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Association of Global DNA Hypermethylation with Hypertension, 8-oxo-7,8-dihydro-2'-deoxyguanosine, and Uracil Misincorporation: A Cross-Sectional Study on Taxi-Motorbike Drivers Working in Cotonou, Benin

Patrice Hodonou Avogbe[1,2*], Elise Jeannesson-Thivisol[3], Christelle Yelognisse Ahomadegbe[4], Arnaud Zinsou Vinouyon Henry[1], Megnisse Catherine Gwladys Monligui[1], Stella Gloria Quist[1], Gbèna Ulrich Evrard Lokonon[1], Thimoléon Kizito Agbessy[1], Mauril Houtchai[1], Achille Martial Nouchet[1], Sodjiné Déo-Gracias Hountokoude[1], Iré Carine Olodo[1], Ambaliou Sanni[2]

[1]Genomic Epidemiology Research Unit, Tropical Infectious Disease Research Center, Faculty of Sciences and Techniques, University of Abomey-Calavi, Cotonou, Republic of Benin
[2]Laboratory of Biochemistry and Molecular Biology, Department of Biochemistry and Cellular Biology, University of Abomey-Calavi, Cotonou, Republic of Benin
[3]University Hospital of Nancy, Brabois Hospital, Nancy, France
[4]Unit of Pathological Anatomy and Medical Oncology, Porto-Novo, Republic of Benin
Email: *Patrice.avogbe@gmail.com

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Abstract

Background: Occupational exposures are known contributors to oxidative stress and epigenetic alterations, but their interrelationships in highly exposed, vulnerable populations remain poorly characterized. This study investigates the associations between oxidative DNA damage, one-carbon metabolism biomarkers, and global DNA methylation in taxi-motorbike drivers (TMDs) in Cotonou, Benin—an occupational group with high exposure to traffic-related air pollution and a documented high prevalence of cardiometabolic risk factors. **Methods:** In this cross-sectional study, 138 male, non-smoking TMDs (mean age 39.4 ± 7.8 years; median occupational exposure 10 years) were included. We assessed cardiometabolic parameters, nutritional biomarkers [folate, vitamin B12, homocysteine, methylmalonic acid (MMA)], and, using a validated UPLC-MS/MS method, quantified oxidative DNA damage markers [uracil misincorporation (dU) and 8-oxo-2'-deoxyguanosine (8-oxodG)] and

global DNA methylation (5-methyl-2'-deoxycytidine, 5mdC) from white blood cell DNA. Participants were stratified into tertiles based on %5mdC levels. Multivariable logistic regression was used to identify independent predictors of global DNA hypermethylation. **Results:** Markers of oxidative DNA damage were elevated, with median dU at 13.1 nmol/L and 8-oxodG at 5.7 nmol/L. The average level of 5mdC was 5.4%. Participants in the highest %5mdC tertile (>5.6%) had significantly higher systolic (140.0 mmHg, p = 0.010) and diastolic blood pressure (88.8 mmHg, p = 0.012) and a greater 10-year Framingham cardiovascular risk (8.7%, p = 0.013) compared to the lowest tertile. They also exhibited markedly elevated levels of dU and 8-oxodG (p = 0.020 and p = 0.004, respectively). After adjustment for confounders, logistic regression models identified 8-oxodG (aOR = 1.23, 95% CI: 1.04 - 1.45, p = 0.016) and dU (aOR = 1.07, 95% CI: 1.01 - 1.13, p = 0.014) as significant independent predictors of global DNA hypermethylation. Interestingly, MMA was inversely associated with DNA methylation levels (aOR = 0.99, 95% CI: 0.98 - 0.99, p = 0.008). **Conclusion:** Global DNA hypermethylation is significantly associated with increased oxidative DNA damage and adverse cardiometabolic outcomes in TMDs cohort. Our findings suggest oxidative stress as a key driver of epigenetic alterations while highlighting a potential protective role for adequate functional vitamin B12 status. This underscores a crucial interaction between oxidative stress, one-carbon metabolism, and epigenetics. Further longitudinal studies are needed to clarify the temporal sequence of these relationships and to explore interventions targeting oxidative stress or nutritional factors as strategies to mitigate epigenetic risk in high-exposure occupational groups.

Keywords

5-methyl-2'-deoxycytidine, 8-oxo-2'-deoxyguanosine, Methylmalonic Acid, Oxidative Stress, Taxi-Motorbike Drivers, Uracil Misincorporation

1. Introduction

Taxi-motorbike drivers (TMDs) in Cotonou represent a critical occupational cohort with prolonged exposure to traffic-related air pollution (TRAP)—a complex mixture of genotoxicants including benzene, ultrafine particles, and polycyclic aromatic hydrocarbons. Our previous investigations have documented that TMDs experience substantial genotoxic damage, reflected in elevated levels of DNA strand breaks, formamidopyrimidine DNA glycosylase sensitive sites, and DNA adducts [1] [2]. Concurrently, this population exhibits a high prevalence of cardiovascular risk factors, including hypertension (47%), dyslipidemia (30%), insulin resistance (67%), hyperhomocysteinemia (88.3%), and hyperuricemia (24%) [3] [4]. These factors translate to substantial cardiovascular risk, with TMDs exhibiting elevated triglyceride-glucose (TyG) index showing nearly 7-fold increased odds of developing cardiovascular events as predicted by the Framingham Risk Score [4]. Despite these

established links, the molecular mechanisms connecting TRAP exposure to disease pathogenesis, particularly the interplay between oxidative DNA damage and epigenetic dysregulation, remain poorly characterized in this population.

Global DNA methylation, primarily measured as 5-methyl-2'-deoxycytidine (5mdC) is a key epigenetic mechanism that regulates gene expression and genomic stability. Its dysregulation is a known contributor to carcinogenesis [5]. There is growing evidence that environmental contaminants, including components of TRAP, can disrupt DNA methylation patterns, thereby mediating some of the adverse health effects of air pollution, such as metabolic syndrome [6]. DNA methyltransferases (DNMTs) establish and maintain these patterns using S-adenosylmethionine (SAM) as the universal methyl donor to cytosine residues in CpG dinucleotides. Critically, environmental exposures may promote aberrant methylation not only through direct genotoxic stress but also by indirectly taxing the metabolic pathways that supply the essential methyl groups. Through mechanisms like oxidative stress and aberrant DNA repair, such exposures can simultaneously promote hypermethylation and DNA lesions, predisposing exposed individuals to a range of malignancies including leukemia, and other tumors [7]-[9].

The integrity of this epigenetic system, however, is not solely determined by external exposures but is also profoundly influenced by internal nutritional status. Specifically, the establishment and maintenance of DNA methylation patterns are fundamentally dependent on nutritional cofactors from the one-carbon metabolism pathway [10]. Folate and vitamin B12 are essential micronutrients that act as methyl donors and coenzymes, respectively, for the regeneration of SAM. Consequently, deficiencies in folate or vitamin B12 can lead to global DNA hypomethylation by depleting SAM pools and accumulating S-adenosylhomocysteine (SAH), a potent inhibitor of SAM-dependent DNMTs [11] [12]. This metabolic disruption is often reflected by elevated levels of homocysteine, a sensitive functional marker of impaired one-carbon metabolism. Elevated homocysteine not only indicates insufficiency of these vitamins but may also contribute directly to increased oxidative stress, further exacerbating epigenetic dysregulation [13]. Therefore, an individual's nutritional status can significantly modulate their epigenetic response to environmental insults like TRAP.

Beyond these indirect nutritional pathways, TRAP can directly induce epigenetic dysregulation through oxidative stress. Exposure generates reactive oxygen species (ROS), notably the highly reactive hydroxyl radical (·OH). This oxidative stress subsequently promotes both oxidative DNA lesions and dysregulation of epigenetic mechanisms, encompassing DNA methylation and histone modifications [14]. A well-established biomarker of this oxidative damage is 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), which results from the oxidation of guanine and is prone to mispairing with adenine, promoting G→T transversions—a mutagenic event common in carcinogenesis [15]. Notably, 8-oxodG has been proposed for inclusion in medical surveillance programs to identify workers with past or recent carcinogen exposures who may require more intense clinical monitoring

[16]. Mechanistically, the presence of 8-oxodG can inhibit the methylation of adjacent cytosine residues, creating a direct link between oxidative damage and epigenetic alteration [17]. Furthermore, uracil misincorporation into DNA, resulting from oxidative stress or folate deficiency, introduces erroneous bases into DNA, compounding genomic instability.

Despite these insights, the interplay between oxidative DNA damage (8-oxodG, dU) and global DNA methylation, and nutritional markers has not been comprehensively investigated in populations with high occupational TRAP exposure. This gap is particularly relevant for TMDs, who endure chronic, high-level exposure. This study was therefore designed to bridge the existing knowledge gap by integratively assessing both oxidative DNA damage markers and global DNA methylation from TMDs in Cotonou. By evaluating the associations among these biomarkers and key nutritional factors, the study aims to characterize molecular alterations driven by chronic TRAP exposure and to determine whether oxidative stress independently predicts epigenetic changes. The findings will illuminate early mechanistic pathways linking TRAP exposure to long-term disease risk and could inform targeted biomarker surveillance and prevention strategies for this vulnerable occupational group.

2. Methods

2.1. Study Population and Participant Selection

The study population consisted of professional taxi-motorbike drivers (TMDs) in Cotonou, Benin, an occupational cohort with well-documented characteristics and exposure profiles as described in previous publications [3] [4] [18]. A cross-sectional design was employed, with participants recruited at random from high-traffic waiting areas where drivers gather between fares.

Inclusion criteria were: 1) male gender, age >18 years, current non-smoker, and actively engaged as a professional TMD; 2) no self-reported history of CVD or chronic conditions known to interfere with vitamin B12 or homocysteine metabolism; and 3) completion of a full health assessment, including questionnaire, clinical examination, and biospecimen collection.

For the present biochemical analysis, an additional criterion of data completeness was applied. Specifically, we included only participants with valid measurements for all primary outcome variables: white blood cell 8-oxodG, global DNA methylation (5mdC), and uracil misincorporation.

After applying these filters, the final analytical sample comprised 138 TMDs. The median duration of occupational exposure in this group was 10 years (interquartile range: 7.0 - 16.0). The study was approved by the institutional review board of the Benin Environmental Agency and was conducted in strict adherence to the ethical standards outlined in the Declaration of Helsinki.

2.2. Data Collection and Biological Sampling

Data and samples were collected using standardized protocols for all participants.

Trained research staff conducted face-to-face interviews using a structured questionnaire to gather comprehensive data on sociodemographic characteristics, lifestyle factors (including tobacco use and alcohol consumption), occupational history, and personal and familial medical history.

Anthropometric and blood pressure measurements were performed according to a standardized protocol. After a resting period of at least five minutes in a seated position, blood pressure was measured twice on the right arm using a calibrated sphygmomanometer; the average of the two readings was used for analysis. The 10-year risk of a general CVD event was estimated for each participant using the Framingham Risk Score (FRS), which incorporated age, sex, blood pressure, lipid parameters, and smoking status, as previously described [18].

Following an overnight fast of at least 8 hours, venous blood samples (approximately 5 mL) were drawn from each participant. Samples were processed within two hours of collection by centrifugation at 4000 rpm for 10 minutes. The resulting plasma and buffy-coat fractions were aliquoted into cryovials and stored at -20˚C until subsequent biochemical and genetic analyses. Plasma concentrations of vitamin B12, folate, homocysteine (Hcy), and methylmalonic acid (MMA) were quantified using established methods, as detailed in previous publications from our group [4] [19].

2.3. DNA Isolation and Quantitation

Genomic DNA was extracted from frozen buffy-coat fractions using the Nucleon BACC3 kit (GE Healthcare, France) according to the manufacturer's instructions. The purified DNA was dissolved in 200 µL Tris-HCl buffer (10 mmol/L, pH 8.0) containing 1 mmol/L EDTA and stored at −20˚C until use. DNA concentrations and purity were measured by using PicoGreen dSDNA quantification kit (Invitrogen, Carlsbad, CA, USA). All DNA samples were diluted to a uniform working concentration for downstream applications.

2.4. Enzymatic Hydrolysis of DNA

A comprehensive enzymatic hydrolysis was performed to quantify global DNA methylation, 8-oxodG, and uracil misincorporation levels. This method was adapted from the protocol previously published by Chango *et al.* [20]. Briefly, 2 µg of extracted DNA was denatured by heating at 100˚C for 3 min, followed by immediate cooling of the tubes on ice. The denatured DNA was then digested with 2 units of nuclease P1 and incubated for 2 hours at 45˚C to cleave the DNA into 5'-mononucleotides. Digestion continued with phosphodiesterase I (0.002 units) for 2 hours at 37˚C, which further ensures complete hydrolysis to deoxynucleoside-5'-monophosphates, followed by alkaline phosphatase (0.5 units) at 37˚C for 1 hour to convert the monophosphates into free deoxynucleosides. After digestion, DTT (10 mM final concentration) was added to the hydrolysate to prevent oxidation of nucleosides. The hydrolysate which was then filtered using filtration plates (Pall Corporation, PN 5034, AcroPrep 96-well Filter Plates, 350 µL, 10K) to remove

any precipitated proteins or enzymes and stored at −20˚C until LC-MS/MS analysis.

2.5. Simultaneous Determination of Global m5dC, Uracil, and 8-oxodG Levels in Genomic DNA by UPLC-MS/MS

Chromatographic separation of deoxy-nucleosides—including dA, dT, dG, dC, 5mdC, dU, and 8-oxodG—was achieved using ultra-high-performance liquid chromatography (UPLC Acquity, Waters) equipped with a C18 column (ACQUITY UPLC HSS T3, 1.8 µm, 2.1 × 100 mm; Waters) maintained at 35˚C. The mobile phase consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in methanol, delivered at a flow rate of 0.27 mL/min with a gradient elution program. Detection was performed with tandem mass spectrometry (4000 QTrap LC-MS/MS, Sciex) operated in positive electrospray ionization (ESI+) mode in Multiple Reaction Monitoring (MRM) mode. The optimized MS parameters were as follows: ion spray voltage, 5500 V; source temperature, 305˚C; curtain gas, 15 psi. The specific MRM transition pairs for the key analytes were set as follows: dC: 228/112 (retention time, RT: 0.93 min), 5mdC: 242/126 (RT: 1.43 min), dU: 229/113 (RT: 1.87 min), and 8-oxodG: 284/168 (RT: 4.70 min).

2.6. Method Validation and Quantification

To ensure analytical rigor and the robustness of the results, the method was validated according to several key parameters: 1) nucleoside stability, 2) sensitivity and calibration, 3) accuracy and precision, and 4) reproducibility and repeatability. Stability assays confirmed that all analyzed nucleosides were perfectly stable in 10 mM DTT; consequently, all standard and sample solutions were prepared using this medium. Sensitivity and calibration were established using a seven-point calibration curve generated with pure deoxynucleoside standards (Sigma-Aldrich), which demonstrated excellent linearity ($R^2 > 0.99$). Precision (repeatability) and accuracy were determined by repeatedly analyzing (n = 10 per batch) a pool of hydrolyzed DNA as a quality control (QC) sample. The average intra- and inter-assay coefficients of variation (CVs) for each nucleotide were <5%, and the accuracy was >95%, confirming the method's reliability. It should be noted that an internal standard was not used in this assay. Quantification of all deoxynucleosides was performed using these curves. The global level of 5mdC was expressed as the relative percentage of total cytosine bases using the formula: %5mdC = [5mdC/(5mdC + dC)] × 100. The levels of dU and 8-oxodG were expressed in nmol/L. Laboratory analyses were performed at the NGERE Research Unit, ("Nutrition-Génétique-Exposition aux risques environnementaux"), Faculty of Medicine, Nancy University, France, following strict quality control protocols and manufacturer specifications.

2.7. Definition of "Hypermethylation" in the Context of This Study

Hypomethylation and hypermethylation are relative terms denoting "less" or "more" methylation compared to a standard reference, such as DNA from healthy

tissues. In this study, 'hypermethylation' is used as a relative classification within the cohort's distribution rather than implying an absolute pathological state. This operational definition was necessary because, in the context of global DNA methylation—which is typically in the range of ~3% - 5% of all cytosine [21]—a universal clinical threshold for a 'hyper' state is not well-defined. In the absence of an established cut-off for our specific cohort of Beninese TMDs, we adopted a data-driven approach, defining hypermethylation as the highest tertile of 5mdC levels (*i.e.*, 5.6%). This standard methodological approach identifies individuals with the relatively highest methylation burden to investigate its association with studied genetic alterations and cardiovascular risk outcomes.

2.8. Statistical Analysis

All statistical analyses were performed using IBM SPSS Statistics version 27. Continuous data are presented as mean ± standard deviation (SD) or median and interquartile range (IQR), as appropriate. Categorical variables are summarized as frequencies and percentages.

To analyze the relationship between global DNA methylation (5mdC) and other variables, participants were categorized into tertiles based on their 5mdC levels: T1 (<5.1%, lowest), T2 (5.1% - 5.6%), and T3 (>5.6%, highest). Differences in continuous and categorical variables across tertiles were assessed using the Kruskal-Wallis test and the Chi-square test, respectively. Bivariate correlations between continuous variables were examined using Pearson's correlation coefficient.

To identify independent predictors of high global DNA methylation, we performed binary logistic regression. The outcome variable was hypermethylation status, which was dichotomized into a binary format: participants with 5mdC levels >5.6% (the highest tertile, T3) were classified as having hypermethylation and were compared against the reference group comprising the combined lower two tertiles (T1 + T2, 5mdC ≤ 5.6%). The model was adjusted for potential confounders selected *a priori*: age, body mass index, systolic and diastolic blood pressure, and key nutritional biomarkers (folate, vitamin B12, methylmalonic acid, and homocysteine). The Framingham Risk Score was also included in the model to assess its predictive value independent of the oxidative stress markers. Results are reported as adjusted odds ratios (aOR) with 95% confidence intervals (CI). A two-tailed p-value < 0.05 was considered statistically significant.

3. Results

3.1. Demographics, Oxidative Stress, and Global DNA Methylation Biomarkers in the Study Population

The study cohort was a population of 138 TMDs who were relatively young (mean age of 39.4 years ±7.8) but occupationally mature population, with a median job duration of 10 years (IQR: 7 - 16) (**Table 1**). Systolic blood pressure averaged 134.2 mmHg (±18.2) and diastolic 84.8 mmHg (±12.7), with a high prevalence of hy-

pertension (46.4%, 95% CI: 38.1 - 54.7).

Nutritional biomarkers showed considerable individual variation: the median folate level was 6.4 nmol/L (IQR: 5.4 - 7.8), and vitamin B12 was 381.5 pmol/L (IQR: 313.8 - 487.0). Homocysteine levels were substantially elevated (median 24.3 µmol/L).

Oxidative stress markers showed substantial DNA damage, with uracil misincorporation (dU) at median 13.1 nmol/L and the oxidative lesion 8-oxodG at median 5.7 nmol/L. Global DNA methylation, measured as 5mdC, showed a mean value of 5.4% and exhibited considerable interindividual variation, ranging from 4.2% to 8.5%.

Table 1. Demographic characteristics, oxidative stress biomarkers, and global DNA methylation levels in the study population.

	Mean (SD)	Median (IQR)	Min	Max
Age (years)	39.4 (7.8)	39.0 (34.0 - 44.0)	22.0	59.0
BMI (kg/m²)	23.5 (3.9)	22.6 (20.8 - 25.7)	17.5	37.2
SBP (mmHg)	134.2 (18.2)	130.0 (120.0 - 145.0)	100.0	190.0
DBP (mmHg)	84.8 (12.7)	80.0 (80.0 - 90.0)	60.0	120.0
Job duration (years)	11.7 (5.9)	10.0 (7.0 - 16.0)	1.0	27.0
Folate (nmol/L)	6.6 (2.0)	6.4 (5.4 - 7.8)	2.6	12.2
Vitamin B12 (pmol/L)	410.2 (156.5)	381.5 (313.8 - 487.0)	146.0	1020.0
Homocysteine (µmol/L)	30.9 (19.3)	24.3 (18.0 - 37.5)	11.8	143.0
Methylmalonic acid (nmol/L)	178.4 (82.6)	157.0 (126.5 - 208.0)	0.0	552.0
5mdC (%)	5.4 (0.6)	5.3 (5.0 - 5.7)	4.2	8.5
dU (nmol/L)	14.5 (8.9)	13.1 (7.1 - 20.1)	2.7	52.6
8-oxodG (nmol/L)	6.2 (3.2)	5.7 (3.6 - 8.1)	1.9	15.9
FRS (10-year CVD)	7.3 (4.8)	5.6 (3.9 - 9.4)	1.6	25.0
	n/N (%)	**95% CI**		
Hypertension, n (%)	64/138 (46.4)	38.1 - 54.7		
Alcohol use, n (%)	55/138(39.9)	31.7 - 48.1		

Values are expressed as mean (standard deviation) or median (interquartile range, IQR). BMI: Body mass index, SBP: Systolic blood pressure, DBP: Diastolic blood pressure, 5mdC: 5-methyl-2'-deoxycytidine, 8-oxodG: 8-oxo-2'-deoxyguanosine, dU: deoxyuracil, FRS: Framingham risk score, IQR: Interquartile range, SD: Standard deviation.

3.2. Cardiometabolic and Oxidative Stress Biomarkers Stratified by Global DNA Methylation Tertiles

The data reveal a clear and significant relationship between global DNA methylation (5mdC) levels and cardiovascular risk profile, with the most pronounced differences observed in blood pressure. Participants in the highest methylation tertile (>5.6%) exhibited substantially higher mean systolic and diastolic blood pressure

(140.0/88.8 mmHg) compared to the middle (130.2/81.4 mmHg) and lowest (134.1/85.1 mmHg) tertiles, with these differences being statistically significant (SBP p = 0.010; DBP p = 0.012) (Table 2). This clinical finding is corroborated by a significantly higher prevalence of hypertension (56.4%) in the highest 5mdC group compared to the middle group (35.2%), further strengthening the link between hypermethylation and elevated cardiovascular risk.

Furthermore, oxidative stress biomarkers increased significantly across the 5mdC tertiles in a dose-dependent manner. Both uracil misincorporation (dU) and the oxidative lesion 8-oxodG were lowest in the low methylation group and rose progressively to their highest levels in the hypermethylation group (dU: 11.9 vs. 17.0 nmol/L, p = 0.020; 8-oxodG: 5.1 vs. 7.6 nmol/L, p = 0.004). This suggests that global DNA hypermethylation is closely associated with increased oxidative DNA damage. The 10-year cardiovascular disease risk, as predicted by the Framingham Risk Score, was also significantly different across groups (p = 0.013), being highest in the top 5mdC tertile (8.7%), which aligns with the group's worse blood pressure profile (Table 2).

Table 2. Cardiometabolic, oxidative stress, and epigenetic biomarkers stratified by global DNA methylation tertiles.

	Global DNA methylation levels						
	T1: 5mdC <= 5.1% (n = 45)		T2: 5.1 < 5mdC < 5.6% (n = 54)		T3: 5mdC > 5.6% (n = 39)		
	Mean (SD)	95% CI	Mean (SD)	95% CI	Mean (SD)	95% CI	P value
Age (years)	38.6 (8.5)	36.1 - 41.1	38.5 (6.7)	36.7 - 40.3	41.3 (8.0)	38.6 - 43.9	0.247
BMI (kg/m²)	24.5 (4.8)	23.0 - 25.9	22.6 (3.2)	21.8 - 23.5	24.0 (3.4)	22.9 - 25.2	0.110
SBP (mmHg)	134.1 (18.2)	128.6 - 139.5	130.2 (18.8)	125.0 - 135.3	140.0 (17.0)	134.5 - 145.6	0.010
DBP (mmHg)	85.1 (12.9)	81.2 - 89.0	81.4 (13.2)	77.8 - 85.0	88.8 (11.4)	85.1 - 92.6	0.012
Job duration (years)	12.0 (6.4)	10.0 - 13.9	11.0 (5.6)	9.5 - 12.5	11.9 (6.0)	9.9 - 13.9	0.775
Folate (nmol/L)	6.9 (1.9)	6.3 - 7.5	6.8 (2.0)	6.2 - 7.3	6.1 (1.8)	5.6 - 6.7	0.127
Vitamin B12 (pmol/L)	412.6 (184.9)	357.0 - 468.1	414.5 (153.8)	372.1 - 456.9	392.8 (108.5)	356.6 - 429.0	0.904
Homocysteine (µmol/L)	28.5 (15.5)	23.9 - 33.2	31.8 (23.8)	25.2 - 38.3	32.1 (17.0)	26.6 - 37.7	0.458
Methylmalonic acid (nmol/L)	201.8 (104.4)	170.4 - 233.2	154.7 (62.0)	137.7 - 171.6	182.4 (70.1)	159.7 - 205.1	0.061
5mdC (%)	4.8 (0.3)	4.7 - 4.8	5.4 (0.2)	5.3 - 5.4	6.0 (0.5)	5.9 - 6.2	<0.001
dU (nmol/L)	11.9 (7.5)	9.5 - 14.2	15.0 (9.4)	12.4 - 17.6	17.0 (8.9)	14.1 - 19.9	0.020
8-oxodG (nmol/L)	5.1 (2.9)	4.1 - 6.1	6.0 (2.8)	5.2 - 6.8	7.6 (3.5)	6.4 - 8.8	0.004
FRS (10-year CVD)	7.49 (5.1)	5.9 - 9.0	6.1 (4.0)	5.0 - 7.2	8.7 (4.9)	7.1 - 10.3	0.013
Hypertension, n (%)	23 (51.1)	36.5 - 65.7	19 (35.2)	22.4 - 48.0	22 (56.4)	40.8 - 72.0	0.045
Alcohol use, n (%)	15 (33.3)	19.6 - 47.0	24 (44.4)	31.1 - 57.6	16 (40.1)	25.6 - 56.4	0.279

Values are expressed as mean (standard deviation) or median (interquartile range, IQR). BMI: Body mass index, SBP: Systolic blood pressure, DBP: Diastolic blood pressure, 5mdC: 5-methyl-2'-deoxycytidine, 8-oxodG: 8-oxo-2'-deoxyguanosine, dU: deoxyuracil, FRS: Framingham risk score, 95% CI: 95% confidence interval, SD: Standard deviation.

Notably, this association appears specific, as other potential confounders showed no significant variation across the methylation groups. Age, BMI, job duration, and

key nutritional biomarkers (folate, vitamin B12, and homocysteine) were statistically similar across all three tertiles. Methylmalonic acid showed a trend toward significance (p = 0.061), with its lowest levels in the middle tertile, hinting at a potential non-linear relationship with methylation that warrants further investigation.

A significant positive correlation was observed between global DNA methylation and the level of deoxyuracil (dU) in white blood cells. This relationship is indicated by a correlation coefficient (r) of 0.309, which is highly statistically significant (p < 0.001). In contrast, the relationship between 5mdC and white blood cell 8-oxo-2'-deoxyguanosine shows a weaker, though still statistically significant, positive correlation (r = 0.235, p < 0.007, Figure 1).

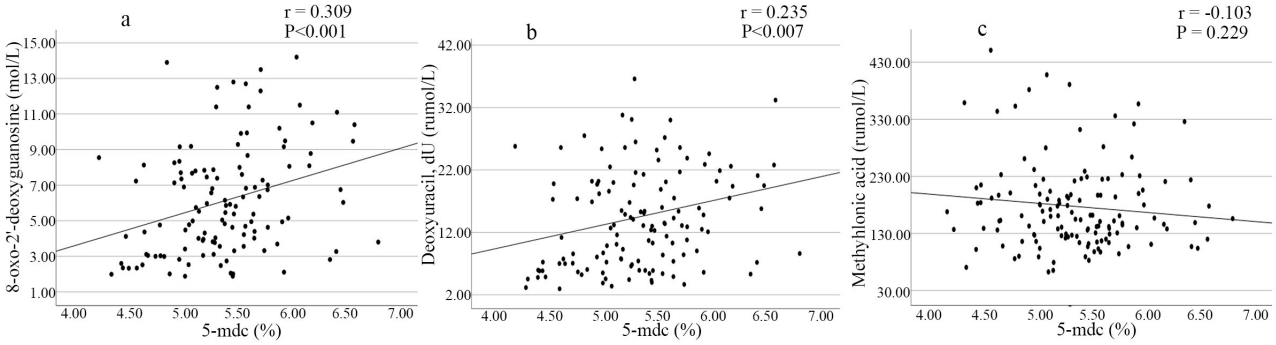

Figure 1. Scatter plots illustrating the relationships between global DNA methylation levels and (a) white blood cell 8-oxo-2'-deoxyguanosine (8-oxodG), (b) deoxyuracil (dU), and (c) methylmalonic acid (MMA) in the study population.

3.3. Predictors of Global DNA Hypermethylation

Table 3. Predictors of elevated global DNA methylation identified by multivariable logistic regression analysis.

Dependent variable	Residual determinants	aOR	95% CI	P value
5mdC > 5.1%	**8-oxodG**	1.23	1.04 - 1.45	0.016
	dU	1.07	1.01 - 1.13	0.014
	MMA	0.99	0.98 - 0.99	0.008

aOR: adjusted odd ratios, 5mdC: 5-methyl-2'-deoxycytidine, 8-oxodG: 8-oxo-2'-deoxyguanosine, dU: deoxyuracil, MMA: methylmalonic acid, 95% CI: 95% confidence interval. Logistic regression model was adjusted for age, BMI, SBP, DBP, folate, MMA, and vitamin B12.

Logistic regression analysis revealed that oxidative stress markers were significant independent predictors of global DNA hypermethylation (5mdC > 5.1%) in TMDs, after adjustment for age, BMI, blood pressure, folate, vitamin B12, and MMA. Each unit increase in 8-oxodG was associated with a 23% increased odds of DNA hypermethylation (aOR = 1.23, 95% CI: 1.04 - 1.45, p = 0.016), while each unit increase in uracil misincorporation (dU) was associated with a 7% increased odds (aOR = 1.07, 95% CI: 1.01 - 1.13, p = 0.014) (Table 3). In contrast to oxidative markers, MMA demonstrated a significant inverse association with global DNA hypermethylation status (aOR = 0.99, 95% CI: 0.98 - 0.99, p = 0.008). In the ad-

justed model, the Framingham Risk Score was not a significant predictor of elevated DNA methylation status (aOR = 0.93, 95% CI: 0.81 - 1.01, p = 0.367, data not shown).

4. Discussion

This study provides novel insights into the interplay between oxidative stress, epigenetic alterations, and cardiovascular risk in a young, high-risk occupational cohort. We demonstrate for the first time in TMDs that global DNA hypermethylation is significantly associated with elevated blood pressure and increased oxidative DNA damage (8-oxodG and dU). Importantly, oxidative stress markers were independent predictors of this epigenetic shift, whereas MMA, a functional marker of vitamin B12 deficiency showed a significant inverse association. Collectively, these results suggest that chronic environmental exposures may contribute to early cardiovascular dysregulation through interconnected pathways that simultaneously drive oxidative stress and alter epigenetic patterns.

4.1. Global DNA Hypermethylation Is Linked to Cardiovascular Risk and Is Independently Driven by Oxidative DNA Damage Biomarkers

A key finding is the dose-dependent association between higher levels of global DNA methylation and an increasingly adverse cardiovascular risk profile. Participants in the highest 5mdC tertile exhibited significantly higher systolic and diastolic blood pressure and a greater prevalence of hypertension, aligning with the well-documented, though complex, role of epigenetic dysregulation in hypertension pathogenesis [22] [23]. Our results are consistent with a growing body of epidemiological evidence linking DNA hypermethylation to cardiovascular disease. This includes findings that LINE-1 hypermethylation in blood DNA predicts a higher risk of CVD mortality, suggesting its role as a biomarker of accelerated vascular aging [24]. Furthermore, methylation levels at specific CpG sites have been associated with incident coronary heart disease (CHD) across diverse populations, underscoring its potential as an informative tool for understanding CHD development [25].

The concomitant, graded increase in oxidative stress biomarkers—8-oxodG and uracil misincorporation—across methylation tertiles offers a plausible mechanistic link. 8-oxodG, a hallmark of oxidative stress to DNA, has been shown to directly influence the DNA methylation machinery by altering the binding affinity of methyl-CpG-binding proteins and DNA methyltransferases [26]-[28]. Similarly, uracil misincorporation, often a consequence of folate deficiency or oxidative stress, can disrupt DNA-protein interactions and trigger repair processes that may inadvertently reshape the epigenetic landscape. Our data suggest that the cumulative oxidative DNA damage reflected by these biomarkers may be a key driver of the observed global hypermethylation. This hypothesis is strongly supported by our multivariable analysis, which identified 8-oxodG and uracil mis-

incorporation as significant, independent predictors of global DNA hypermethylation, even after adjusting for key nutritional and cardiometabolic confounders. The persistence of these associations underscores the robustness of the link between oxidative damage and epigenetic alteration. The specificity of this relationship is further highlighted by the lack of significant association between hypermethylation and other potential confounders, such as age, BMI, and homocysteine, which are often implicated in epigenetic studies. However, the cross-sectional nature of our study precludes definitive conclusions on causality, and we cannot rule out the possibility of a bidirectional relationship, wherein hypermethylation of genes involved in antioxidant defense or DNA repair genes could impair cellular defenses and exacerbate oxidative stress.

MMA (aOR = 0.99, 95% CI: 0.98 - 0.99, p = 0.008) shows a small but statistically significant inverse association with global hypermethylation. Because higher MMA reflects poorer vitamin B12 status, this indicates that better functional B12 status (lower MMA) is associated with a higher likelihood of global DNA hypermethylation, whereas impaired B12 status is linked to relatively lower 5mdC. The modest effect size nonetheless points to a subtle but meaningful role of efficient one-carbon metabolism in shaping the epigenetic landscape, and although these cross-sectional data cannot prove causality, they support further longitudinal work to clarify whether improving B12 status can modify methylation patterns relevant to health and disease.

The dissociation between DNA hypermethylation and the FRS is not necessarily contradictory but rather adds a critical nuance to our findings. While DNA hypermethylation was linked to the immediate, measurable phenotype of hypertension, it was not an independent predictor of the 10-year composite risk score. This suggests that in this relatively young cohort, DNA hypermethylation may act as an early marker of a specific pathogenic process—namely, oxidative stress-induced vascular dysfunction leading to hypertension—rather than a broad predictor of multifactorial long-term risk. The FRS, which heavily weights age and lipid profiles, may be less sensitive to this specific oxidative-epigenetic pathway in a population where its effects are just emerging. This aligns with the concept of epigenetic changes as early, modifiable responders to environmental stress, potentially preceding the full manifestation of clinical risk captured by traditional algorithms. Finally, it is also important to note that global methylation measures, while informative, do not reveal which specific genomic loci are affected. Future longitudinal studies employing epigenome-wide association studies are needed to achieve two key goals: first, to identify the specific genes whose altered methyla tion underlies the hypertensive phenotype, and second, to determine if these epigenetic markers can predict incident cardiovascular events.

4.2. A Mechanistic Link between DNA Hypermethylation and Uracil Misincorporation

Increased global DNA methylation may promote uracil misincorporation in white

blood cell DNA of exposed TMDs through several interrelated mechanisms involving nucleotide metabolism, DNA repair, and oxidative stress. DNA methylation, primarily occurring at cytosine residues within CpG dinucleotides, utilizes S-adenosylmethionine (SAM) as a methyl donor ("one-carbon metabolism"). This pathway is critically dependent on folate and vitamin B12 status, which not only regulate methyl group availability but are also critical for de novo thymidylate (dTMP) synthesis. Specifically, they facilitate the methylation of deoxyuridine monophosphate (dUMP) to form dTMP, thereby limiting the pool of dUTP available for misincorporation into DNA. It is plausible that in TMDs, chronic genotoxic and oxidative stress creates a high demand for SAM, potentially diverting one-carbon flux towards methylation at the expense of dTMP synthesis. This shift could elevate the dUTP/dTTP ratio in the nucleotide pool, favoring uracil misincorporation during DNA replication and repair [29].

Additionally, high global DNA methylation may induce a more condensed chromatin structure, which can sterically hinder the access of DNA repair machinery. This chromatin compaction may specifically impair the base excision repair (BER) pathway, reducing the efficiency of uracil removal by enzymes like uracil-DNA glycosylase. When the rate of uracil incorporation outpaces repair—a likely scenario under sustained oxidative stress and increased cellular turnover—uracil persists in DNA, leading to genomic instability and increased risk of DNA strand breaks. Consequently, changes in nucleosome structure and function are critical during double-strand break repair, as they help regulate the processing of DNA breaks in these less accessible, methylated regions [30]. While our data are consistent with this proposed mechanistic model, it is important to state that this pathway is inferred from the observed associations and the existing literature.

In TRAP-exposed TMDs, many of whom are hyperhomocysteinemic, oxidative stress creates a vicious cycle that exacerbates uracil misincorporation into DNA. Air pollutants generate ROS that directly damage DNA (forming 8-oxodG) and can impair the enzymes of folate metabolism, which are crucial for normal dTMP synthesis. This disruption further elevates dUTP pools, favoring uracil incorporation during DNA replication. Furthermore, oxidative stress can interfere with DNA methyltransferases, linking global DNA hypermethylation to nucleotide imbalance [31]. Consequently, uracil in DNA becomes a key marker of both metabolic disruption and failed repair in these oxidatively stressed, hypermethylated cells of exposed TMDs.

In summary, our findings support a model in which chronic exposure to TRAP drives uracil misincorporation in TMDs through three interconnected pathways: 1) increased methylation demand that diverts resources from nucleotide biosynthesis; 2) chromatin-mediated suppression of uracil repair efficacy; and 3) oxidative disruption of both metabolic and epigenetic homeostasis. This model underscores the value of concurrently monitoring epigenetic and DNA damage biomarkers in high-risk occupational health surveillance.

4.3. Limitations and Strengths

This study benefits from detailed molecular phenotyping in a unique occupational cohort with well-characterized exposure histories. The study also addresses a critical gap by integrating oxidative endpoints, epigenetic, and cardiometabolic biomarkers, enabling a comprehensive view of molecular pathways. However, the cross-sectional design precludes causal inference about the directionality of observed relationships. The observed associations, while robust and adjusted for key confounders, may be influenced by residual confounding or reverse causality. The sample size, while sufficient for initial exploration, limited our ability to explore more complex interactions or stratify by potential effect modifiers. Additionally, we lacked direct measures of air pollution exposure or genetic variants that might influence oxidative stress or methylation processes. Furthermore, the measurement of global DNA methylation, while functionally relevant, does not provide locus-specific information that could more directly link epigenetic changes to gene regulation and pathophysiology. An additional consideration is the generalizability of our findings, which may be constrained by the unique occupational profile of the TMD cohort. Specifically, the exclusion of smokers, while strengthening the internal validity of the results by removing a major confounder, may limit the applicability of our findings to the broader, smoking-included population.

4.4. Public Health Implications

These findings have important implications for occupational health policies and clinical practice, positioning TMDs as a distinct occupational group requiring urgent and targeted health strategies. This cohort exhibits a high prevalence of hypertension, moderate hyperhomocysteinemia and substantial oxidative DNA damage at a relatively young age, moving their status from a general concern to a documented, high-risk population. This elevated risk is mechanistically explained by the identified pathway in which chronic exposure to TRAP induces oxidative stress, which in turn drives global DNA hypermethylation and contributes directly to elevated blood pressure. This model provides a biologically plausible link between occupational exposure and early cardiovascular damage, suggesting a pathway that warrants testing in longitudinal and interventional studies.

Consequently, traditional health surveillance for this group is likely inadequate. The inability of the Framingham Risk Score to capture this epigenetic risk indicates that conventional assessments miss critical subclinical pathology. We therefore propose the prospective evaluation of oxidative and epigenetic biomarkers in occupational health screenings to determine if their use can improve early detection and shift the paradigm from late-stage treatment to pre-emptive intervention. While implementing such a screening program has an initial cost, it is likely to be highly cost-effective in the long term for this high-risk group, as the early identification and mitigation of cardiovascular risk would avert the far greater expenses associated with managing advanced CVD, including hospitalizations, procedures,

and long-term medication.

This evidence directly informs a multi-tiered prevention strategy. Primary prevention must focus on reducing exposure at its source through stricter occupational standards, including promoting cleaner vehicles, and providing high-quality protective masks. Secondary prevention requires the establishment of mandatory, enhanced health surveillance that includes not only blood pressure monitoring but also the aforementioned biomarkers to proactively identify individuals on a detrimental health trajectory. Finally, given the role of folate and vitamin B12 in one-carbon metabolism and DNA repair, nutritional guidance or enhanced antioxidant supplementation programs could be explored as a practical intervention to strengthen metabolic resilience against the constant oxidative insult.

In summary, this study provides a scientific foundation to advocate for TMDs as a priority group in occupational health. It calls for a fundamental shift from a reactive treatment model to a proactive, biomarker-informed public health framework designed to preserve the cardiovascular health of this essential workforce.

5. Conclusion

Our study indicates a notable association between oxidative DNA damage, global DNA hypermethylation, and elevated blood pressure among occupationally exposed TMDs. These findings suggest that chronic occupational exposures to air pollutants may contribute to oxidative stress, which in turn drives epigenetic changes that could increase susceptibility to cardiovascular dysfunction. While this cross-sectional study cannot establish causality, it provides a mechanistic framework that warrants further investigation. Future longitudinal studies are needed to validate this proposed pathway and to assess whether biomarkers of oxidative stress and DNA methylation can serve as early predictors of cardiovascular risk in young, high-risk populations. Targeted interventions to reduce oxidative stress or modify epigenetic patterns should also be explored as potential strategies for primary prevention of CVD in these vulnerable groups.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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