patient each testing positive for *KRAS* and *GNAS*, the latter developing new worrisome features warranting surgical reconsideration.

A significant correlation was detected between *GNAS* positivity and surgery recommendation ($p=0.024$), as well as *KRAS* positivity and worrisome features in branch duct IPMN ($p=0.043$). *GNAS* status was linked to IPMN type ($p=0.031$). Despite their low sensitivity in identifying high-risk IPMN, *KRAS* and *GNAS* mutations showed high specificity (95.7%) in the surveillance cohort.

However, within our surgical cohort, only two out of 25 cases proved to be high grade dysplasian (HGD) IPMNs.

No CECs were detected in any of the 31 analyzed patients using the Parsortix™ system.

Table 1 *GNAS* results (summarized)

		patients n=48	***GNAS* negative n=39**	**%**	***GNAS* positive n=9**	**%**	**p-value**
Surgery	**yes**	25	17	68.0	8	32.0	**0.024**
	no	23	22	95.7	1	4.3	
Type of IPMN (patho)	Main/mixed branch	21	14	66.6	7	33.3	**0.031**
		27	25	92.6	2	7.4	
Grade of dysplasia (resected IPMN n=25)	low grade	23	16	69.5	7	30.4	n.s.
	high grade	2	1	50.0	1	50.0	
Worrisome features (only branch duct n=31)	yes	8	6	75.0	2	25.0	0.060
	no	23	23	100.0	0	0.0	

Table 2 KRAS results (summarized)

		patients n=48	***KRAS* negative n=43**	**%**	***KRAS* positive n=5**	**%**	**p-value**
Surgery	**Yes**	25	21	84.0	4	16.0	0.350
	no	23	22	95.7	1	4.3	
Type of IPMN (patho)	Main/mixed branch	21	18	85.7	3	14.3	0.641
		27	25	92.6	2	7.4	
Grade of dysplasia (resected IPMN n=25)	low grade	23	18	78.2	5	21.7	1.000
	high grade	2	2	100.0	0	0.0	
Worrisome features (only branch duct n=31)	yes	8	5	62.5	3	37.5	**0.043**
	no	23	22	95.7	1	4.3	

The following results are presented in addition to the results in the manuscript.

1.3.1 Results on the *KRAS* Primer Bio-Rad '*KRAS* G12/G13 Screening assay' (*KRAS* mutations G12A, G12C, G12D, G12R, G12S, G12V, G13D) and *GNAS* Primer Bio-Rad assay '*GNAS* p.R201C'

Prior to conducting testing on samples from patients with IPMNs using the *KRAS* primer, a preliminary assessment was undertaken to establish the primer's efficacy in accurately identifying positive *KRAS* samples while correctly classifying healthy samples as wildtype. Positive samples were acquired from cfDNA obtained from a PDAC tissue sample known to harbor *KRAS* mutations, specifically hotspot mutations G12D, G12R, and G12V.
Presenting in the PCR assessment run (Figure 2) a distinct bubble of droplets (blue) above the 10.000 mark indicated positive outcomes, while the wildtype remained negative (green).

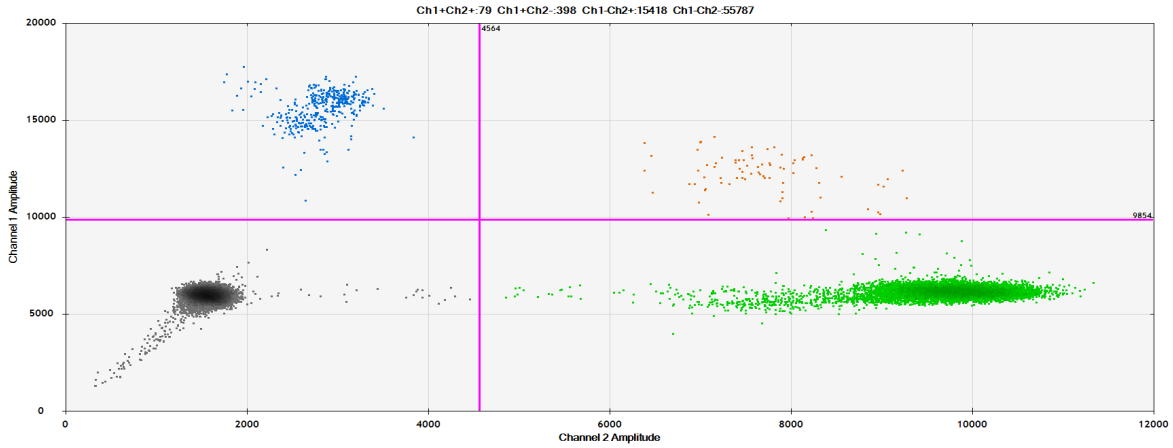

Figure 2: Establishment ddPCR-run of the *KRAS* primer: blue droplets indicate positive outcomes, green droplets indicate negative outcomes

A parallel preliminary evaluation was conducted to determine the effectiveness of the *GNAS* primer. Lacking a positive sample, we utilized cyst fluid from a diagnosed IPMN resection, which exhibited high positivity. Although numerous positive drops were observed, their count did not surpass 10.000. Subsequently, the threshold was adjusted to 6.000.

For both mutations a criterion was established wherein a sample would be count as positive only if two or more drops per patient exhibited mutation positivity. This adjustment, set at two drops, served to eliminate any potential accidental false positives with single drops.

1.3.2 Results cyst fluid samples IPMN patients

Furthermore, the study included five cyst fluid samples from IPMN patients. The cyst fluid was obtained during surgical resection. The isolation of cfDNA from these cyst fluid patient samples matched to the plasma isolation protocol outlined in the methods in the manuscript. The average concentration of cfDNA was determined to be 239.24 ng/ml. The notably higher concentration observed in comparison to the liquid biopsy samples (0.5 ng/ml) can be attributed to the proximity to the cyst and the direct sourcing from the cyst itself. For *GNAS*, a positivity rate of 60% was observed, while *KRAS* exhibited a 100% positivity rate.

Table 3: Results *GNAS* and *KRAS* outcomes of five IPMN cyst fluid samples

Pseudonym	**cfDNA**	**GNAS**	**KRAS**
49O	1024	pos	pos
53O	0.792	pos	pos
66O	73.6	neg	pos
82O	94.4	pos	pos
88O	3.42	neg	pos

1.4 Discussion

In addition to the discussion presented in the paper, I further explore more limitations of our study, the analysis of cyst fluid and tissue samples, discuss the existing clinical guidelines regarding surgical removal of IPMNs, and a more detailed exploration of our method for isolating and staining CECs.

In discussing the limitations of our study, it is necessary to address the challenges we faced with the *GNAS* primer. Primarily, one significant limitation was the absence of a proper positive control sample to validate and establish the *GNAS* primer effectively. Furthermore, we observed that the positive droplets for *GNAS* did not surpass 10,000 which is why we had to adjust the threshold to 6,000. To justify our choice, we refer to the results by Hata et al [13]. Their work with IPMN liquid biopsy samples and ddPCR demonstrated threshold settings ranging between 3,000 and 8,000 for *GNAS*. This suggests that our decision to apply a 6,000 threshold is within an acceptable range.

In our study, our findings revealed a 60% positivity rate for *GNAS* mutations and a 100% positivity rate for *KRAS* mutations in the cyst fluid of patients with IPMN. These patients all underwent surgical resection of their IPMNs. Other studies – as presented in the paper - have

reported a broad range of positivity rates for both mutations in cyst fluid from IPMN patients, with *KRAS* ranging from 26% to 82% and *GNAS* from 27% to 66% [14-19]. One possible explanation for the discrepancy in *KRAS* mutation detection is the high amount of DNA in the cystic fluid. In our cohort, the average concentration of cfDNA in cyst fluid was 239.24 ng/ml. This high cfDNA concentration may contribute to analytical challenges and potentially leading to false positive results.

An additional notable limitation of our study is the absence of an analysis of resected tissue samples in the surgical cohort. Such analyses could have provided insights into the detectability rate of *KRAS* and *GNAS* mutations in liquid biopsies when corresponding tissue is positive. This would provide with sensitivity and specificity numbers of liquid biopsy in IPMN meaning to assess how likely liquid biopsy detect the mutations when the corresponding tissue is positive. Further, it could then show any potential correlation between liquid biopsy positivity rates and advanced stages of IPMNs.

Considering the pathological outcomes of the resected IPMNs, it is important to evaluate the existing clinical recommendations for IPMN surgery. Out of the 25 IPMNs examined, only 2 exhibited HGD. Retrospectively, the necessity of surgical removal and invasive procedures for the remaining 23 low-risk cases is debatable. Though, existing literature on this subject reflects this low incidence of HGD within surgically treated IPMNs. Khoury et al. conducted an evaluation of 478 resected IPMNs, revealing HGD in only 23% of cases [20]. Similarly, smaller studies have reported similar rates. Litchinko et al. examined 18 cases of IPMN resection, with 68,4% demonstrating benign histopathology [21]. Izumo et al. analyzed 295 resected IPMNs, finding that 47% exhibited low grade dysplasia [22]. However, one study reported higher rates of HGD among resected IPMNs. Djordjevic et al. observed a HGD rate of 73.5% among 68 resected IPMN in their study [23].

Numerous guidelines address the surgical indications for pancreatic cysts and IPMNs, with the Kyoto Guidelines (an update of the Fukuoka Guidelines, released in 2024) being the most widely used. However, given our finding on histopathological outcomes of HGD in the surgical cohort and the data from the literature, there are doubts regarding the preoperative diagnostic accuracy of the IPMN guidelines in identifying HGD IPMNs.

Recent studies have also addressed this issue by evaluating the Fukuoka guidelines. Dbouk et al. reported a sensitivity of 40% and a specificity of 85% for the Fukuoka guidelines in resected IPMNs [24]. Vanden Bulcke et al. found a sensitivity of 66.8% and a specificity of 26.8% for the Fukuoka Guidelines in resected cyst (of which 51/72 were IPMNs in the study's cohort). They also stated an overtreatment of 48.4% according to the Fukuoka guidelines [25]. Djordjevic et al. concluded a sensitivity of 68% and a specificity of 55.8% for at least one high-risk stigmata according to the Fukuoka guidelines [23].

The Kyoto guidelines introduce some modifications to the Fukuoka guidelines. They maintain the classification of high-risk (HR) stigmata while incorporating additional factors such as the presence of an associated mass and suspicious or positive cytology derived from cyst fluid. The criteria for surgical referral or resection have also been adjusted. The Kyoto guidelines now suggest HR if surgical intervention is deemed appropriate and include any worrisome features (WF) as criteria for referral, differing from the Fukuoka guidelines, which specified that any WF must be followed by an endoscopic ultrasound (EUS) with typical features. Moreover, the Kyoto guidelines advocate for surgical consideration in cases of repeated acute pancreatitis, multiple WF, and among younger, fit patients [26].

While the Kyoto guidelines represent a step forward, their overall performance remains to be evaluated. Given that the modifications to the existing guidelines are relatively modest, we do not anticipate a significant change in sensitivity and specificity as a result of these updates.

Our study likewise must suggest that the presence of established clinical high-risk stigmata and worrisome features do not appear to effectively identify HGD.
Due to the insufficient diagnostic performance with the clinical guidelines on IPMN, there is an increasing demand for improved biomarkers to predict the risk of malignancy in IPMNs.
Our study aimed to address this need. However, it appears that only the specificity of *GNAS* mutations in liquid biopsy (95.7%) demonstrates a level of accuracy. Concluding that a *GNAS* negativity can be used to confirm the surveillance approach. Both *KRAS* nor *GNAS* mutations, as indicated by our findings, do not appear to be effective in identifying IPMNs with HGD.

Furthermore, our investigation did not detect any CECs in our samples, contrary to findings in other studies. We utilized Parsortix^{TM}, a size-based isolation system for potential CEC isolation.

Franses et al. utilized a system known as iChip (self-developed), which employs magnetic-based cell sorting and microfluidics for rare cell manipulation. The study utilized cytokeratin and EpCam as positive markers for staining [8]. Poruk et al. conducted CEC filtration based on size isolation and stained the cells with cytokeratin and Pdx1 [10]. Kuvendjiska et al. employed the ScreenCell system for CEC isolation, which employs microfilters to capture cells on small metal-rimmed filters via low-pressure vacuum. Subsequently, they stained the cells with EpCam, L1Cam, Vimentin, and PDX1 [9].

Two of the above-mentioned studies also employed size-based isolation methods. Hence, the absence of detected CECs in our study cannot be attributed to the Parsortix™ system. Rather, it is likely that our pan-keratin antibody proved ineffective in staining the CECs. While all studies utilized a cytokeratin antibody, it's worth noting that they were sourced from different manufacturers.

In overall conclusion of our study, clinical guidelines lack the ability to effectively distinguish IPMNs with high grade dysplasia. Testing new IPMN biomarkers to predict malignancy risk, the absence of *GNAS* mutations in liquid biopsy samples shows promise in reliably identifying IPMNs with low grade dysplasia. Neither *KRAS* nor *GNAS* mutations, nor CECs, prove reliably for identifying IPMN with high grade dysplasia.
Further research into new biomarkers should be conducted for accurately identifying HGD in IPMNs, thereby improving patient care in IPMN treatment.

Molecular Oncology

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KRAS and *GNAS* mutations in cell-free DNA and in circulating epithelial cells in patients with intraductal papillary mucinous neoplasms—an observational pilot study

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Intraductal papillary mucinous neoplasms (IPMNs) are potential precursor lesions of pancreatic cancer. We assessed the efficacy of screening for KRAS proto-oncogene, GTPase (*KRAS*), and GNAS complex locus (*GNAS*) mutations in cell-free DNA (cfDNA)—using digital droplet polymerase chain reaction (ddPCR) and circulating epithelial cell (CEC) detection—as biomarkers for risk stratification in IPMN patients. We prospectively collected plasma samples from 25 resected patients at risk of malignant progression, and 23 under clinical surveillance. Our findings revealed *KRAS* mutations in 10.4% and *GNA*S mutations in 18.8% of the overall cohort. Among resected IPMN patients, *KRAS* and *GNAS* mutation detection rates were 16.0% and 32.0%, respectively, whereas both rates were 4.0% in conservatively managed IPMN. *GNAS* mutations in cfDNA were significantly more prevalent in resected IPMN ($P = 0.024$) compared with IPMN under surveillance. No CECs were detected. The absence of *KRAS* and *GNAS* mutations could be a reliable marker for branch duct IPMN without worrisome features. The emergence of *GNAS* mutations could prompt enhanced imaging surveillance. Neither the presence of established worrisome features nor *GNAS* or *KRAS* mutations appear effective in identifying high-grade dysplasia among IPMN patients.

1. Introduction

Intraductal Papillary Mucinous Neoplasms (IPMN) of the pancreas are the most commonly encountered cystic lesions in clinical practice. They have been recognised as significant precursor lesions for invasive

Abbreviations
AB, antibody; CEC, circulating epithelial cell; cfDNA, cell-free DNA; CTC, circulating tumour cell; ctDNA, circulating tumour DNA; ddPCR, digital droplet polymerase chain reaction; IPMN, intraductal papillary mucinous neoplasm; MAF, mutant allele frequency; MRCP, magnetic resonance cholangiopancreatography; PDAC, pancreatic ductal adenocarcinoma; PFA, paraformaldehyde; SPSS, Statistical Package for the Social Sciences.

pancreatic ductal adenocarcinoma (PDAC). This connection underscores the critical need for precise risk assessment and timely intervention in managing IPMN patients to mitigate the progression to more severe pancreatic conditions effectively [1].

IPMN can be classified into three primary subtypes based on the involvement of the pancreatic duct system: main duct, branch duct and mixed type. Notably, the main duct and mixed type IPMN, which affect both the main and branch ducts, present the highest risk of progression to malignancy, with rates up to 70.0% [2]. Accurately distinguishing IPMN from other pancreatic cystic lesions and assessing their malignancy risk are critical steps in determining each patient's most appropriate management strategy. This strategy typically involves surgical resection for patients in the higher-risk category or active surveillance for those with IPMN at a lower risk of malignant transformation.

Patients diagnosed with IPMN require close monitoring or surgical resection, depending on the IPMN subtype and other risk factors [3]. The medical imaging methods of choice for characterising the lesion and guiding the decision for surgical intervention are magnetic resonance cholangiopancreatography (MRCP) and endoscopic ultrasound. These techniques are instrumental in identifying IPMN subtypes and evaluating worrisome features, thereby determining which patients are most likely to benefit from surgical resection [4]. According to current guidelines, worrisome features are, for instance, the size of the cyst, the presence of solid components within the lesion, and the degree of dilatation of the pancreatic duct [3]. These features are particularly crucial in managing branch duct IPMN, as they correlate with an increased risk of malignant progression. However, reliance on imaging and definition of main duct/branch duct classification and worrisome features alone for decision-making regarding surgical intervention may lead to inaccuracies and rushed decisions, resulting in a considerable number of unnecessary surgeries. This is a substantial concern, given the intrinsic risks and complications associated with pancreatic surgery [5–7].

There is a growing need for more precise and reliable diagnostic methods to address this issue to inform surgical decision-making for IPMN. In recent years, liquid biopsy has emerged as a non-invasive diagnostic method that has gained significant importance in cancer research [8]. This technique involves the analysis of disease-related markers in various biofluids, such as blood or plasma, to obtain valuable information about the biology of the disease. Liquid biopsy holds great potential for contributing to personalised therapy by providing real-time and minimally invasive monitoring of cancer progression [9].

The subject of many current studies is the detection of circulating tumour cells (CTCs) and circulating tumour DNA (ctDNA) through liquid biopsy in manifest PDAC [10]. One molecular alteration that plays a pivotal role in the progression of PDAC and its precursor lesions is the presence of *KRAS* mutation. *KRAS* is a G-protein that regulates cell growth and differentiation. Mutations in the *KRAS* gene result in losing control over the protein, leading to unregulated cell proliferation, found in > 80.0% of PDAC patients [11]. Monitoring ctDNA with *KRAS* mutations has emerged as an independent prognostic marker with adverse implications for early and advanced PDAC cases [10]. In addition, mutations in the *GNAS* gene play a crucial role in IPMN development, affecting the cGMP-mediated receptor signalling pathways [12].

Our study specifically targeted hotspot mutations present in *KRAS* (G12A, G12C, G12D, G12R, G12S, G12V and G13D) and *GNAS* (R201C) genes, which allow for highly sensitive and quantitative detection methods in IPMN patients [10,13]. We utilised digital droplet polymerase chain reaction (ddPCR) to detect these specific mutations in cell-free DNA (cfDNA) and concurrently screened for the presence of circulating epithelial cells (CECs) in patients either under surveillance or scheduled for surgery [14]. The primary objective was to evaluate the significance of cfDNA-based analyses for assessing malignancy risk—particularly through the detection of *KRAS* and *GNAS* mutations—and to explore the role of CECs in enhancing clinical decision-making strategies for IPMN treatment.

2. Materials and methods

2.1. Patient cohort

Our explorative study cohort prospectively included patients diagnosed with IPMN between November 2020 and June 2023 at the University Hospital Hamburg-Eppendorf. During this period, $n = 48$ adult IPMN patients were enrolled in the study. Among them, $n = 25$ patients presented with main duct IPMN, mixed type or branch duct IPMN with worrisome features, and underwent surgery—representing the surgical cohort. The remaining $n = 23$ patients with branch duct IPMN and no worrisome features were closely conservatively monitored through our outpatient clinic—representing the conservative cohort. Clinicopathological data were collected from all patients. Additional data were provided from the prospectively surgical database for patients with pancreatic

resections, which was in concordance with the General Data Protection Regulation guidelines. Clinical characteristics (e.g., age, gender, risk classification), preoperative diagnostics (e.g., imaging, tumour marker), operative details (e.g., type of resection performed), and follow-up data were extracted from the database. The Ethics Commission of Hamburg approved collecting data and patient material for this study (PV3548). The study methodologies conformed to the standards set by the Declaration of Helsinki. The experiments were undertaken with the understanding and written consent of each subject.

2.2. Sample collection

Blood samples were collected from each patient in the study before initiating any treatment interventions. For each patient, peripheral blood samples were taken using four 7.5-mL EDTA tubes to obtain plasma samples for the cfDNA analysis and for CEC detection.

2.3. cfDNA isolation from plasma

Plasma samples were isolated from whole blood through standard two-step centrifugation (10 min 300 ***g*** and 10 min 1800 ***g***) and were stored at −80.0 °C. The isolation of cfDNA from plasma was performed using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) following the corresponding protocol [10]. The concentration of cfDNA was quantified using the Qubit 4 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and was stored at −20.0 °C.

2.4. ddPCR for *KRAS* and *GNAS* mutations

DdPCR was employed to detect the hotspot mutations in the *KRAS* and *GNAS* genes. The ddPCR analysis was conducted using the Bio-Rad ddPCR system and the manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA, USA) [15]. Specific primers (assays) and probes (qPCR Supermix) targeting the *KRAS* and *GNAS* hotspot mutations were utilised in the ddPCR reactions. For *KRAS*, the Bio-Rad '*KRAS* G12/G13 Screening assay' (*KRAS* mutations G12A, G12C, G12D, G12R, G12S, G12V, G13D) and for *GNAS* the Bio-Rad assay '*GNAS* p.R201C' were applied. Due to the limited amount of cfDNA in IPMN patients, our study focusses on the most prevalent *KRAS* mutations, such as G12D and G12V—and *GNAS* R201C [16,17]. Each sample was tested in duplicates except for $n = 10$ samples due to insufficient cfDNA concentration (one cell tested). Each run included a nontemplate control, a wild-type control, and a positive control (mutation known). To evaluate the positive mutant droplets, the PCR plates were read by the QX100 Droplet Reader using Quantasoft® software version 1.7.4 (Bio-Rad Laboratories, USA). For *KRAS*, a sample was considered positive if two or more droplets were positive (cut-off 10 000); for *GNAS*, a sample was considered positive if two or more droplets were positive (cut-off 6000). As described before, the absolute number of copies per mL of plasma was calculated [10].

2.5. CEC detection from EDTA blood

CECs in the blood samples for the first analysed $n = 31$ patients were detected through the marker-independent microfluid-based Parsortix™ cell separation system. Previous studies have shown that the Parsortix™ device provides size and deformability-based enrichment by capturing cells sized > 6.5 µm [18]. Data on detecting CTCs and Cancer-associated Macrophage-like cells in blood samples from patients with PDAC through Parsortix™ have been recently reported [19].

All harvested cells were analysed via immunofluorescence staining for the nuclear staining DAPI, pan-keratins as an epithelial marker for positive selection, and CD45 for negative enrichment. The enriched cell fraction was first fixed with 4.0% paraformaldehyde (PFA, Sigma, Ronkonkoma, NY, USA) for 10 min at room temperature for the immunofluorescence staining. Then, it was permeabilised with 0.2% Triton X-100 (Sigma Aldrich, St. Louis, MO, USA) for 10 min, blocked with 10.0% AB-Serum (Bio-Rad, Contra Costa County, CA, USA), and incubated with DAPI (1 : 500), conjugated pan-keratins C11 (1 : 80, AlexaFluor546 Cell Signalling, Danvers, MA, USA) and AE1/3 (1 : 80), Anti-Pan-CytokeratinAlexa Fluor 546 Clone (Invitrogen, Waltham, MA, USA), and allophycocyanin (APC) conjugated CD45 antibodies (1 : 150, Alexa Fluor 647 anti-human CD45 Clone H130 BioLegend, SanDiego, MA, USA) for 60 min. The consecutive analysis was performed using immunofluorescence microscopy. A CEC was enumerated as positive using the definitions $DAPI^+$, $Keratin^+$, and $CD45^-$.

After $n = 31$ patients, an interim analysis was performed, and due to negative findings, the CEC detection was terminated.

2.6. Statistical data analysis

The statistical analyses were performed using SPSS version 29 (SPSS Inc., Chicago, IL, USA). For the evaluation of a potential association between the *GNAS*

and *KRAS* mutation status and clinicopathological parameters (including current risk classification), the chi-squared/Fisher's exact test was used. Significant statements refer to *P*-values of two-tailed tests that were < 0.05. The sensitivity of mutations in detecting a surgical resected IPMN was determined by dividing the number of positive cases in the surgical cohort by the total number of cases in the surgical cohort. Similarly, the specificity in detecting low-risk IPMNs in the surveillance cohort as wildtype mutations (negative) was calculated by dividing the number of negative cases in the surveillance cohort by the total number of cases in the surveillance cohort.

3. Results

The study cohort included 48 patients (25 resected IPMN patients and 23 conservatively managed IPMN patients). We analysed KRAS and GNAS mutations in their plasma samples using ddPCR (cfDNA) and quantified total cfDNA concentrations ($ng{\cdot}mL^{-1}$) in these plasma samples. We also examined the existence of CECs in the plasma samples of 31 IPMN patients.

3.1. *KRAS* and *GNAS* mutation status

Among all the patients, 10.4% ($n = 5$) were tested positive for *KRAS* mutations, 18.8% ($n = 9$) for *GNAS* mutations, and 25.0% ($n = 12$) for either *KRAS* and/or *GNAS* mutations in cfDNA (Fig. 1). No tissue samples were analysed.

In the surgical cohort ($n = 25$), 16.0% ($n = 4$) of patients tested positive for *KRAS* mutations, 32.0% ($n = 8$) for *GNAS* mutations, and 40.0% ($n = 10$) for either *KRAS* and/or *GNAS* mutations. In the conservative cohort ($n = 23$), for both *GNAS* and *KRAS* mutations we had each one positive patient (4.3%) (Fig. 1). The *GNAS*-positive patient's clinical diagnostics in the conservative cohort revealed a new onset of worrisome features during surveillance, necessitating reconsideration of surgery soon after the blood draw. In contrast, the *KRAS*-positive patient had an additional neurinoma of the appendix.

3.2. Correlation with clinicopathological data

Our findings revealed a significant correlation between surgery recommendation and *GNAS* positivity ($P = 0.024$). A significant correlation was found between *KRAS* and worrisome features in branch duct IPMN ($P = 0.043$). In patients without worrisome features, 22 out of 23 patients were *KRAS* negative. The same results were evident for *GNAS*-negative patients.

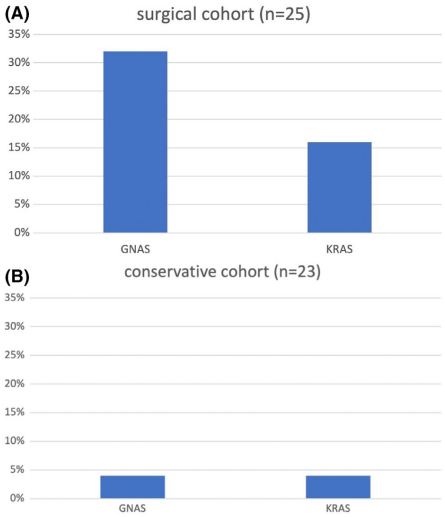

Fig. 1. Positivity rate of *KRAS* and *GNAS* mutations in plasma. (A) Positive rates of *KRAS* and *GNAS* mutations in the surgical cohort. (B) Positive rates of *KRAS* and *GNAS* mutations in the conservative cohort.

All 23 patients without worrisome features were *GNAS* negative. The *GNAS* status was additionally associated with the type of IPMN ($P = 0.031$). No further correlations with other clinicopathological parameters were evident (Tables 1 and 2). Notably, the grade of dysplasia did not show a significant correlation with *KRAS* and *GNAS* detection in our sample size of $n = 25$ resected patients.

The sensitivity of *GNAS* and *KRAS* mutations for identifying IPMN with a higher risk of malignancy (surgical cohort) is relatively low, at 32.0% and 16.0%, respectively. However, the specificity of a negative result for these mutations in cases recommended for surveillance (surveillance cohort) is notably high, at 95.7% in both instances.

3.3. Mutant copies per mL plasma *KRAS* and *GNAS* mutations

For all *GNAS* positive samples, the mean mutant copy number was 4.3 mL^{-1} plasma, and for all *KRAS* positive patients, it was 7.3 mL^{-1} plasma. In the surgical cohort, the mean mutant copy number for *GNAS*-positive samples was 4.5 mL^{-1} plasma and for *KRAS* 8.2 mL^{-1} plasma, – while in the conservative cohort,

Table 1. *KRAS* mutant status and clinicopathological data. Ca 19-9, Carbohydrate Antigen 19-9; CEA, Carcinoembryonic Antigen. Significant *P*-values < 0.05 are bolded; * for $n = 8$ patients no CA 19-9 and CEA values were available.

	Patients, $n = 48$	*KRAS* negative, $n = 43$	%	*KRAS* positive, $n = 5$	%	*P*-value
Age						
≤ 67 years	16	15	93.7	1	6.3	0.652
> 67 years	32	28	87.5	4	12.5	
Gender						
Male	19	16	84.2	3	15.8	0.372
Female	29	27	93.1	2	6.9	
Type of IPMN (patho)						
Main/mixed branch	21	18	85.7	3	14.3	0.641
	27	25	92.6	2	7.4	
Grade of dysplasia (resected IPMN $n = 25$)						
Low grade	23	18	78.3	5	21.7	1.000
High grade	2	2	100.0	0	0.0	
Worrisome features (only branch duct $n = 31$)						
Yes	8	5	62.5	3	37.5	**0.043**
No	23	22	95.7	1	4.3	
Surgery						
Yes	25	21	84.0	4	16.0	0.350
No	23	22	95.7	1	4.3	
CA19-9, $n = 40$*						
< 37 U·mL^{-1}	35	30	85.7	5	14.3	0.355
≥ 37 U·mL^{-1}	5	5	100.0	0	0.0	
CEA, $n = 40$*						
< 2 mg·L^{-1}	28	25	89.3	3	10.7	0.488
> 2 mg·L^{-1}	12	10	83.3	2	16.7	

the mean mutant copy number for *GNAS* was 2.7 mL^{-1} plasma and for *KRAS* 3.7 mL^{-1} plasma (Fig. 2). The average mutant copies per mL plasma were higher in the surgical cohort throughout both mutations (Fig. 2).

The Mann–Whitney-*U*-Test was applied to compute a possible correlation between surgery recommendation and the number of *GNAS* or *KRAS* mutant copies per ml plasma. The correlation between surgery recommendation and *GNAS* mutant copies per mL plasma was significant ($P = 0.011$).

3.4. cfDNA concentration in IPMN patients

The mean cfDNA concentration among all patients was 0.5 ng·mL^{-1} plasma. Within the surgical cohort, the mean cfDNA concentration was 0.8 ng·mL^{-1} plasma; in the conservative cohort, it was 0.3 ng·mL^{-1} plasma. We observed a significant correlation ($P = 0.003$) between surgery recommendation and cfDNA concentration. The cfDNA concentration was, on average, 2.2-fold higher in the surgical cohort.

3.5. CECs status

We used the marker-independent microfluid-based Parsortix™ cell separation system for CEC detection. No CECs were detected in the analysed cohort of $n = 31$ IPMN patients ($n = 14$ resected and $n = 17$ conservatively managed).

4. Discussion

Our study analysed liquid biopsy samples from IPMN patients for *KRAS* and *GNAS* mutations and CECs. Our data show that *GNAS* and *KRAS* mutations (18.8% and 10.4%) can already be found in the cfDNA of IPMN patients in general, concluding that IPMNs, as preneoplastic disease, can shed mutated *KRAS* and *GNAS* cfDNA into the bloodstream.

We could furthermore show that both the *GNAS* positivity rate and mutational load (mutant copies·mL^{-1}) were statistically significantly higher in the surgical cohort compared to the surveillance cohort (32% vs. 4% ($P = 0.024$) and 4.5 vs. 2.7 mut copy·mL^{-1} ($P = 0.011$) respectively). For *KRAS*, only the mutation load was higher in the surgical cohort (8.2 vs. 3.7 mut copy·mL^{-1}).

In our study, a further interesting observation was the 2.2-fold increase in total mutant cfDNA concentrations within the surgical cohort ($P = 0.003$), underscoring the potential significance of cfDNA-based analysis in evaluating malignancy risk. However, it is

Table 2. *GNAS* mutant status and clinicopathological data. Ca 19-9, Carbohydrate Antigen 19-9; CEA, Carcinoembryonic Antigen. Significant *P*-values < 0.05 are bolded; * for $n = 8$ patients no CA 19-9 and CEA values were available.

	Patients, $n = 48$	*GNAS* negative, $n = 39$	%	*GNAS* positive, $n = 9$	%	*P*-value
Age						
≤ 67 years	16	14	87.5	2	12.5	0.697
> 67 years	32	25	78.1	7	21.9	
Gender						
Male	19	13	68.4	6	31.6	0.127
Female	29	26	89.7	3	10.3	
Type of IPMN (patho)						
Main/mixed branch	21	14	66.7	7	33.3	**0.031**
	27	25	92.6	2	7.4	
Grade of dysplasia (resected IPMN $n = 25$)						
Low grade	23	16	69.6	7	30.4	1.000
High grade	2	1	50.0	1	50.0	
Worrisome features (only branch duct $n = 31$)						
Yes	8	6	75.0	2	25.0	0.060
No	23	23	100.0	0	0.0	
Surgery						
Yes	25	17	68.0	8	32.0	**0.024**
No	23	22	95.7	1	4.3	
CA19-9, $n = 40$*						
< 37 U·mL^{-1}	35	28	80.0	7	20.0	0.498
≥ 37 U·mL^{-1}	5	5	100.0	0	0.0	
CEA, $n = 40$*						
< 2 mg·L^{-1}	28	23	82.1	5	17.9	0.881
> 2 mg·L^{-1}	12	10	83.3	2	16.7	

noteworthy that most resected IPMN (23 out of 25) were pathologically classified as low-grade dysplasia. In the subset of patients with high-grade dysplasia ($n = 2$), one exhibited a *GNAS* mutation, while neither showed *KRAS* mutations, limiting the scope for definitive clinical correlations or conclusions. Due to our limited sample size, establishing a significant correlation between *KRAS* or *GNAS* mutations and the grade of dysplasia in resected patients was not feasible. Nonetheless, this preliminary observation suggests that a more extensive study could reveal more definitive patterns. A further limitation of our study is the exclusive analysis of the *GNAS* hotspot mutation R201C, caused by the limited amount of cfDNA found in the IPMN patients. It is important to note that the positivity rate and discrimination power could have potentially been even higher than reported when both *GNAS* R201C and R201H mutations were analysed [20].

Our results align with other mainly smaller liquid biopsy studies on IPMN. Berger et al. [21] had a sample size of $n = 21$ and reported positivity rates for *KRAS* of 0% and *GNAS* of 71% in samples from IPMN patients under surveillance. In contrast, in their resected cohort of previously stored serum samples ($n = 16$), the positive rates were 0% for *KRAS* and 25% for *GNAS*. Hata et al. [22] had a sample size of $n = 34$ and reported positivity rates for *KRAS* of 6% and *GNAS* of 32% in samples from resected IPMN patients. Furthermore, Okada et al. [23] analysed a larger IPMN follow-up cohort of $n = 112$ patients regarding cfDNA quantification and mutant allele frequency (MAF)—concluding that the MAF of *KRAS* in IPMN patients was significantly increased compared with healthy controls. At the same time, the MAF of *GNAS* did not show significant changes. Finally, Park et al. [24] did detect neither *KRAS* nor *GNAS* mutations in their cohort of $n = 15$ resected IPMN patients, possibly due to the limited amount of input plasma (300–500 μL). None of the studies compared prospectively *KRAS* and *GNAS* mutations in liquid biopsy samples between two IPMN patient cohorts with different clinically IPMN risk stratifications (surgical resected vs. under surveillance) (Table S1).

Reading tissue status, the current literature reports a wide range of positivity rates for *KRAS* and *GNAS* mutations, with *KRAS* mutations detected in 31% to 88% of cases and *GNAS* mutations in 41% to 79% of resected IPMN patients [13,24–29]. Similarly, cyst fluid analysis in IPMN patients shows variable positivity

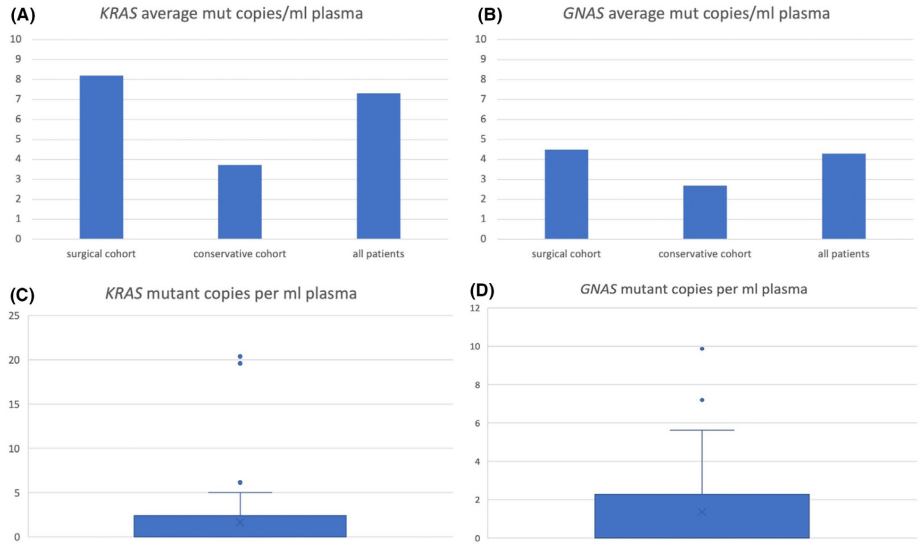

Fig. 2. *KRAS* and *GNAS* mutant copies per mL plasma. (A) *KRAS* average mutant copies per mL plasma for *KRAS* positive patients, in the surgical and conservative cohorts and the average of all *KRAS* positive patients. (B) *GNAS* mutant copies per mL plasma for *GNAS* positive patients, in the surgical and conservative cohorts and the average of all *GNAS* positive patients. (C) *KRAS* mutant copies per mL plasma for all patients; the blue box contains the minimum to 75th percentiles, the whisker marks the 95th percentile, and the upper values are the outliers of the dataset; x represents the mean. (D) *GNAS* mutant copies per mL plasma for all patients; the blue box contains the minimum to 75th percentiles, the whisker marks the 95th percentile, and the upper values are the outliers of the dataset; x represents the mean.

rates, ranging from 26% to 82% for *KRAS* and 27% to 66% for *GNAS* mutations [17,30–34]. In the pancreatic juice of patients with IPMN, current literature indicates a positive detection rate ranging from 39% to 62% for *KRAS* and 31% to 65% for *GNAS* mutations [29,35–38]. Thus, not surprisingly, *KRAS* and *GNAS* mutations in both high- and low-risk IPMN patients are less frequently detected in liquid biopsy than in other analytes. Nevertheless, our study highlights that even in its preneoplastic stage, IPMN can lead to the presence of *KRAS* and *GNAS* mutations in the bloodstream of patients. Blood analytes are more accessible and are also a frequent monitoring tool.

Regarding translational significance, our study indicates that detecting *GNAS* mutations in liquid biopsy may correlate with clinical imaging-based recommendations for assessing the malignancy risk in IPMN patients. Given the relatively low sensitivity but high specificity of *KRAS* and *GNAS* detection in cfDNA, our primary conclusion is that the absence of these mutations supports a conservative surveillance approach, particularly for branch duct IPMN lacking worrisome features. This suggests that *KRAS* and *GNAS* mutations in liquid biopsy samples from IPMN patients could primarily serve to reinforce the decision for conservative surveillance in cases of branch duct IPMN without worrisome features—which underlines the study's clinical relevance, providing a more transparent basis for risk stratification and management decisions. Within our study cohort, *GNAS* and *KRAS* mutations in cfDNA were each detected in only one case in patients without worrisome features. Notably, the one *GNAS*-positive patient, initially diagnosed with a branch duct IPMN without worrisome features, had worrisome features identified in subsequent surveillance imaging, leading to a surgical recommendation. Moreover, a shift to *GNAS* positivity could potentially prompt more intensive imaging surveillance. However, this hypothesis warrants further validation in larger study cohorts to solidify its clinical applicability.

In our study, unlike the cfDNA findings, we did not observe any CECs in our IPMN cohort. This contrasts with other studies that reported CEC detection rates in IPMN samples/pancreatic cystic lesions ranging

from 33% to 88% [39–43]. The previous research has utilised various microfluidic- and size-based cell isolation systems to isolate and identify CECs in cystic pancreatic lesions and PDAC, indicating that numerous conditions might be associated with CEC detection [42,43]. Our study is the first to implement the Parsortix™, a size-based cell separation system for CEC isolation. This discrepancy suggests that our cell separation method, or the pan-keratin antibody, might be less effective for isolating and detecting CECs in IPMN cases. Interestingly, our platform has proven capable of detecting CTCs in a significant proportion of PDAC patients [44]. This raises the possibility that CECs and CTCs may differ in their physical properties, potentially affecting their detectability using our current methodology. The lack of standardised CEC and CTC detection methods, especially for malignancies of the pancreas [43], indicates that ctDNA-based mutation analyses rather qualify for biomarker research in IPMN settings than CEC analysis. Here, sensitive and cost-effective *KRAS* and *GNAS* based mutation analyses offer a more standardised and objective approach.

5. Conclusions

In summary, our study represents the first to investigate the differences in the frequency of *GNAS* and *KRAS* mutations between two distinct IPMN cohorts —one undergoing conservative monitoring and the other with recommendations for surgical intervention. Our findings highlight the potential utility of assessing *KRAS* and *GNAS* mutations in cfDNA to guide IPMN treatment decision-making. Notably, the absence of these mutations appears to be a reliable indicator for branch duct IPMN patients without worrisome features, supporting a conservative management approach in low-risk cases. Conversely, a shift to *GNAS* positivity could be considered a significant marker for enhanced imaging surveillance, helping to identify patients at a higher risk of malignant transformation who may require surgical resection. However, it is worth noting that a substantial majority of patients within the surgical cohort exhibited only low-grade dysplasia, raising questions about the necessity of surgery in these cases. Neither the presence of established worrisome features nor *GNAS* or *KRAS* mutations appear effective in identifying high-grade dysplasia among IPMN patients, which is the only true indication for surgery. Future research involving larger patient cohorts and longer follow-up periods is essential to solidify these preliminary observations.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

The corresponding author takes full responsibility that all authors on this publication have met the following required criteria of eligibility for authorship: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; (c) final approval of the published article; and (d) agreement to be accountable for all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved. Nobody who qualifies for authorship has been omitted from the list. CN, MT, HW and FGU conceived and designed the project. CN, MT, PW, KM, MG, JK, AWB, JRI, FN, TH, KP, HW and FGU acquired the data and provided the samples. CN, MT, HW and FGU analysed and interpreted the data. CN and MT wrote the paper.

Data accessibility

The data that support the findings of this study are available from the corresponding author [c.nitschke@uke.de] upon reasonable request.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Comparison of cfDNA analysis data in IPMN.

3 Summary

This study assessed the efficacy of liquid biopsy techniques, particularly the detection of *KRAS* and *GNAS* mutations in cell-free DNA (cfDNA) and circulating epithelial cells (CEC), for risk stratification in patients with IPMN of the pancreas. The research included 48 patients (25 surgical, 23 conservative cases) from the University Hospital Hamburg-Eppendorf, utilizing ddPCR and a size-based Parsortix™ system for analysis.

GNAS mutations were found in 18.8% of total patients and 32% in those requiring surgery, indicating significant relevance in high-risk cases (p=0.024).

CfDNA mutation analysis provided low sensitivity (*GNAS* 32%, *KRAS* 16%) but high specificity (95.7%), highlighting its suitability for confirming conservative management in non-worrisome cases.

The absence of resected tissue sample analysis limited insights into the correlation of liquid biopsy findings with corresponding tissue pathology.

No CECs were detected using Parsortix™, a difference from other studies possibly due to the ineffective staining by the pan-keratin antibody.

Only two of 25 surgically resected IPMNs had high grade dysplasia, questioning current surgical criteria, including those outlined in the Kyoto Guidelines, which display limited diagnostic accuracy (sensitivity 40-68%).

While *KRAS* and *GNAS* detection did not reliably identify high grade dysplasia IPMNs, the absence of *GNAS* mutations notably supported a conservative approach, reinforcing its potential as a biomarker for low-grade dysplasia.

Diese Studie untersuchte die Wirksamkeit von Liquid-Biopsy-Techniken, insbesondere die Erkennung von *KRAS*- und *GNAS*-Mutationen in zell-freier DNA (cfDNA) und zirkulierenden epithelialen Zellen (CEC), zur Risikostratifizierung bei Patienten mit IPMN des Pankreas. Die Forschung umfasste 48 Patienten (25 chirurgische, 23 konservative Fälle) vom Universitätsklinikum Hamburg-Eppendorf, wobei ddPCR und ein größenbasiertes Parsortix™ -System zur Analyse verwendet wurden.

GNAS-Mutationen wurden bei 18,8 % der Gesamtpatienten und 32 % der Patienten in der operativen Kohorte, welches auf eine signifikante Relevanz bei Hochrisikofällen hinweist (p=0,024).

Die cfDNA-Mutationsanalyse zeigte eine geringe Sensitivität (*GNAS* 32 %, *KRAS* 16 %), aber eine hohe Spezifität (95,7 %) und unterstreicht damit ihre Eignung zur Bestätigung eines konservativen Managements in low risk-IPMNs.

Das Fehlen einer Analyse von resezierten Gewebeproben ließ keine Korrelation von Liquid-Biopsy-Befunden mit der entsprechenden Gewebepathologie zu.

Mit Parsortix™ wurden keine CECs detektiert. Dies stellt einen Unterschied zu anderen Studien dar und ist möglicherweise auf eine ineffektive Färbung durch den Pan-Keratin-Antikörper zurückzuführen.

Nur zwei von 25 chirurgisch resezierten IPMNs wiesen eine hochgradige Dysplasie (HGD) auf, was die aktuellen chirurgischen Richtlinien zur Resektion von IPMNs in Frage stellt - einschließlich der Kyoto-Richtlinien, die eine begrenzte diagnostische Genauigkeit aufweisen (Sensitivität 40-68 %).

Während die Detektion von *KRAS* und *GNAS* Hochrisiko-IPMNs nicht zuverlässig identifizierte, unterstützte das Fehlen von *GNAS*-Mutationen einen konservativen Ansatz und stärkte dessen Potenzial als Biomarker für low-risk Dysplasien.

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5 List of Abbreviation

CEC - circulating epithelial cell

cfDNA - cell-free DNA

ddPCR - digital droplet polymerase chain reaction

GNAS - a gene that encodes the G-protein alpha subunit which is involved in cell signaling

HGD - high-grade dysplasia

HR – high risk

IPMN - intraductal papillary mucinous neoplasm

KRAS - a gene that codes for a protein involved in regulating cell division and growth

PDAC - pancreatic ductal adenocarcinoma

WF – worrisome features

6 List of Figures

7 List of Tables

8 Declaration of own contribution

CN, MT, HW and FGU conceived and designed the project. CN, MT, PW, KM, MG, JK, AWB, JRI, FN, TH, KP, HW and FGU acquired the data and provided the samples. CN, MT, HW and FGU analysed and interpreted the data. CN and MT wrote the paper.

My personal contribution to the presented original paper is reflected appropriately from the declaration of contribution.

The work on which the promotion thesis is based was mainly carried out in cooperation with members of the staff from the Institute of Tumor Biology and the Department of General, Visceral and Thoracic Surgery at the University Medical Center Hamburg-Eppendorf.

The research involved sample collection and isolating plasma and CECs using the Parsortix system on the same day as collection.
The following process included the isolation and quantification of cfDNA from the plasma. The isolated CECs were analyzed microscopically to identify potential CECs. The whole process was conducted by me in person.

In relation to the cfDNA, I executed PCR runs aimed at detecting mutations in KRAS and GNAS. All data generated from the CEC analysis and the PCR assays were subjected to processing and presenting the data and its statistical evaluation, which I performed under appropriate supervision.

The writing of this paper, including the statistical analysis, literature review, and interpretation of the results, was completed by me.

9 Eidesstaatliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe, insbesondere ohne entgeltliche Hilfe von Vermittlungs- und Beratungsdiensten, verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe. Das gilt insbesondere auch für alle Informationen aus Internetquellen.

Soweit beim Verfassen der Dissertation KI-basierte Tools („Chatbots“) verwendet wurden, versichere ich ausdrücklich, den daraus generierten Anteil deutlich kenntlich gemacht zu haben. Die „Stellungnahme des Präsidiums der Deutschen Forschungsgemeinschaft (DFG) zum Einfluss generativer Modelle für die Text- und Bilderstellung auf die Wissenschaften und das Förderhandeln der DFG“ aus September 2023 wurde dabei beachtet.

Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.

Ich erkläre mich damit einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

30.05.2025
Datum Unterschrift

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