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Temporal variability of global DNA methylation and hydroxymethylation in buccal cells of healthy adults: Association with air pollution

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ABSTRACT

Background: Epigenetic changes, such as DNA methylation, are observed in response to environmental exposure and in the development of several chronic diseases. Consequently, DNA methylation alterations might serve as indicators of early effects. In this context, the aim of this study was to assess the temporal variability of global DNA methylation and hydroxymethylation levels in buccal cells from healthy adult volunteers.

Methods: Global DNA methylation (%5mdC) and hydroxymethylation (%5hmdC) levels in human buccal cells, collected from 26 healthy adults at different time points, were quantified by UPLC-MS/MS. Associations between %5mdC and %5hmdC, respectively, and short-term exposure (1–7 days) to air pollutants $PM_{2.5}$ and PM_{10} were tested with mixed-effects models including various covariates.

Results/Discussion: Dynamic short-term changes in DNA methylation and hydroxymethylation levels in buccal cells were observed, which were inversely associated with exposure to $PM_{2.5}$ and PM_{10}. An IQR increase in $PM_{2.5}$ over a 7-day moving average period was significantly associated with a decrease of −1.47% (−1.74%, −1.20%) and −0.043% (−0.054%, −0.032%) in %5mdC and %5hmdC, respectively. Likewise, for PM_{10}, a decrease of −1.42% (−1.70, −1.13) and −0.040% (−0.051%, −0.028%) was observed.

Conclusion: Global DNA methylation and hydroxymethylatation varied over a time period of three weeks. The observed temporal variability was associated with exposure to ambient $PM_{2.5}$ and PM_{10} levels. This should be taken into account when interpreting epigenetic alterations in buccal cells.

1. Introduction

Since it is not always possible to obtain a sample of the target tissue of choice without invasive procedures that require trained personnel (Hansen et al., 2007; Langie et al., 2017), there is an increasing need to identify a suitable surrogate tissue. Large-scale human biomonitoring studies (HBM) often include hundreds of participants. Hence, the surrogate tissue needs to be easy to collect and the procedure should be more cost-efficient for repeated sampling. HBM studies routinely use peripheral blood and/or urine as target tissue, but also saliva has shown its value in exposure assessment (Angerer et al., 2007; Langie et al., 2017).

Buccal cells are an easy accessible, reliable and non-invasive source of DNA. Furthermore, the collection of buccal cell samples is straightforward, inexpensive and does not bring discomfort to the patient (Burger et al., 2005; Milne et al., 2006). It was already proposed that for non-blood based diseases or phenotypes, buccal cells are a more informative tissue for HBMs than blood cells since buccal cells are more likely to display dynamics that are more representative of other tissues than blood (Lowe et al., 2013; Teschendorff et al., 2015). Additionally, buccal cells are becoming increasingly used for genetic and forensic endpoints including DNA damage (Bolognesi et al., 2015; Holland et al., 2008; Jovanovich et al., 2015), as well as for epigenetic endpoints, in newsborns and very young children (Hagerty et al., 2016; Jiang et al., 2015; Novakovic et al., 2014; Pauwels et al., 2017; Torrone et al., 2012; Verma et al., 2014). It is nowadays of major interest to assess the potential of DNA methylation and hydroxymethylation as biomarkers (Leenen et al., 2016). Hence, studies have led to an increase in knowledge of the DNA methylome in pathophysiological conditions, highlighting the importance of DNA methylation with regard to

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underlying mechanisms of diseases (Lovinsky-Desir and Miller, 2012; Sanchez-Mut et al., 2016; Witte et al., 2014). More recently, a great breakthrough was achieved by the discovery of a novel epigenetic mark, namely 5-hydroxymethylcytosine (5hmC), formed by the enzymatic oxidation of 5-methylcytosine (5mC) (Tahiliani et al., 2009). It was first thought that 5hmC was only an intermediate in DNA demethylation pathway, but there is an increase in evidence that also supports its role as a regulator of gene expression (Bachman et al., 2015).

Even if these findings are crucial for the suitability of using DNA methylation and hydroxymethylation as biomarkers, little is known about the temporal behavior of the DNA methylome under normal physiological conditions. Almost every biomonitoring study assessed DNA methylation in samples collected at a single-time point, and thus accounting for the inter-individual variability (Giuliani et al., 2016; Talens et al., 2012). In contrast, no information about temporal trends or the intra-individual variability were provided (Yamada and Chong, 2016).

Furthermore, DNA methylation changes were associated with changes in gene expression induced by environmental exposures such as air pollution (Leenen et al., 2016). In this sense, it has already been shown that particulate air pollution is associated with oxidative stress (Grevendonk et al., 2016; Li et al., 2016) that may further affect both global and gene-specific DNA methylation in blood and placental tissue (Baccarelli et al., 2009; Janssen et al., 2015; Madrigano et al., 2011). Nevertheless, associations between environmental exposures and global DNA (hydroxy)methylation levels in surrogate tissues such as buccal cells have never been investigated.

In this context, the objectives of this study were to evaluate whether global DNA (hydroxy)methylation levels in buccal cells of healthy adult individuals are dynamic on short-term and whether these levels are influenced by factors such as life-style and pollution.

2. Materials and methods

2.1. Study design

A group of 26 Caucasian persons were enrolled in this study. Study participants were recruited among students (n = 23) and researchers (n = 3) from the University of Leuven, and were not occupationally exposed to air pollutants. Most volunteers resided in Leuven. The volunteers were not allowed to eat or brush their teeth 2 h before sample collection in order to minimize the loss of cells on the buccal mucosa. All participants received information about the purpose and objectives of the study, gave written informed consent and filled in a short 'life-style' questionnaire (Godderis et al., 2012). Several factors that are known for their potential influence on the epigenome, including sex, alcohol consumption and smoking behavior were assessed using a standardized questionnaire (Christensen and Marsit, 2011; Hagerty et al., 2016). Also aging is known to alter DNA methylation (Bollati et al., 2009; Jones et al., 2015). Therefore, only volunteers aged between 18 and 27 years were included. The study was approved by the Commission for Medical Ethics of University Hospitals Leuven (reference number: S57170) and registered at ClinicalTrials.gov (ID: NCT02297009).

2.2. Sample collection

Buccal cells were collected using a cytobrush, provided in the DNA extraction kit (QIAGEN, Venlo, The Netherlands). Three samples were taken at four different time points separated by one week starting from 9 February 2015 until 9 March 2015. All samplings were scheduled between 8:00 h and 12:00 h. The cytobrush was twirled and rubbed for 30 s against the buccal mucosa from the right inner cheek, when at the same time counter pressure was applied on the outer cheek. The brush was separated from the stick with sterile scissors in a 1.5 mL Eppendorf. Samples were stored at − 80 °C before DNA extraction.

The turnover time of the surface layer of the oral mucosal epithelium is about 2.7 h and the whole epithelium is replaced after approximately 4.5 days (Dawes, 2003). Thus, it is highly unlikely that the weekly collection of buccal swabs as such can induce an effect on DNA methylation, neither directly by mechanical forces applied onto the cheek, nor indirectly by disrupting the oral epithelium, since buccal swabs contain mainly exfoliated cells.

2.3. DNA extraction

DNA was extracted using the Gentra Puregene® Buccal Cell Kit (QIAGEN), according to the manufacturer's protocol with minor adjustments. An extra protein precipitation and DNA washing step was performed to improve DNA purity. Each sample was extracted separately. DNA concentration and A_{260}/A_{280} ratio were determined using the GeneQuant™ 100 (GE Healthcare, Diegem, Belgium). A minimum of 0.75 µg DNA was needed in order to perform ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) analysis of global DNA methylation and hydroxymethylation levels. Sufficient DNA for all samples of all time points was extracted. The average A_{260}/A_{280} ratio was 1.53 ± 0.25. Samples were stored at − 80 °C before analysis.

2.4. DNA methylation and hydroxymethylation analysis

Samples were prepared according to the method described by Godderis et al. (Godderis et al., 2014) with minor changes and adaptations. Briefly, isolated DNA (1 µg) was enzymatically hydrolyzed to individual deoxyribonucleosides by a simple one-step DNA hydrolysis procedure. A digest mix was prepared by adding phosphodiesterase I, alkaline phosphatase and benzonase® Nuclease to Tris-HCl buffer. Extracted DNA was spiked with a mixture containing the internal standards (IS), dried and then hydrolyzed at 37 °C for at least 8 h in presence of 10 µL digest mix. After hydrolysis, 490 µL acetonitrile (ACN) was added to each sample, to a total volume of 500 µL. Daylight was avoided at maximum over the entire sample preparation procedure, in order to minimize potential deamination of the target compounds.

An ultra-performance UPLC-MS/MS method was used for the identification and quantification of 2′-deoxycytidine (2dC), 5-methyl-2′-deoxycytidine (5mdC) and 5-hydroxymethyl-2′-deoxycytidine (5hmdC) as described in Cardenas et al. (2017). A 20 µL aliquot was injected on a hydrophilic interaction liquid chromatography (HILIC) column (Phenomenex® Kinetex 2.6 µm Hilic, 50 mm × 4.6 mm), held at a temperature of 60 °C. The mobile phase used for the chromatographic separation was a mixture of 20 mM Ammonium Format Buffer pH 3 (A) and ACN (B) using the following gradient: the program starts at 7%A, was increased linearly to 20%A for 2.2 min, then was hold from 2.2 to 2.4 min at 20%A, brought back to the initial status from 2.4 to 2.6 min and allowed to equilibrate for one minute prior to the next injection. A flow rate of 0.4 mL/min was applied. The analyses were performed using electrospray ionization (ESI) in positive mode and the compounds were determined using multiple reactions monitoring (MRM), with argon as the collision gas.

Stock solutions of 2dC, 5mdC, 5hmdC and [15N3]-2dC were prepared by dissolution of commercial solid reference standards in water. The stock solutions were used to prepare the calibration standards. To compensate for the matrix effects, the validation was conducted using an artificial matrix simulating a mammalian DNA hydrolysate comprising three 2′-deoxyrubonuleosides (2′-deoxiguanisine, 2′-deoxyadenosine, and thymidine). The correlation coefficients, R^2, of the regression equations exceeded the value of 0.98, demonstrating a good correlation between the measured response (peak area) and the concentration of the target compounds. The limits of quantification (5mdC: 0.482 ng/mL, 5hmdC: 0.023 ng/mL, and 2dC: 1.856 ng/mL) were determined based on the lowest calibration levels analyzed in five