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RESEARCH ARTICLE

Peripheral blood collection: the first step towards gene expression profiling

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ABSTRACT

A crucial challenge for gene expression analysis in human biomonitoring studies on whole blood samples is rapid sample handling and mRNA stabilization. This study was designed to evaluate the impact of short bench times (less than 30 min) on yield, quality and gene expression of mRNA in the presence of different stabilization buffers (TempusTM Blood RNA tube and RNeasy[®] Stabilization Reagent). Microarray analyses showed significant changes over short periods of time in expression of a considerable part of the transcriptome (2356 genes) with a prominent role for NFκB-, cancer- and glucocorticoid-mediated networks, and specifically interleukin-8 (IL-8). These findings suggest that even short bench times affect gene expression, requiring to carry out blood collection in a strictly standardized way.

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Introduction

Transcriptional profiling of whole blood has emerged as a powerful tool for biomarker discovery in clinical medicine and health research (Bai et al., 2013; Ellinger-Ziegelbauer et al., 2009; McHale et al., 2010; Sullivan et al., 2006; Tang et al., 2005; Taurino et al., 2010). This technology provides the opportunity to explore altered expression of large numbers of genes simultaneously by use of e.g. quantitative polymerase chain reaction (qPCR), RNA Sequencing or microarrays; and may lead to a better understanding of the regulatory events involved in health and disease processes. In the field of environmental health, human biomonitoring studies are used to investigate possible associations between lifestyle, environmental factors and public health. Also in this domain, researchers have adopted genome-wide transcriptome analysis of human matrices such as whole blood in order to identify expression signatures reflecting early biological responses (Hebels et al., 2013; Remy et al., 2014; van Leeuwen et al., 2008). Peripheral blood is the most practical tissue to profile gene expression of the human immune system due to accessibility, whereas the site of primary infection in certain diseases may not be easy to access, accurate reflection of *in vivo* processes at the time of blood collection and the possibility to obtain reproducible blood transcriptional profiles from volumes as small as that obtained from a finger prick (Blankley et al., 2014; Vartanian et al., 2009; Weber et al., 2010). Due to *ex vivo* instability of RNA transcripts (Baechler et al., 2004; Fleige & Pfaffl, 2006a; Fleige et al. 2006b; Hartel et al., 2001), a crucial challenge for gene expression analysis on blood samples is rapid sample handling and mRNA stabilization by

adding RNA preservative (e.g. PAXgeneTM Blood RNA Tube, TempusTM Blood RNA tube, RNeasy[®] Stabilization Reagent). The latter stabilizes RNA and prevents induction of new transcripts (Asare et al., 2008; Rainen et al., 2002). Quick addition or direct collection of blood in RNA preservative is a significant challenge during fieldwork in biomonitoring studies and is even not always possible for example in case of cord blood cell collection or prior fractionation of blood cell populations.

Recent studies have investigated gene instability in whole blood collected immediately in a blood tube versus different bench times (hours [hr] or days), collection with or without RNA preservative or anticoagulants, and storage at different temperatures or for extended periods of time. In this context, bench time is considered as the time between blood collection and sample stabilization, either through addition of RNA preservative or by storage at low temperature. Differences in gene expression were mainly observed between different bench times (2 hr minimum), followed by anticoagulants and to a much lesser extent storage temperatures and duration (Duale et al., 2012, 2014; Hebels et al., 2013). Yet, no study has evaluated the effects of short bench times (as short as 10 minutes [min] to 30 min after collection) on whole blood gene expression profiling while this actually is a realistic scenario in sampling protocols of large-scale population studies.

This study focuses on the effect of short bench times (less than 30 min) and RNA stabilization buffer on RNA yield, RNA quality and specific gene expression changes in whole blood obtained from three healthy donors, requiring the comparison of two commercially available stabilizing technologies i.e. TempusTM Blood RNA tube and RNeasy[®] Stabilization Reagent. In order to obtain an overall picture of the influence

of short bench time on gene expression, whole transcriptome and pathway analysis was performed on RNA derived from whole blood collected directly in a Tempus™ Blood RNA Tube versus blood that was mixed with RNeasy Lysis Buffer Stabilization Reagent 30 min after collection. In a next step, the effect of even shorter bench times (as short as 10 min), was evaluated for the most affected molecule using qPCR.

Methods

Ethical statement

This study protocol was approved by the ethical committee of the University of Antwerp (B300201316329).

Sample collection and storage

Whole blood samples were obtained via phlebotomy from three healthy adult female donors aged 33.4, 32.4 and 23.5 years. From each participant peripheral blood was collected in two Tempus™ Blood RNA tubes and in two dipotassium ethylenediaminetetraacetic acid (EDTA) Vacutainer™ tubes (Becton Dickinson, Franklin Lakes, NJ). The duplicate Tempus™ Blood RNA tubes from one donor (donor A) were further used in the experiment as technical replicates. After collection, the EDTA blood sample kept at room temperature was processed in different ways: (1) 3 mL was added to the Tempus™ Blood RNA tubes after 10 min and 30 min; and (2) 0.5 mL blood was mixed with 1.3 mL RNeasy Lysis Buffer Stabilization Reagent immediately after blood drawing and after 10 min, 20 min and 30 min (Table 1). A similar sampling procedure was used for both stabilization buffers in order to compare RNA yield, RNA quality and specific gene expression changes between the two stabilization methods 10 min or 30 min after blood collection. To this end, the blood was collected in a plain EDTA tube and after 10 min the Tempus™ Reagent or the RNeasy Lysis Buffer Stabilization Reagent was added. After sample collection, the Tempus™ Blood RNA tubes were kept at ambient temperature and the blood- RNeasy Lysis Buffer Stabilization Reagent aliquots were transferred to dry ice, as indicated by the respective manufacturers. Upon arrival at the laboratory, the Tempus™ Blood RNA tubes were stored at -20°C and the blood-RNeasy Lysis Buffer Stabilization Reagent aliquots were stored at -80°C as recommended by the manufacturers. The duration of cold storage of the stabilized blood fraction prior to transcriptome analysis was one week.

Table 1. Sampling conditions for each individual.

RNA stabilization buffer	Time between collection and addition of RNA preservative (bench time)			
	0 min	10 min	20 min	30 min
Tempus	x*	x	—	x
RNeasy	x	X	x	x*

Whole blood was directly collected in a Tempus™ Blood RNA Tube or mixed with RNeasy Lysis Buffer Stabilization Reagent. After 10, 20 and/or 30 min, whole blood was added to the Tempus™ Blood RNA tubes or was mixed with RNeasy Lysis Buffer Stabilization Reagent. All sampling conditions were used for quantitative qPCR; sampling conditions applied for microarray analysis are indicated with *.

RNA extraction and QC

Frozen samples were thawed at room temperature and RNA was isolated immediately using the Tempus™ Spin RNA Isolation Kit (Ambion, Austin, TX, USA) and the RiboPure Blood Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. The concentration of extracted total RNA was measured before and after DNase I treatment (DNA-free™ Kit, Invitrogen/AQ2) using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA purity was estimated by examining the OD 260/280 and the OD 260/230 ratios. After DNase I treatment, RNA integrity was assessed by the Agilent 2100 Bioanalyzer using the Agilent RNA 6000 Nano Kit (Agilent Technologies, Palo Alto, CA, USA). The RNA integrity numbers (RIN) were derived using the Agilent 2100 Expert Software (RIN = 1; low RNA quality to RIN = 10; highest RNA quality).

RNA amplification and labelling

Total RNA was amplified and labelled to generate complementary RNA (cRNA) using the Low Input Quick Amp Labelling (two colour) kit from Agilent Technologies. Briefly, an aliquot of 75–100 ng of RNA was reverse transcribed into complementary DNA (cDNA) using T7-promotor primer and the Affinity Script enzyme. The cDNA was transcribed into cRNA which was fluorescently labelled by simultaneous incorporation of cyanine 3-CTP (Cy3) for the Tempus™ Blood RNA tubes and cyanine 5-CTP (Cy5) for the blood-RNeasy Lysis Buffer Stabilization Reagent aliquots. The single-stranded, labelled cRNA was purified with Qiagen's RNeasy mini spin columns (Qiagen, KJ Venlo, Netherlands). cRNA yield and Cy-incorporation efficiency (specific activity) were determined using the NanoDrop spectrophotometer.

Microarray analysis and data pre-processing

From each donor, equal amounts (825 ng) of Cy3- and Cy5-labelled cRNA derived from whole blood collected directly in Tempus™ Blood RNA Tubes or mixed with RNeasy Lysis Buffer Stabilization Reagent 30 min after collection were competitively hybridized onto 4x44K Agilent Whole Human Genome microarrays (design 014850) for 17 hr using the automated HS4800TM Pro Hybridization Station according to the manufacturer's instructions (Tecan, Männedorf, Switzerland). The arrays were scanned on an Agilent DNA microarray scanner (G2565BA) and further processed using Agilent Feature Extraction Software (Version 10.7). The software automatically finds and places microarray grids, rejects outlier pixels, accurately determines feature intensities and ratios, and calculates statistical confidences. For each feature on the array, the software accurately determines the intensity for the red (derived from Cy5 fluorescence) and the green signal (derived from Cy3 fluorescence), which were used as input for further processing and analysis (Matlab, version R2012b). Normalization includes both linear scaling and lowest normalization to a rank invariant set of microarray features. Changes in gene expression relative to the control Tempus™ Blood RNA tube (also defined as 2-base logarithmic fold change [LFC]) were

obtained by calculating the ratio between rProcessedSignal (RNAlater[®] Stabilization Reagent) and gProcessedSignal (Tempus[™] Blood RNA tube). Fold changes (FC) were transformed by a 2-base logarithm in order to have distributions that are closer to Gaussian ones. Note that a strictly positive or negative LFC means an up- or down-regulation of the corresponding gene expression level. A LFC of 1 and -1 is representative for a fold change of 2 and 0.5, respectively. The control probes were removed and data files with the expression signals of 41 000 unique probes were obtained.

Pathway and network analysis

Functional and pathway enrichment assessment was conducted using Ingenuity Pathway Analysis (IPA) software (Ingenuity[®] Systems, Inc., Mountain View, CA, USA). Expression change differences between collection in a tube with RNAlater[®] Stabilization Reagent after 30 min and direct collection in a Tempus[™] Blood RNA Tube within one donor with a p value ≤ 0.01 (from the Feature Extraction files) and a LFC ≥ 1 were considered significant. For the union of significant genes, the median LFC for each transcript was calculated over the three donors. Network relationships between the genes were created based on information contained in Ingenuity's Knowledge Base by entering these values in the IPA core analysis. Three criteria were set before adding relationships and molecules. First, only direct relationships were allowed. Second, only a subset of molecules that was previously described in human peripheral blood cells (blood platelets, hematopoietic progenitor cells and immune cells) was considered for pathway analysis. And third, all types of interaction molecules were accepted in the network. Upstream Regulator Analysis (URA) was used to identify the cascade of upstream transcriptional regulators (transcription factors, enzyme, cytokine, growth factor, miRNA, compound or drug) that could explain the observed gene expression changes in the dataset. For each potential transcriptional regulator an overlap p value was computed with Fisher's Exact test to assign a score to each subnetwork that indicates the likelihood of the input genes in the network being found together due to random chance; and an activation z-score as well to infer the activation status of the predicted transcriptional regulators. Significance is generally attributed to p values < 0.01 and z-scores ≤ -2 or ≥ 2 . Further details of these calculations can be obtained at: <http://pages.ingenuity.com/IngenuityUpstreamRegulatorAnalysisWhitepaper.html>. Moreover, Canonical Pathway Analysis was used to identify the known pathways according to the Ingenuity Knowledge Base associated with metabolism and signalling that were most significant to the dataset. The significance of the association between the dataset and the canonical pathway was measured in two ways: 1) A ratio of the number of molecules from the dataset that map to the pathway divided by the total number of molecules that map to the canonical pathway was calculated. 2) Fisher's Exact test was used to calculate a p value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone (Cohen et al., 2014).

p Values were adjusted for multiple testing using the Benjamini-Hochberg method (B-H p value) (Benjamini & Hochberg, 1995).

Quantitative PCR

Total RNA from samples was used as template for the synthesis of cDNA using the Transcriptor First Strand cDNA Synthesis Kit following the instructions of the manufacturer (Roche, Vilvoorde, Belgium). The amplification reactions were carried out in a Veriti 96-well Thermal Cycler (Applied Biosystems). qPCR was performed in 384-well plates using a Roche LightCycler480. The reaction mixtures consisted of 2.5 μ L cDNA sample and 7.5 μ L reaction mix (5 μ L LightCycler480 Probes Master Mix (2x; Roche), 0.5 μ L PrimeTime[®] qPCR Assays (20x; Integrated DNA Technologies, Leuven, Belgium) and 2 μ L nuclease-free water). Details of the 5' exonuclease PrimeTime[®] qPCR Assays that were used are given in Table 2. The qPCR programme was set to one cycle of pre-incubation at 95 °C for 10 min followed by 45 cycles of amplification (10 seconds [s] at 95 °C, 30 s at 62 °C and 1 s at 72 °C) and one cycle of cooling at 40 °C for 10 s. qPCR data processing was performed using the LightCycler software (Bos et al., 2012). Gene expression changes were analysed using the Biogazelle qBasePlus software (www.qbaseplus.com; Biogazelle, Ghent, Belgium) and were calculated as relative FC with the $\Delta\Delta C_t$ method (Hellemans et al., 2007). The stability of three reference genes (*GNB2L1*, *RPLP0*, *RPL13A*) was assessed as described by Vandesompele et al. (2002). To achieve accurate FC, normalization was performed to a set of at least three stable reference genes. Subsequently, FCs were transformed by a 2-base logarithm and addressed as LFC.

Statistical data analysis

Statistical analyses were performed using SPSS (version 22.0, IBM Corp, Armonk, NY, USA) and Matlab R2012b (MathWorks, Natick, MA, USA). The values of RNA yield and RNA integrity were visually inspected and presented in a table as the mean $\pm$ standard deviation (SD) for all conditions. Statistical comparisons were made using a two-way repeated measures analysis of variance (ANOVA) with time and tube serving as within-subjects factors and the data (RNA yield and RIN values) logarithmic transformed to be a better fit to the normal distribution. Regarding the qPCR data, statistical differences between the different stabilization systems were evaluated using a paired t -test. Significant between-group differences were tested by using an independent sample t -test. The level of significance of all statistical analyses was set at $p = 0.05$.

Results

The effect of short bench times on RNA yield and quality

To evaluate the effect of short bench times on the stability of total RNA in whole blood, obtained from three healthy donors, RNA yield and quality of all RNA samples were determined. Bench time was defined as the time between blood

Table 2. Sequences of the 5' exonuclease PrimeTime® assays.

<i>IL-8: NM_000584 (reference gene)</i>	
PrimeTime Probe	/56-FAM/AGGACAAGA/ZEN/GCCAGGAAGAAACCAC/3IABkFQ/
PrimeTime Primer 2 (reverse)	ACACAGAGCTGCAGAAATCAG
PrimeTime Primer 1 (forward)	TTTCAGAGACAGCAGAGCAC
<i>GNB2L1: NM_006098 (reference gene)</i>	
PrimeTime Probe	/56-FAM/AGTGGCTCT/ZEN/CATCCTGGACAGTGTAT/3IABkFQ/
PrimeTime Primer 2	CAAGCTATGGAATACCCTGGG
PrimeTime Primer 1	AGAAGCGGACACAAGACAC
<i>RPLP0: NM_001002 (reference gene)</i>	
PrimeTime Probe	/56-FAM/CCCTGTCTT/ZEN/CCCTGGGCATCAC/3IABkFQ/
PrimeTime Primer 2	TCGCTCTTAAACCCTGCGTG
PrimeTime Primer 1	TGCTCTGCTCCCAATGAAAC
<i>RPL13A: NM_012423 (reference gene)</i>	
PrimeTime Probe	/56-FAM/CTTCAGACG/ZEN/CACGACCTTGAGGG/3IABkFQ/
PrimeTime Primer 2	CTGTCACTGCCTGGTACTTC
PrimeTime Primer 1	TGTTTGACGGCATCCAC

Table 3. The effect of collection conditions on RNA yield and integrity.

	Bench time				<i>p</i> Value RMA*		
	0 min	10 min	20 min	30 min	Tube	Bench time	Tube × Bench time
RNA-yield (μg)					0.015	0.243	0.138
Tempus	83.10 ± 15.11	99.71 ± 9.14	–	90.86 ± 3.94			
RNAlater	74.20 ± 10.56	56.80 ± 8.38	54.35 ± 4.62	52.57 ± 2.21			
RIN-value					0.788	0.506	0.542
Tempus	8.23 ± 1.24	8.63 ± 0.72	–	8.53 ± 0.90			
RNAlater	8.60 ± 0.10	8.67 ± 0.06	8.43 ± 0.06	8.63 ± 0.65			

The average yields and RINs (mean ± SD) of RNA samples from three independent donors subjected to different collection methods (Tempus™ Blood RNA tube and RNAlater® Stabilization Reagent) in relation to bench time (0–30 min).

**p* value two-way Repeated Measures ANOVA.

collection at room temperature and storage mostly preceded by the addition of RNA preservative. The mean values and standard deviations of RNA yield and RNA integrity subjected to stabilization system (Tempus™ Blood RNA tube and RNAlater® Stabilization Reagent) and bench time (0–30 min) are presented in Table 3. A statistically significant difference in RNA yield was observed between whole blood collected in a Tempus™ Blood RNA Tube versus blood that was mixed with RNAlater® Stabilization Reagent ($p = 0.015$). There was no statistically significant difference in RNA yield over different bench times ($p = 0.243$). The integrity and purity of RNA was assessed to evaluate the quality of sample handling and processing. No significant differences in RIN values between the two stabilizing systems ($p = 0.788$) and over different bench times ($p = 0.506$) could be observed. All samples from both collection methods had a RIN value above 7.5, except for one sample 6.8 (direct collection of peripheral blood in a Tempus™ Blood RNA tube for donor C). This quality was considered sufficient as a standard RIN above 5 was set as a cut-off for downstream applications including microarray analyzes and qPCR (Fleige et al., 2006a; Opitz et al., 2010). The 260/280 ratios for all RNA samples ranged between 1.92 and 2.16, indicating little or no protein contamination.

Bench-time induced whole transcriptome profile changes

To obtain an overall picture of the influence of short bench time and RNA stabilization buffer on gene expression, whole transcriptome analysis was performed on RNA derived from whole blood collected directly in a Tempus™ Blood RNA Tube versus blood that was mixed with RNAlater®

Stabilization Reagent 30 min after collection. Figure 1 shows the number of genes differently expressed ($p \leq 0.01$) for each donor (A, B, C) with an absolute LFC [abs(LFC)] greater than or equal to 1 (left) or 2 (right). For donor A, B and C, respectively 1170, 954 and 1709 genes had a p value ≤ 0.01 and an $\text{abs(LFC)} \geq 1$, with the majority of differentially expressed genes for each individual having very small p values ($p \leq 0.00001$). In all donors, for approximately 90% of the differentially expressed genes an abs(LFC) between 1 and 2 (number of genes, A: 1054; B: 838; C: 1477) was observed. The technical control T presents the number of genes ($n = 65$) that differs between two blood collections directly in a Tempus™ Blood RNA Tube from donor A. In total, 2356 genes were differentially expressed between RNA stabilization immediately in a Tempus™ Blood RNA Tube and after 30 min in RNAlater® Stabilization Reagent [$p \leq 0.01$; $\text{abs(LFC)} \geq 1$], 634 of these genes were overlapping among the donors. The change in expression of all 2356 genes was visualized in a heat map in which each bar represents the LFC of a gene for each individual. For 92.4% of the genes the same gene expression regulation (up- or down-regulated) in the three donors was observed (Figure 2).

Bench-time induced networks and pathways

Using the gene set that was altered in expression by the 30' bench time, enriched pathways and biological processes were identified. The transcriptional regulator that can explain the observed gene expression changes in the three donors was the Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (NFκBIA) with an overlap p value of

Figure 1. Differences in gene expression between the two stabilization systems. Number of genes significantly different between collection in a tube with RNeasy[®] Stabilization Reagent at 30 min and in a Tempus[™] Blood RNA Tube at 0 min for the three donors (A, B, C) with a p value ≤ 0.01 and an $\text{abs(LFC)} \geq 1$ (left) or 2 (right). The technical control T includes genes that differ between two Tempus[™] Blood RNA Tubes derived from individual A at 0 min.

Figure 2. Expression change of the significantly different genes ($n = 2356$) between collection in a tube with RNeasy[®] Stabilization Reagent after 30 min and direct collection in a Tempus[™] Blood RNA Tube. The gene expression changes are indicated as LFC by a gradient of blue and red colour, as shown in the color scale on the left side of the heat map. The red colour indicates upregulated gene expression, the blue colour indicates downregulated gene expression. Additionally, the genes were clustered based on their LFC (y-axis).

7.99E – 07 and an activation z-score of 2.65. An activation z-score greater than 2 is considered highly statistically significant. NF κ BIA was associated with 79 differentially expressed genes in our dataset, and the expression profiles of these genes reflected NF κ BIA activation. The NF κ BIA-driven mechanistic network, involving 357 genes of our dataset, consisted of 12 transcription regulators. The molecules are coloured in Figure 3 by their predicted activation state: activated (orange) or inhibited (blue). The canonical pathways most significantly associated with the 2356 differentially expressed genes are presented in Table 4. Additionally, IPA calculated a ratio of genes differentially expressed within each canonical pathway and also included in the dataset. The two pathways most significantly associated with the differentially expressed genes are the Molecular Mechanisms of Cancer pathway (B-H p value = 1.2E – 03; ratio = 73/328) and the Glucocorticoid Receptor Signalling pathway (B-H p value = 2.6E – 03; ratio = 59/259).

The effect of shorter bench times on interleukin-8 gene activity

The gene, with the maximum abs(LFC) in the microarray experiment, is the pro-inflammatory marker gene interleukin-8 (*IL-8*) with a LFC of 5.083. To further scrutinize the effect of short bench times before RNA stabilization, the activity of *IL-8* in whole blood that was collected in a Tempus[™] Blood RNA Tube or in a tube with RNeasy[®] Stabilization Reagent after several minutes (0–30 min) was evaluated using qPCR. Figure 4 presents the mean expression change of *IL-8* (LFC) for all three donors in whole blood collected in a Tempus[™] Blood RNA Tube (grey bars) or in a tube with RNeasy[®] Stabilizing Reagent (white bars) after different bench times. The mean expression values are considered relative to the Tempus[™] Blood RNA Tube at 0 min. The response of the three donors is shown in orange (A), blue (B) and green (C). Significant differences in expression values of *IL-8* between

Figure 3. NFkBIA-driven mechanistic network. This network, involving 357 genes of the 2356 dataset genes, consists of 12 transcription regulators. The lines connecting the nodes are coloured orange when leading to activation of the downstream node, blue when leading to its inhibition, and yellow if the findings underlying the relationship are inconsistent with the state of the downstream node. Pointed arrowheads indicate that the downstream node is expected to be activated if the upstream node connected to it is activated, while blunt arrowheads indicate that the downstream node is expected to be inhibited if the upstream node that connects to it is activated.

Table 4. Top ingenuity canonical pathways related to the 2356 differentially expressed genes.

Canonical pathway	B-H <i>p</i> value*	Ratio
Molecular mechanisms of cancer	1.2E – 03	73/328 (22.3%)
Glucocorticoid receptor signalling	2.6E – 03	59/259 (22.8%)
Role of NFAT in cardiac hypertrophy	4.09E – 02	37/158 (23.4%)
Role of tissue factor in cancer	4.44E – 02	26/100 (26.0%)
IL-22 signalling	4.44E – 02	10/24 (41.7%)
RAR activation	4.44E – 02	37/165 (22.4%)
Aryl hydrocarbon receptor signalling	4.44E – 02	29/120 (24.2%)
Role of JAK family kinases in IL-6-type cytokine signalling	4.44E – 02	10/25 (40.0%)
p53 signalling	4.57E – 02	23/90 (25.6%)
HMGB1 signalling	4.57E – 02	27/112 (24.1%)

The significance of the association between the dataset and the canonical pathway was measured by a *p* value determining the probability that the association is explained by chance alone, and a ratio of the number of molecules from the dataset that map to the pathway divided by the total number of molecules that map to the canonical pathway.

*Benjamini-Hochberg corrected *p* value.

the two stabilizing systems ($p=0.004$) and over different bench times ($p=0.028$) were observed. Stabilization with RNeasy Lysis Buffer compared to stabilization in a TempusTM Blood RNA Tube resulted in a ~2-fold higher induction of *IL-8*. The difference in mean expression value between the two stabilizing systems and expressed in LFC was largest at 0 min i.e. 1.12 ($p=0.0083$). When leaving the blood sample on the bench at room temperature for 10 min and 30 min, the mean *IL-8* expression was increased with respectively 28.3% ($p=0.0300$) and 15.4% ($p=0.0259$) in RNeasy Lysis Buffer-stabilized samples compared to TempusTM-stabilized samples. Since no TempusTM Blood RNA solution was added to whole blood after 20 min, no comparison with RNeasy Lysis Buffer could be made for this condition. Noteworthy, there was no statistical difference in absolute gene expression values of *IL-8* between the qPCR and the above-mentioned microarray experiment ($p=0.762$).

Discussion

Transcriptome analysis of peripheral blood as surrogate tissue has become an important approach in clinical and health

Figure 4. Comparison of *IL-8* expression in TempusTM and RNeasy[®] stabilization buffer after different bench times. The mean *IL-8* expression was examined after the addition of whole blood in a TempusTM Blood RNA Tube (grey) or in a tube with RNeasy[®] Stabilization Reagent (white) after several minutes (0–30 min; x-axis). The response of donor A (orange), B (blue) and C (green). The y-axis shows the LFC, the difference in expression compared to the TempusTM Blood RNA Tube at 0 min. Significant differences between collection of whole blood in a TempusTM Blood RNA Tube or mixed in a tube with RNeasy[®] Stabilization Reagent within an individual are indicated with * ($p < 0.05$). The error bars on the graph represent the mean standard deviation of the three donors for each collection condition.

monitoring studies. However, application of gene expression profiling in routine settings requires careful consideration of the influence of sample handling and mRNA stabilization on gene expression profile outcome. These results are often biased by decreased sensitivity and reliability due to *ex vivo* instability of RNA transcripts and no possibility of immediate mRNA fixation. To this end, we evaluated the influence of short bench times (less than 30 min) on RNA yield, RNA quality and specific gene expression levels in whole blood samples, while comparing two commercially available stabilizing

technologies, i.e. TempusTM Blood RNA tube and RNeasy[®] Stabilization Reagent. The key findings can be summarized as follows:

RNA yield and quality

In both stabilization systems, no differences in RNA quality and integrity were observed over different short bench times. This was consistent with the recent findings of Hebels et al. that focused on the impact of larger bench times (0–24 hr) on gene expression profiles (Hebels et al. 2013). All samples had RIN values of sufficient quality for transcriptome analysis. A significant difference in RNA yield can most probably be explained by differences in initial blood volume: 3 mL whole blood for the TempusTM Blood RNA tube versus 0.5 mL for the RNeasy[®] Stabilization Reagent aliquots.

Gene expression

In order to obtain an overall picture of the influence of short bench time on gene expression, whole transcriptome analysis was performed on blood collected directly in a RNA stabilizing Tempus tube compared with transfer of the collected blood in RNeasy[®] Stabilization Reagent after 30 min of incubation. Microarray analyses showed significant changes in expression of a considerable part of the transcriptome (2356 genes), with almost all genes the same expression regulation in the three donors. In this gene set, enriched pathways and biological processes were identified with a prominent role for Nuclear factor of kappa light polypeptide gene enhancer in B-cells (NFκB)-, cancer- and glucocorticoid-mediated networks. Similar findings were published by Hebels et al. when looking at larger bench times. The study revealed pathways mainly involved in apoptosis, stress signalling and DNA damage repair (Hebels et al. 2013). In the current study, network analysis reveals NFκB as the most significant and activated upstream regulator of the genes whose expressions were altered by the 30' bench time. Induction of the NFκB protein results in inhibition of NFκB, which are key regulators in immune-mediated inflammatory processes, by trapping the activated NFκB in inactive cytoplasmic complexes (Auphan et al., 1995; Ghosh et al., 1998; Hoesel & Schmid, 2013; Jacobs & Harrison, 1998; Mogensen, 2009). The two pathways most significantly associated with the differentially expressed genes are the Molecular Mechanisms of Cancer pathway and the Glucocorticoid Receptor Signalling pathway. Glucocorticoids are among the most potent anti-inflammatory and immunosuppressive agents. They inhibit synthesis of almost all known cytokines and of several cell surface molecules required for immune function; and are known to be dominant inhibitors of NFκB activation (Auphan et al., 1995). Besides, inhibition of NFκB is an important molecular pathway in the protection against cancers (Lin et al., 2010).

The gene mainly altered in the microarray experiment and also involved in NFκB- and glucocorticoid-mediated networks, is the pro-inflammatory marker gene *IL-8*. In a next step, the effect of bench times even shorter than 30 minutes was evaluated for this molecule. *IL-8* expression levels were

demonstrated to be increased over short transcending bench times (0–30 min) in whole blood collected in both stabilization systems. Previous studies demonstrated that *IL-8* was induced within 4 hr *ex vivo* (Barnes et al., 2010; Rainen et al., 2002). Baechler et al. confirmed this dramatic upregulation of *IL-8* in overnight samples (Baechler et al. 2004). In our study, at each time point, a ~2-fold higher induction of *IL-8* was observed in RNeasy[®] Stabilizing Reagent compared to the TempusTM system. It has been shown that mRNA concentrations after 24 hr storage in PAXgeneTM or TempusTM blood collection tubes much more closely approximated the initial values than the grossly induced concentrations in EDTA tubes, indicating that the choice of RNA collection tube greatly influences the transcriptional profiles obtained from peripheral blood (Rainen et al., 2002).

Our data indicated that a large amount of genes are dysregulated by *ex vivo* incubations of blood as short as 10 min (as demonstrated for *IL-8*). The specific cause of the identified gene expression changes should still be determined although several explanations can be hypothesized. Within 30 min after phlebotomy, the environment of the blood sample can change significantly by e.g. glucose depletion, pH drop, lactate accumulation, hypoxia and temperature change, causing biological stresses (Barnes et al., 2010; Burczynski & Rockett, 2006). Even anticoagulant in the collection tube may play a role in modulating cell activation, triggering the release of cytokines (ElHabbal et al., 1995; Pahl & Brune, 2002). The findings in the present study thus indicate that blood cells that are not immediately stabilized at the moment of phlebotomy, start to compensate for this potentially lethal microenvironment. Their complex regulatory machineries (like e.g. the Glucocorticoid Receptor Signalling pathway) may be activated to regulate the expression of many stress-associated genes, genes associated with important physiological functions like cell cycle, proliferation, transcription and metabolism and genes associated with immune function (e.g. chemokines, cytokine receptors and cell surface receptors). These biological processes and functions can therefore only be interpreted when measured in studies with an appropriate experimental design.

Conclusion

Overall these findings demonstrate that even short bench times (as short as 10 min) affect the human blood transcriptome. The optimal condition for gene expression profiling, in the context of monitoring health and disease processes in large-scale population studies, is direct collection of RNA in a stabilizing system (e.g. TempusTM Blood RNA Tube). When direct collection in a RNA stabilization system isn't possible due to practical reasons e.g. volume requirements, RNeasy[®] Stabilization Reagent can be used instead. For the latter, researchers must take into account that certain genes e.g. stress-associated genes can have higher gene expression levels due to technical variation instead of biological factors, and therefore, caution should be taken when interpreting expression profiles. Although the results based on three individuals are very clear, a larger sample size could add power to fully

comprehend the impact of short bench time on the human blood transcriptome.

Disclosure statement

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