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The Impact of potential 'confounders' on the diagnostic sensitivity of circulating free DNA in management of FIT+ patients: a pilot study

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ABSTRACT

Cell-free DNA (cfDNA) has long been established as a useful diagnostic and prognostic tool in a variety of clinical settings, ranging from infectious to cardiovascular and neoplastic diseases. However, non-neoplastic diseases can act as confounders impacting on the amount of cfDNA shed in bloodstream and on technical feasibility of tumour derived free circulating nucleic acids selecting patients with cancer. Here, we investigated the potential impact of other pathological processes in the clinical stratification of 637 FIT+ patients. A single and multiple logistic regression yielded similar results. Crude sensitivity was 75.9% versus adjusted sensitivity of 74.1%, relative risk 0.9761 (0.8516 to 1.1188), risk difference 0.0181 (−0.0835 to 0.1199) and OR 0.9079 (0.5264 to 1.5658). Potential confounding effect from other source of cfDNA plays a pivotal role in the clinical stratification of FIT+ patients.

INTRODUCTION

Metastatic colorectal cancer (mCRC) is still one of the leading causes of death worldwide.[1] Despite improvements in technical and clinical approaches, the vast majority of patients with cancer are diagnosed in advanced stage with poor prognosis and few therapeutic options.[2] Early detection of pre-malignant lesions may represent the most feasible approach to improve clinical outcome of patients with mCRC.[3] Carcinoembryonic antigen and carbohydrate antigen-19–9 are routinely employed for the monitoring of CRC lesions but their diagnostic role in clinical practice is limited by the low correlation between biomarkers level and tumour disease.[4] In addition, organised screening programmes for the early detection of pre-malignant lesions are currently based on FIT (Faecal Immunochemical Test) that identifies blood traces in stool.[3] Of note, a non-negligible number of FIT+ patients are referred to colonoscopy, returning most often a negative result.[3 4] Given these critical issues, the identification of non-invasive biomarkers is currently underway to optimise the clinical management of FIT+ patients. In this scenario, liquid biopsy, consisting in the peripheral blood collection, emerged as a valid biological matrix to analyse molecular mechanisms behind tumour progression.[5 6] Particularly, circulating tumour DNA (ctDNA), which represents a small fraction of circulating cell-free DNA (cfDNA), may be considered a multi-informative analyte already approved in the clinical management of patients with CRC and non-small cell lung cancer.[7 8] Somatic mutations

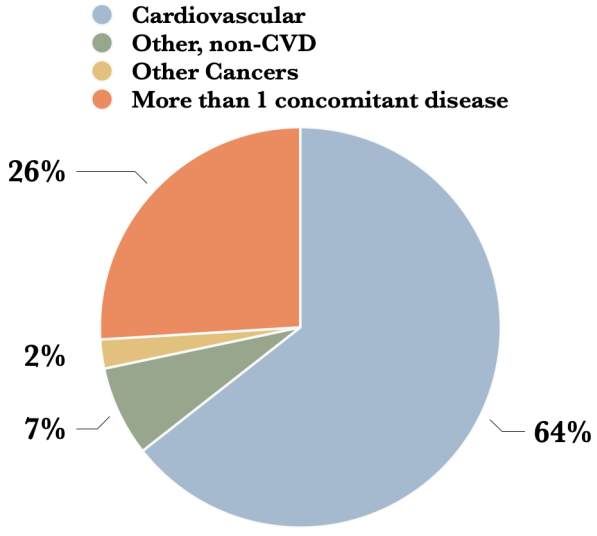

Figure 1 Schematic representation of concomitant diseases in tumour patients. CVD, cardiovascular disease.

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found in ctDNA may also select patients with tumour to the best therapeutical option or molecularly monitor clonal evolution in neoplastic cells.[9] It was also observed that, compared with healthy individuals, the quantification of cfDNA levels is different in patients with tumour.[10 11] Fragment size distribution in healthy individuals highlighted a median value of 167 bp; conversely, nucleic acids fragmentation from tumour cells showed a distinct distribution pattern due to tumour-related epigenetic and genetic modifications.[10 11] In addition, cfDNA median amount may also play a pivotal role in the clinical management of patients with tumour. It has been demonstrated that a considerable number of clinical variables, namely 'confounders', may impact cfDNA amount shed in the bloodstream.[12 13] In this scenario, age did not show any statistically relevant correlation with cfDNA amount variation, while literature data regarding sex and body mass index are controversial.[12 13] Moreover, it should be kept in mind that non-neoplastic diseases may also contribute to increase cfDNA levels in torrent blood.[12 13] The highest contribution to cfDNA in blood is provided by a significant increase of nucleosomes in severe inflammatory diseases. Furthermore, cfDNA quantification heavily depends on inflammation severity.[13] A massive release of cfDNA in the bloodstream is also caused by cardiovascular diseases (CVDs).[14] The exposure to damaging factors and the rapid turnover of cell renewal at the basis of inflammation and chronic diseases may also be considered high-impact factors on changes in cfDNA in patients with tumour.[13–15] We investigated the role of cfDNA as a diagnostic biomarker in FIT+ patients to evaluate the role of several clinical variables.

MATERIALS AND METHODS

Study design

637 FIT+ individuals of both genders, aged 50–74, participating in a CRC screening programme managed by ASL-NA-3-SUD (Naples, Italy) were enrolled in a cross-sectional study aimed at investigating the role of QuantiDNA test (DiaCarta, Pleasanton, California, USA), an assay designed to detect long cfDNA fragments (247 bp) from plasma in the triage of FIT+ subjects. Each participant was also interviewed for medical history. According to the study protocol (DIA-001), all FIT+ (or FOBT+) patients, willing to sign an informed consent and eligible for the study, received both the QuantiDNA test and gold standard colonoscopy. A whole-blood sample was collected from each patient in the ASL-NA-3-SUD endoscopy centre, processed and stored at the Department of Public Health of the Federico II University of Naples (Italy); long fragments of plasma cfDNA were measured using QuantiDNA test at the CLIA Lab of DiaCarta, Pleasanton, California, USA.

An ROC curve was run for cfDNA long fragments establishing a cut-off of 6.27 ng/mL to discriminate subjects with colorectal neoplasia (CN) from those without it.

The sensitivity for CN of cfDNA long fragments was assessed using a single predictor logistic regression. A multiple logistic regression was also run to adjust for potential confounders, namely CVD, metabolic and other diseases and different types of neoplasia. A matrix showing correlations between cfDNA and potential confounders was also used. Comparison of ORs, assay sensitivity and specificity between unadjusted and adjusted values of the predictor was conducted. The areas under the curve (AUCs) for adjusted and unadjusted slopes of the predictor were also compared.

Study population

385 FIT+ subjects (60.4%) had one or more concomitant disease (CD) (figure 1). Of these, 67 (21.1%) had also CN. In the remaining 252 subjects without CD, there were 68 cases of CN (27.0%). Among the 385 subjects with CD, 248 (64.4%, of which hypertension was by far the most prevalent) had a CVD, 28 (7.3%, of which diabetes was the predominant one) had other non-CVDs, 9 (2.3%) had other cancers (non-CN) and 100 (26.0%, of which HTA (hypertension) and diabetes were more often reported together) had more than one of these CDs.

Technical workflow

All enrolled subjects underwent an eligibility check (subject information, visit information, inclusion/exclusion criteria, demographics, medical history, subject history, substance use, vital signs, family history). 20 mL of whole blood were collected from each subject for molecular analysis, of which 2 mL were used for cfDNA evaluation. Whole blood specimens were collected in Streck Cell-free DNA BCT blood collection tubes (La Vista, Nebraska, USA) capable of storing whole blood samples up to 7 days at room temperature. Sample collection took place on the day subjects underwent pre-colonoscopy procedures (such as blood coagulation tests). Samples were shipped on the same day of collection to the processing laboratory at the University of Naples where standardised technical procedures to collect and store samples were carried out. Whole blood samples underwent two centrifugations, first at 1600×g and second at 16 000×g at room temperature for 10 min each. Following centrifugation, plasma samples were stored at −80°C. Following enrollment conclusion, samples were shipped to the DiaCarta (Pleasanton, California, USA), CLIA/CAP-certified laboratory for QuantiDNA testing. Plasma samples were stored at −80°C prior to being thawed and tested with the QuantiDNA test at the DiaCarta laboratory. Relative light units data were measured by a DiaCarta Luminometer and converted into ng/mL concentration values using a point-to-point calibration. We analysed the assay's performance for OR, sensitivity and specificity.

Statistical analysis

A single-predictor logistic regression was used to derive crude sensitivity of 75.9% (95% CI: 68.0% to 82.7%), previously described. To investigate a potential confounding effect of CD, we used a multiple logistic regression with the following predictors: concentration of long fragments of cfDNA (coded as 1 if ng/mL ≥6.27, as 0 if not), presence of more than one CD (coded as 1 if true, as 0 if subject had just one or less CD), presence of CVD (coded as 1 if true, as 0 if not and if subject had more than one CD), presence of other diseases (coded as 1 if true, as 0 if not and if subject had more than one CD) and presence of other types of cancers (coded as 1 if true, as 0 if not and if subject had more than one CD).

The statistical analysis software was R-studio V.2022.12.0-353 under MacOS Monterey V.12.4 for internal analysis and SAS V.9.4 under Windows 2016 Terminal.

Results

The adjusted sensitivity (table 1) of cfDNA long fragments, derived from the multiple logistic regression, was 74.1% (95% CI: 66.1% to 80.7%). Risk difference between crude and adjusted estimates was 0.02 (−0.08 to 0.12). Relative risk was 0.98 (0.85 to 1.12) and the OR was 0.91 (0.53 to 1.57) (tables 1–5). These three indicators were not statistically significant. The odds of disease for a subject with adjusted cfDNA+

Table 1 Adjusted estimate of sensitivity for colorectal neoplasia (74.1%) by using R-studio V.2022.12.0-353 under MacOS Monterey V.12.4

	Disease	No disease	Total
Test positive	100	324	424
Test negative	35	178	213
Total	135	502	637

value was 73.0% higher than a subject with a cfDNA− value, with a p value=0.013, while the unadjusted OR was 1.76, with p value=0.009. The AUCs were 56% for the unadjusted form and 55% for the adjusted form. The specificity was 35.8% in the unadjusted analysis, while it was 35.5% in the adjusted one.

Finally, the correlation between cfDNA and the potential confounders (table 3), as reported by the matrix generated adopting the multiple logistic regression, was: 0.095 versus more than one CD, 0.054 versus CVD, −0.075 versus other diseases and 0.028 versus other cancers (figure 1, table 4),

DISCUSSION

To date, liquid biopsy is considered a reliable diagnostic tool integrating tissue specimen analysis for the analysis of clinically informative molecular alterations able to stratify patients with solid tumour.[5 6] In this scenario, a plethora of analytes derivable from plasma samples may be isolated (circulating nucleic acids, circulating tumour cells, extracellular vesicles, non-coding RNA) but only cfDNA is currently available in clinical practice as a diagnostic tool supporting molecular profiling of predictive biomarkers.[5] Recently, cfDNA has proved pivotal in distinguishing between healthy individuals and patients with tumour both in terms of molecular assessment and total amount of nucleic acids relapsed by tumour cells in torrent blood.[5 6] As previously demonstrated, cfDNA may also play a pivotal role to detect early-stage asymptomatic CRC lesions.[16] Of note, cfDNA shedding in peripheral blood is also guided by physiological turnover process both of malignant and normal cells.[13–15] It has been ascertained that cfDNA levels in torrent blood are directly dependent from the clinical frame of collecting sample for patients.[11 12] In this scenario, direct measurement of cfDNA levels may represent a key weapon establishing the best clinical administration of advanced or early-stage asymptomatic tumour patients.[8 11] To date, a plethora of commercially available assays enable quantitative and/or qualitative evaluation of cfDNA fragments from biological fluids, but these approaches require different pre-analytical and analytical managing procedures, like as starting input of analyte, volume of blood, isolation and purification of cfDNA to indirectly measure cfDNA.[17] QuantiDNA testing assay was designed on branched DNA technology (SuperbDNA) to directly measure cfDNA levels removing isolation and purification procedures.[18] It has been demonstrated that several non-neoplastic diseases (like inflammatory processes) dramatically impact on the total amount of nucleic acids detectable in bloodstream.[13–15] As a consequence, screening programmes based on cfDNA measurement may be discouraged due to this intrinsic limitation. In the previous study, we investigated the usefulness of long fragments of cfDNA in the triage of FIT+ subjects undergoing a colorectal cancer screening programme, with a view to improve the efficiency of those programmes, by reducing the number of unnecessary colonoscopies. In doing so, we aimed to maximise the assay's sensitivity and demonstrate non-inferiority towards the standard of care, in order to ensure the greatest degree of safety for patients. As a result of such strategy, the specificity of 35.8%, together with a sensitivity for CN of 75.9%, was enough to show non-inferiority and ensure a drop in colonoscopies equal to 33.3%.

In this study, we aimed to dig deeper into the elevation of cfDNA brought about by potential confounders, which could impact the assay's performance. All of the key performance indicators did not appear to be affected. CIs for the difference in sensitivities between crude and adjusted estimates, passed through the 0, suggesting non-significance, this was further confirmed by CIs for relative risk and ORs, both passing through the unit. Also, the AUCs were similar in size. Finally, there was a weak correlation between the long fragment's predictor and all of the confounders, both positive and negative.

This study has limitations. Several other confounders may exist which were not investigated here, and more scientific contributions are encouraged in this domain.

CONCLUSIONS

Long fragments of cfDNA are a reliable predictor of CN within the context of the triage of FIT+ patients. The age groups normally enrolled in colorectal cancer screening programmes carry a risk of several CDs, namely hypertension, atrial fibrillation, diabetes, other inflammatory conditions and cancers, which could theoretically raise total plasma cfDNA levels. The QuantiDNA assay, which measures long fragments of plasma cfDNA, appears robust to the potential confounding effects generated by CDs, and could thus be implemented in clinical practice. More

Table 2 Unadjusted estimate of sensitivity for colorectal neoplasia (75.9%) by using R-studio V.2022.12.0-353 under MacOS Monterey V.12.4

	Disease	No disease	Total
Test positive	107	340	447
Test negative	34	190	224
Total	141	530	671

Table 3 Correlation between long fragments cfDNA values and concomitant diseases

	Fragments	More than one CD	CVD	Other CD	Other cancers
Fragments	1.000	0.09548	0.05409	−0.07527	0.02846
More than one CD	0.09548	1.000	−0.3446	−0.09253	−0.05166
CVD	0.05409	−0.3446	1.000	−0.1712	−0.09559
Other CD	−0.07527	−0.09253	−0.1712	1.000	−0.02567
Other cancers	0.02846	−0.05166	−0.09559	−0.02567	1.000

CD, concomitant disease; cfDNA, cell-free DNA; CVD, cardiovascular disease.

Table 4 Breakdown of concomitant diseases

Disease	Absolute frequency	Relative frequency (%)
Cardiovascular	248	64
Other, non-CVD	28	7
Other cancers	9	2
More than one concomitant disease	100	26
Total	385	100

CVD, cardiovascular disease.

Table 5 Risk difference, relative risk and OR between unadjusted and adjusted evaluation in FIT+ patients

	Estimate	95% CI
Risk difference	0.02	−0.08 to 0.12
Relative risk	0.98	0.85 to 1.12
OR	0.91	0.53 to 1.57

and different studies are needed to establish its effectiveness in primary screening.

Handling editor Runjan Chetty.

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