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Serum IgG4 levels outperform IgG4/IgG RNA ratio in differential diagnosis of IgG4-related disease

To the Editor:

IgG4-related disease (IgG4-RD) is a chronic inflammatory-fibrosing disorder affecting virtually any organ, but most frequently the pancreaticobiliary system. Differential diagnosis is challenging and relies on multiple criteria rather than on single markers. Serum IgG4-levels (sIgG4) are elevated in IgG4-RD, correlate with disease activity, yet lack sensitivity. Malignancies can also display elevated sIgG4 levels that reduce its specificity.[1,2] The correct differential diagnosis of IgG4-RD from malignancies is critical to implement treatment and avoid unnecessary resections. Thus, reliable biomarkers are urgently warranted.[3] Previously, qPCR-based measurements of the IgG4/IgG mRNA ratio that originate from dominant $IgG4^{+}$-B-cell receptor (BCR) clones have been proposed to fill this gap with "ideal" test characteristics (AUC 0.991, sensitivity 94%, specificity 98.7%).[4] In contrast, recent work from Beuers and colleagues found relevant limitations of IgG4/IgG qPCR ratio in distinguishing between IgG4-RD and pancreaticobiliary malignancies.[5] Our data strongly support this observation from an independent referral center in distinct cohorts comprising the diagnostic spectrum of pancreaticobiliary IgG4-RD: Specifically, we analyzed IgG4/IgG qPCR ratios in blood samples from our prospectively collecting biobank (ethics vote No. 159/19 and 87/20) in a cohort of 98 patients with IgG4-RD, cholangiocarcinomas, pancreatic ductal adenocarcinomas and benign pancreaticobiliary diseases such as chronic pancreatitis (CP) and primary sclerosing cholangitis (PSC) (Fig. 1A). mRNA was extracted from leucocytes, followed by qPCR analyses and IgG4/IgG ratio calculation (cut-off range from 3.5–6.0%) (Fig. 1B[4]). Remarkably, false positive test results became particularly evident in patients with cholangiocarcinomas (12–18 out of 25; 48–72%) and pancreatic carcinomas (9–10 out of 21; 42.9–47.6%). However, false-positive diagnoses were also made in CP and PSC groups (Fig. 1B,H). In contrast, 10-13 out of 16 patients with confirmed IgG4-RD could be correctly diagnosed leading to a sensitivity of 62.5–81.3% depending on the 3.5 or 6.0% cut-off, respectively (Fig. 1B). However, our IgG4-RD cohort was not therapy-naïve, lowering sensitivity as previously reported.[4] To circumvent this problem, we challenged our non-IgG4-RD patients with the treatment naïve IgG4-RD cohort from Doorenspleet *et al.* during area under the receiver-operating characteristic curve (AUC) analysis. Still, AUC dropped from 0.987 to 0.863 (Fig. 1C), pointing to a lower test performance, independent of the treatment status of IgG4-RD patients. There might be several reasons for this observation: (i) Most patients in de Vries *et al.*[5] or in our study have not received chemotherapy, in contrast to the original publication.[4] Hence, chemotherapy might also suppress existing BCR-clones in malignancies, therefore, limiting diagnostic accuracy, as shown for BCR-counterparts in IgG4-RD.[4] (ii) We hypothesized that technical and methodological constraints in the qPCR-reaction might hamper test performance. In particular, a common single nucleotide polymorphism (RS10137020; Fig. 1D), with a minor allele frequency of 30% in one of the primer binding sites, could reduce the efficacy of PCR. Furthermore, separated reactions and primer sets for IgG4 and total IgG mRNA measurement, declining polymerase activity, PCR efficacy and pipetting errors could negatively influence the test performance. To circumvent this, we designed a multiplex digital droplet PCR assay simultaneously measuring IgG4 and total IgG: Specifically, the IgG4/IgG ratio was calculated based on Poisson distribution using a single pair of primers implemented together with allele-specific TaqMan®-probes (Fig. 1E; IgG4/IgG digital droplet PCR). Intriguingly, the digital droplet PCR approach did not outperform the standard qPCR-assay[4] for determining IgG4/IgG ratios. This excludes technical constraints in the method but instead suggests reduced test performance (Fig. 1F). To further probe IgG4/IgG qPCR ratio against the current gold standard in diagnostic scorings, AUC analysis for pre-therapeutic sIgG4 levels was performed in our cohorts and compared to Doorenspleet *et al.*[4] Conversely, AUC analysis for sIgG4 levels across various comparisons displayed similar accuracy values, underpinning the superiority of sIgG4 levels compared to IgG4/IgG ratios in the differential diagnosis of IgG4-RD (Fig. 1C,F,G;AUC 0.902 *vs.* 0.916 *vs.* 0.950). False positive diagnosis of IgG4-RD in case of pancreaticobiliary malignancy can delay diagnosis, potentially curative surgery or timely chemotherapy. Also, in case of benign disease unnecessary steroid pulse treatment can cause side effects. Therefore, false positive test results pose the strongest threat to non-IgG4-RD patients and need to be imperatively avoided, likewise underpinned by de Vires *et al.*[5] In turn, we compared false positive rates for IgG4/IgG mRNA ratios with sIgG4 levels head-to-head for differential diagnosis of (non-)IgG4-RD. Strikingly, there were lower false positive rates for sIgG4 levels compared to IgG4/IgG mRNA ratios in both patients with malignant and benign non-IgG4-RD (Fig. 1H). Collectively, we demonstrated (i) in concordance with de Vires *et al.* (ii) on independent cohorts and (iii) with different methodological setups that the IgG4/IgG mRNA ratio is prone to false-positive results, which could cause misdiagnosis of pancreaticobiliary cancer. Furthermore, we found that sIgG4 levels were more accurate, although still not perfect. Therefore, our study questions the clinical benefit of

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Patient characteristics (total cohort)	n = 98
Sex: male	70.4%
Sex: female	29.6%
Age	61.1 (±13.9)
Autoimmune pancreatitis	28
- Type 1	16
-- on glucocorticosteroids	7
-- on rituximab	2
- Type 2	12
Other chronic pancreatitis	18
Cholangiocarcinoma	25
Primary sclerosing cholangitis	6
Pancreatic ductal adenocarcinoma	21
Leukocyte count G/L	8.7 (±3.47)
Infections	4
Additional chronic inflammatory diseases (not IgG4 Related)	20
- Inflammatory bowel diseases	13
- Other	7

Characteristics of subgoups	IgG4-RD patients (n = 16)	Non-IgG4-RD patients (n = 82)
Sex: male	87.5%	67%
Sex: female	12.5%	33%
Age	60.1 (±15.6)	61.3 (±13.7)
Chemotherapy (current)	0 (0%)	10 (12%)
Corticosteroid treatment - current - former	 7 (44%) 16 (100%)	 13 (16%) n.a.
Immunosuppression	3 (27%)	0
Malignancy: treatment naive	(0%)	79%
G-CSF treatment	0	2

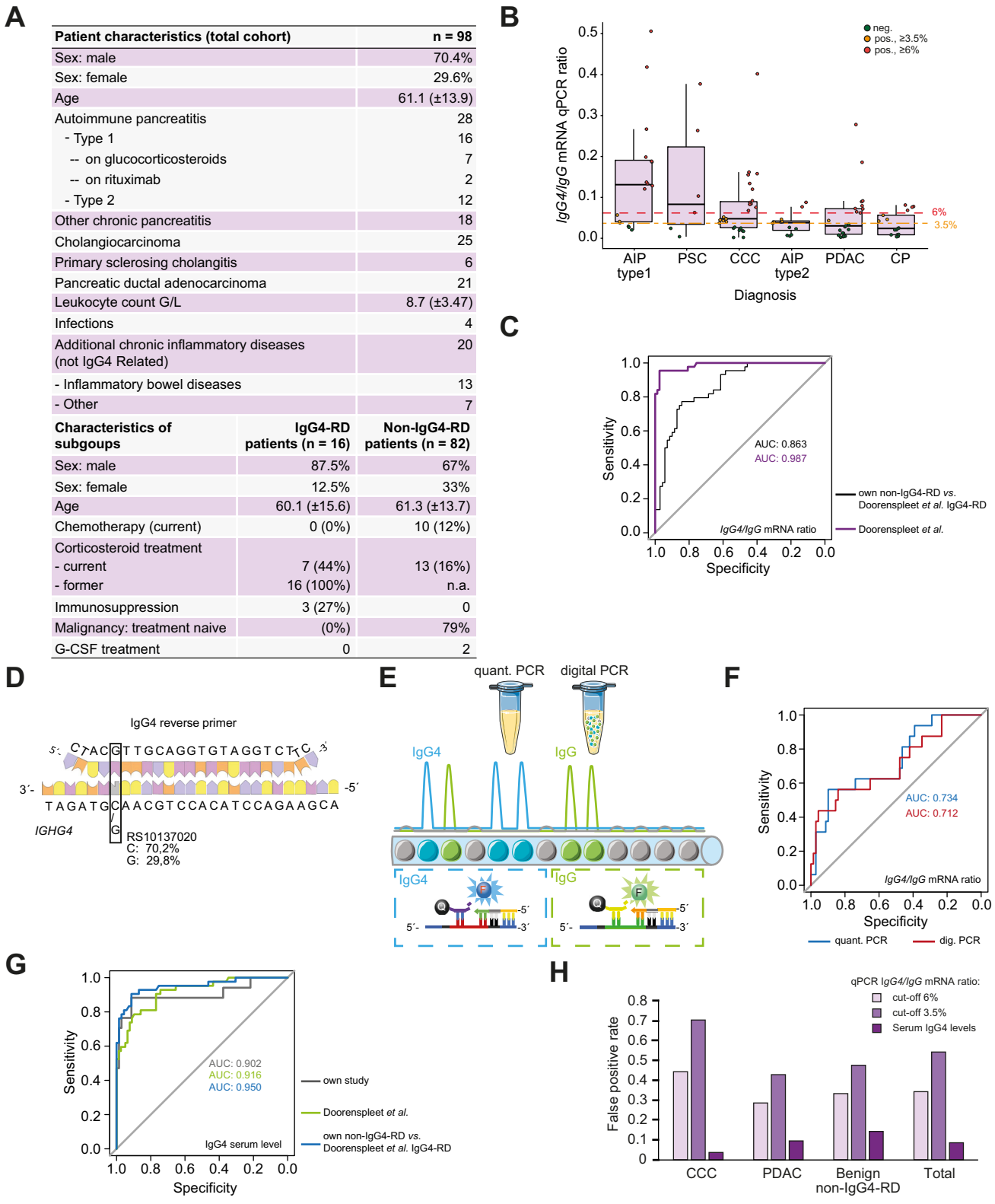

Fig. 1. IgG4/IgG mRNA ratios are not suitable for differential diagnosis of IgG4-RD. (A) Patient characteristics of internal study cohort: total cohort, IgG4-RD and non-IgG4-RD subgroups. For non-IgG4-RD patients, data on former glucocorticoid treatment was not available. Immunosuppression refers to 2 cases of rituximab and 1 case of azathioprine treatment. Infections were 1 case each of infected walled-off necrosis (IgG4-RD patient), bronchitis, acute flare of ulcerative colitis and post-ERCP pancreatitis (each in non-IgG4-RD patients) (B) IgG4/IgG mRNA expression in patients with IgG4-RD or non-IgG4-RD differential diagnosis, measured by qPCR. Dashed lines: Cut-off values (6.0% and 3.5% relative expression of IgG4). Green, orange or red dots: Value below, between or above cut-off values, respectively. (C) AUC of IgG4/IgG mRNA ratio. Green line: Test results as published by Doorenspleet *et al.* (IgG4-RD *vs.* non-IgG4-RD; external data[4]); black line: IgG4/IgG mRNA ratio of IgG4-RD patients from[4] compared to non-IgG4-RD patients from own cohort. (D) Schematic illustration of the SNP RS10137020 with a minor allelic frequency of ~30%. (E) Principle of IgG4/

IgG4/IgG mRNA ratios in the differential diagnosis of IgG4-RD in support of de Vires *et al.* but concurrently underpins the necessity for more reliable biomarkers to differentially diagnose IgG4-RD.

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Conflict of interest
The authors declare no conflicts of interest that pertain to this work.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions
L.S.: Experiments, data evaluation, text drafting, figures, clinical data collection. F.A.: Experiments, data evaluation, figures. F.S.: Experiments. J.B., L.P., A.B, G.B., F.U.W.: Clinical data collection. T.S. text editing, approval. M.M., A.K.: Supervision, idea, data evaluation, text drafting, figures, clinical data collection.

Supplementary data
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Author names in bold designate shared co-first authorship

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IgG based digital droplet PCR approach. Concentration was calculated based on Poisson distribution (BioRad® QuantaSoft™ 1.7.4.0917) (F) AUC of IgG4/IgG mRNA ratio either assessed by quantitative PCR (quant. PCR, blue line) or by digital droplet PCR (dig. PCR, red line). (G) AUC analysis of sIgG4 levels in distinct cohorts. Black line: Own cohort of IgG4-RD *vs.* non-IgG4-RD patients. Green line: Data from Doorenspleet *et al.* on sIgG4 levels in IgG4-RD *vs.* non-IgG4-RD;[4] Blue line: sIgG4 levels of IgG4-RD patients from[4] compared to non-IgG4-RD patients from own cohort in analogy to (C). (H) False positive rates of IgG4/IgG mRNA ratio (qPCR) and serum IgG4 levels (sIgG4; cut-off: 135 mg/dl) according to published cut-off values (patients included when both sIgG and IgG4/IgG mRNA qPCR were available, n = 69). AIP, autoimmune pancreatitis; AUC, area under the receiver-operating characteristic curve (R 3.6.0, pROC package); CCC, cholangiocarcinoma; CP, chronic pancreatitis of other causes; ERCP, endoscopic retrograde cholangiopancreatography; IgG4-RD, IgG4-related disease; PDAC, pancreatic ductal adenocarcinoma; PSC, primary sclerosing cholangitis; SNP, Single nucleotide polymorphism.