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ORIGINAL ARTICLE

RNA integrity in post-mortem samples: influencing parameters and implications on RT-qPCR assays

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Abstract Messenger RNA (mRNA) profiling in post-mortem human tissue might reveal information about gene expression at the time point of death or close to it. When working with post-mortem human tissue, one is confronted with a natural RNA degradation caused by several parameters which are not yet fully understood. The aims of the present study were to analyse the influence of impaired RNA integrity on the reliability of quantitative gene expression data and to identify ante- and post-mortem parameters that might lead to reduced RNA integrities in post-mortem human brain, cardiac muscle and skeletal muscle tissues. Furthermore, this study determined the impact of several parameters like type of tissue, age at death, gender and body mass index (BMI), as well as duration of agony, cause of death and post-mortem interval on the RNA integrity. The influence of RNA integrity on the reliability of quantitative gene expression data was analysed by generating degradation profiles for three gene transcripts. Based on the deduced cycle of quantification data, this study shows that reverse transcription quantitative polymerase chain reaction (RT-qPCR) performance is affected by impaired RNA integrity. Depending on the transcript and tissue type, a shift in cycle threshold values of up to two cycles was observed. Determining RNA integrity number of 136 post-mortem samples revealed significantly different RNA qualities among the three tissue types with brain revealing significantly lower integrities compared to skeletal and cardiac muscle. The body mass index was found to influence RNA integrity in skeletal muscle tissue (M. iliopsoas). Samples originating from deceased with a BMI>25 were of significantly lower integrity compared to samples from normal weight donors. Correct data normalisation was found to partly diminish the effects caused by impaired RNA quality. Nevertheless, it can be concluded that in post-mortem tissue with low RNA integrity numbers, the detection of large differences in gene expression activities might still be possible, whereas small expression differences are prone to misinterpretation due to degradation. Thus, when working with post-mortem

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samples, we recommend generating degradation profiles for all transcripts of interest in order to reveal detection limits of RT-qPCR assays.

Keywords RNA integrity · Post-mortem tissue · RT-qPCR

Introduction

When performing RNA profiling by means of reverse transcription quantitative PCR, mainly two factors are known to influence the quality and reliability of the data obtained: Firstly, a high assay performance needs to be ensured by optimising parameters influencing the PCR efficiency, e.g. amplicon size, primer sequences and locations, and priming conditions. Secondly, the integrity of the target RNA is a crucial factor for the performance of gene expression analysis because varying tissue qualities may have an impact on the data obtained by reverse transcription quantitative polymerase chain reaction (RT-qPCR) [1, 2]. In studies focussing on animal models, ante-mortem conditions, manner of death and post-mortem tissue storage can be controlled [3]. This is impossible for studies using post-mortem human tissue resulting in rather inhomogeneous sample collections showing a high degree of biological variance (e.g. [1, 3–6]). Additionally, partial RNA degradation has to be expected [4]. Thus, it is very important to analyse the influence of reduced RNA quality on RT-qPCR data and to reveal further information on parameters leading to low RNA integrities [7].

Beside the photometric assessment of yield and purity in total RNA extracts, the calculation of the degradation status of each sample is regarded as indispensable [8]. For a long time, agarose gel electrophoresis with ethidium bromide [9] or SYBR green staining [10] was the method of choice to estimate RNA integrity. Considering the ratio of the intensity of 28S and 18S ribosomal RNA bands, a ratio of approximately 2 suggests high integrity of the particular RNA [11, 12]. In the current literature, a lack of sensitivity and specificity of this method, particularly concerning slight differences in RNA degradation, is discussed [13, 14]. Meanwhile, the prevalent method for the determination of total RNA quality is the highly sensitive microfluidic chip-based capillary electrophoresis [15, 16] using a Bioanalyzer (Agilent Technologies, USA) or Experion (Biorad, München, Germany) instrument. Based on electrophoretical data, an RNA integrity number (RIN) or RNA quality index is generated. Thus, compared to conventional gel electrophoresis, the advantages of this method are its reproducibility and its user-independent and impartial measurements [17, 18].

Since RNA from post-mortem tissues is expected to be at least partially degraded, the aims of the present study were to analyse the influence of different RNA integrities on data obtained with RT-qPCR and to identify parameters influencing the post-mortem integrity of total RNA. For the first part, commercially available human brain, heart and skeletal muscle total RNA was artificially degraded, and the cycle of quantification values (C_q, also known as C_t, nomenclature according to Bustin et al. [8]) of three gene expression assays were determined by RT-qPCR. By doing so, a specific degradation profile of these three gene transcripts was achieved. For the second part, our study analysed the possible influence of the tissue type as well as several pre- and post-mortem parameters on RNA integrity in human brain, cardiac muscle and skeletal muscle samples obtained during routine forensic autopsy. These parameters include “post-mortem interval” and “cause of death”, and also parameters mentioned in the relevant literature, e.g. “duration of agony” [5, 19] and “gender” [4, 6]. Additionally, the individual parameters “age at death” and “body mass index” were included in the present study. Finally, we aimed to analyse whether a carefully validated normalisation strategy as described previously [20] is suitable to balance the influence of low RNA integrity on RT-qPCR results.

Material and methods

RNA degradation

Aliquots of commercially available intact human brain, heart and skeletal muscle total RNA (200 ng/μL, Stratagene, Heidelberg, Germany) were gradually degraded at 70°C [21] for different times ranging from 0 to 175 min and immediately transferred to ice. For each type of tissue, we obtained 10 different degradation degrees.

Samples

During forensic routine autopsies (vote 272/05 of the ethical committee of the Freiburg University), a total of 136 samples from 49 individuals were collected in RNAlater (Ambion, Austin, TX, USA) and transferred to −80°C for long time storage. Three tissue types without obvious pathological changes were included in this study. The tissue types were selected following the findings from a previous study showing that brain, cardiac muscle and skeletal muscle might be promising tissue types for post-mortem gene expression analysis [22]. The following total numbers of samples were available: 45 brain samples (frontal lobe), 47 cardiac muscle samples (left ventricle, posterior myocardial wall) and 44 skeletal muscle (M. iliopsoas) samples. The discrepancy between the total number of individuals and numbers of each tissue can be explained by the fact that not all tissue types were available from all individuals—

for example, in cases of severe head injury, no brain tissue could be collected. Only individuals without macroscopic signs of putrefaction were considered for sampling. ESM Table 1 (electronic supplementary material) gives an overview of the parameters characterising the individuals. Regarding "age at death", samples were assorted into three evenly sized groups named "<35 years", "35–50 years" and ">50 years", respectively. For "body mass index", samples were grouped according to the World Health Organization (WHO) standards defining body mass index (BMI)>25 as pre-obesity or obesity, respectively. The influence of the parameter "cause of death" was analysed by assorting a subset of the samples into four groups. Only those samples that could be clearly attached to one of the following groups were included in the analysis: "central dysregulation" (deaths due to CNS depression, such as in opiate overdose), "suffocation" (deaths due to mechanical airway obstruction and external chest compression), "sudden cardiac death" (deaths due to acute myocardial ischemia and infarction) or "bleeding to death" (fatal internal or external haemorrhage due to traumatic or natural causes). We furthermore grouped our samples into "<1 min" and ">1 min" duration of agony. The former comprises cases of sudden and unexpected traumatic events leading to immediate death. Given this very short (or nonexistent) time frame, it can be assumed that gene expression shows the lowest possible response, if any. The group of ">1 min" comprises samples with longer agonal phases. Since in most cases the exact duration of agony cannot be determined, no further subgroups were distinguishable. The analysis of this parameter was also restricted to a subset of the sample collection, because in some cases no reliable information on the duration of agony was available. In accordance with previous recommendations [7], it was decided to use small well-defined groups rather than large heterogeneous groups for the parameters "cause of death" and "duration of agony". Regarding the parameter "post-mortem interval" only individuals with a post-mortem interval (PMI) of less than 50 h were included in the analysis.

RNA extraction

Tissue samples (~200 mg) were cut from post-mortem tissue and total RNA was isolated using RNagents Total RNA Isolation System and phenol/chloroform/isoamyl alcohol (25:24:1, pH 4.7, both from Promega, Mannheim, Germany) according to the manufacturer's recommendations. Samples were homogenized in denaturing solution with an IKA-T10 homogenizer (IKA, Staufen, Germany) and finally dissolved in 100 μL nuclease-free water. DNase treatment and additional RNA cleanup were performed as described previously [20]. To minimise possible nuclease-mediated RNA degradation during experimental workflow, all materials and working surfaces were cleaned using RNase Away (Molecular Bioproducts Inc., San Diego, CA, USA). Extraction yield and purity were assessed using NanoDrop ND-1000 (Peqlab, Erlangen, Germany).

Determination of RNA integrity

RNA integrity was assessed by on-chip capillary electrophoresis using Agilent RNA 6000 Nano kit and Bio-Analyzer 2100 (Agilent Technologies, Boeblingen, Germany). Voltage-induced RNA is shifting in gel-filled micro channels and separated according to its molecular weight. The laser-induced detection of fluorescence signals reveals electropherograms, and the amount of fluorescence detected correlates with the amount of RNA of a specific size [18]. The ratio of the fluorescence signals corresponding to 28S and 18S ribosomal RNA (rRNA) molecules, their height and width as well as the lower marker, the "fast area" ratio and several intermediate regions of the electropherogram, are used to calculate RINs ranging from 1 to 10, with 1 representing highly degraded and 10 representing intact total RNA [4, 13, 17, 18]. Thus, RIN values can be used as a measure of the degradation status of the sample. If necessary, samples were diluted to meet the chip's working range of 50–500 ng/μL.

RT-qPCR

RT-qPCR was performed using a two-step protocol applying the High Capacity cDNA Reverse Transcription kit with random hexamer primers and Multi Scribe Reverse Transcriptase (Applied Biosystems, Darmstadt, Germany) for complementary DNA (cDNA) synthesis. cDNA from post-mortem human brain, cardiac muscle and skeletal muscle tissue was diluted with HPLC grade water (VWR International, Fontenay sous Bois, France) to a concentration equivalent to 5 ng/μL of total RNA, whereas cDNA generated from accurately degraded commercial human brain, heart and skeletal muscle total RNA had a final concentration equivalent to 10 ng/μL of RNA. For all samples, the transcript amount of beta-actin (ACTB, Hs00357333_g1), beta-2-microglobulin (B2M, Hs00187842_m1) and 18S rRNA (Hs99999901_s1, all from Applied Biosystems) was detected using real-time PCR as described previously [20]. For the 18S rRNA assay, an additional dilution step of 1 to 10 was necessary prior to qPCR. Technical replicates were performed as follows: (1) Commercially available human RNAs were gradually degraded (see above) and samples of each degradation degree were reverse-transcribed in three replicates. Furthermore, each resulting cDNA was used for qPCR in three replicates. Thus, for each degradation step of each tissue, nine C_q values were obtained. In the degradation study, a total number of 90 C_q values for

each of the three tissues and each assay was measured. (2) For the 136 post-mortem samples, each sample was analysed in duplicates or triplicates starting from the reverse transcription step.

Data analysis

RIN values of samples assorted according to the abovementioned parameters are presented as box–whisker plots. Significances were calculated using Mann–Whitney *U* test.

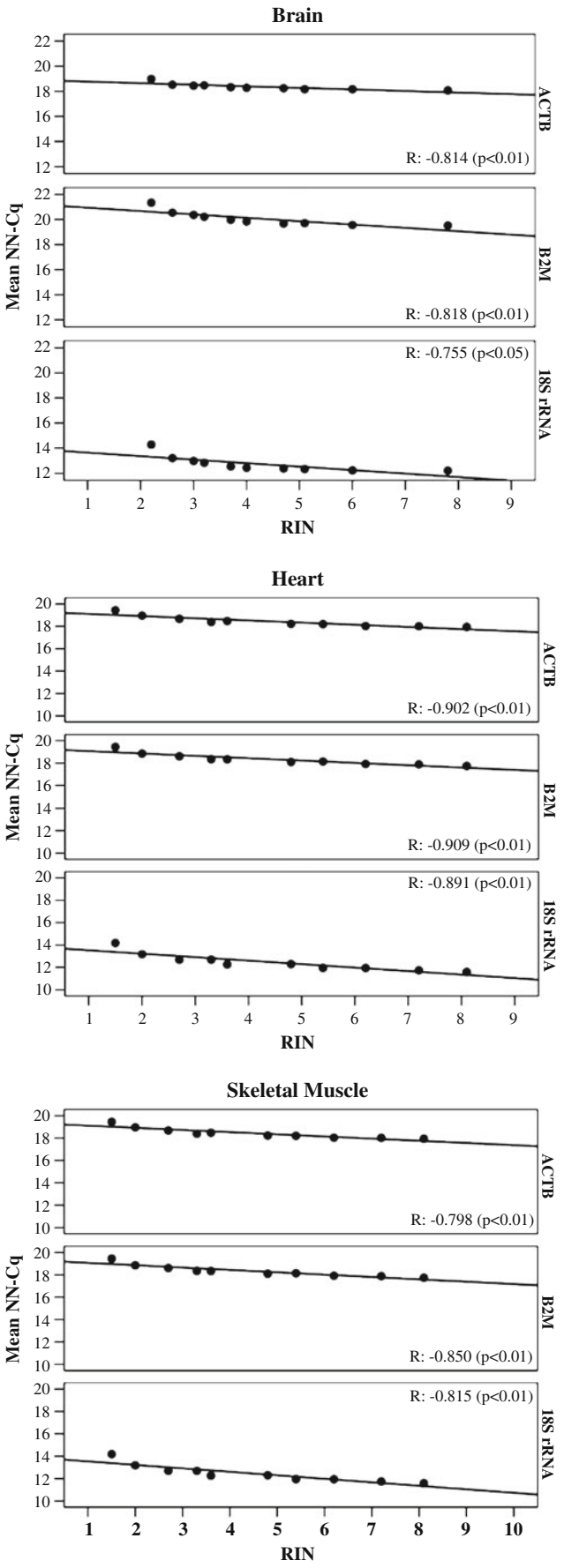
In addition to the data generated in the present study, data published previously were reanalysed: Non-normalised C_q values obtained from RT-qPCR of three gene transcripts (B2M, ACTB and 18S rRNA) in skeletal muscle, cardiac muscle and brain tissue cDNA of 37 individuals, as well as the associated calibrated normalised relative quantities (CNRQ values) calculated using qBasePlus software [23] in Koppelkamm et al. [20], were reanalysed to determine whether a data normalisation step can eliminate the influence of impaired RNA integrity. The statistical correlation between the RNA integrity number and C_q values or CNRQ data, respectively, was determined by Pearson correlation (*R*). All calculations were performed using SPSS versions 15.0 or 18.0.

Results and discussion

Because conventional methods assessing RNA quantity and quality are known not to be sensitive enough [13, 14, 24], this study applied the innovative lab-on-a-chip technology using an Agilent 2100 Bioanalyzer (Agilent Technologies). Thus, based on the electropherogram, a RIN was calculated taking into account the 18S/28S rRNA peaks as well as the background and possible degradation products [18].

Influence of RIN on RT-qPCR performance

Accurate degradation of commercially available human brain, heart and skeletal muscle total RNA came out with RNA of 10 degradation degrees ranging from RIN 7.8 to RIN 2.2 in brain, from RIN 8.1 to RIN 1.5 in heart and from RIN 7.6 to RIN 2.1 in skeletal muscle total RNA (ESM Table 2). RT-qPCR data were analysed for three different transcripts: ACTB, B2M and 18S rRNA. C_q shifts between the highest and the lowest RIN values were found to be 0.91 (ACTB), 1.83 (B2M) and 2.06 (18S rRNA) in brain; 1.51 (ACTB), 1.69 (B2M) and 2.60 (18S rRNA) in heart; and 1.06 (ACTB), 1.04 (B2M) and 1.65 (18S rRNA) in skeletal muscle cDNA. The statistically significant correlation coefficients suggest that RT-qPCR performance is affected by RNA integrity (Fig. 1), which is concordant



◀ **Fig. 1** The correlation is shown between RNA integrity numbers (RINs) and mean non-normalised cycles of quantification (NN-C_q) for three commercially available human RNAs (brain, heart and skeletal muscle) after gradual degradation. *P*earson correlation (*R*) and significances (*p*) were calculated using SPSS

with several other studies [4, 13, 18, 24]. Thus, even though the RIN reveals the integrity of total RNA and the messenger RNA (mRNA) fraction is only approximately 3% of total RNA, the RIN was found to be a very good indicator for mRNA integrity, too. The highest observed shift was 2.60 (18S rRNA in heart), which corresponds to an approximately 7-fold change in the amount of the starting target, because the PCR efficiency was found to be close to 105% [20], which corresponds to an amplification rate of 2.05 per cycle. Thus, with this assay, slight *n*-fold expression changes of below 7 cannot be distinguished from differences caused by degradation. On the other hand, ACTB shows shifts of approximately one C_q corresponding to a 2-fold difference in starting material. Thus, with this assay, the limit of detection is lower, and thus, also slight expression changes can be detected.

From this rather small experiment, we can learn that the influence of degradation is assay dependent as well as partly tissue dependent. Degradation profiles might rather be influenced by the assay than by the transcript itself because it is very well possible that primers might be located in a particularly well-preserved or poorly preserved area of a certain transcript. Thus, a further validation of the assays with relocation of the primer binding sites might help to decrease the limit of detection. Revalidation might also include further reduction of the amplicon size to decrease the influence of degraded target material (e.g. [25]). However, most commercially available assays show sizes which are well below the threshold of 400 bp calculated by Fleige et al. [24].

Parameters influencing RNA integrity

When working with post-mortem human autopsy tissue, one is confronted with samples of various storage and environmental conditions, which obviously leads to a highly varying and overall lower integrity of total RNA compared to fresh samples. To examine different parameters, which are assumed to have an impact on RNA quality, samples were assorted to different groups (see above).

First, analysis using the Mann Whitney *U* test came out with a statistically significant difference in RIN values between post-mortem brain and cardiac muscle, and brain and skeletal muscle, respectively (Fig. 2). Mean RIN value was 2.8 in brain tissue (range from 1.0 to 6.2), 3.8 in cardiac muscle tissue (range from 1.2 to 8.0) and 4.4 in skeletal muscle tissue (range from 1.6 to 7.6), whereas

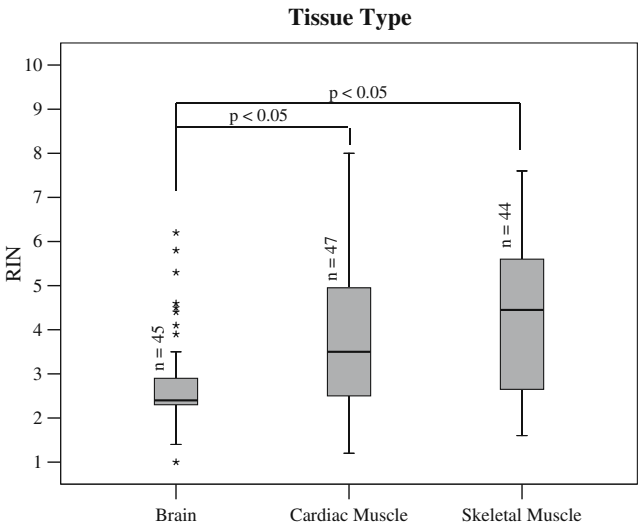


Fig. 2 Box–whisker plots of RIN values observed from the 136 post-mortem samples. RIN values of brain samples were found to be significantly lower ($p<0.01$) compared to cardiac and skeletal muscle samples, respectively

RNA extracted from cardiac muscle and skeletal muscle tissues showed higher variation in RNA integrity than RNA isolated from post-mortem human brain tissue. Thus, RNA integrity showed tissue specificity in the present study, which is consistent with the findings of previous publications [25–27]. Skeletal muscle seems to be highly suited for gene expression analysis because extracted RNA showed the overall highest RNA integrity. Overall, the lowest RNA integrity was observed in post-mortem brain tissue. One factor known to reduce RNA integrity in brain tissue is ante-mortem brain acidosis caused by prolonged agony [4, 5, 19, 28–32].

Second, the influence of the post-mortem interval was investigated. In post-mortem cardiac muscle tissue, RNA shows increased degradation with longer PMI (ESM Fig. 1). Thus, Pearson correlation between RNA integrity and PMI came out to be statistically significant. However, given the high degree of variation in these data points, it remains unclear whether this statistically significant finding can be explained by one biological parameter only (in this case PMI), because a high degree of interpersonal variance has to be assumed. There was no obvious correlation in skeletal muscle and brain, which confirms several studies proposing that RNA integrity does not decrease due to prolonged post-mortem delay only [5]. Age of the deceased, gender, duration of agony and cause of death were not found to have a statistically significant influence on the post-mortem RNA integrity (ESM Fig. 2a–d). Concerning the cause of death, following myocardial infarction, RNA integrity seems to be slightly more impaired in cardiac muscle tissue (outside the area of

infarction) and skeletal muscle tissue, whereas in brain tissue, no difference between examined causes of death was obvious. In any case, this consideration does not show statistical significance (ESM Fig. 2d). Elevated BMI seems to affect the integrity of RNA in M. iliopsoas (Fig. 3). It was shown that there is a temperature gradient between body core and shell during cooling of the body [33], and thus, the anatomical location of the M. iliopsoas and the reduced cooling rate of a body with a higher BMI during storage of 4°C might be an explanation for this finding.

Data normalisation

Normalisation by stable reference genes may reduce the influence of impaired RNA integrity on RT-qPCR experiments in order to obtain biologically relevant results [24, 34]. In the present study, this was verified by reanalysis of data obtained in a former study [20]: For each tissue and each of the three genes analysed, Pearson correlation (R) was assessed between non-normalised C_q values (NN-C_q) and the calculated RIN values of the same sample as well as between CNRQ and RIN. The only statistically significant correlation was observed for B2M in cardiac muscle tissue (Fig. 4 and ESM Table 3). Normalising B2M against four stable reference genes, as performed in the previous study [20], indeed balanced the influence of RIN (Fig. 4). Surprisingly,

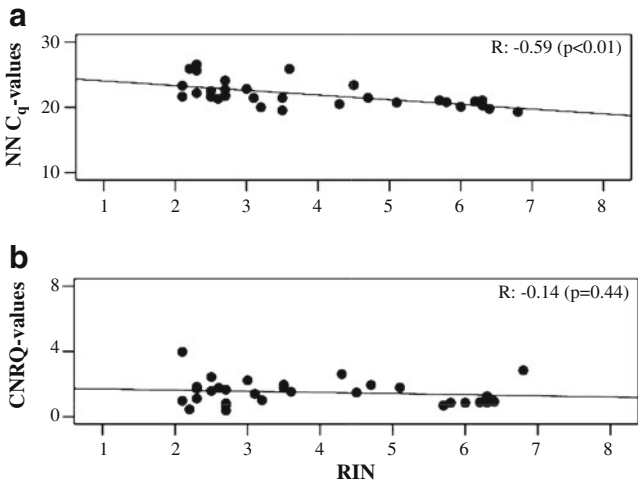


Fig. 4 Normalisation of beta-2-microglobulin (B2M) in cardiac muscle tissue: **a** Pearson correlation (R) between RNA integrity numbers (RIN) and non-normalised cycles of quantification (NN-C_q) shows high statistical significance ($p<0.01$). **b** After normalisation, no significant correlation between RIN and calibrated normalised relative quantities (CNRQ) can be detected

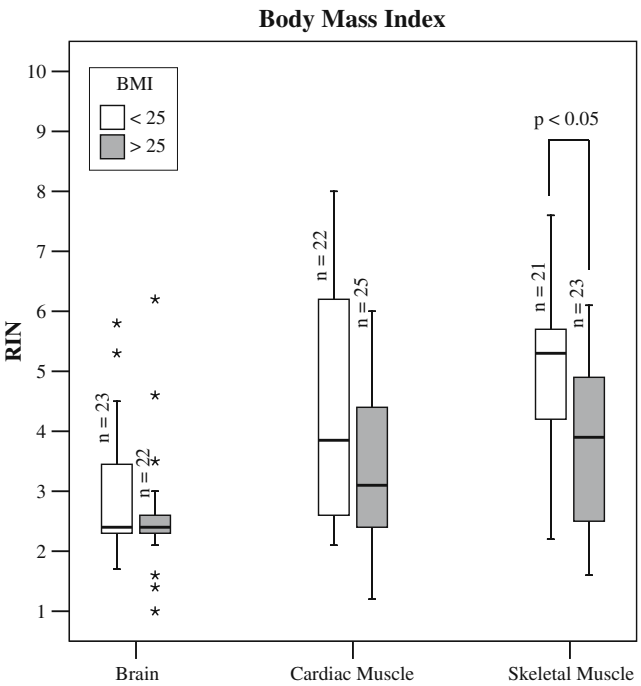


Fig. 3 Box–whisker plots of RNA integrity numbers (RIN) with respect to the body mass index. Samples were grouped according to the WHO standards defining BMI>25 as pre-obesity or obesity, respectively. Significances (p) were calculated using SPSS

for ACTB and 18S rRNA, the strong correlation between RIN and NN-C_q observed for the degradation curve could not be confirmed in real samples. It is very well possible that the heterogenic nature of the pool of samples incorporates many parameters influencing the amount of transcripts, like, e.g. the cause and circumstances of death, so that the effect of RIN may be masked. Nevertheless, the strong influence of RIN on RT-qPCR performance has to be kept in mind and should be considered during data interpretation (see below).

Conclusions and recommendations

Our results emphasize the importance of RNA quality control when gene expression analysis is performed using post-mortem tissue samples. Besides the yield and purity of the extracts, the integrity of total RNA is of particular importance for the generation of biologically meaningful data. In fact, quantitative data should always be verified for the influence of various ante- and post-mortem parameters in the particular set of samples. Thus, it is essential to check whether obvious differences in gene expression are really related to the questions being asked in the particular study or whether they are explainable to some extent by a certain bias in the composition of the sample set concerning the abovementioned parameters.

In our study, the tissue type and the body mass index were found to have a statistically significant influence on post-mortem RNA integrity. Thus, these parameters should

be taken into account when adequate biological replicates are selected and gene expression data are interpreted.

Recent works in RT-qPCR recommend the use of RIN thresholds, below which samples should be excluded from analysis. For example, Fleige et al. [17] proposed RIN≥5 as a cut-off value in different bovine tissues and cell cultures, while in human brain tissue, RIN≥3.95 was calculated as threshold by Weis et al. [14]. This surely helps to minimise the impact of integrity and it is recommendable to use samples with the highest possible RINs. Unfortunately, when working with post-mortem tissue, it is difficult to collect sufficient samples that meet the threshold. Thus, at least biological replicates showing similar RINs should be used.

Our data show that the influence of RNA integrity on RT-qPCR results might not be immediately visible in the analysis of samples because masking by other parameters might occur (including those mentioned in this study as well as general biological variation defined by, e.g. health, medication or lifestyle). Nevertheless, this influence has to be considered in gene expression studies. Ho-Pun-Cheung et al. [21] proposed the use of an integrity normalisation factor to correct for the influence of impaired integrity. This is an interesting approach but might bear the risk of data extenuation. When working with impaired samples, we strongly recommend establishing a degradation profile for each RT-qPCR assay applied in a certain study in order to determine the limit of detection by means of a threshold of *x*-fold changes in gene expression, below which the changes cannot be distinguished from differences caused by varying integrities. In our study, we showed the differences of this threshold between the three assays analysed (see above). Thus, with our 18S rRNA assay, we are not able to detect minor changes in gene expression, because up to 7-fold changes may be caused by impaired integrity. On the other hand, the detection of strong changes, e.g. 20-fold or higher, might really be biologically meaningful. As a rule of thumb, we suggest detection of the C_q shift that has to be expected from impaired integrity and calculate the threshold of detection by doubling of the *x*-fold change corresponding to this shift. For our assays, we would recommend a limit of detection of 14-fold for 18S rRNA, 4-fold for ACTB and 7-fold for B2M.

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