Plasma circular RNA hsa_circ_0001445 and coronary artery disease: Performance and stability as a biomarker

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ABBREVIATIONS AND ACRONYMS

ACS: Acute coronary syndrome

CAD: Coronary artery disease

CTA: Computed tomographic angiography

CHAID: Chi-squared automatic interaction detector

circRNA: Circular RNA

hs-CRP: High-sensitive C-reactive protein

hs-TnT: High-sensitive troponin T

IDI: Integrated discrimination improvement index

miRNA: microRNA

ncRNA: noncoding RNA

NRI: Net reclassification improvement index

SIS: Segment involvement score

SMARCA5: SWI/SNF-related matrix-associated actin-dependent regulator of chromatin

subfamily A member 5 gene

SSS: Segment stenosis score

ABSTRACT

The role of circular RNAs (circRNAs) as biomarkers remains poorly characterized. Here, we investigated the performance of the circRNA hsa_circ_0001445 as a biomarker of coronary artery disease (CAD) in a real-world clinical practice setting. Plasma hsa_circ_0001445 was measured in a study population of 200 consecutive patients with suspected stable CAD who had undergone coronary computed tomographic angiography (CTA). Multivariable logistic models were constructed combining conventional risk factors with established biomarkers and hsa_circ_0001445. Model robustness was internally validated by bootstrap technique. Biomarker accuracy was evaluated using the C-index. The integrated discrimination improvement (IDI) and net reclassification improvement (NRI) were also calculated. Risk groups were developed via classification tree models. The stability of plasma hsa_circ_0001445 was evaluated under different clinical conditions. hsa_circ_0001445 levels were associated with higher coronary atherosclerosis extent and severity with a 2-fold increase across tertiles (28.4%-50.0%). Levels of hsa_circ_0001445 were proportional to coronary atherosclerotic burden, even after comprehensive adjustment for cardiovascular risk factors, medications and established biomarkers (fully adjusted OR=0.432 for hsa_circ_0001445 as a continuous variable and fully adjusted OR=0.277 for hsa_circ_0001445 as a binary variable). The classification of patients was improved with the incorporation of hsa_circ_0001445 into a base clinical model (CM) composed of conventional cardiovascular risk factors, showing an IDI of 0.047 and NRI of 0.482 for hsa_circ_0001445 as a continuous variable and an IDI of 0.056 and NRI of 0.373 for hsa_circ_0001445 as a binary variable. A trend towards higher discrimination capacity was also observed ($C\text{-index}_{CM}=0.833$, $C\text{-index}_{CM+\text{continuous hsa_circ_0001445}}=0.856$ and $C\text{-index}_{CM+\text{binary hsa_circ_0001445}}=0.855$). Detailed analysis of stability showed that hsa_circ_0001445 was present in plasma in a remarkably stable form. *In vitro*, hsa_circ_0001445 was downregulated in extracellular vesicles secreted by human coronary smooth muscle cells upon exposure to atherogenic conditions. In patients with suspected stable CAD referred for coronary CTA, plasma hsa_circ_0001445 improves the identification of coronary artery atherosclerosis.

Keywords: Atherosclerosis; Biomarker; Chronic coronary syndrome; Circular RNA; circSMARCA5; Coronary artery disease; Coronary heart disease; hsa_circ_0001445; Ischemic heart disease; SMARCA5; Stability.

INTRODUCTION

Patients with stable coronary artery disease (CAD) are naturally at higher cardiovascular risk and are recommended standardized secondary prevention measures, including lifestyle modifications and management of risk factors, or even percutaneous coronary intervention. There is widespread interest among the medical community in identifying suitable tools to facilitate the clinical management of patients with symptoms suggestive of stable CAD (1, 2). At present, there is no generally accepted or used circulating biomarker for the detection of the disease. Indeed, biomarker-driven diagnostic strategies are currently not recommended by the recent guidelines on chronic coronary syndromes due to the lack of robust evidence (3).

Multiple approaches have been explored to identify noninvasive indicators of stable CAD that may provide complementary diagnostic and prognostic information. Most efforts have been focused on protein-based and gene-based biomarkers (4, 5). Promising results point to the new generation high-sensitivity cardiac troponin assays (6). In this context, the noncoding transcriptome constitutes a novel and poorly explored source of diagnostic and prognostic tools for the management of cardiovascular disease (CVD) (7). In particular, circular RNAs (circRNAs), a subclass of the noncoding RNA (ncRNA) superfamily, have recently gained great interest in biomarker development (8, 9). Due to their covalently closed loop structure, circRNAs are protected from exonuclease-mediated degradation and have a longer half-life than linear RNAs (10). CircRNAs have also been detected in clinical specimens (11) and their expression is dynamically regulated in response to stress and disease (12, 13).

Despite the knowledge of the chemical and biological properties, the role of circRNAs as biomarkers of CVD (14), and more specifically CAD (15), remains poorly characterized. Recent independent reports have identified the circular isoform derived from exons 15 and 16 of the gene SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 5 (SMARCA5); circBase ID hsa_circ_0001445 (<http://www.circbase.org/>), also named circSMARCA5, as one the most abundantly

expressed circRNAs in the human heart (13, 16-18). hsa_circ_0001445 has also been consistently detected in clinically relevant samples, including plasma (19), serum (20) and whole blood (21). We speculated that hsa_circ_0001445 would constitute a candidate biomarker for monitoring the coronary atherosclerotic burden, similar to troponins or myocardium-enriched microRNAs (miRNAs) (22). Therefore, this study aimed to evaluate the performance of plasma hsa_circ_0001445 as an indicator of stable CAD.

METHODS

Study population

The study included 200 consecutive patients with clinically suspected stable CAD who underwent a coronary computed tomographic angiography (CTA) exam. With this sample size, the study was able to detect standardized differences of 0.45 with a power of 0.80 ($\beta=0.20$) at standard significant levels of 5% ($\alpha=0.05$). The study design, study procedures and characteristics of the study population have been previously described (23). In short, participants were recruited from the Hospital de la Santa Creu i Sant Pau (Barcelona, Spain). Exclusion criteria were suspected or current acute coronary syndrome (ACS), any severe infectious disease or any survival-limiting disease. Detailed demographic, anthropometric, clinical and pharmacological information was abstracted from the electronic medical record at the time of the coronary CTA study. High-sensitive troponin T (hs-TnT) and C-reactive protein (hs-CRP) levels were determined by standardized procedures at the clinical laboratory of the Biochemistry Service of the Hospital de la Santa Creu i Sant Pau.

Ethical aspects

The study was performed in accordance with the Declaration of Helsinki II and with approval by the Research Ethics Committee. All individuals provided written informed consent prior to study participation.

Coronary computed tomography angiography

Coronary CTA exams were performed using a 256-row CT system (iCT Philips Healthcare) in the Cardiac Imaging Unit at the Hospital de la Santa Creu i Sant Pau as previously described (23). CT images were analyzed by two level-3 cardiac computed tomography readers, blinded to the biomarker study, who interpreted the data and assigned the patients to the study groups. The coronary arteries were assessed using a 16-segment model (modified 15-segment model with segment 16 being the intermediate branch). Luminal diameter stenosis severity was graded as 0% (none), 1–29% (very mild), 30–49% (mild), 50–69% (moderate), 70–99% (obstructive) and 100% (totally occluded). The segment involvement score (SIS) and segment stenosis score (SSS) were calculated to determine the extent and severity of coronary atherosclerosis, respectively, as measures of coronary atherosclerotic burden. The SIS score, ranging from 0 to 16, was calculated as the total number of individual coronary artery segments exhibiting plaques irrespective of the degree of luminal stenosis. The SSS score was calculated for each individual coronary segment as 0 points if luminal stenosis was 0 to 29%, 1 point if it was 30 to 49%, 2 points for 50 to 69%, and 3 points for stenosis equal to or greater than 70%. Then, the individual segment scores of all 16 segments were summed to yield a total score ranging from 0 to 48. The prognostic value of these coronary artery plaque scores has been previously demonstrated in patients with suspected stable CAD without current ACS (24) and validated by independent groups (25-27). According to the cutoff proposed by Min et al. (24), patients were separated into groups with SIS or SSS of ≤ 5 versus > 5 . Finally, a 3-vessel plaque score was calculated as 0 or 1 based on the absence or presence of plaques in the left anterior descending, left circumflex and right coronary arteries, irrespective of severity.

Blood collection

Blood collection and processing were performed in accordance with the Standard Operating Procedures for Serum and Plasma Collection proposed by the Early Detection Research Network Consensus Statement and Standard Operating Procedure Integration

Working Group (28). Venous whole blood samples were collected into 10 mL Vacutainer K₂EDTA tubes (BD) by venipuncture after a night of fasting and immediately prior to CTA procedures. The tubes were immediately inverted 8-10 times. Blood was processed within 2 hours of extraction. Blood samples were fractionated by centrifugation at 1,300 x g for 15 min at room temperature. After centrifugation, the plasma phase was immediately transferred to 1.5 mL DNA LoBind tubes, leaving approximately 1 mL of plasma on top of the buffy coat, and stored at -80°C.

Circular RNA stability assays

The stability of hsa_circ_0001445 was tested under different experimental conditions in samples from five subjects. The assays included hsa_circ_0001445 quantification in plasma processed as described above and storing the samples at room temperature and at 4°C for 4, 8, 12, 24, 48, 72 hours and 7 days. The influence of 1, 2, 4, 6 and 8 repetitive freeze/thaw cycles was tested in plasma aliquots stored at -80°C. Hemolysis was evaluated in plasma samples processed from whole blood hemolyzed using a syringe (21 gauge needle).

Plasma circular RNA quantification

circRNA quantification was conducted in the same laboratory and under the same conditions. Experienced staff blinded to clinical data performed all laboratory measurements. RNA isolation was performed using aliquots that had been thawed ones. Total RNA was isolated from 150 µL of frozen plasma samples using the miRNeasy Serum/Plasma Kit (Qiagen), according to the manufacturer's instructions. After thawing at room temperature (15-25°C), plasma samples were recentrifuged at 16,000 x g for 5 min at 4°C. Then, 5 volumes of QIAzol Lysis Reagent were mixed with 1 volume of each plasma sample and incubated for 5 min at room temperature. The synthetic *Caenorhabditis elegans* miR-39-3p (cel-miR-39-3p) was added as an external reference miRNA (1.6×10^8 copies/µL) to normalize for differences in the RNA isolation efficiency. The mixture was supplemented with

1 µg of carrier RNA from bacteriophage MS2 (Roche) to improve RNA yield as previously reported (29). Both reagents were spiked into samples during RNA isolation after incubation with the denaturing solution. Subsequently, one volume of chloroform was added, and after 3 min at room temperature, the mixture was centrifuged at 12,000 x g at 4°C for 15 min. The upper aqueous phase was transferred to a fresh reagent tube, and 1.5 volumes of ethanol were added. RNA purification was performed with RNeasy MinElute spin columns according to the manufacturer's recommendations and was eluted in a final volume of 15 µl of nuclease-free H₂O. RNA was stored in a -80°C freezer until further analysis.

RNA concentration cannot be accurately determined in plasma samples. Accordingly, the input RNA amount for subsequent analysis was based on starting volume rather than RNA quantity. For hsa_circ_0001445, 2 µl of RNA was reverse transcribed in 10 µl using the iScript select cDNA synthesis kit (Bio-Rad). The RT reaction was performed with the following conditions: 5 min at 25°C, 30 min at 42°C, 5 min at 85°C and immediately cooling to 4°C. cDNA was stored at -20°C. For qPCR, cDNA was diluted (1:3 ratio) and 2 µl was amplified in 10 µl qPCR reactions using the iQ™ SYBR® Green Supermix (Bio-Rad) and containing 1:50 diluted ROX (Invitrogen). The NCBI (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and Primer 3 (<http://primer3.ut.ee/>) online tools were used to design the divergent primers. PCR primer sequences are shown in **Supplementary Figure S1**. The detection of the specific sequences was validated by conventional PCR and agarose gel electrophoresis (**Supplementary Figure S1**). Amplified transcripts showed specific bands of the correct size for intracellular and extracellular samples. Melting curve analysis and sequencing of PCR products corroborated the specificity and the presence of the back splicing site (**Supplementary Figure S1**). cel-miR-39-3p was quantified according to the protocol for the miRCURY LNA Universal RT microRNA PCR System (Exiqon). Briefly, 2 µl of RNA was reverse transcribed in 10 µl reactions using the Universal cDNA synthesis kit II (Exiqon). The RT reaction was performed with the following conditions: incubation for 60 min at 42°C, heat inactivation for 5 min at 95°C, and immediate cooling to 4°C. cDNA was stored at -20°C. For

qPCR, cDNA was diluted 40x and 4 µl was used in 10 µl qPCR reactions containing 1:50 diluted ROX (Invitrogen).

Both hsa_circ_0001445 and cel-miR-39-3p were quantified in 384-well PCR Plates (Life Technologies). qPCR was performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems) with the following cycling conditions: 3 min at 95°C and 40 cycles of 15 s at 95°C, 30 s at 60°C and 40 s at 72°C for hsa_circ_0001445, and 10 min at 95°C and 40 cycles of 10 s at 95°C and 1 min at 60°C for cel-miR-39-3p. Both procedures were followed by a melting curve analysis. Negative controls excluding the template from the RT and PCRs and the enzyme mix in the RT reaction were included. A negative control with 1 µg MS2 carrier RNA as the mock template for the RT reaction was also analyzed. The circular transcript was undetectable in controls. SDS v2.3 software was used for both the determination of the quantification cycle (Cq) and for the melting curve analysis. The Cq was defined as the fractional cycle number at which the fluorescence exceeded a given threshold. The specificity of the PCR was checked by the analysis of the melting curves and negative controls. The circRNA was considered to be expressed when Cq values <35. The relative hsa_circ_0001445 expression level was calculated using a standard curve generated by plotting the Cq against the log of the starting quantity of known dilutions. The quantity was calculated from standard dilutions (1:1, 1:5, 1:25 and 1:125) of a random subset of samples (n=10). The Cq value of each sample was used to calculate the expression level in relation to the standard curve. The PCR efficiencies varied between 2.07 and 2.11. The expression of hsa_circ_0001445 was normalized to the expression of cel-miR-39-3p. Natural logarithmic (log) transformations were performed for hsa_circ_0001445 expression levels. Methods for circRNA quantification in human coronary smooth muscle cells (SMCs) and human coronary SMC-derived extracellular vesicles are described in the **Supplemental Material**.

Statistical analysis

The characteristics of the studied population were summarized through standard descriptive statistics. Data are presented as the median (first quartile, Q1–third quartile, Q3)

for continuous variables and as frequency (percentage) for categorical variables. The demographic, clinical and biochemical characteristics were compared across hsa_circ_0001445 tertiles using the Kruskal-Wallis test for continuous variables and Chi-squared test for categorical variables. The same characteristics were compared between SSS groups using the Wilcoxon rank-sum test for continuous variables and Fisher's exact test for categorical variables. Spearman's rho coefficient was used to assess the correlation between continuous variables. The analysis strategy considered the hsa_circ_0001445 results as both continuous (plasma levels of hsa_circ_0001445) and binary (first tertile *versus* second and third tertiles of hsa_circ_0001445 levels) variables. Logistic regression was used to assess the relationship between SSS and hsa_circ_0001445. In model 1, age, sex, body mass index, history of hypertension, dyslipidemia, diabetes mellitus and smoking were included as covariates. To establish whether the associations could be influenced by other confounding factors, model 1 was also adjusted by medication: statin use, antiplatelet therapy, ACE inhibitor use and beta blocker use (model 2). In model 3, model 1 was also adjusted for the clinical indicators hs-TnT and hs-CRP. The results are presented as odds ratios (OR) and 95% confidence intervals (CI). Receiver operating characteristic (ROC) curves were constructed to estimate the C-index. Data are presented as the C-index and 95% CI.

A base clinical model of CAD, including age, sex, body mass index, history of hypertension, dyslipidemia, diabetes mellitus and smoking, was constructed using multivariable logistic regression. The clinical covariates were selected among conventional risk factors and the differences observed between study groups (comparison SSS $\leq$ 5 *versus* SSS $>$ 5). Discrimination accuracy was compared between the base clinical model and the base clinical model with hsa_circ_0001445 using the DeLong test (30). The internal validity of the final models was tested using 500 bootstrap samples using the 'rms' package (31) in the R Project for Statistical Computing. The calibration of the models was assessed using the same package. Reclassification analyses (32); the Integrated Discrimination Improvement

(IDI) and continuous Net Reclassification Improvement (NRI) indexes were implemented to quantify the added value of hsa_circ_0001445.

Classification tree models were developed using a Chi-squared automatic interaction detector (CHAID) algorithm (33), as previously described (34). The CHAID algorithm utilizes statistical significance from Chi-square tests to establish a hierarchy of predictors that best discriminate between study groups, here the variables that composed the base clinical model, hs-TnT, hs-CRP and hsa_circ_0001445. CHAID analysis identifies potential interactions among the predictors and selects the optimal combination of variables and cutoff values for classification. Classification accuracy was evaluated using the average variance of the terminal nodes (impurity).

The two-tailed significance level was set at <0.05. The statistical software package R version 3.5.2 (www.r-project.org) was used for statistical analyses.

RESULTS

Plasma levels of the circular RNA hsa_circ_0001445 are lower in patients with higher coronary atherosclerotic burden

The demographic, clinical and biochemical data as a function of plasma hsa_circ_0001445 tertiles are detailed in **Table 1**. Patients in the lowest tertile of hsa_circ_0001445 levels showed higher coronary atherosclerotic extension and severity, as determined by the higher percentage of patients with SSS>5 (first tertile=50%, second tertile=32.8%, third tertile=28.4%, $p=0.024$). A trend towards higher coronary atherosclerotic burden was also observed for other coronary atherosclerosis plaque scores. The group of patients in the highest tertile of hsa_circ_0001445 levels showed a higher percentage of former and current smokers (first tertile=19.7%, second tertile=34.3%, third tertile=46.3%, $p=0.002$). hsa_circ_0001445 levels were not correlated with hs-TnT or hs-CRP concentrations (**Supplemental Table S1**).

The characteristics of the study population according to SSS groups ($SSS \leq 5$ versus $SSS > 5$) are shown in **Table 2**. As expected, the patients with $SSS > 5$ were typically older, more likely to be male, had a substantial burden of cardiovascular risk factors and showed a worse profile of coronary atherosclerosis scores than the patients with $SSS \leq 5$. The use of cardiovascular medications was also common in this group of patients. Plasma levels of hsa_circ_0001445 were lower in patients with $SSS > 5$ compared to patients with $SSS \leq 5$. Consequently, the percentage of patients in the first tertile of hsa_circ_0001445 levels was higher in patients with $SSS > 5$.

In multivariable analysis, plasma levels of hsa_circ_0001445 were inversely associated with SSS, even after adjusting for the impact of cardiovascular risk factors, medication use and the biochemical indicators hs-TnT and hs-CRP (**Table 3**). An independent association between the first tertile of hsa_circ_0001445 levels and $SSS > 5$ was also observed after adjusting for all covariates.

Performance of circular RNA hsa_circ_0001445 as a biomarker of stable coronary artery disease

ROC curves and the C-index were used to assess the discriminative accuracy of plasma hsa_circ_0001445. The C-indices for discriminating $SSS \leq 5$ from $SSS > 5$ were low-moderate: C-index (95% CI)=0.589 (0.506-0.671) for hsa_circ_0001445 as a continuous variable and C-index=0.592 (0.509-0.675) for hsa_circ_0001445 as a binary variable (**Supplemental Figure S2**).

To further explore the possible role of plasma hsa_circ_0001445 as a biomarker of CAD, we evaluated the effect of adding circRNA to a multiparameter clinical model including the following cardiovascular risk factors: age, sex, body mass index, history of hypertension, dyslipidemia, diabetes mellitus and smoking (**Table 4**). The addition of hsa_circ_0001445 to the base model allowed a significant reclassification of the patients with IDI (95% CI)=0.047 (0.016-0.077) and NRI (95% CI)=0.482 (0.198-0.766) for hsa_circ_0001445 as a continuous variable and IDI (95% CI)=0.056 (0.022-0.091) and NRI (95% CI)=0.373 (0.092-0.655) for

hsa_circ_0001445 as a binary variable. The C-indices for the models including hsa_circ_0001445 (as continuous or binary predictor) tended to be higher than for the clinical model alone, with C-index (95% CI) for the base model=0.833 (0.775-0.892), C-index (95% CI) including hsa_circ_0001445 as a continuous variable=0.856 (0.800-0.912) and C-index (95% CI) including hsa_circ_0001445 as a binary variable= 0.855 (0.800-0.910). Bootstrap internal validation supported the robustness of the models that included the circRNA **(Supplemental Figure S3)**. Adding hs-TnT or hs-CRP did not improve the discrimination of the clinical model or allow reclassification of the patients **(Table 4)**.

Classification tree models were constructed including clinical data, established biomarkers and hsa_circ_0001445. As shown in **Supplemental Figure S4**, the classification tree model used plasma hsa_circ_0001445 for classification. For females older than 63 years, being in the second or third tertile of plasma hsa_circ_0001445 levels allowed the enrichment of patients with SSS≤5 from 67.3% to 77.8%, and being in the first tertile allowed the enrichment of patients with SSS>5 from 32.7% to 55.2%. This group was not divided if we did not include the circRNA. Nevertheless, the new nodes generated using the circRNA did not substantially improve the classification accuracy with impurity for clinical variables=0.36 *versus* impurity for clinical variables and plasma hsa_circ_0001445=0.34.

Stability of circRNAs hsa_circ_0001445 in plasma samples

A prerequisite for circulating biomarkers is their stability. To this end, the levels of hsa_circ_0001445 were evaluated in plasma samples under different experimental conditions **(Figure 1)**. Storage at room temperature or 4°C did not alter hsa_circ_0001445 levels for up to 24 hours **(Figure 1A & 1B)**. At room temperature, minimal effects were observed at 48 hours and 72 hours. At 4°C, levels of hsa_circ_0001445 remained relatively stable for up to 7 days after blood collection. Serial freeze/thaw cycles (up to 8 cycles) did not significantly affect hsa_circ_0001445 levels **(Figure 1C)**. Hemolysis caused a negligible increase in circRNA levels **(Figure 1D)**.

The circular RNA hsa_circ_0001445 is expressed in human coronary smooth muscle cells, and its secretion is reduced in atherosclerotic conditions

To explore the potential mechanism implicated in the hsa_circ_0001445 downregulation observed in patients with higher coronary atherosclerotic burden, we used an *in vitro* model previously validated for the evaluation of ncRNAs as biomarkers of coronary atherosclerosis (35). hsa_circ_0001445 levels were assessed in human coronary SMCs and extracellular vesicles secreted by human coronary SMCs under atherogenic lipoprotein stimulation to mimic the atherosclerotic environment in the vascular wall. hsa_circ_0001445 was detected in the supernatant, indicating that this circRNA is secreted by coronary SMCs. Under stimulation with nLDL or agLDL, decreased levels of hsa_circ_0001445 were detected in extracellular vesicles (**Figure 2A**). The intracellular expression levels were not affected by nLDL incubation (**Figure 2B**). Exposure to agLDL caused a downregulation of intracellular hsa_circ_0001445 levels.

DISCUSSION

Using a real-world clinical scenario, the present study suggests the circRNA hsa_circ_0001445 as a blood-based biomarker of stable CAD in patients with clinical suspicion of the disease.

The main findings of this study can be summarized as follows. Patients with suspected stable CAD in the lower tertile of the distribution of plasma hsa_circ_0001445 levels showed higher coronary atherosclerosis extension and severity. hsa_circ_0001445 was inversely proportional to coronary atherosclerotic burden, even after accounting for confounding factors. In the ROC analysis, hsa_circ_0001445 evaluated separately had poor discriminative power. Nonetheless, a detailed analysis demonstrated that hsa_circ_0001445 correctly reclassified the patients misclassified by a multiparameter model of stable CAD exclusively based on clinical history and cardiovascular risk factors. A trend towards a higher

discrimination capacity was also observed when hsa_circ_0001445 was added to the clinical model. Finally, hsa_circ_0001445 showed remarkable stability in clinical specimens.

To our knowledge, this study is the first clinical evaluation of a circulating circRNA as a diagnostic biomarker of stable CAD. Despite the poor performance of plasma hsa_circ_0001445 when evaluated separately, due to the overlap between patients with stable CAD and patients with suspicion of the disease but nonsignificant CAD, plasma hsa_circ_0001445 provided additional information beyond conventional risk factors in terms of reclassification of patients into more appropriate diagnostic groups. The association between hsa_circ_0001445 and coronary atherosclerotic burden persisted even after multivariable adjustment that included clinical history and established biomarkers. The information provided by the circRNA may complement rather than replace the available data in the health record. hsa_circ_0001445 levels likely reflect other mechanisms associated with the disease not accounted for by cardiovascular risk factors. Additionally, the performance as a biomarker seemed to be higher than for contemporaneous test. Although hs-TnT provided higher independent information, its incremental value in terms of discrimination and reclassification was lower than that observed for hsa_circ_0001445. The comparison with previous studies on circRNAs and CAD is limited by the differences in the experimental designs and study populations. Nonetheless, these findings support the notion that circRNAs could be used for the assessment of cardiovascular disease (36) and coronary atherosclerotic disease (15, 37, 38).

This study provides a rationale for further investigations of circRNA quantification in the evaluation of suspected stable CAD. Similar to what has been observed for other ncRNAs (39), our results suggest a substantial opportunity to improve clinical assessment and guide clinical decision-making using a model based on readily available clinical data and circRNAs. SSS has been proposed as a useful tool for risk evaluation in patients with suspected CAD (24, 27). The combination of hsa_circ_0001445 with clinical factors may therefore assist in the stratification of patients according to their coronary atherosclerotic burden, which ultimately could guide management based on cardiovascular risk (e.g.,

recommendation of secondary prevention measures or consideration of invasive and expensive interventions). The inclusion of hsa_circ_0001445 in triage strategies also constitutes an interesting strategy. The measurement of hsa_circ_0001445 may potentially have utility in deciding whether imaging, such as CTA, is suitable for a definite diagnosis in patients with suspected stable CAD. Since the major drawbacks of this technique are the availability of specialized centers and personnel, the exposure to ionizing radiation and the high cost (40), the implementation of a noninvasive test based on circRNAs could be useful. The small number of variables included in the model and the easy access to samples for hsa_circ_0001445 analysis could facilitate its implementation in routine care.

Exonic circRNAs, such as hsa_circ_0001445, are long-lived transcripts under intracellular conditions with transcripts exhibiting a half-life over 48 hours (41). However, the stability of circRNAs in biological fluids is a matter of debate (16). Li et al. (20) reported that incubation of serum at room temperature for up to 24 hours had minimal effect on circRNA levels. In contrast, other authors have proposed that exonic circRNAs are rapidly degraded in serum, with a half-life of seconds, due to the presence of circulating endonucleases (42, 43). Here, the results of our detailed testing indicated a long life of hsa_circ_0001445 in plasma samples, even after prolonged storage at room temperature or 4°C or repetitive freeze/thaw cycles. Therefore, hsa_circ_0001445 has remarkable stability in standard clinical samples. Furthermore, the levels of hsa_circ_0001445 were not significantly affected by hemolysis. These data reinforce the potential of hsa_circ_0001445 for implementation in clinical laboratories.

hsa_circ_0001445 is a circRNA highly enriched in the human myocardium (18) and left ventricle (17). Considering the elevation of troponin peptides in the bloodstream (44), we expected that events such as myocardial ischemia or subclinical myocardial necrosis would cause an increase in hsa_circ_0001445 in those patients with higher coronary atherosclerotic burden (45). Strikingly, we showed a robust and inverse association between plasma hsa_circ_0001445 and the extent and severity of coronary atherosclerosis. Furthermore, no correlation was observed between hs-TnT and hsa_circ_0001445. It seems

that the myocardium is not implicated, or is slightly implicated, in the alteration of the circulating hsa_circ_0001445 pool. Previous data from our group demonstrated a decrease in the miRNA content of extracellular vesicles secreted by an *in vitro* model of human coronary SMC in response to atherogenic conditions (35). These results have been reproduced in the plasma of patients in different population-based studies (23, 35). Thus, we tested whether hsa_circ_0001445 may have a similar behavior in coronary atherosclerotic conditions. Supporting our previous findings, an atherogenic environment promoted the unloading of hsa_circ_0001445 into the extracellular vesicles secreted by coronary SMCs, the main cellular type in the vascular wall and a key player in coronary atherosclerosis development (46). These results are noteworthy since a biomarker of CAD should reflect the pathological processes occurring in the coronary artery wall. At this point, hsa_circ_0001445 may therefore be considered a reported biomarker of CAD.

The strengths of the study are the use of a real-world setting, the rigorous control of covariates, the evaluation of the circRNA in conjunction with electronic health data and contemporaneous tests and the use of CTA. The inclusion of consecutive patients who underwent a coronary CTA exam avoided selection bias and ensured representability. Contrary to the case-control approach, which could overestimate the potential of novel biomarkers due to the comparison of extreme cases and healthy controls, here we included a cohort of patients with suspected stable CAD that has been referred for further evaluation using imaging techniques. The comparison of biomarker performance with clinical variables and established measurements is also fundamental to obtaining robust evidence for the clinical applicability of hsa_circ_0001445. In addition, CTA is a well-established technique proposed by clinical guidelines to determine the presence of coronary atherosclerotic burden in patients with stable CAD (3, 47). Some limitations of the study merit consideration. First, we used a single-center study design without conducting external validation. Although internal validation was performed, the incremental value of hsa_circ_0001445 warrants evaluation in independent cohorts. Second, the incorporation of circRNAs in clinical laboratories is hampered by the fact that research technologies are required for circRNA

quantification. Third, possible confounders cannot be ruled out despite adjustment (48, 49). Fourth, our results reflect an association. The role of hsa_circ_0001445 in the causal pathway of the disease cannot be inferred from our study design, although this knowledge is not prerequisite for a biomarker. Finally, the contribution of other cells or tissues to the circulating pool of hsa_circ_0001445 cannot be discarded.

In conclusion, in patients with suspected stable CAD referred for a coronary CTA, the circRNA hsa_circ_0001445 provides additional useful information over available clinical data and has potential as a predictor of coronary atherosclerotic burden. Plasma hsa_circ_0001445 constitutes a novel biomarker of stable CAD.

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DISCLOSURES

DdGC and VLI-C have licensed a patent on microRNAs as biomarkers (PCT/ES2018/070697). TT has filed patents in the field of noncoding RNAs and is founder and holds shares of Cardior Pharmaceuticals GmbH. The other authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1. Stability of plasma hsa_circ_0001445. A) CircRNA levels after sample storage at room temperature for the indicated time. B) CircRNA levels after sample storage at 4°C for the indicated time. C) CircRNA levels after the indicated number of repetitive freeze/thaw cycles. D) Effect of hemolysis on circRNA levels. hsa_circ_0001445 levels were measured by qPCR, calculated from standard dilutions (1:1, 1:5, 1:25 and 1:125) of a random subset of samples (n=10) and normalized to cel-miR-39-3p. The results are expressed as a relative to the control in each experiment (n=5). The results are shown as the mean (standard deviation).

Figure 2. hsa_circ_0001445 in human coronary SMCs and human coronary SMC-derived extracellular vesicles. Effect of exposure to atherogenic lipoproteins on extracellular vesicle-derived (A) and cellular (B) hsa_circ_0001445. Human coronary SMCs were incubated for 24 hours in the absence or presence of nLDL or agLDL (100 µg/mL). hsa_circ_0001445 levels were measured by qPCR and calculated from standard dilutions

807 (1:1, 1:5, 1:25 and 1:125) of a random subset of samples (n=10). For extracellular vesicle
808 experiments, cel-miR-39-3p was used for normalization. For coronary SMC experiments,
809 SNORD48 was used for normalization. The results are expressed relative to the control in
810 each experiment (n=6). Between-group differences were analyzed using the Wilcoxon rank-
811 sum test. P values describe the significance level. The results are shown as the mean
812 (standard deviation). agLDL: aggregated LDL; EV: extracellular vesicles, nLDL: native LDL,
813 SMC: smooth muscle cells.

Table 1. Characteristics of the patients stratified by tertiles of plasma hsa_circ_0001445 level.

	Tertile 1 (n=66)	Tertile 2 (n=67)	Tertile 3 (n=67)	p-value
Clinical Characteristics				
Age (years), median [Q1-Q3]	67.5 [58.0-72.3]	67.0 [58.0-76.0]	66 [57.0-73.0]	0.529
Male, n (%)	38 (57.6)	43 (64.2)	32 (47.8)	0.156
Body mass index (Kg/m ²), median [Q1-Q3]	26.8 [25.0-30.3]	27.4 [24.8-29.4]	26.0 [24.2-31.3]	0.813
Medical history of hypertension, n (%)	38 (57.6)	49 (73.1)	39 (58.2)	0.108
Medical history of dyslipidemia, n (%)	38 (57.6)	37 (55.2)	37 (55.2)	0.952
Medical history of diabetes mellitus, n (%)	14 (21.2)	12 (17.9)	17 (25.4)	0.574
Former or current smoker, n (%)	13 (19.7)	23 (34.3)	31 (46.3)	0.002
Glomerular filtration rate < 60 mL/min/1.73 m ² , n (%)	10 (15.2)	7 (10.4)	6 (9.0)	0.506
Medications				
Statins, n (%)	35 (53.0)	35 (52.2)	31 (46.3)	0.803
Antiplatelet therapy, n (%)	34 (51.5)	22 (32.8)	30 (44.8)	0.103
Angiotensin-converting-enzyme inhibitors, n (%)	34 (51.5)	43 (64.2)	30 (44.8)	0.071
Beta blockers, n (%)	21 (31.8)	21 (31.3)	26 (38.8)	0.521
Diuretics, n (%)	18 (27.3)	21 (31.3)	16 (23.9)	0.640
Biomarkers				
hs-TnT (ng/L), median [Q1-Q3]	12.2 [6.4-20.6]	10.5 [7.1-18.5]	11.5 [7.0-19.1]	0.972
hs-CRP (mg/L), median [Q1-Q3]	4.4 [3.3-5.7]	3.6 [2.9-5.6]	4.2 [3.3-5.2]	0.338
Coronary artery disease				
Presence of stenosis ≥ 50%, n (%)	23 (34.8)	17 (25.4)	16 (23.9)	0.312
SIS, median [Q1-Q3]	4.0 [0.8-8.0]	3.0 [1.0-7.0]	2.0 [0.0-6.0]	0.096
SIS > 5, n (%)	27 (40.9)	21 (31.3)	19 (28.4)	0.278
SSS, median [Q1-Q3]	5.0 [0.8-9.0]	3.0 [1.0-7.0]	2.0 [0.0-6.0]	0.130
SSS > 5, n (%)	33 (50.0)	22 (32.8)	19 (28.4)	0.024
3-vessel plaque score, n (%)	28 (42.4)	22 (32.8)	19 (28.4)	0.219
hsa_circ_0001445 (au), median [Q1-Q3]	1.1 [0.9-1.2]	1.7 [1.5-2.1]	2.6 [2.4-2.7]	<0.001

Data are presented as the median [Q1-Q3] for continuous variables and as frequencies (percentages) for categorical variables. Differences between groups were analyzed using Kruskal-Wallis test or Chi-squared test. hs-TnT: High-sensitivity Troponin T, hs-CRP: High-sensitivity C Reactive Protein, SIS: Segment involvement score, SSS: Segment stenosis score, au: arbitrary units.

Table 2. Characteristics of the patients stratified by segment stenosis score (SSS) groups.

	SSS ≤ 5 (n=126)	SSS > 5 (n= 74)	p-value
Clinical Characteristics			
Age (years), median [Q1-Q3]	62.0 [52.0-71.3]	70.5 [65.0-76.3]	<0.001
Male, n (%)	59 (46.8)	54 (73.0)	<0.001
Body mass index (Kg/m ²), median [Q1-Q3]	25.9 [24.2-29.6]	28.7 [25.7-30.9]	0.002
Medical history of hypertension, n (%)	70 (55.6)	56 (75.7)	0.006
Medical history of dyslipidemia, n (%)	60 (47.6)	52 (70.3)	0.002
Medical history of diabetes mellitus, n (%)	18 (14.3)	25 (33.8)	0.002
Former or current smoker, n (%)	33 (26.2)	34 (45.9)	0.003
Glomerular filtration rate < 60 mL/min/1.73 m ² , n (%)	13 (10.3)	10 (13.5)	0.500
Medications			
Statins, n (%)	54 (42.9)	47 (63.5)	0.008
Antiplatelet therapy, n (%)	40 (31.7)	46 (62.2)	<0.001
Angiotensin-converting-enzyme inhibitors, n (%)	55 (43.7)	52 (70.3)	0.001
Beta blockers, n (%)	31 (24.6)	37 (50.0)	<0.001
Diuretics, n (%)	29 (23.0)	26 (35.1)	0.100
Biomarkers			
hs-TnT (ng/L), median [Q1-Q3]	9.6 [6.0-17.4]	15.5 [9.2-22.6]	<0.001
hs-CRP (mg/L), median [Q1-Q3]	1.8 [1.0-3.6]	2.2 [1.0-5.3]	0.224
Coronary artery disease			
Presence of stenosis ≥ 50%, n (%)	7 (5.6)	49 (66.2)	<0.001
SIS, median [Q1-Q3]	0.0 [0.0-3.0]	7.0 [6.0-9.0]	<0.001
SIS > 5, n (%)	1 (0.8)	66 (89.2)	<0.001
SSS, median [Q1-Q3]	0.0 [0.0-3.0]	9.0 [7.0-11.3]	<0.001
SSS > 5, n (%)	0 (0.0)	74 (100.0)	<0.001
3-vessel plaque score, n (%)	5 (4.0)	64 (86.5)	<0.001
hsa_circ_0001445 (au), median [Q1-Q3]	1.9 [1.3-2.4]	1.5 [1.1-2.4]	0.037
hsa_circ_0001445 Tertile 1, n (%)	33 (26.2)	33 (44.6)	0.009

Data are presented as the median [Q1-Q3] for continuous variables and as frequencies (percentages) for categorical variables. Differences between groups were analyzed using Wilcoxon rank-sum test or Fisher's exact test. hs-TnT: High-sensitivity Troponin T, hs-CRP: High-sensitivity C Reactive Protein, SIS: Segment involvement score, SSS: Segment stenosis score, au: arbitrary units.

Table 3. Association of plasma hsa_circ_0001445 with the segment stenosis score (SSS).

	Model 1		Model 2		Model 3	
	OR (95% IC)	p-value	OR (95% IC)	p-value	OR (95% IC)	p-value
hsa_circ_0001445	0.313 (0.212, 0.725)	0.003	0.432 (0.222, 0.839)	0.013	0.400 (0.216, 0.740)	0.003
hsa_circ_0001445 Tertile 1	0.240 (0.105, 0.549)	0.001	0.277 (0.115, 0.670)	0.004	0.245 (0.107, 0.560)	0.001

Model 1 is adjusted for age, sex, body mass index, hypertension, dyslipidemia, diabetes mellitus and smoking; Model 2 includes the components of model 1 and statin use, antiplatelet therapy, angiotensin-converting-enzyme inhibitor use and beta blocker use; Model 3 includes the components of model 1 and hs-TnT and hs-CRP. OR: Odd ratio, 95% CI 95% confidence interval.

Table 4. Performance of plasma hsa_circ_0001445 as a biomarker of coronary atherosclerosis extent and severity (SSS).

	Discrimination		Reclassification			
	C-index (95% CI)	p-value (vs CM)	IDI (95% CI)	p-value	NRI (95% CI)	p-value
Clinical Model (CM)	0.833 (0.775, 0.892)	NA	Reference model	NA	Reference model	NA
CM + hs-TnT	0.834 (0.775, 0.892)	0.318	0.0002 (-0.0032, 0.0036)	0.894	0.111 (-0.163, 0.385)	0.429
CM + hs-CRP	0.835 (0.777, 0.893)	0.547	0.005 (-0.004, 0.014)	0.284	-0.139 (-0.381, 0.103)	0.262
CM + hsa_circ_0001445	0.856 (0.800, 0.912)	0.105	0.047 (0.016, 0.077)	0.003	0.482 (0.198, 0.766)	<0.001
CM + hsa_circ_0001445 Tertile 1	0.855 (0.800, 0.910)	0.126	0.056 (0.022, 0.091)	0.002	0.373 (0.092, 0.655)	0.009

Clinical Model (CM): age, sex, body mass index, hypertension, dyslipidemia, diabetes mellitus and smoking. hs-CRP: high-sensitivity C-Reactive Protein, hs-TnT: high-sensitivity Troponin T. 95% CI: 95% confidence interval; IDI: Integrated discrimination improvement index; NRI: Continuous net reclassification improvement index. NA: Not applicable.

Supplemental Table S1. Correlation between hsa_circ_0001445 and established biomarkers.

	Spearman's rho	p-value
hs-TnT	-0.012	0.868
hs-CRP	0.106	0.136

Results are presented using the Spearman's rho. hs-TnT: High-sensitivity Troponin T, hs-CRP: High-sensitivity C Reactive Protein.

FIGURE 1

All Figures

A

B

C

D

FIGURE 2

All Figures

All Figures

SUPPLEMENTAL FIGURE S2

Supplemental Figure S2. C-index for coronary atherosclerosis severity (SSS). 95% CI: 95% confidence interval. hs-CRP: high-sensitivity C-Reactive Protein, hs-TnT: high-sensitivity Troponin T.

All Figures

SUPPLEMENTAL FIGURE S3

Supplemental Figure S3. Calibration plots (with internal validation). Calibration of the base clinical models including hsa_circ_0001445 as continuous variable (A) and binary variable (B). Plots suggested a good calibration.

All Figures

SUPPLEMENTAL FIGURE S4

Supplemental Figure S4. Classification tree model for segment stenosis score (SSS). The classification tree model was calculated by Chi-square Automatic Interaction Detector (CHAID) analysis. The variables considered in the model were: age, sex, body mass index, hypertension, dyslipidemia, diabetes mellitus, smoking, hs-TnT, hs-CRP and hsa_circ_0001445. The results are presented in a tree with several splits based on the selected variables and cutoffs. Numbers indicate the frequencies (percentages) of cases and controls in each node.

SUPPLEMENTAL MATERIAL

Cell culture

Human coronary smooth muscle cells

Primary cultures of human coronary smooth muscle cells (SMCs) were obtained from the media layer of macroscopically healthy coronary artery segments collected from patients undergoing cardiac transplantation at the Hospital de la Santa Creu i Sant Pau (n=6). Coronary SMCs were isolated through a modification of the explant technique, as previously described (1). Cell monolayers were grown in medium M199 (Life Technologies) supplemented with 20% fetal bovine serum (FBS), 2% human serum, 2 mM L-glutamine, 100 U/mL penicillin G and 100 µg/mL streptomycin. Cells used in the experiment were between the fourth and sixth passages. Quiescence was induced by maintaining cells with 0.2% FBS for 24 hours. Coronary SMCs were incubated in the absence or presence of native and aggregated LDL (nLDL and agLDL) in medium deprived of serum (100 µg/mL, 24 hours). Each experiment was performed at the same passage. Human nLDL ($d_{1.019-d_{1.063}}$ g/mL) was obtained from the pooled sera of normocholesterolemic volunteers through sequential ultracentrifugation in a KBr density gradient (1). AgLDL was prepared by vortexing nLDL in PBS at room temperature. The percentage of nLDL (in aggregated form) was calculated by measuring the fraction of protein recovered in the pellet obtained after centrifugation at 10,000 x g for 10 min (2). The extracellular vesicle isolation protocol was performed as previously described by our group (3, 4). The purity and characteristics of the isolated extracellular vesicles were evaluated using transmission electron microscopy, flow cytometric analysis and immunoblotting, as previously described (3, 4).

Primer validation

Cell culture was performed under standard conditions in a CO₂ incubator (5%). Cells were grown in the following media: HEK293T cells in DMEM (Gibco) supplemented with 10% FCS and 1% Pen-Strep; HUVECs in EBM-2 + specific supplements (Lonza)

and 10% FCS; hiPSCs in StemMACS iPS-Brew XF plus supplements; hiPSC-CM in RPMI 1640 Medium, GlutaMAX™ Supplement (Gibco) and with HEPES + B27. hiPSC-CM were generated from hiPSCs by directed cardiac differentiation as described in Lian et al. (5).

Circular RNA quantification (coronary SMCs and coronary SMC-derived extracellular vesicles)

Total RNA extraction was performed using the *miRNeasy Serum/Plasma Kit* (Qiagen) for extracellular vesicles and the *miRNeasy Mini Kit* (Qiagen) for cells. Isolation was performed according to the manufacturer's instructions. The synthetic *Caenorhabditis elegans* miR-39-3p (cel-miR-39-3p) was spiked in vesicle samples. The mixture was also supplemented with 1 µg of MS2 carrier RNA (Roche) as recommended by the manufacturer. For cells, total RNA concentration was determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies).

For hsa_circ_0001445, 2 µl of RNA (extracellular vesicles) or 200 ng (cells) were reverse transcribed using the *iScript Select cDNA Synthesis Kit* (Bio-Rad). The RT reaction was performed with the following conditions: 5 min at 25°C, 30 min at 42°C, 5 min at 85°C and immediate cooling to 4°C. For qPCR, cDNA was diluted (1:3 ratio) and 2 µl was used in 10 µl qPCR reactions with the *iQ™ SYBR® Green Supermix* (Bio-Rad) and 1:50 diluted ROX (Invitrogen). For internal (small nucleolar RNA SNORD8) and external controls (cel-miR-39-3p), cDNA was synthesized using the universal cDNA synthesis kit II (Exiqon). In cellular samples, the RNA concentration was adjusted to 5 ng/µL using nuclease-free water. For vesicles, we used the same starting sample volume (2 µl) rather than RNA quantity (similar to plasma samples). Controls were quantified by RT-qPCR using the *ExiLent SYBR Green master mix* (Exiqon). qPCR was performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems) with the following cycling conditions: 3 min at 95°C, 40 cycles of 15 s at 95°C, 30 s at 60°C and 40 s at 72°C for hsa_circ_0001445, and 10 min at 95°C, 40 cycles of 10 s at 95°C and 1 min at

60°C for controls. The relative hsa_circ_0001445 expression level was calculated using a standard curve generated by plotting the Cq against the log of the starting quantity of known dilutions. The quantity was calculated from standard dilutions (1:1, 1:5, 1:25 and 1:125) of a random subset of samples (n=10). The Cq value of each sample was used to calculate the expression level in relation to the standard curve. PCR efficiencies were between 1.93 and 1.98 for extracellular vesicles and cells, respectively. The expression of hsa_circ_0001445 was normalized to the expression of cel-miR-39-3p (vesicles) or SNORD48 (cells).

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