Introduction
Changes in miRNA expression can be an early biological event in disease development. Furthermore, a toxicological stress can damage tissues and lead to the leakage of biological material in the bloodstream, including miRNAs. Plasma miRNA have been used as markers for early diagnosis of lung cancer and show differential expression in other tobacco-related diseases such as COPD and cardiovascular diseases.

Objective
We asked whether miRNAs have the potential to be used as early biomarker of biological effect in healthy smokers and if miRNAs candidates were correlated with biomarkers of tobacco smoke exposure.

Materials and Methods
We profiled by qRT-PCR a panel of 84 disease-associated plasma miRNAs in 30 smokers, 20 non-smokers and 20 ex-smokers. The smokers smoked the same cigarette brand. A robust statistical strategy was applied with samples collected in duplicate at two days interval and processed by different operators. Differentially expressed miRNA were correlated with two biomarkers of exposure with a long half-life (CeVal, blood-adduct marker for exposure to acrylonitrile) and a short half-life (TNEQ, total nicotine in urine).

Results
miRNA qRT-PCR data distribution according to operator

Figure 1: Sample data distribution (ΔCt) prior to normalization by operator (A) and data distribution of samples showing ΔCt post-normalization by operator (B). Colours are representative of operators. Red indicates Operator 1 and blue indicates Operator 2.

Figure 2: (A) Hierarchical cluster representing the expression profiles of 84 miRNAs commonly found in plasma of smokers (n=30), non-smokers (n=20) and ex-smokers (n=20) profiled in duplicates. Columns represent individual subjects and replicates and rows represent miRNA. Green black and red indicate high signal intensity, moderate signal intensity and low to no signal intensity in normalized expression data (ΔCt). (B) Principal component analysis of plasma miRNA expression colored by smoking status and operator.

Mixed model statistical analysis for miRNA expression adjusted by age and gender

Figure 3: Volcano plots showing the difference in miRNA expression between smokers vs non-smokers (A) and smokers vs ex-smokers (B). Fold change is plotted on the x-axis (log2 scale) (dotted vertical line marks the 2-fold increase threshold), and false discovery rate (FDR)-Bonferroni adjusted significance is plotted on the y-axis (log10 scale) (dotted horizontal line marks the p-value=0.05 threshold). Up-regulated miR-124 is indicated in red. Let-7a was also significantly different between smokers and non-smokers but using the less stringent qFDR method (Data not shown).

Correlation of significant miRNA with two biomarkers of exposure with a long half-life (CeVal, acrylonitrile adduct) and a short half-life (TNEQ, total nicotine in urine)

Table 1: Correlation between miRNAs and biomarkers of cigarette smoke exposure. miR-124 significant at qFDR<0.05 in smokers vs non-smokers and let-7a significant at qFDR<0.05 in smokers vs ex-smokers were the two miRNA used for the correlation study. The data from the smoking group only was used for correlation between the two miRNA and a biomarker of effective dose CeVal on one hand and a biomarker of internal dose TNEQ on the other hand. A linear mixed model was applied without adjustment (A) and with age, gender and pack years as fixed effects and subject as a random effect (B, C).

Conclusions
- The serum level of miR-124 and let-7a is associated with smoking status
- miR-124 & let-7a are regulators of the EGFR-Receptor and tumor suppressors
- miR-124 is differentially expressed in rats lung exposed to smoke (Izzotti et al., 2009)
- Exosome secretion and leakage from damaged tissues is a source of plasma miRNA (Figure 4)
- Limited correlation with markers of exposure due to different half-life

Conflict of interest statement: This study was a collaboration between BAT and Meddietmics Ltd. The study was funded by BAT Meddietmics Ltd. as a commercial contract.

Clinical Study compliance statement: The study protocol and informed consent forms were approved by the Ethics Committee of the Antwerpse instelling, Belgium and the clinical study was conducted in accordance with the World Medical Association Declaration of Helsinki (World Medical Association, 2008). The study was registered in the Current Controlled Trials database under the reference ISRCTN78124626.
Quantification of plasma miRNAs in a group of healthy smokers, ex-smokers and non-smokers and correlation to biomarkers of tobacco exposure

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Therefore we asked whether miRNAs have the potential to be used as early biomarker of biological effect in healthy smokers and if miRNAs candidates were correlated with biomarkers of tobacco smoke exposure.

We profiled by qRT-PCR a panel of 84 disease-associated plasma miRNAs in 30 smokers, 20 non-smokers and 20 ex-smokers. A robust statistical strategy was applied with replicate samples to account for reproducibility of the results. We identified differential expression of miR-124 (fold changer=2, Bonferroni adjusted p-value<0.05) between the smoking and control groups. The Storey method also revealed that let-7a was a potential miRNA associated with smoking. We investigated for the first time the dose correlation between the miRNA biomarkers (miR-124 & let-7a) and two biomarkers of tobacco exposure with a long half-life (Cr/Val, blood marker for exposure to acrylonitrile) and a short half-life (TNEQ, total nicotine in urine). miR-124 was correlated with the biomarker of acrylonitrile exposure (p<0.01) but not with the biomarker of nicotine exposure whilst let-7a was correlated with nicotine exposure (p<0.05).

Although miR-124 and let-7a show a correlation with biomarkers of tobacco exposure, we found that the relationship is dependent on other confounding factors. In future, it might be worth investigating the correlation in a larger group of subjects with a broader biomarker of exposure panel representing different chemical families.

Related publication:

Conflict of interest statement: The study was funded by BAT, all the authors were BAT employees at the time this study was conducted.

Clinical Study compliance statement: The study protocol and informed consent forms were approved by the Ethics Committee of the Ärztekammer Hamburg, Germany and the clinical study was conducted in accordance with the World Medical Association Declaration of Helsinki (World Medical Association, 2004). The study was registered in the Current Controlled Trials database under the reference ISRCTN12862886.