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ABSTRACT

Left Ventricular Hypertrophy (LVH) is a cardiovascular disease and the identification of biomarkers that could permit the prediction and stratification of patient's risk for developing LHV would be very helpful. A group of small non-coding RNAs, MicroRNAs (miRNAs), have been described as possible rising disease biomarkers and association of miRNAs with risk of LVH have been reported. Despite this, internal reference genes are necessary for measuring miRNA expression by RT-qPCR and their choice can have a serious impact on the expression level quantitation but, so far, there is no consensus on how to normalize miRNAs for analysis of plasma miRNAs in LVH. We evaluated a set of miRNAs as endogenous controls aiming the identification of which of these would be proper stable internal control genes in expression analysis on Essential Hypertension (EH) and LVH blood samples. We evaluated candidate's expression levels in 26 healthy controls, 32 hypertensive patients without LVH and 14 LVH patients using Norm Finder and RefFinder. These analyses pointed MiR-342-3p as the most stable gene and the analysis of the effect of gene normalization on the expression of miR-328-3p

and miR-185-5p, demonstrated that data interpretation could be very affected by the use of different normalization strategies. Our. results demonstrated that miR-342-3p is a good candidate to be used as reference gene for plasma miRNA analysis in LVH.

Keywords: MicroRNA, Left Ventricular Hypertrophy, Essential Hypertension, Circulating MicroRNAs, Endogenous Reference Genes.

RESUMO

A Hipertrofia Ventricular Esquerda (LVH) é uma doença cardiovascular e a identificação de biomarcadores que poderiam permitir a previsão e estratificação do risco do doente para o desenvolvimento do VHL seria muito útil. Um grupo de pequenos RNAs não codificadores, MicroRNAs (miRNAs), foram descritos como possíveis biomarcadores de doença em ascensão e foi relatada a associação de miRNAs com risco de HVL. Apesar disto, são necessários genes de referência internos para medir a expressão de miRNA por RT-qPCR e a sua escolha pode ter um sério impacto na quantificação do nível de expressão, mas, até agora, não há consenso sobre como normalizar os miRNAs para análise de miRNAs plasmáticos em LVH. Avaliámos um conjunto de miRNAs como controlos endógenos com o objetivo de identificar quais destes seriam genes de controlo interno estáveis adequados na análise de expressão em amostras de sangue de Hipertensão Essencial (EH) e LVH. Avaliámos os níveis de expressão dos candidatos em 26 controlos saudáveis, 32 doentes hipertensivos sem LVH e 14 doentes com LVH, utilizando NormFinder e RefFinder. Estas análises apontaram o MiR-342-3p como o gene mais estável e a análise do efeito da normalização genética na expressão de miR-328-3p e miR-185-5p, demonstrou que a interpretação dos dados poderia ser muito afetada pela utilização de diferentes estratégias de normalização. Os nossos resultados demonstraram que miR-342-3p é um bom candidato a ser utilizado como gene de referência para a análise do miRNA plasmático em LVH.

Palavras-chave: MicroRNA, Hipertrofia Ventricular Esquerda, Hipertensão Essencial, MicroRNAs Circulantes, Genes Endógenos de Referência.

1 INTRODUCTION

Cardiovascular diseases (CVDs) are a major cause of global deaths and are a leading and increasing contributor of the global disease burden. In terms of mortality, CVDs are the leading noncommunicable disease (NCD) and claimed 17.5 million lives in 2012 (46% of all NCD deaths), 6 million of which were people under age 70 [1]. Common chronic disease, like Essential hypertension (EH), may also increase the risk of diverse CVDs, for example coronary heart disease, stroke, kidney failure and heart failure, that may have serious consequences for human health [2].

Under pressure overload, conditions as in EH, hypertrophic cardiomyopathy (HCM) develops and when pressure overload persists, maladaptative left ventricular (LV) remodeling can lead to the development of heart failure [3]. Its typical features on cellular level are reactivation of fetal genes, assembly of sarcomeres and increase in cardiomyocyte size [4], but we currently have few information about the molecular events that are involved these distinct forms of cardiac hypertrophy.

Although physiologic hypertrophy indicates an improvement in cardiac function, hypertrophy in response to pathophysiologic stimuli such as hypertension is pathologic and increases the risk of heart failure or sudden death, also being a major risk factor for stroke and coronary artery disease. HCM is characterized by left ventricular hypertrophy (LVH), with predominant involvement of the interventricular septum [5][6] that results from cardiac growth and wall thickening and often yields a substrate for left ventricular diastolic dysfunction [7][8]. LVH is associated with a risk of cardiovascular events 2 to 4 times greater than in patients without cardiac hypertrophy [9], highlighting the importance of early stage screening and diagnosis of cardiac hypertrophy to minimize the impact of CVDs.

Electrocardiography, echocardiography, computed tomography and magnetic resonance imaging are the actual methods for diagnosing cardiac hypertrophy, but their effectiveness can be hindered by low sensitivity and specificity and lengthy diagnostic time, so novel methods for accurate, uncomplicated and early detection of hypertrophy are needed. Incorporating biomarker analysis into risk stratification of people with EH would aid in early diagnosis and guide treatment methods for those with LVH. A biomarker can be described as a characteristic that can be objectively quantified and evaluated as an indicator of normal biological processes, pathogenic processes or response to a therapeutic intervention and the measurement of such biomarkers must be applicable for day-to-day clinical decision making.

Circulating microRNAs (miRNAs) have been cited as good non-invasive biomarkers for evaluation of disease progression and prognosis. MicroRNAs are single stranded, short length (21 to 23 nucleotides) non-coding RNA molecules that play roles in regulating gene expression in plants and animals by binding to the complementary sites of targets mRNA [10][11][12]. Recently, scientists have found the presence of miRNAs in the circulation of humans that developed a pathology and differential expression of circulating miRNAs has been detected in a wide range of pathological conditions including cancer [13][14], diabetes [15][16], neurodegenerative [17] and cardiovascular [18][19][20]diseases. They also play important roles in cardiac hypertrophy and dysfunction [21][22][23]. Their altered expression was described in cardiac hypertrophy by a series of high-throughput [24][25][26] miRNA microarray analyses. They were recently demonstrated in the circulation in a highly stable, cell-free form in body fluids including serum [27][28], plasma [29][30] , saliva [31], urina [32] and milk [33] and these findings have generated a great interest on using extracellular circulating miRNAs as non-invasive biomarkers for molecular diagnostics and prognostics.

Circulating miRNAs can be detected and quantified by different methods, but reverse transcription quantitative polymerase chain reaction (RT-qPCR) is one of the most sensitive.

However, the lack of a consolidated normalization strategy to account for interindividual or intergroup variability is a limitation of some of the reported works. Different reference genes or a set of multiple stable reference genes [34] for normalization were used in the majority of the obtained qPCR results. Typically, housekeeping genes have been used for the normalization of qPCR data because they represent endogenous controls that are affected by the same sources of variability as the target genes. Variations in the amount of starting material, sample collection, sample storage, RNA isolation, RT and PCR efficiency may introduce potential bias and contribute to quantification errors and could affect miRNA use as biomarkers and in their functions determination, so accurate quantification of miRNA levels is necessary. Given these concerns, the development of a solid normalization strategy is critical for quantifying circulating miRNAs expression and the choice of normalization method plays a fundamental role and needs to be strongly evaluated, because it can minimize measurement errors and technical variability in experiment. The mean global expression [35] and exogenous spike-in artificial synthetic oligonucleotide [36] are also described as normalization methods. However, to date, there is no consensus on normalization of circulating miRNAs in RT-qPCR amplification, especially because their expression is highly influenced by the different physiological and pathological conditions, so there is no reference miRNA which can be appropriate for all types of samples and diseases. Thus, researchers have to conduct a careful validation of the endogenous reference genes according to different study populations in order to obtain reliable data regarding miRNA expression [37]. Until date, there is no consensus on reference miRNAs for RT-qPCR analysis of plasma miRNAs in cardiovascular diseases or LVH.

Herein, we investigated candidate miRNA reference genes in hypertensive patients with LVH aiming to find specific miRNAs that could serve as reliable and reproducible endogenous reference for plasma miRNAs quantification in healthy people, hypertensive patients without LVH and LVH patients to promote the further application of miRNA analysis. We previously employed miRNA PCR array to screen plasma miRNAs profiles of patients with LVH, EH and healthy subjects [18]. After an accurate selection, we screened 10 equally expressed miRNAs and select 6 as endogenous reference genes candidates (MiR-320a, MiR-133, MiR-27, MiR-99a, MiR-342 and RNU6B) for large sample validation in 62 patients to validate the most expressed endogenous reference genes to normalize RT-qPCR data. Then, the expression stability of 3 candidate genes was assessed using two statistical tools: RefFinder [38] and NormFinder [39]. Finally, we tested the effect of using different combinations of reference genes on target miRNA expression.

2 MATERIALS AND METHODS

2.1 PATIENTS AND CONTROL SUBJECTS

As previously reported, 14 hypertensive patients with LVH aged 55–60 years (average 61.6±11.2years), 32 hypertensive patients without LVH aged 37–65 years (average 51±12 years) and 26 healthy subjects aged 41–55 years (average 48.7±5.6 years) were recruited. Patients with a high age range were included in all the three groups of the study, to minimize age influence on microRNA expression. All patients with LVH had previous diagnosis of hypertension, all hypertensive patients were under treatment and had blood pressure control in the last six months. The medications used for hypertension and LVH treatment are shown in table 1. All echocardiographic studies were performed using a commercial ultrasound machine (HD 11, Philips, Andover, Massachusetts). LV end-diastolic and -systolic diameters were measured and indexed to the body surface area, according to American Society of Echocardiography guidelines. As an additional simple estimate of LVH, the maximal wall thickness (MWT) measured at any level in the LV wall was also considered. LVH was defined by left ventricular mass indexed to body surface area > 125 g/m^2 in men and > 110 g/m^2 in women. Patients were classified as hypertensive according to WHO (World Health Organization) criteria, [40] [41] when presenting a systolic blood pressure (SBP) of 140 mmHg or more, or a diastolic blood pressure (DBP) of 90 mmHg or more.

LVH, hypertensive and control subjects had no other concomitant diseases, including another form of hypertension in addition to essential hypertension, body mass index greater than 35 kg/m^2, cancer, heart valve disease, acute coronary artery disease or acute myocardial infarction, Chagas disease, bundle branch block and ventricular pre excitation syndromes. The institutional Ethics and Clinical Research Committee of the Santa Cruz State University approved the study and all patients gave written informed consent.

Table 1. Clinical characteristics of the patients.

Characteristics	Control Subjects	Hypertensive subjects	Left ventricular Hypertrophy subjects	P value
n	26	32	14	
Age (years)	48.7±5.6	51±12	61.6±11.2	0.13
SBP (mmHg)	112.2±8.3	129.6±7.4	128.3±7.5	0.0002*
DBP (mmHg)	73.3±7	84±5.1	86.6±5.1	0.0004
LV mass index (g/m^2)	50,6±17.4	58.8±15	126±76	<0.0001*
LV Wall Thickness (mm)	9.33±0.57	8.88±0.83	13.0±1.88	0.0002*
LV Dimension (mm)	31.3±4.6	32.2±2.8	38.2±3.11	0.01*
Ejection Fraction (%)	74.6±5.5	71.2±6.9	69.3±5.6	0.5
Glucose (mg/dL)	79.3±13	88.2±11.4	88.6±5.2	0.14
Creatinine (Mg/dL)	0.75±0.14	±0.15	0.80±0.2	0.23
Uric acid (MG/dL)	4.26±1.8	4.27±0.5	3.9±0.7	0.83
Current Smoker	2	2	3	
Medication				
ACEI/ARBs (%)		75%	100%	
B-blockers (%)		25%	20%	
Diuretic (%)		25%	25%	*

*P<0.05
SBP: systolic blood pressure; DBP: diastolic blood pressure; LV: left ventricular; ACEI: angiotensin converting enzyme inhibitor; ARB: angiotensin receptor blocker.

2.2 BLOOD SAMPLES, RNA ISOLATION AND CDNA SYNTHESIS

Venous blood samples (5mL) were collected from each donor in BD vacutainers containing 10 mg dipotassium EDTA anticoagulant and processed within one hour. Separation of the plasma was accomplished by centrifugation at 800 g for 10 min at room temperature to remove cell debris. Supernatant plasma was recovered and those plasma samples with pink/red discolouration were considered haemolysed and were excluded. Plasma sample of 300 µL was mixed with TrizolLS (Invitrogen) with a ratio of 1:3 in 1.5 mL microcentrifuge tube and incubated at room temperature for 5 minutes. Then, 250 µL of chloroform was added and mixed vigorously by vortex. The mixture was incubated at room temperature for 15 min and centrifuged at 12000 rpm for 20 min at 4°C. The supernatant (approximately 400 µL) was transferred to a microcentrifuge tube. Subsequently, the RNA was precipitated by adding 800 µL isopropanol to the aqueous phase. After being incubated at -80°C for 12 hours and centrifuged at 12000 rpm for 20 minutes at 4°C, the RNA pellet was rinsed, air-dried and resuspended in 20 µL RNAase-free water. RNA concentrations were determined with a NanoDrop 1000 (Thermo Scientific). Only RNA samples with a 260/280 ratio of ≥1.8 were included. Total RNA (500 ηg) was reverse transcribed using miR-specific primers and Taqman miRNA Reverse Transcription Kit (Applied Biosystem) in a scaled down volume of 15 µL RT reaction, according to the manufacturer's instructions. The

thermal cycling parameters of reverse transcription were 30 min at 16°C, 30 min at 42°C and 5 min at 85°C. The cDNA samples were diluted in nuclease-free water and stored at -20°C.

2.3 QUANTITATIVE REAL-TIME PCR

Expression levels of individual miRNAs were detected by subsequent RT-qPCR using Taqman MicroRNA assays (Applied Biosystems) and a QuantStudio3 Instrument (ThermoFisher Scientific) using standard thermal cycling conditions in accordance with manufacturer recommendations. RT-qPCR amplification mixtures contained 20 ηg template cDNA, 10 µL Taqman master mix (Applied Biosystems) and probes for RNU6B (assay ID: 001093), MiR-99a (assay ID: 000435), MiR-27b (assay ID: 000409), MiR-342-3p (assay ID: 002260), MiR-320a (assay ID: 002277) and MiR-133 (assay ID: 002246) in a final volume of 20 µL. The PCR protocol was applied as follows: incubation for 10 min at 95°C, followed by 40 cycles of 10 s at 95°C and 1 min at 60 °C. The Ct values for RT-qPCR were determined using the QuantStudio™ Design & Analysis Software (Applied Biosystems) and the single-threshold method. PCR reactions were performed in a triplicate and experiments with coefficients of variation greater than 5% or that displayed unusual amplification curves were excluded from further analysis. A no-template control (NTC) and no reverse transcription controls (No-RT) were also included.

2.4 SELECTION OF CANDIDATE GENE AND STABILITY ANALYSIS

A two test was designed to select and validate reference miRNAs for LVH with greater accuracy. In the initial screening, the expression of 6 miRNAs (MiR-320a, MiR-133, MiR-27, MiR-99a, MiR-342 and RNU6B) was detected in plasma samples of 10 subjects of each group. Then, miRNAs without significant difference between LVH, HAS and healthy group (fold change <1) were selected for validation in all collected samples, as most suitable candidate reference genes in LVH. Their stability was analyzed by Reffinder [38] and NormFinder [39] softwares.

NormFinder is a Microsoft Excel-based application that uses an algorithm for identifying the optimal normalization gene, accounting for intra- and inter-group variation. Exponentially transformed data (2^{-Ct} value) were used as input data in the NormFinder software. The lower stability value, the higher stability of the candidate.

The second tool used in this study is the online-based tool RefFinder (http://leonxie.esy.es/RefFinder/). It comprises four different commonly used normalization tools, namely Bestkeeper[41], comparative Delta Ct [42], NormFinder [38] and GeNorm [43],

working with different algorithms to evaluate the most stably expressed gene or gene pair of a specific sample set.

Bestkeeper [41] analysis is based on the raw Ct values of each sample and determines the most stably expressed gene based on the Pearson coefficient (r) of the Bestkeeper Index. GeNorm [43] analysis is based on the average pairwise variation of a gene compared with all other genes and evaluates the stability of candidate reference genes. The lowest M value indicates the most stable expression. The overall stability ranking of candidate genes was determined using the geometric mean of the rankings generated from all four analyses.

2.5 EFFECT OF NORMALIZATION

In an independent experiment, we tested the effect of using MiR-342-3p alone and the average of all miRNAs displaying Ct<32 cycles as showed in previous studies [44] on relative expression values using MiR-328-3p (assay ID: 000543) and MiR-185-5p (assay ID: 002271) as targets to verify the effect of candidate reference genes on the accuracy of RT-qPCR results. Fold change of miRNAs were calculated using the $2^{-\Delta CT}$ method. The ANOVA *Analysis* of variance test and post-hoc Tukey test were used to determine statistically significant differences in expression levels between LVH, HAS and control groups. Statistical analysis was performed with GraphPad Prism 5.0 (GraphPad Software). P-values <0.05 were considered statistically significant.

3 RESULTS

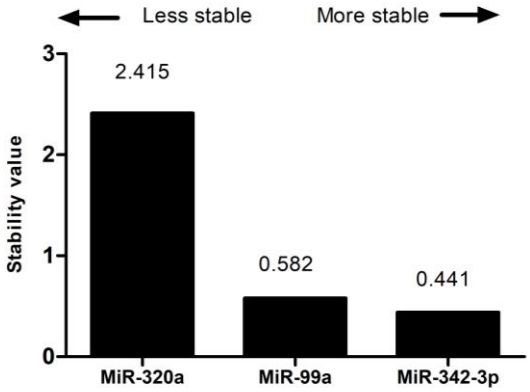
3.1 SELECTION OF CANDIDATE REFERENCE MIRNAS AND STABILITY ANALYSIS

Initially, the expression of 6 miRNAs (MiR-320a, MiR-133, MiR-27, MiR-99a, MiR-342 and RNU6B) was evaluated in plasma samples of 10 subjects of each group. For RNU6B, widely used as a normalizer for intracellular miRNA studies as well as for circulating miRNA studies, miR-27b and miR-133 we could not detect any expression, indicated by raw Ct values ≥39 (data not shown). The expression levels of the three remaining candidate endogenous normalizers (MiR-320a, MiR-99a and MiR-342-3p) were compared in the remaining 16 healthy controls, 22 hypertensive patients withou LVH and 4 LVH patients to validate candidate endogenous reference genes.

For stability analysis, the whole data set of input RNA and inter-run calibrator-normalized Ct-values was analyzed with the NormFinder software to calculate stability values derived from the intra and inter-group variability. This analysis revealed that among the 3 candidate genes, miR-342-3p was the most stable gene (Stability value = 0.441) (Figure 1). The best combination

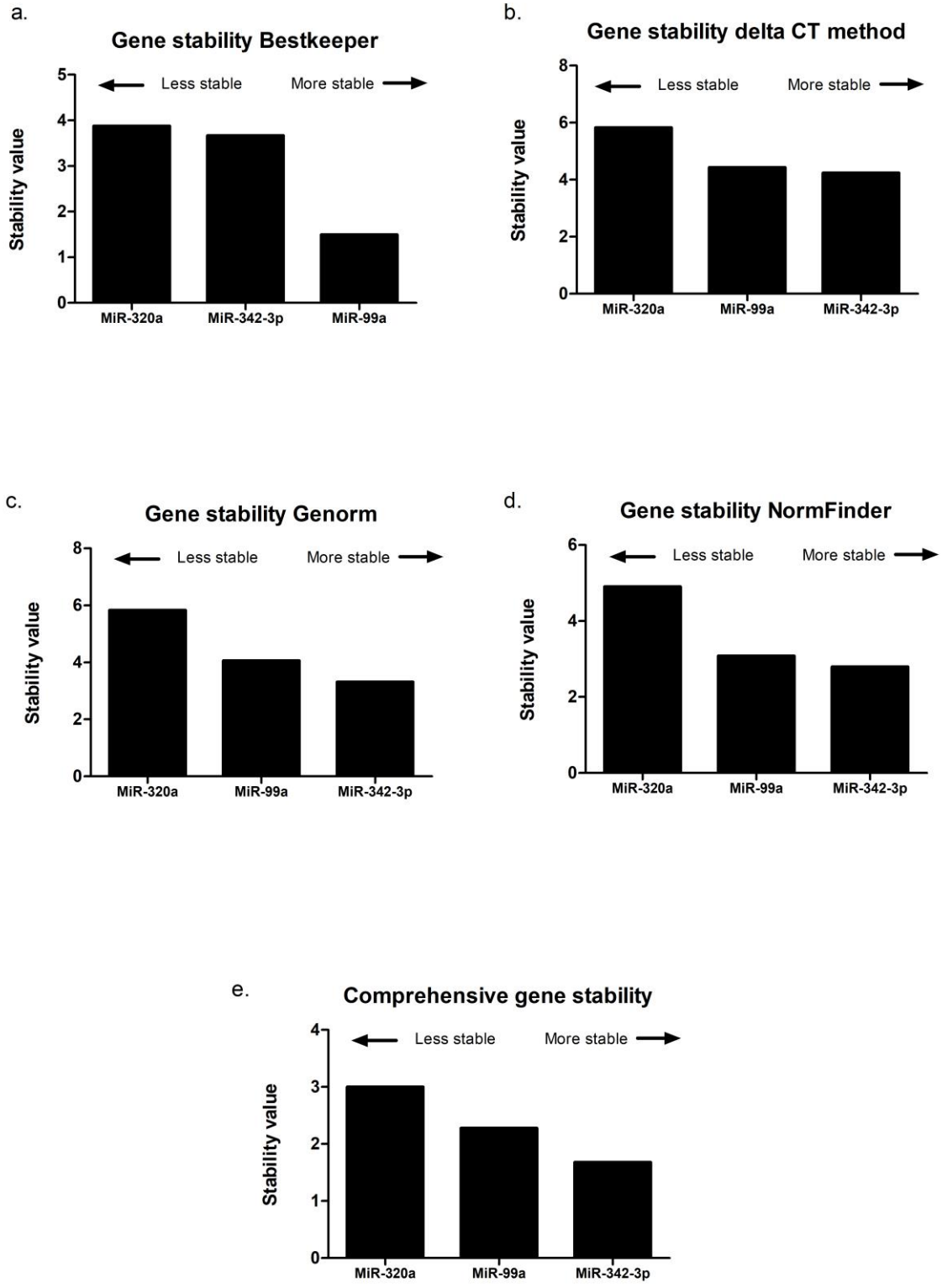
recommended by NormFinder was a set of miR-328-3p and MiR-342-3p (Stability value = 0.953).

Figure 1. NormFinder analysis of the candidate endogenous normalizers showing the expression stability of three candidate endogenous normalizers that was evaluated by the stability values that the algorithm generated by comparing plasma samples from LVH, HAS and healthy control groups: the lower the stability value, the more stable the candidate endogenous normalize. MiR-342-3p is the most stable candidate normalizer within the data set.



Additionally, the data set was analyzed with the online tool RefFinder, that combines the normalization determination algorithms GeNorm, BestKeeper, DeltaCt and NormFinder. BestKeeper analysis identified miR-99a as the most stable normalization candidate in the presented data set (Fig. 2a). MiR-99a was followed by MiR-342-3p as the second stable candidate, which in turn was followed by MiR-320a. The comparative Delta Ct method (Fig. 2b) showed the same results as the Genorm software (Fig. 2c) or the Normfinder analysis (Fig. 2d) that stated miR-342-3p is the most stable normalization candidate, followed by MiR-99a and MiR-320a. The NormFinder results obtained from the software were also confirmed by the online version included in RefFinder. Putting the output of all the algorithms together by calculating the mean rank for each of the 4 candidate endogenous normalizers, MiR-342-3p followed by MiR-99a and MiR-320a were the best (Fig. 2e).

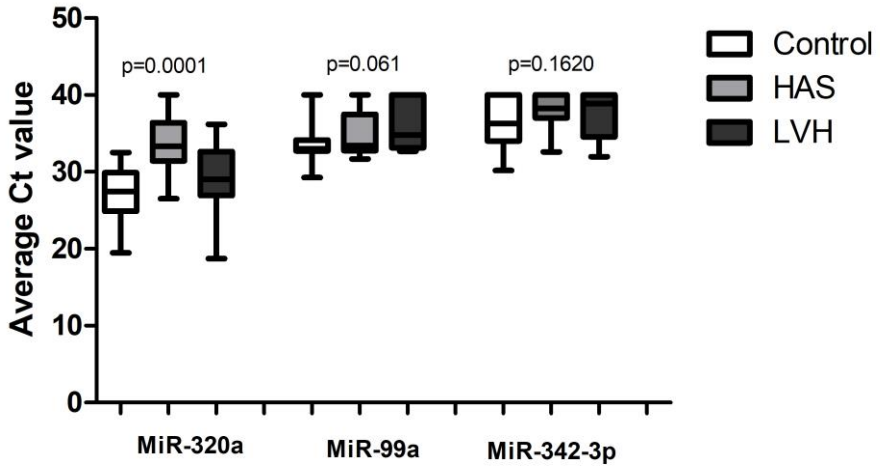
Figure 2. Stability of endogenous reference candidates determined with different algofithms. Stability values were calculated by the online available tools BestKeeper, Delta Ct, Genorm and NormFinder. The lower stability value, the higher stability. Each tool reveals MiR-342-3p as the most stable one as also stated by the comprehensive ranking.



3.2 EXPRESSION DIFFERENCES BETWEEN LVH, HAS AND HEALTHY CONTROL GROUPS

Since it is essential for an endogenous reference gene to be stably expressed in diseased and healthy states, we analyzed our data for differences in mean Ct values between the three groups. No significant differences between LVH, HAS and healthy control groups were observed in the mean expression of MiR-342-3p and MiR-99a (all p-values >0.05), but significant diffence between these groups was observed with respect to the expression of MiR-320a (Fig. 3).

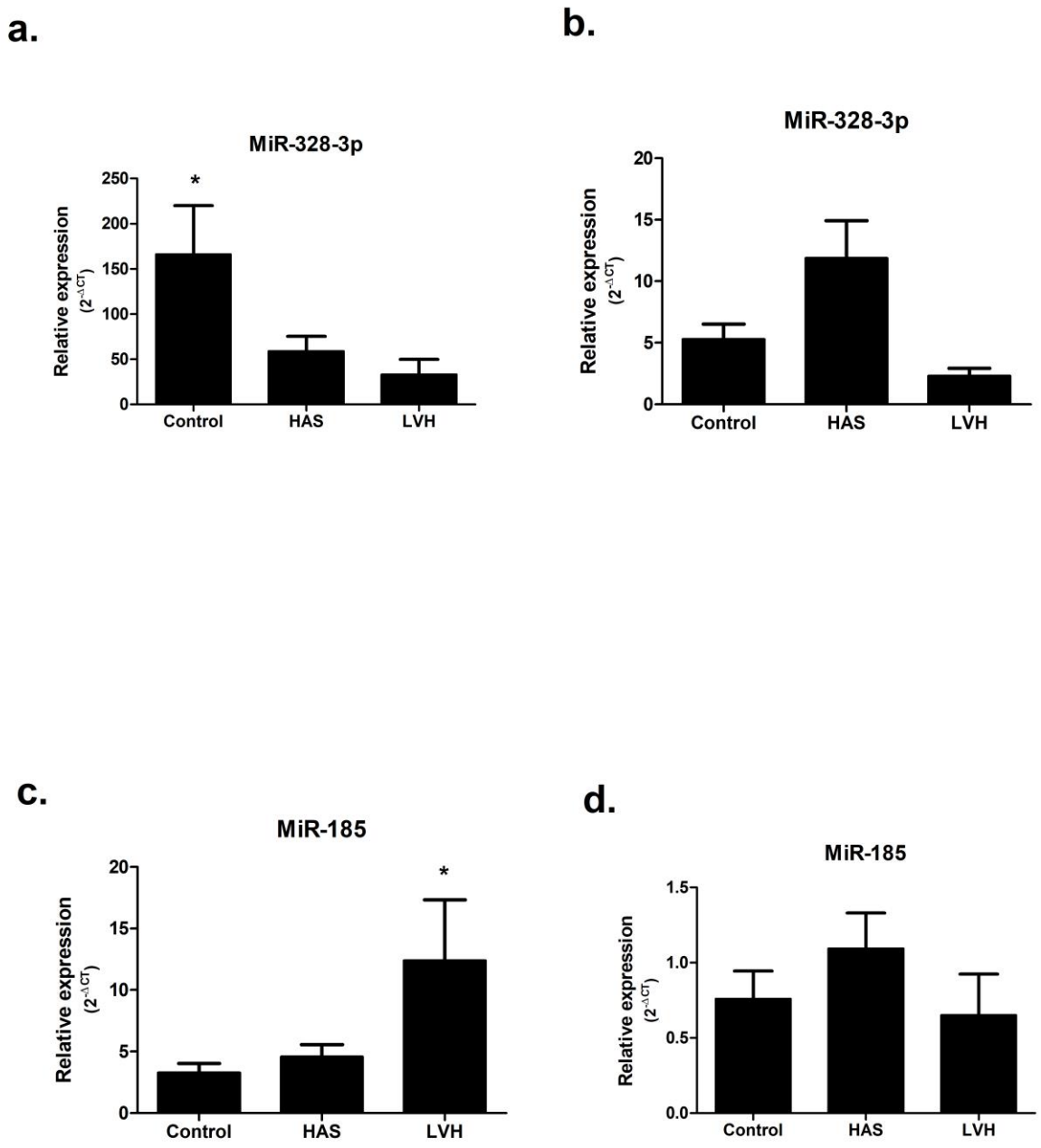
Figure 3. Expression differences of the endogenous reference candidates between LVH, HAS and healthy control patients. The expression levels of three candidate normalizers were compared between plasma of the patients from the three groups.



3.3 COMPARATIVE QUANTIFICATION OF TARGET MIRNA RELATIVE TO REFERENCE ENDOGENOUS CONTROL GENE

For normalization purposes, we tested the effect of using MiR-342-3p alone and the average of all miRNAs displaying Ct<32 cycles as showed in previous studies [45] on relative expression values using MiR-328-3p and MiR-185-5p as targets. Normalizing the expression values of MiR-328-3p to MiR-342-3p, which was shown to be the best reference gene, shows significant downregulation in LVH group (Fig. 4a). However, the expression of MiR-328-3p normalized to the average of all miRNAs displaying Ct<32 revelead no detectable expression difference between LVH, HAS and healthy control groups (Figure 4b). Normalizing the expression values of MiR-185-5p to MiR-342-3p revealed significant upregulation in LVH group (Fig. 4c). However, the expression of MiR-185-5p normalized to the average of all miRNAs displaying Ct<32 revelead no detectable expression difference between LVH, HAS and healthy control groups (Figure 4d). These results demonstrate that different normalization strategies significantly influence the expression level results.

Figure 4. Effect of normalization on the expression of MiR-328-3p and MiR-185-5p in LVH, HAS and healthy control patients, using MiR-342-3p alone (A and C) or the average of all miRNAs displaying Ct<32 cycles (B and D). Relative expression of LVH patients (n = 14) was compared with HAS (n = 32) and healthy control patients (n = 26). The expression levels ($2^{-\Delta Ct}$) of MiR-328-3p and MiR-185-5p are presented as mean fold changes ± standard errors. Significance was calculated by one-way ANOVA test with post-hoc Tukey test. Results with p values <0.05 were considered significant.



4 DISCUSSION

Circulating miRNAs have been described as promising and minimally invasive biomarkers and there are many evidences they play significative roles in physiological and pathological processes, also serving as candidate biomarkers in multiple conditions. qRT-PCR is an important technology and the gold standard method for miRNA quantitation because its sensitivity and reproducibility, but this high sensitivity demands a suitable reference gene to correct the non-biological variation. Data normalization in the analysis of circulating miRNAs has been conducted using various strategies, including the use of miRNA or small RNA controls or external spike-in molecules. The substantial differences found represent a major problem in comparing expression levels between different studies and can possibly bias the ability of identifying differential expression between groups.

The use of an accurate normalization strategy would guarantee better reproducibility and the identification of internal reference miRNAs would empower the feasibility of large-scale assay on selected markers. An ideal endogenous reference gene should be expressed at a similar level across all analyzed samples, exhibiting relatively stable expression levels between samples and groups, besides having no known association with the condition or disease under inspection. Traditionally used endogenous reference genes for tissue/cell miRNAs are no suitable to normalize circulating miRNA levels because the efficiency of their extraction, reverse transcription and PCR amplification are different from circulating miRNAs. Therefore, circulating miRNAs themselves may be the sole suitable reference genes for normalization of circulating miRNAs. However, the reference endogenous circulating miRNAs must be carefully selected and systematically validated to avoid inaccurate normalization. Until date, there is no consensus on reference miRNAs for RT-qPCR analysis of plasma miRNAs in LVH or EH patients.

RNU6 is a universal reference gene in tissue and cell samples. But in our study, we could not detect any expression, indicated by raw Ct values ≥39, confirming recent findings that RNU6 was not stably expressed in plasma and serum samples and it is not a suitable control [28][46].

In this work, we validated that miR-342-3p could be used as a suitable reference in plasma of LVH patients for qRT-PCR, based on the combination of four statistical approaches: BestKeeper, GeNorm, NormFinder and RefFinder, which have been developed to identify optimal endogenous reference genes in a given set of samples. Bestkeeper considers standard deviation and correlation of Ct values to assess stability [41], whereas geNorm uses a pairwise comparison approach and assumes that the expression ratio of ideal endogenous reference genes is identical in all samples, regardless experimental conditions [43]. NormFinder can account for

heterogeneity among samples groups as it estimates intra- and inter-group variability and allows the calculation of the stability values for a set of genes [39]. The overall stability ranking genes was determined using the geometric mean of the rankings generated from all four analyses. The selection of the best endogenous reference gene for gene expression studies in LVH blood samples from those tested was based on the efficiency of the Taqman MicroRNA Assays, the quality of the related expression data and on the expression stability analysis. Using these metrics of efficiency, quality and stability, MiR-342-3p ranks top of the list as the best reference gene in our experimental conditions, but miRNA expression stability must be carefully assessed in each specific experimental setting to avoid erroneous results.

Alternatively, qPCR data for specific miRNAs expression may be normalized following strategies that take into account the total miRNA expression in the samples, proposing the use of the mean expression value of whole miRNAs in a sample to normalize miRNA qPCR data. Zampetaki et al. assessed 300 patients with diabetic retinopathy (DR) in two randomized clinical trials using a candidate microRNA approach, identifying miR-27b as associated with decreased risk of DR, using the average of all miRNAs displaying Ct<32 cycles for normalization purposes [44]. The effect of these different normalization strategies was compared in our study. When data were normalized to MiR-342-3p, miR328-3p showed to be downregulated in LVH patients compared to HAS and control groups. However, when the data were normalized to the average of all miRNAs displaying Ct<32, no significant difference between the three analyzed groups of patients was found. MiR-185-5p showed to be upregulated in LVH patients when normalized to MiR-342-3p, but no expression difference was observed when normalized to the average of all miRNAs. These results have drawn particular attention to the effect of reference genes in normalizing the results, demonstrating the urgent need for identification os suitable reference genes to produce reliable data.

5 CONCLUSION

In summary, our findings constitute the first report describing the rigorous identification and validation of a suitable reference gene for normalization of miRNA in plasma of LVH and EH patients and that this has important implications for proper experimental desing and accurate data interpretation.

DATA AVAILABILITY

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest that are directly relevant to the content of this study.

FUNDING

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REFERENCES

1. Organization. WH (2015) Health in 2015: from MDGs, Millennium Development Goals to SDGs, Sustainable Development Goals

2. Williams L (2003) 2003 World Health Organization (WHO)/ International Society of Hypertension (ISH) statement on management of hypertension World Health Organization , International Society of Hypertension Writing Group. J Hypertens 1983–1992. https://doi.org/10.1097/01.hjh.0000084751.37215.d2

3. Sanchez-ruderisch H, Queirós AM, Fliegner D, et al (2019) Sex-specific regulation of cardiac microRNAs targeting mitochondrial proteins in pressure overload. 1:1–10

4. Bell D, Campbell M, Wang X, et al (2010) Adrenomedullin Gene Delivery is Cardio-protective in a Model of Chronic Nitric Oxide Deficiency Combining Pressure Overload, Oxidative Stress and Cardiomyocyte Hypertrophy. Cell Physiol Biochem 26:383–394. https://doi.org/10.1159/000320562

5. Bos JM, Towbin JA, Ackerman MJ (2009) Diagnostic, Prognostic, and Therapeutic Implications of Genetic Testing for Hypertrophic Cardiomyopathy. J Am Coll Cardiol 54:201–211. https://doi.org/10.1016/j.jacc.2009.02.075

6. Richardson P, McKenna W, Bristow M, et al (1996) Report of the 1995 World Health Organization/International Society and Federation of Cardiology Task Force on the Definition and Classification of cardiomyopathies. Circulation 93:841–2

7. Opie LH, Commerford PJ, Gersh BJ, Pfeffer MA (2006) Controversies in ventricular remodelling. Lancet (London, England) 367:356–67. https://doi.org/10.1016/S0140-6736(06)68074-4

8. Dorn GW, Robbins J, Sugden PH (2003) Phenotyping hypertrophy: eschew obfuscation. Circ Res 92:1171–5. https://doi.org/10.1161/01.RES.0000077012.11088.BC

9. Vakili BA, Okin PM, Devereux RB (2001) Prognostic implications of left ventricular hypertrophy. Am Heart J 141:334–341. https://doi.org/10.1067/mhj.2001.113218

10. Bartel DP (2004) MicroRNAs. Cell 116:281–297. https://doi.org/10.1016/S0092-8674(04)00045-5

11. Ambros V (2004) The functions of animal microRNAs. Nature 431:350–355. https://doi.org/10.1038/nature02871

12. He L, Hannon GJ (2004) MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet 5:522–531. https://doi.org/10.1038/nrg1415

13. McDermott AM, Kerin MJ, Miller N (2013) Identification and validation of miRNAs as endogenous controls for RQ-PCR in blood specimens for breast cancer studies. PLoS One 8:1–11. https://doi.org/10.1371/journal.pone.0083718

14. Niu Y, Wu Y, Huang J, et al (2016) Identification of reference genes for circulating microRNA analysis in colorectal cancer. Sci Rep 6:1–9. https://doi.org/10.1038/srep35611

15. Chien H-Y, Lee T-P, Chen C-Y, et al (2014) Circulating microRNA as a diagnostic marker

in populations with type 2 diabetes mellitus and diabetic complications. J Chinese Med Assoc 78:204–211. https://doi.org/10.1016/j.jcma.2014.11.002

16. Satake E, Pezzolesi MG, Md Dom ZI, et al (2018) Circulating miRNA Profiles Associated With Hyperglycemia in Patients With Type 1 Diabetes. Diabetes 67:1013–1023. https://doi.org/10.2337/db17-1207

17. Serafin A, Foco L, Blankenburg H, et al (2014) Identification of a set of endogenous reference genes for miRNA expression studies in Parkinson's disease blood samples. BMC Res Notes 7:715. https://doi.org/10.1186/1756-0500-7-715

18. Kaneto CM, Nascimento JS, Moreira MCR, et al (2017) MicroRNA profiling identifies miR-7-5p and miR-26b-5p as differentially expressed in hypertensive patients with left ventricular hypertrophy. Brazilian J Med Biol Res 50:. https://doi.org/10.1590/1414-431X20176211

19. Liu Y, Chen S, Zhang J, et al (2015) Analysis of serum MicroRNAs as potential biomarker in coronary bifurcation lesion. Dis Markers 2015:. https://doi.org/10.1155/2015/351015

20. Wang L, Liu Y, Du L, et al (2015) Identification and validation of reference genes for the detection of serum microRNAs by reverse transcription-quantitative polymerase chain reaction in patients with bladder cancer. Mol Med Rep 12:615–622. https://doi.org/10.3892/mmr.2015.3428

21. Chen J-F, Murchison EP, Tang R, et al (2008) Targeted deletion of Dicer in the heart leads to dilated cardiomyopathy and heart failure. Proc Natl Acad Sci 105:2111–2116. https://doi.org/10.1073/pnas.0710228105

22. da Costa Martins PA, Bourajjaj M, Gladka M, et al (2008) Conditional Dicer Gene Deletion in the Postnatal Myocardium Provokes Spontaneous Cardiac Remodeling. Circulation 118:1567–1576. https://doi.org/10.1161/CIRCULATIONAHA.108.769984

23. Rao PK, Toyama Y, Chiang HR, et al (2009) Loss of Cardiac microRNA-Mediated Regulation Leads to Dilated Cardiomyopathy and Heart Failure. Circ Res 105:585–594. https://doi.org/10.1161/CIRCRESAHA.109.200451

24. Sayed D, Hong C, Chen I-Y, et al (2007) MicroRNAs Play an Essential Role in the Development of Cardiac Hypertrophy. Circ Res 100:416–424. https://doi.org/10.1161/01.RES.0000257913.42552.23

25. Thum T, Galuppo P, Wolf C, et al (2007) MicroRNAs in the Human Heart. Circulation 116:258–267. https://doi.org/10.1161/CIRCULATIONAHA.107.687947

26. Zhou S, Liu Y, Prater K, et al (2013) Roles of microRNAs in pressure overload- and ischemia-related myocardial remodeling. Life Sci 93:855–862. https://doi.org/10.1016/j.lfs.2013.08.023

27. Lawrie CH, Gal S, Dunlop HM, et al (2008) Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma. Br J Haematol 141:672–675. https://doi.org/10.1111/j.1365-2141.2008.07077.x

28. Chen X, Ba Y, Ma L, et al (2008) Characterization of microRNAs in serum: A novel class of biomarkers for diagnosis of cancer and other diseases. Cell Res 18:997–1006. https://doi.org/10.1038/cr.2008.282

29. Solayman MHM, Langaee T, Patel A, et al (2016) Identification of Suitable Endogenous Normalizers for qRT-PCR Analysis of Plasma microRNA Expression in Essential Hypertension. Mol Biotechnol 58:179–187. https://doi.org/10.1007/s12033-015-9912-z

30. Mitchell PS, Parkin RK, Kroh EM, et al (2008) Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A 105:10513–8. https://doi.org/10.1073/pnas.0804549105

31. Park NJ, Zhou H, Elashoff D, et al (2009) Salivary microRNA: Discovery, Characterization, and Clinical Utility for Oral Cancer Detection. Clin Cancer Res 15:5473–5477. https://doi.org/10.1158/1078-0432.CCR-09-0736

32. Lange T, Stracke S, Rettig R, et al (2017) Identification of miR-16 as an endogenous reference gene for the normalization of urinary exosomal miRNA expression data from CKD patients. PLoS One 12:e0183435. https://doi.org/10.1371/journal.pone.0183435

33. Floris I, Billard H, Boquien CY, et al (2015) MiRNA analysis by quantitative PCR in preterm human breast milk reveals daily fluctuations of hsa-miR-16-5p. PLoS One 10:1–15. https://doi.org/10.1371/journal.pone.0140488

34. Chen X, Liang H, Guan D, et al (2013) A combination of Let-7d, Let-7g and Let-7i serves as a stable reference for normalization of serum microRNAs. PLoS One 8:. https://doi.org/10.1371/journal.pone.0079652

35. Wylie D, Shelton J, Choudhary A, Adai AT (2011) A novel mean-centering method for normalizing microRNA expression from high-throughput RT-qPCR data. BMC Res Notes 4:555. https://doi.org/10.1186/1756-0500-4-555

36. Roberts TC, Coenen-Stass AML, Wood MJA (2014) Assessment of RT-qPCR Normalization Strategies for Accurate Quantification of Extracellular microRNAs in Murine Serum. PLoS One 9:e89237. https://doi.org/10.1371/journal.pone.0089237

37. Schwarzenbach H, da Silva AM, Calin G, Pantel K (2015) Data Normalization Strategies for MicroRNA Quantification. Clin Chem 61:1333–1342. https://doi.org/10.1373/clinchem.2015.239459

38. Xie F, Xiao P, Chen D, et al (2012) miRDeepFinder: a miRNA analysis tool for deep sequencing of plant small RNAs. Plant Mol Biol 80:75–84. https://doi.org/10.1007/s11103-012-9885-2

39. Andersen CL, Jensen JL, Ørntoft TF (2004) Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res 64:5245–50. https://doi.org/10.1158/0008-5472.CAN-04-0496

40. Williams L (2003) 2003 World Health Organization (WHO)/ International Society of Hypertension (ISH) statement on management of hypertension World Health Organization , International Society of Hypertension Writing Group. 1983–1992. https://doi.org/10.1097/01.hjh.0000084751.37215.d2

41. Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP (2004) Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper--Excel-based tool using pair-wise correlations. Biotechnol Lett 26:509–15

42. Pfaffl MW, Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45. https://doi.org/10.1093/nar/29.9.e45

43. Vandesompele J, De Preter K, Pattyn F, et al (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3:RESEARCH0034

44. Zampetaki A, Willeit P, Burr S, et al (2015) Angiogenic MicroRNAs Linked to Incidence and Progression of Diabetic Retinopathy in Type 1 Diabetes. Diabetes db150389. https://doi.org/10.2337/db15-0389

45. Zampetaki A, Willeit P, Burr S, et al (2015) Angiogenic MicroRNAs Linked to Incidence and Progression of Diabetic Retinopathy in Type 1 Diabetes. Diabetes db150389. https://doi.org/10.2337/db15-0389

46. Zhang Y, Tang W, Peng L, et al (2016) Identification and validation of microRNAs as endogenous controls for quantitative polymerase chain reaction in plasma for stable coronary artery disease. Cardiol J 23:694–703. https://doi.org/10.5603/CJ.2016.0109