Effects of storage time and tissue volume in the yield and quality of RNA, and expression level of RNU6, from formalin-fixed paraffin-embedded prostate cancer tissues

Efectos del tiempo de almacenamiento y el volumen de tejido en rendimiento y calidad del ARN, y nivel de expresión de RNU6, de tejidos con cáncer de próstata fijados con formalina e incluidos en parafina

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ABSTRACT

Background: Molecular analyses of tumor RNA expression have become widely used both for research and clinical purposes. Tumoral tissue preservation is a critical step to ensure accuracy of molecular-based diagnostics, for which, formalin-fixed and paraffin-embedded (FFPE) tissues represent a valuable source of clinical samples. MicroRNAs are ideal biomarkers in FFPE-tissues, in whose expression evaluation RNU6 is one of the genes used as a normalizer. Our aim was to determine, in FFPE tissue samples, the effects of length of storage and corresponding volume of each studied sample, on the RNA retrieval, quality and concentration, as well as their correlation to the expression level of RNU6. **Methods:** Fifty tissue blocks with a mean length of tissue storage of 30 months (SD=±12.07, 95% CI= 27.4-34.3). were included. Total RNA was isolated, absorbance and concentrations were determined and correlated with length of storage and volume of tissue. RT-qPCR for RNU6 was performed and their Ct results were correlated to the same parameters. **Results:** There was a direct correlation between the concentration and quality of the obtained RNA, and an inverse correlation between the tissue storage time and the RNA quality. The volume of tissue studied was not correlated with the RNA quality or concentration. The RNA quality and the length of tissue storage directly correlated to the RNU6 expression level, while RNA concentration and the volume of tissue studied did not affect it. **Conclusions:** There is an association between longer FFPE tissue storage time with lower RNA quality and lower RNU6 expression level.
Keywords: RNA; microRNA; nucleic acids; tissues.

RESUMEN

Introducción: Los análisis moleculares de la expresión tumoral de ARN se usan ampliamente tanto con fines clínicos como de investigación. para ello, los tejidos fijados con formalina e incluidos en parafina (FFPE, por su sigla en inglés) representan una valiosa fuente. Los microARNs son biomarcadores ideales en tejidos FFPE, siendo RNU6 uno de los normalizadores usados en la evaluación de su expresión. Nuestro objetivo fue determinar, en muestras de tejido FFPE, los efectos del tiempo de almacenamiento y el volumen de tejido estudiado, sobre la recuperación, calidad y concentración de ARN, en relación con el nivel de expresión de RNU6. **Métodos:** se incluyeron 50 bloques de tejido con almacenamiento medio de 30 meses (DE=±12.07, IC 95%=27.4-34.3). Se extrajo el ARN total, se determinaron absorbancia y concentración y se correlacionaron con tiempo de almacenamiento y volumen de tejido. Se realizó RT-qPCR para RNU6, sus resultados se correlacionaron con los mismos parámetros. **Resultados:** Hubo correlación directa entre concentración y calidad del ARN, e inversa entre el tiempo de almacenamiento del tejido y la calidad del ARN. El volumen de tejido no se correlacionó con la calidad o concentración del ARN. La calidad del ARN y la duración del almacenamiento de tejido se correlacionaron con el nivel de expresión de RNU6, pero no se vio afectada por la concentración de ARN y el volumen de tejido estudiado. **Conclusiones:** A mayor tiempo de almacenamiento del tejido FFPE se observa menor calidad y concentración del ARN, y menor nivel de expresión de RNU6.
Palabras clave: ARN; microRNA; ácidos nucleicos; tejidos.

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I. INTRODUCTION

The molecular diagnosis of genetic alterations and mRNA, microRNAs or protein expression profile of neoplasms are essential in diagnosis and treatment of cancer patients; some RNA-based gene expression profilings are used to subclassify tumors into gene expression signatures, and have proven to be useful prognostic and predictive biomarkers of response to anticancer therapies; while, microRNA signatures are being investigated as diagnostic and prognostic biomarkers in several tumor types (1). Accuracy and reproducibility of this molecular diagnosis depends on the quantity and quality of the biomolecules obtained from tumoral tissue specimens (2). The techniques used for tissue preservation represent critical steps to ensure a suitable, high-quality and adequate amount of these biomolecules (3,4).

The fixation, used to preserve tissue morphology, has also focused on preserving the tissue molecular integrity (5). The fixator used routinely is formalin at 10%, due to its low cost, fast and complete penetration (4), and excellent conservation of morphology (5,6). The tissues are denominated formalin-fixed and paraffin-embedded (FFPE), given the processing they go through following fixation, that facilitates its conservation at room temperature for long periods of time (7,8). The FFPE tissues represent a valuable source of clinical samples archived in pathology laboratories, they are a useful tool for risk stratification, identification of prognostic markers (8,9), and a highly valuable source of genetic material for molecular analyses both in research and clinical diagnostics (10).

The ideal fixation method should provide a balance between the preservation of tissue morphology, and the quantity and quality of nucleic acids obtained, especially RNA (11), but the active ingredient in formalin, formaldehyde, leads to cross-linking between proteins and nucleotides and causes DNA denaturation (2,12). This can lead to inconsistent results or failures in molecular analysis, resulting from partial degradation or chemical modifications to DNA, RNA, or proteins. Other factors that can affect the quality of nucleic acids are the tissue fixation time and tissue exposure to oxidation, extreme temperatures or light (13). It has been suggested that these alterations are accentuated with prolonged fixation, leading to additional degradation during the storage period (4,5,9).

Results in relation to the RNA degradation during the FFPE tissue storage time have been divergent and RNA obtained from archival FFPE tissue samples can vary widely both within and across studies; previously have been reported that it is possible to extract mRNA from minute FFPE samples but, the quality of the mRNA in these samples significantly decline with increasing sample age (14); while others, reported no correlation between age of the FFPE tissue blocks neither RNA yield or integrity of the extracted RNA, from archival FFPE prostate biopsies (15), and that RNA retrieved from FFPE tissues may be successfully used for molecular analysis (16).

MicroRNAs are ideal biomarkers in FFPE-tissues because, although RNA can potentially be degraded in them, the possibility of development of the described cross-links is greater in longer RNA molecules; thus, they can be obtained more easily and it is possible to evaluate their expression level from these samples (9,17). Considering this and that molecular analyses of RNA expression using FFPE tissue have become widely used, both for research and clinical purposes, the objective of this study was to determine, in FFPE prostatic tissue samples, the effects of length of storage and the volume of tissue studied, on the RNA retrieval, quality and

concentration, as well as, on the expression level of the U6 small nuclear RNA reference gene (RNU6), which is used as a normalizer to evaluate microRNAs expression, as an important step in the molecular diagnosis of the neoplasms.

II. METHODS

2.1. Study design

The study was conducted at the School of Medicine, University of Cartagena, Colombia. A group of FFPE prostate tissue blocks and their data were obtained from the Department of Pathology at the Hospital Universitario del Caribe. The FFPE tissue blocks were manually dissected and cut, total RNA was isolated, the quality and concentration of RNA obtained from FFPE tissues were evaluated in relation to the length of tissue storage and the volume of each studied sample, and, to determine the influence of these parameters in the expression level of RNU6, quantitative real-time-PCR assays were done, and their correlations to these samples Ct of RNU6 were calculated. The assays were conducted at the School of Medicine, University of Cartagena. The study was approved by the local ethics committees, no patient data appear in this article, and no experiments were performed on humans or animals for this study.

2.2 Samples selection

Fifty FFPE tissue blocks were selected from the Department of Pathology at the Hospital Universitario del Caribe (Cartagena, Colombia). All samples were from radical prostatectomy and trans-urethral prostatectomy specimens resected from patients with a previous diagnosis of localized prostate cancer, who underwent surgical resection as their primary treatment. For each patient, H&E stained slides were previously revised and specific tissue areas were selected and dissected in the way that is described below.

2.3 Laboratory assays

Obtaining manually dissected tissue from FFPE prostate tissue

The manual dissection of the FFPE tissue blocks, to obtain specific tissue areas, was performed in the Histopathology Laboratory at the School of Medicine, University of Cartagena. Excess of paraffin was removed from the blocks, the area to be studied was drawn and measured in the H&E slide and the corresponding block by a Pathologist expert in prostate cancer diagnosis; the blocks were manually carved to cut only that area. The tissue blocks were placed in a Leica® microtome (Buffalo Grove, IL, USA), which blades were cleaned after cutting each block to avoid contamination; after discarding the first three cuts, two to six cuts with a thickness between 10 and 15 um were taken (depending on the tissue area chosen), which were directly deposited into a 1.5 ml microcentrifuge tube previously labeled, for each of which the volume of tissue was calculated.

RNA isolating from manually dissected FFPE prostate tissue sections

Total RNA from these samples was extracted using the miRNeasy FFPE® commercial kit (Qiagen® Germantown, MD, USA), specially designed to purify RNA from FFPE samples. Each

tissue cut was incubated for fifteen minutes, at 56 ºC, in 160 µL of deparaffinization solution® (Qiagen® Germantown, MD, USA) to melt the paraffin. Then, 150 µL of lysis buffer (PKD) was added, centrifuged for one minute at 11,000 g, the lower clear phase was removed, 10 µL of proteinase K was added and it was first incubated for 60 minutes at 56 ºC to release the RNA from tissue samples, and then for fifteen minutes at 80 ºC to reverse the modifications to the RNA produced by formaldehyde. The clear lower phase was transferred to another tube and centrifuged for fifteen minutes at 20,000 g; the supernatant was transferred to another tube and incubated at room temperature for fifteen minutes with DNAse and DNase booster buffer to eliminate genomic DNA. Subsequently, the samples were mixed with 320 µL of RBC buffer, and 1120 µL of ethanol, placed in the elution column, washed twice with RPE buffer, and finally the RNA was eluted with 20 µL of RNAse-free water.

The concentration and quality of total RNA were evaluated spectrophotometrically to determine their absorbance at wavelengths of 260/280 using NanoDrop 2000c® (Thermo Scientific® Waltham, MA, USA).

RNU6 detection

The miScript PCR® kit (Qiagen® Germantown, MD, USA) which allows the detection of multiple microRNAs from a single cDNA, was used to determine the expression level of RNU6.

cDNA synthesis

For each sample, was obtained cDNA from total RNA isolated from the FFPE tissues. cDNA synthesis was performed using the miScript II RT® commercial kit (Qiagen® Germantown, MD, USA), whereby mature microRNAs are polyadenylated by a poly-A polymerase and then reverse transcribed into cDNA using oligo-dT primers. For this, the 5X miScript HiSpec buffer included in the kit, specific to prepare cDNA for the subsequent quantification of mature microRNAs was used. In detail, 2 µg of RNA were taken from each sample to prepare the reverse transcription master mix: 4 µL of 5X miScript HiSpec Buffer, 2 µL of 10X miScript Nucleics Mix, 2 µL of miScript Reverse Transcriptase Mix, RNA volume equivalent to 2 µg, and variable volume of water, for a final reaction volume of 20 µL. Incubated at 37 ºC for 60 minutes, and then incubated at 95 ºC for 5 minutes to inactivate the miScript Reverse Transcriptase Mix. The cDNA was stored at -20 ºC until it was used in the RT-qPCR assays.

Quantitative real-time PCR

RNU6 expression levels from total RNA isolated from each sample were determined by quantitative real-time PCR using the commercial kit miScript SYBR Green PCR® (Qiagen®, Germantown, MD, USA), in a StepOne Real-Time PCR System® (Applied Biosystems, Beverly, MA, USA). Each sample was analyzed in duplicate and a blank was used in each reaction. Melting curves were acquired to monitor the quality of the reaction.

2.4 Statistical analysis

The data were registered using Microsoft Excel® software (Washington, USA). Differences between the groups of tissue with different storage time an volume were determined by ANOVA test. To study the influence of length of tissue storage and volume of tissue in the quality and

concentration of RNA, and also their effects in the expression levels of RNU6, were calculated correlations using Spearman's correlation. Statistical analyses were performed using GraphPad Prism® v7.00 software (Graph-Pad Software Inc, San Diego, CA); a p value <0.05 was considered statistically significant..

III. RESULTS

RNA was isolated from manually dissected FFPE tissue samples from fifty blocks with prostate tissue, with a mean length of tissue storage of 30 months (SD= ±12.07), and a mean volume of tissue studied of 1.38 mm3 (SD= ± 0.791), (**Table 1**).

The mean value of the RNA absorbance at wavelengths of 260/280 was 1.75 (± SD 0.115) (Table I). When comparing the absorbance between the tissues with different storage times, a significant difference was found (p = 0,0076, ANOVA test); in the post hoc test a significant difference in absorbance ratios at 260/280 wavelengths was observed between the RNA obtained from blocks stored for less than 18 months, and those obtained from blocks stored for 43 to 48 months (p = 0,0069, Tukey test) (**Table 2**).

Table 1. Characteristics of the FFPE prostate tissue blocks, purity and concentration of the RNA obtained

Characteristic	Mean (± SD) (n=50)	95% CI
Length of tissue storage		
(months)	30.9 (± 12.07)	27.47 - 34.33
(years)	2.53 (± 1.003)	2.24 - 2.81
Volume of tissue studied (mm^3)	1.38 (± 0.791)	1.15 – 1.60
Absorbance relation at 260/280 wavelenght	1.75 (± 0.115)	1,72 - 1,79
RNA concentration (ng/µL)	221.8 (± 214.3)	160.9 - 282.7

Source: authors' elaboration

The RNA concentrations were between 15.5 and 1008 ng/µL, (mean 221.8 ng/µL), No significant difference was found between the RNA concentrations from the blocks stored for different periods of time (<18 months to 48 months), (ANOVA Test) (Table 2).

The correlation between RNA concentration and the RNA purity, determined by their absorbance at wavelengths of 260/280, and the correlation of these parameters with length of tissue storage, and corresponding volume of each studied sample, were determined by Spearman's correlation. The results show a clear direct and statistically significant correlation between the RNA concentration and the RNA quality (Rho=0.612, moderate positive correlation, 95% CI=0.396 to 0.765, p<0.0001), (Figure 1). A statistically significant and negative correlation between length of tissue storage and RNA purity was observed (Rho= -0.393, low negative correlation, 95% CI=-0.611 to -0.120, p=0.048), while the RNA concentration was not correlated to the length of tissue storage (Rho= -0.174, 95% CI= -0.438 to 0.119, p=0.228), (Figure 2).

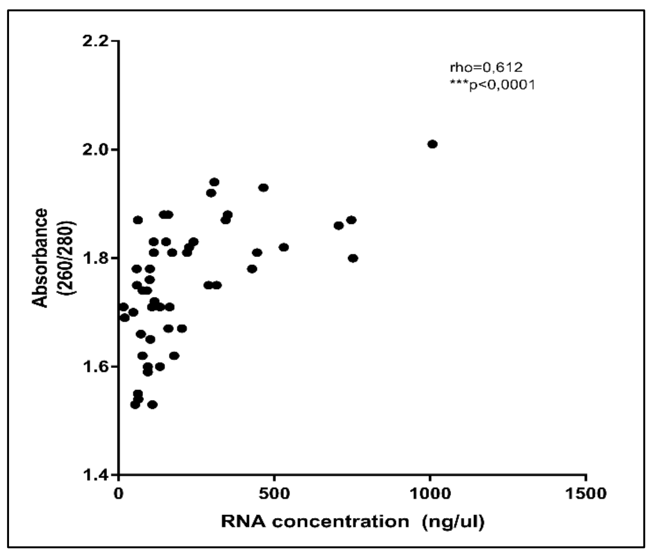
Table 2. Characteristics of the RNA obtained in relation to the length of FFPE tissue storage.

		Length of FFPE prostate tissue storage (months)						p Value	
		< 18 months	18-24 months	25-30 months	31-36 months	37-42 months	43-48 months		
		(n=9)	(n=6)	(n=10)	(n=8)	(n=5)	(n=12)		
		RNA characteristics						p value	*Post hoc* test Adjusted p value
Relation 260/280	Mean ($\pm$ SD)	1,848 (0,051)	1,732 (0,061)	1,757 (0,103)	1,809 (0,135)	1694 (0,061)	1,680 (0,127)	0,0076*	
	CI 95%	(1,808-1,888)	(1,668-1,796)	(1,683 – 1,831)	(1,695-1,922)	(1,617-1,771)	(1,599-1,761)		
	Mean ($\pm$ SD)	1,848 (0,051)					1,680 (0,127)		0,0069**
Concentration (ng/μL)	Mean ($\pm$ SD)	297,6 (131,1)	75,11 (46,25)	260,5 (208,8)	291,0 (320,3)	77,26 (44,3)	220,3 (242,2)	0,1918*	
	CI 95%	(196,8-398,3)	(26,57-123,7)	(111,1-409,9)	(23,15-558,8)	(22,14-132,4)	(66,41-374,1)		

*ANOVA test, **Tukey test

Source: authors' elaboration

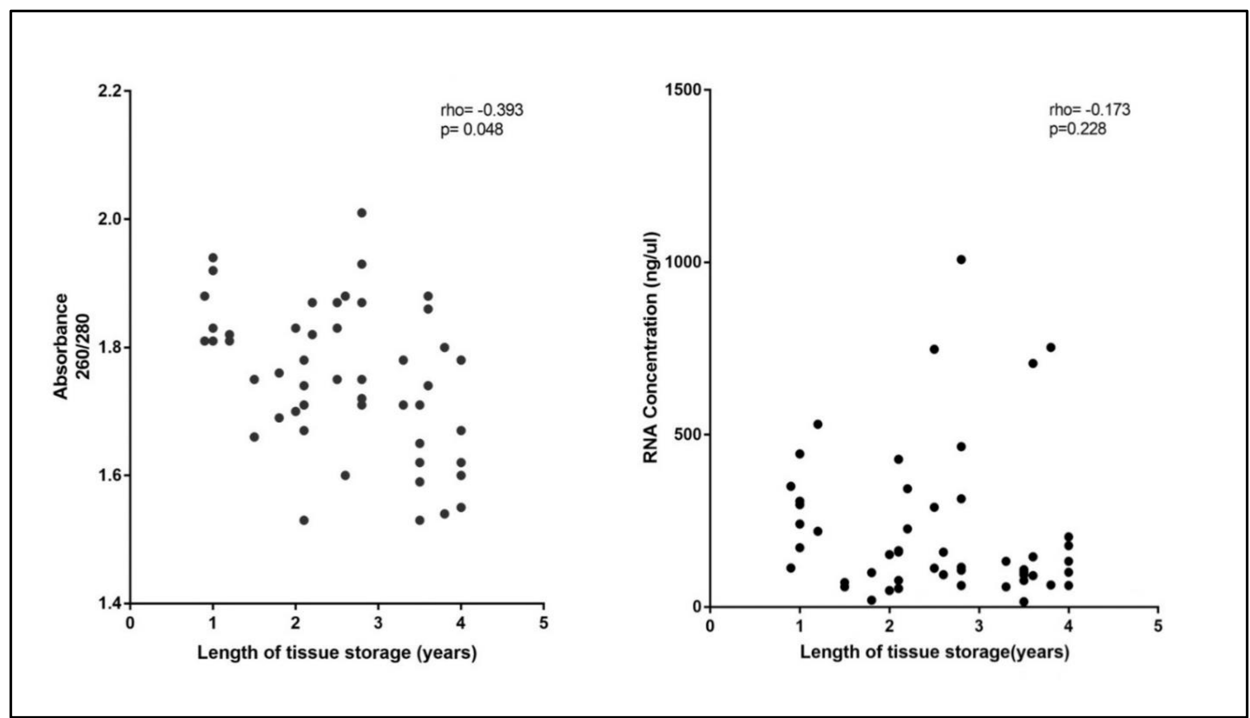
Figure 1. Association between quality and concentration of the RNA obtained from the FFPE tissue samples



Source: authors' elaboration

The concentration and quality (determined by their absorbance at wavelengths of 260/280) of the RNA obtained from the FFPE prostate tissue samples, were directly associated. Correlation coefficients (rho) are indicated, statistical significance is represented by asterisks, (Spearman's correlation).

Figure 2. Association between length of tissue storage, and, purity and concentration of the RNA obtained from the FFPE tissue samples



Source: authors' elaboration

The purity of the RNA obtained from the FFPE prostate tissue samples stored for 1 to 4 years was correlated with the length of tissue storage. The RNA absorbance at wavelengths of 260/280 ratios were negatively correlated with the length of tissue storage (left), while the RNA concentration was not correlated to the time of tissue storage (right). Correlation coefficients (rho) are indicated, statistical significance is represented by asterisks, (Spearman's correlation).

The volume of tissue studied was not associated with the RNA quality (Rho= 0.273, 95% CI= -0.163 to 0.400, p=0.054), nor the RNA concentration obtained (Rho= 0.128, 95% CI= -0.163 to 0.400, p=0.372).

RNU6 expression levels in the prostate tissue samples

To study the influence of RNA quality and concentration, volume of tissue studied and length of tissue storage, in the expression level of RNU6, the correlations between Ct of RNU6 and those parameters were calculated using Spearman's correlation. RNA absorbance ratio at wavelengths of 260/280 was negatively correlated with the Ct of RNU6, indicating that RNU6 expression level could be affected by the RNA quality. Meanwhile, there was a trend towards a negative correlation between RNA concentration and RNU6 expression level (Rho= -0.276,

p = 0.510). The length of tissue storage was positively correlated with the Ct of RNU6, indicating that the longer the storage time, the lower the obtained RNU6 expression level (Fig. 3). No correlation was observed between the volume of tissue studied and the RNU6 expression level (Rho= -0.095, 95% CI= -0.371 to 0.196, p=0.510).

The correlations between the Ct of RNU6, with the concentration and quality (determined by the absorbance at wavelengths of 260/280) of the RNA obtained from the FFPE prostatic tissue samples, and, with the length of FFPE tissue blocks storage, were determined. The RNA absorbance at wavelengths of 260/280 was negatively correlated with the Ct of RNU6 (top), while the RNA concentration showed a trend towards a negative correlation with the Ct of RNU6 (middle). The length of tissue storage was negatively correlated with the Ct of RNU6 (bottom). Correlation coefficients (rho) are indicated, statistical significance is represented by asterisks, (Spearman's correlation).

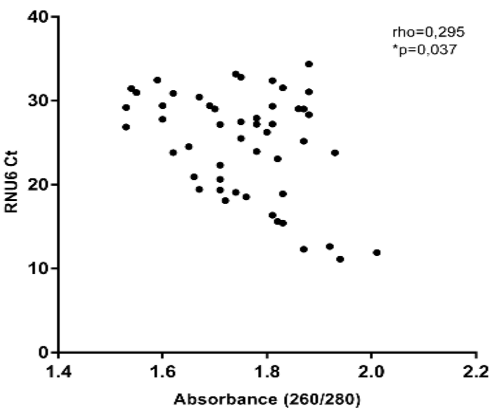
IV. DISCUSSION

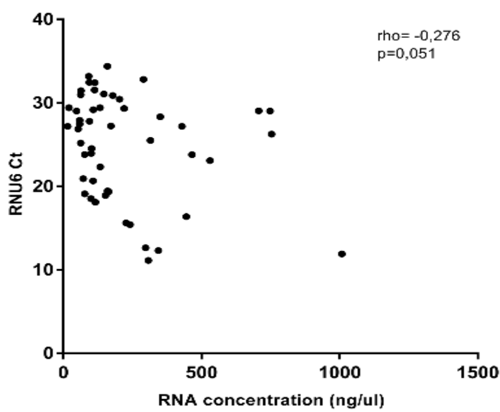
The archives of FFPE tissues are a valuable resource to study genetic alterations, changes in gene expression of mRNA, microRNAs, and proteins in tumor tissue. These samples can be stored for a long time at room temperature without loss of integrity and offer an historic register of tumor histology and molecular profile that could be correlated to disease evolution (18,19). However, manipulation of human tissue specimens is known to cause changes in their composition and structure, including nucleic acids (20), and processing of long-term FFPE stored samples demonstrates changes in their nucleic acids content by degradation (13,16). In this study, we observed a mild loss in the quality of the transcripts obtained from FFPE samples, which increases with longer time of tissue storage; conversely, the RNA concentration was not correlated to the length of tissue storage (**Figure 2**).

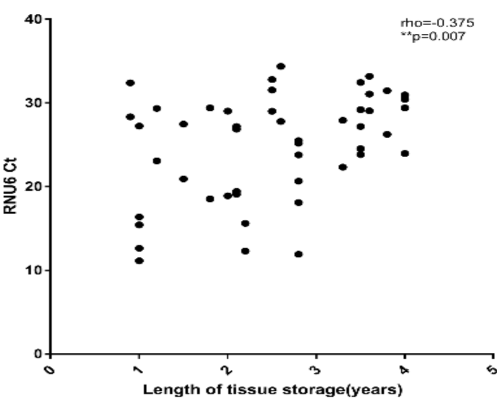
Previously, Nam et al (2), amplified shorts DNA or RNA sequences in most of their FFPE tissue samples, they obtained the best yield in RNA quality from recently processed samples compared to the samples stored for prolonged time. They reported that RNA integrity was less affected during the first year, than during longer periods of storage time (2). Von Ahlfen also describes that only after storing for more than twelve months, or at elevated temperatures, the RNA integrity becomes a limiting factor for the PCR performance (21). Similar findings were reported by Scorsato and Telles, who did not observe loss of material, or changes in RNA purity related to the age of the tissue block (22).

In relation to the influence of the tissue area studied on the yield of RNA obtained, Doleshal et al, reported that some types of tissue with a larger area on the block cut surface had higher performance than specimens with smaller surfaces, such as skin and mammary gland (23). In this study instead, there was not variation in the yield of RNA obtained in relation to the volume of tissue studied, this could be related to the fact that it was tried to obtain similar volumes of tissue, increasing the thickness of the cuts in the samples with less surface area. Similar to the results of Carlsson in their study on prostate biopsies (15), there was not difference in the RNA quality between samples with different volume, which rules out the need for large amounts of tissue for adequate molecular identification.

Figure 3. Association between length of tissue storage, purity and concentration of the RNA obtained from the FFPE tissue samples, with RNU6 Ct.







Source: authors' elaboration

The Johns Hopkins Pathology group demonstrated that processing to which FFPE tissue is subjected resulted in lower performance in obtaining RNA than an equivalent amount of frozen

tissue. But expression profile analysis showed close to 80% agreement in the differences in expression between recently FFPE tissues and frozen tissues. They proposed that cylinders of FFPE tissue could be taken to store at lower temperatures, or, to store RNA obtained shortly after tissue processing to allow molecular studies without compromising current surgical pathology routines (24).

The alteration in amplification generated by formalin seems to correlate positively to the fixation time and the amplicon length (25). However, despite this, biomolecules can be recovered and analyzed using specific extraction protocols, optimized for FFPE tissues (7,26), as many of the modifications can be reversed by heating before hybridization, and reverse transcription (25), and the assays based on the PCR technique have been optimized to overcome the technical difficulties generated by this type of samples (27). For example, Rodrigues Gouveia et al., implemented an additional wash step with PBS in DNase/RNase-free water during FFPE tissue sample preparation for the RNA extraction, with a significant improvement in the RNA quality, and the amplification results. They proposed that possibly washing favors the elimination of fixative residues in tissues, which are contaminants that can act as PCR inhibitors, and also reported that amplification was not influenced by the age of tissue block (6).

Comparative analysis of frozen and FFPE tissues suggests that microRNAs are unusually stable and easier to retrieve from FFPE when compared to mRNA transcripts owing to their small size (28-30). However, diverse conclusions have been reported regarding the stability of microRNAs in FFPE tissues stored for long periods of time. Nonn et al., reported good correlation between microRNAs expression profile in frozen tissues and FFPE tissues (17). Siebolts et al., and Szafranska et al., found similar results on microRNAs expression in FFPE tissues compared to frozen tissues (18,31). In contrast, Boisen et al., reported that global mean yield of microRNA is lower in tissues with increased fixation time and in older paraffin blocks (32). And Peskoe et al., found that tissues stored for a long time, greater than twelve years, had a decrease in the amount of microRNA and RNU6 (12,13). In this study, like this previous report, there was an association between longer tissue block storage time with lower RNU6 expression level.

A recent review of the literature concluded that is exceedingly difficult to harmonize the quality criteria for human tissue samples because their great heterogeneity and the large number of pre-analytical factors associated. They proposed to assess the integrity of tissue itself and its derived biomolecules, to evaluate if stored human tissue samples fit for the purpose for which they were collected (20). In this sense, although the use of FFPE samples has increased in biomedical research to evaluate the genetic and molecular basis of diseases and the outcome, survival and new therapies for patients, their handling, processing and storage can alter their characteristics and influence their quality, integrity and/or molecular composition. These aspects should be considered to take advantage of the enormous potential of these samples for their use in precision medicine.

Author Contributions: Conceptualization, I.B. and N.R.; methodology, I.B. and N.R.; validation, I.B. and N.R.; analisis, I.B. and N.R.; research, L.D. ; resources, I.B and L.D..; data curation, I.B and L.D.; writing: preparation of the original draft, L.D.; writing: review and editing, I.B. and L.D. ; visualization, L.D.; supervision, I.B.; project administration, I.B.; acquisition of funds, I.B. All authors have read and accepted the published version of the manuscript.

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