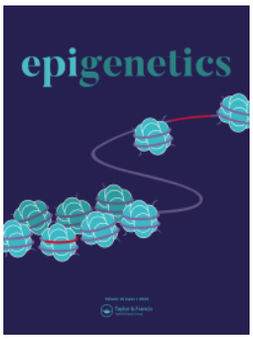

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RESEARCH ARTICLE

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Differential methylation in blood pressure control genes is associated to essential hypertension in African Brazilian populations

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ABSTRACT

While genetic studies have provided insights into essential hypertension (EH, defined by high blood pressure ≥140/90 mmHg), investigation through epigenetics may address gaps in understanding its heritability. This study focused on African Brazilian populations in Vale do Ribeira River region, due to their high hypertension prevalence. We aimed to determine if DNA methylation is linked to hypertension susceptibility, through a genome-wide evaluation of 80 peripheral blood samples from normotensive (39) and hypertensive (41) individuals, with Infinium Methylation EPIC BeadChip platform. Data were analyzed using ChAMP package and cross-referenced with information from databases such as EWAS Atlas, GWAS catalog, GeneCards, literature, and tools such as VarElect and EWAS Toolkit. The comparison between hypertensive and normotensive revealed 190 differentially methylated CpG positions (DMPs) and 46 differentially methylated regions (DMRs), both with *p*-value ≤0.05. Among the DMPs, 27 were found to have a plausible role in blood pressure. Among the DMRs, those mapped to *ABAT, BLCAP, CERS3, EIF4E, FMN1, GABBR1, HLA-DQB2, HOXA5, IL5RA, KCNH2, MIR487B, MIR539, MIR886, MKRN3, NUDT12, PON3, RNF39, RWDD3,* and *TSHBS1* were highlighted because of their lowest *p*-values, current literature, and/or VarElect prioritization. Our findings suggest that differences in methylation contribute to the high susceptibility to essential hypertension in these populations.

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DNA methylation; essential hypertension; Quilombo populations; cardiac epigenetics

Background

Essential hypertension (EH) is a clinical condition defined by the elevation of blood pressure to levels greater than or equal to 140 mmHg (systolic blood pressure, SBP) and/or 90 mmHg (diastolic blood pressure, DBP), according to Brazilian guidelines [1]. It is a multifactorial disease that affects 1.28 billion people globally and is one of the significant risk factors for cardiovascular diseases, kidney failure, and premature death, resulting in substantial financial and personal costs for the population [2]. Although genome-wide linkage and association studies have provided valuable insights into the genetic underpinnings of complex diseases such as essential hypertension (EH), these studies do not completely elucidate the heritability of the condition [3,4]. Increasing evidence suggests that epigenetic modifications may play a crucial role in many complex diseases and disorders by mediating the influences of genetic and environmental factors. Examining EH from an epigenetic perspective can help address this knowledge gap.

Modifications like DNA methylation regulate chromatin accessibility and gene expression, impacting health and disease progression [5,6]. These patterns are shaped by factors such as diet, stress, and toxin exposure [7].

Differential DNA methylation has emerged as a potential biomarker for hypertension and its

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complications. Increasing evidence suggests that epigenetic modifications play a pivotal role in regulating inflammatory pathways, oxidative stress responses, and vascular function, thereby influencing blood pressure dynamics and contributing to the onset and progression of cardiovascular and related diseases, including hypertension, pulmonary arterial hypertension, and heart failure [8–11]. However, the majority of these studies have been conducted in populations of European ancestry or in large-scale cohorts that may not adequately account for the genetic and environmental specificities of underrepresented groups, such as Afro-descendant Brazilians. Recently, the importance of conducting epigenetic studies in underrepresented ethnic groups was reinforced by a study conducted by D'Addario et al., which demonstrated significant differences in the expression of DNA demethylases and methyltransferases between Caucasians and a combined group of Hispanics and African Americans [12]. Moreover, these epigenetic variations were associated with the severity of pulmonary arterial hypertension, highlighting the potential role of DNA methylation in the pathophysiology of the disease and emphasizing the need for more inclusive research to better understand the epigenetic mechanisms underlying hypertension in diverse populations.

Hypertension is particularly prevalent in underserved populations, including Brazilian populations known as 'Quilombos' [13]. 'Quilombos' are communities of African Brazilians, descendants of runaway slaves who established independent settlements during the era of slavery in Brazil [14]. These populations are characterized by a unique cultural heritage and a close relationship to nature but are also marked by poverty, social exclusion, nutrition transition, and limited access to healthcare services, all factors contributing to the high (32.1%) prevalence of hypertension [15]. Nutritional transition is a shift in dietary patterns, from traditional to diets higher in sugars, fat, and animal-source food, which implies increased sodium and processed foods consumption. It is often linked to epidemiological transition, which is characterized by the replacement of infectious and parasitic diseases by urban-related chronic conditions like hypertension, dyslipidemia, and obesity.

The Quilombo remnants from the Ribeira River Valley (Vale do Ribeira), in São Paulo State, Brazil represent a valuable model for studying complex diseases like EH. These populations result from ancestral admixture between Africans, Native Americans, and Europeans (with 40% African, 39% European, and 21% Native American average estimates of ancestry) [16]. They also exhibit a high prevalence of EH, estimated at about 40% [13]. Besides, they are semi-isolated populations, with similar ancestry proportions – partly due to founder effects – and are exposed to environmental similarities compared to populations in large cities. Studying EH in Quilombo remnants helps to reduce biases associated with population heterogeneity. Studies have indicated a higher prevalence of arterial hypertension in African-descendant populations compared to other populations, leading to speculation about the presence of genetic and epigenetic predisposition mechanisms [17–20].

The aim of this study was to investigate the association between patterns of genomic DNA methylation in peripheral blood and the occurrence of EH in Quilombo populations from the Ribeira Valley, in São Paulo State, Brazil. We hypothesize that environmental factors may lead to epigenetic changes, such as DNA methylation, which have the potential to modulate the expression of genes related to blood pressure. To achieve this, we focused on identifying differentially methylated CpG positions (DMPs) and differentially methylated regions (DMRs) in hypertensive and normotensive individuals from these populations using the Infinium Methylation EPIC BeadChip platform and exploring their association with hypertension susceptibility. The results of this research have the potential to offer valuable insights into the comprehension of hypertension among underrepresented populations, thereby contributing to more tailored strategies for the prevention and treatment of the condition.

Methods

An overview of the experimental strategy of the study is presented in Figure 1.

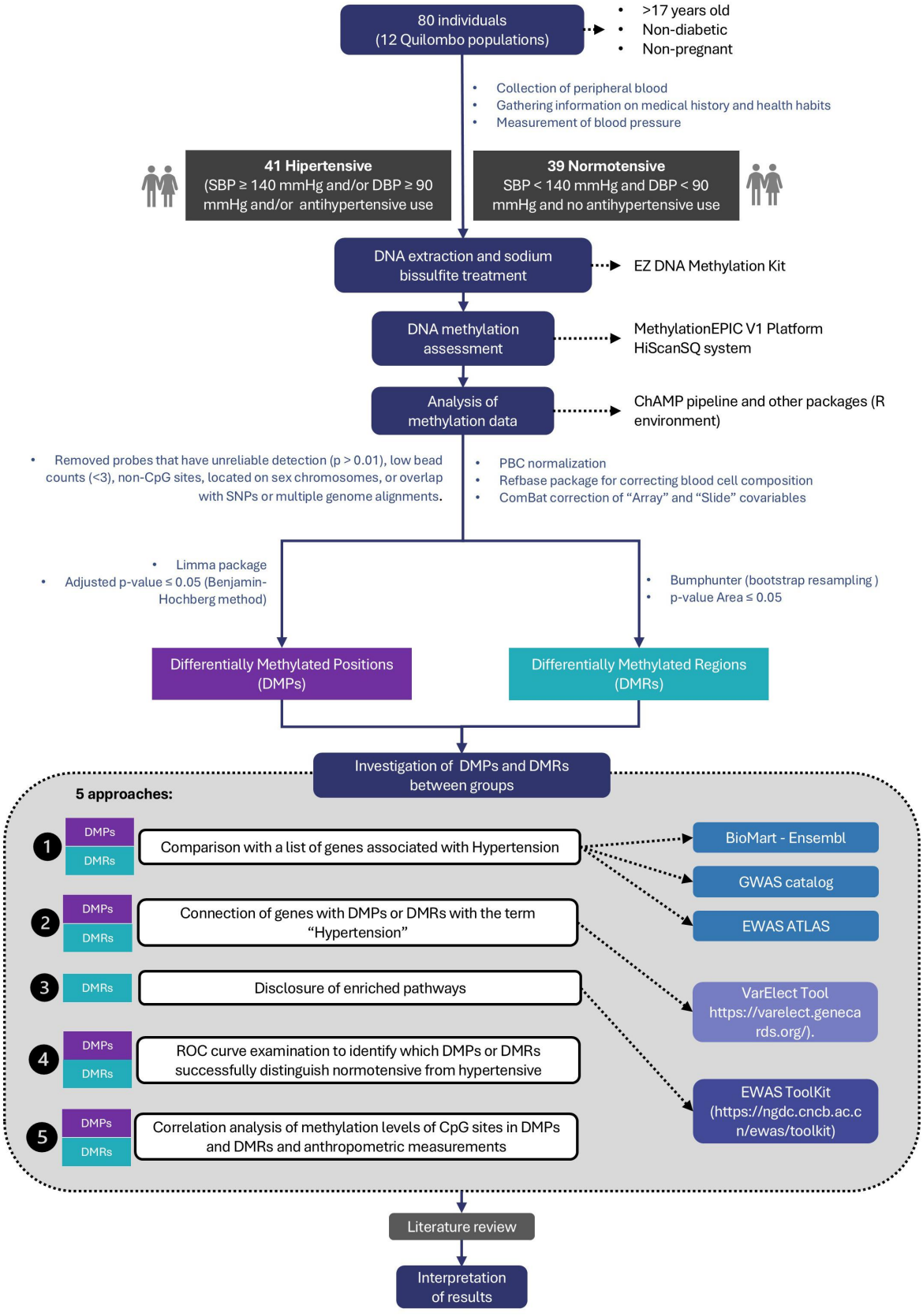

Figure 1. Study design. SBP: systolic blood pressure; DBP: diastolic blood pressure.

Study population

The cohort included 80 individuals aged over 17 y selected from 12 populations of Quilombo remnants from the Ribeira Valley region (São Paulo state, Southeastern Brazil): Abobral, Galvão, São Pedro, Pedro Cubas, Pilões, André Lopes, Nhunguara, Sapatu, Ivaporunduva, Maria Rosa, Poça, and Reginaldo [21]. Clinical evaluation and samples collection took place between 2003 and 2010, with a small subset of individuals having their blood samples recollected in 2019 to meet DNA extraction standards. All biological sample collections were conducted after obtaining informed consents, as part of research projects approved by the Ethics Committees of the Institute of Biomedical Sciences (CEP 012/2004) and the Institute of Biosciences (CEP/IBUSP 034/2005), both from the University of São Paulo (Brazil).

A questionnaire was administered to obtain medical history and health habits. Clinical data collected included age, height, weight, BMI (Body Mass Index), mean waist circumference, mean hip circumference, waist-to-hip ratio, mean systolic (SBP) and diastolic (DBP) blood pressure, and physical activity level (PAL).

Participants were measured and weighed without shoes and in light clothing. Anthropometric data were collected according to [22]. Waist and hip circumferences were measured three times, and the arithmetic mean of these values was used. Blood pressure was measured after a period of rest of 15 min and two measurements from each subject were taken at intervals of 5 min. Both SBP and DBP values were averaged from these two measurements.

Participants were classified into hypertensive and normotensive groups. Hypertension was defined according to the 'Diretrizes Brasileiras de Hipertensão Arterial' (SBP ≥ 140 mm Hg and/or DBP ≥ 90 mm Hg and/or taking antihypertensive medication) [1]. Blood pressure values in participants taking antihypertensive medications were adjusted by adding 15 mmHg and 10 mmHg, respectively, to the SBP and DBP, following [23]. Normotensive individuals were classified as having SBP < 140 mmHg and DBP < 90 mmHg and if there was no history of antihypertensive medication.

PAL was divided into four categories with higher values indicating greater activity: PAL 1 for individuals who remain mostly seated at home throughout the day; PAL 2 for those performing domestic and garden work while mostly standing; PAL 3 for individuals engaged part-time in agricultural work and part-time in domestic tasks; and PAL 4 for those working full time in agriculture, carrying heavy loads.

Only subjects whose peripheral blood sampling occurred on the same day as their blood pressure and anthropometric measurements were included in the study. Samples from diabetics and pregnant women were excluded. To minimize familial relatedness, participants in both the normotensive and hypertensive groups were selected with a kinship coefficient $K \leq 0.05$ ($1/16 = 0.0625$ corresponding to first cousins). The kinship coefficient K was calculated using genotyping data from the Axiom Human Origins 1 Array (Thermo-Fisher, Affymetrix, Massachusetts, EUA), with about 629,000 SNPs.

Global ancestry estimates were performed using the High-Density Array – Axiom Human Origins (Thermo-Fisher, Affymetrix, Massachusetts, EUA), and ancestry proportions were estimated using superpopulations (African, European, and Native American) from the Human Genome Diversity Panel (HGDP) public dataset genotyped with the same SNPs. Ancestry estimates were performed using the software ADMIXTURE v.1.3.0 [24], which assumes independence between genetic markers; therefore, the link imbalance filter ($r^2 = 0.1$) was applied using PLINK v1.9.

Statistical analysis of anthropometric data

Age, height, BMI, waist-to-hip ratio, mean SBP, and Native American ancestry were compared between the hypertensive and normotensive groups using the independent parametric Student's t test (parameters with normal distribution and homogeneous variances). For data not following a normal distribution – such as weight, mean waist circumference, mean hip circumference, mean diastolic blood pressure (adjusted or unadjusted), and African and European ancestry – the nonparametric Mann–Whitney test (Wilcoxon rank sum test) was applied. Differences between the groups were considered significant when $p \leq 0.05$.

Extraction of peripheral blood DNA and bisulfite conversion

DNA samples were extracted from the whole blood (5 mL of peripheral blood) using standard protocols of salt-extraction or with either in the Autopure LS systems (Gentra Systems) or the QIAsymphony DNA Midi Kit (96) (Qiagen, Venlo Limburg, Netherlands). DNA quantification was performed using a Nanodrop ND-1000 spectrophotometer (ThermoScientific) with an average DNA input of 250 ng up to 45 µL. The extracted DNA was subsequently treated with sodium bisulfite, using the EZ DNA Methylation Kit (ZymoResearch, Freiburg, Germany) for downstream methylation analysis.

HumanMethylationEPIC array protocol and data analysis

The hybridization step was performed with the methylation array Infinium MethylationEPIC version 1 BeadChip Kit (Illumina©, San Diego, California, USA), and the samples were scanned with the HiScanSQ system.

Each CpG site was classified based on two criteria: in relation to genes and its CpG context [25]. In relation to genes, they are categorized into TSS200 (up to 200 bases before the start of transcription), TSS1500 (from 200 to 1700 bases before the start of transcription), 5'and 3' UTRs (untranslated regions), IGR (intergenic regions), and Gene Body. In relation to the CpG context, the classifications included CpG island, Shore (up to 2000 bases before and after CpG islands), Shelf (up to 2000 bases before and after Shores), and Opensea (regions more distant than the Shelf regions).

We processed the raw intensity data files and performed a quality and differential methylation analysis [26,27] using the methylation pipeline package ChAMP version 2.20.1 [28–30] within R [31]. Probes were excluded based on unreliable detection value ($p > 0.01$), low beads counts (≤3 counts in at least 5% of samples; beadCutoff = 0.05), non-CpG probes, probes located on sex chromosomes, probes that overlap with SNPs [32], or multiple genome alignments. Infinium I and II probes were normalized using the PBC method [33], and 'Array' and 'Slide' covariables batch effects were corrected using the ComBat method, as implemented in ChAMP [34,35]. The methylation data were also corrected for blood cell composition to account for variations in cell-type proportions using the Refbase method [36].

Differential methylation analyses were carried out for the identification of DMPs between hypertensive and normotensive individuals using the Limma package [37,38] using adjusted p-value≤0.05 as given by the Benjamin–Hochberg test as criteria. Bumphunter was used to identify DMRs [39] as implemented in ChAMP, using bootstrap resampling [40] to estimate the null distribution. Criteria to be considered a DMR included adjPvalDmr = 0.05 and maxGap = 300. This approach preserves the data structure while accounting for variability within each group. For statistical significance assessment, we computed empirical p-values (p-value area) as the fraction of bootstrap-derived areas greater than or equal to the observed DMR area. To control multiple testing, we estimated the family-wise error rate (FWER Area) by comparing each observed area to the maximum areas obtained across bootstrap iterations. This conservative correction reduces the likelihood of false positives. Subsequently, Differentially Methylated Regions (DMRs) were identified as segments within a maximum of 300 base pairs between CpG sites (probes) with a statistical methylation difference between groups and at least 5 CpG sites showing the same methylation tendency. In summary, each DMR comprised a minimum of five probes and extended to several dozens. Methylation levels were quantified as beta values ranging from 0 (completely unmethylated) to 1 (completely methylated).

In silico characterization of differentially methylated positions and regions

All positions (DMPs) and regions (DMRs) identified as differentially methylated between hypertensive and normotensive subjects were analyzed *in silico* to investigate potential associations with the phenotype of interest. Genes that overlapped or were located within these positions and regions were identified using the Infinium MethylationEPIC v1.0 B4 Manifest File (CSV format) provided by Illumina (*https://support.illu*

mina.com/array/array_kits/infinium-methylationepicbeadchip-kit/downloads.html), which includes probe annotations based on the UCSC database (*https://genome.ucsc.edu/*). *In silico* characterization of these regions was performed using several tools and databases: VarElect (*https://varelect.genecards.org/*), BioMart/Ensembl (*https://www.ensembl.org*), GWAS Catalog [41], Ewas Atlas (*https://ngdc.cncb.ac.cn/ewas/atlas*), Ewas ToolKit (*https://ngdc.cncb.ac.cn/ewas/toolkit*) and PubMed (*https://pubmed.ncbi.nlm.nih.gov*).

ROC curve analysis

We conducted a receiver operating characteristic (ROC) analysis to assess the ability of DMPs and DMRs to discriminate between Hypertensive and Normotensive groups. Beta values for each CpG site were obtained from Infinium MethylationEPIC BeadChip data and analyzed using the pROC package [42] in R.

The ROC curves were generated for each DMP and DMR using the roc() function, with the sample group (Hypertensive vs. Normotensive) as the binary outcome variable. For DMRs, average beta values across sites were used. The optimal threshold for classification was determined using Youden's Index, which maximizes the sum of sensitivity and specificity. Sensitivity and specificity values at the optimal threshold were extracted using the coords() function. The area under the curve (AUC) was calculated to assess the predictive performance of each CpG site.

Correlation analysis of methylation and anthropometric data

We assessed the correlation coefficient (r) between DNA methylation levels and anthropometric variables using Spearman's rank correlation test in an in-house R script. Methylation data were transposed and preprocessed to retain only DMPs and DMRs, ensuring a focus on biologically relevant sites. Anthropometric variables were numerically encoded, including sex (1/2), hypertension status (1/2), community (1–12), and antihypertensive medication use (1/2). Methylation and anthropometric datasets were merged by sample ID. Spearman's correlation test, implemented via the cor() function (stats R package v. 3.6.2), was used to evaluate associations between methylation levels and key anthropometric traits, such as body mass index (BMI), age, sex, weight, height, hip and abdominal circumference, waist-to-hip ratio, hypertension status, medication use, and physical activity level (PAL). P-values were computed using the *p*value() and spearman_test(), with false discovery rate (FDR) correction applied via p.adjust() (coin R package v.1.4.3). Spearman's test was selected for its robustness against non-normal distributions and its ability to capture monotonic relationships. Statistical significance was defined as $p < 0.05$, with a correlation threshold of $|0.4|$. Results were structured for downstream analysis and interpretation.

Results

Clinical and anthropometric characterization of the sample

Our cohort consisted of 80 subjects, including 41 hypertensive (19 women and 22 men) and 39 normotensive (17 women and 22 men) individuals from 12 Quilombo populations (Table 1 and Supplementary table S1). There was a higher frequency of males with PAL 3 and 4, while females were more frequent in levels 1 and 2. Then, observing the distribution of hypertensive and normotensive individuals according to the PAL, normotensive individuals had the highest frequency at level 4, whereas hypertensive individuals were most frequent at level 2 (Supplementary table S1). Chi-square (χ^2)-independence tests showed differences between the PAL distribution of based on gender, male or female ($\chi^2 = 25.2$, $\alpha = 0.05$, and $df = 3$), but not regarding blood pressure status, hypertensive or normotensive ($\chi^2 = 2.4$, $\alpha = 0.05$, and $df = 3$) (Supplementary Table S1).

We also divided the cohort based on clinical and anthropometric characteristics and compared parameters between groups (Table 1). Significant differences were observed between hypertensive and normotensive subjects in average weight ($p = 1.06 \times 10^{-3}$), BMI ($p = 7 \times 10^{-4}$), waist circumference ($p = 5.08 \times 10^{-5}$), hip circumference ($p = 6.34 \times 10^{-4}$), systolic pressure ($p = 1.03 \times 10^{-11}$), adjusted systolic pressure ($p = 6.88 \times 10^{-13}$),

Table 1. Anthropometric data of 80 individuals from the Quilombo populations divided in hypertensive/normotensive groups. N represents the number of individuals. P-values are considered significant ($p \leq 0.05$), assuming a 95% confidence interval. **ancestry ranges from 0 to 1, with 1 corresponding to complete African, European, or Native American ancestry. The t-value refers to Student's t-test for comparing means between two independent samples (with or without Welch correction for different variances between groups). The U-value refers to the non-parametric Mann–Whitney test for comparing medians between groups for parameters that do not follow normal distribution. Df refers to degrees of freedom.

	Normotensive							Hypertensive										
Parameters	N	Average	Standart deviation	Median	Interquartile range	Variance	Normal distribution	N	Average	Standart deviation	Median	Interquartile range	Variance	Normal distribution	t-value	U-value	df	P-value	Welch correction
Age (years)	39	41.77	14.35	41.00	22.05	206.03	Yes	41	44.35	15.33	43.9	24.5	235.15	Yes	0.77	–	78	4.39×10^{-1}	No
Height (m)	39	1.62	0.07	1.61	0.12	0.005	Yes	41	1.61	0.1	1.6	0.14	0.01	Yes	0.33	–	78	7.39×10^{-2}	No
Weight (Kg)	39	60.98	11.36	59.15	11.42	129.22	No	41	67.36	10.16	66.35	9.15	103.2	Yes	–	464	–	1.06×10^{-3}	–
BMI (Kg/m^2)	39	23.13	3.36	22.24	4.54	11.35	Yes	41	25.93	3.69	25.32	5.74	13.62	Yes	3.52	–	78	7.00×10^{-4}	No
Mean waist circumference (cm)	39	78.29	9.26	77.00	11.01	85.76	No	41	86.69	9.00	85.67	10.67	81.15	Yes	–	389.5	–	5.08×10^{-5}	–
Mean hip circumference (cm)	39	86.79	11.83	84.83	11.91	140.12	No	41	93.35	9.08	92.17	13.66	82.62	Yes	–	450.5	–	6.34×10^{-4}	–
Waist-hip ratio	39	0.91	0.07	0.91	0.12	0.005	Yes	41	0.93	0.07	0.96	0.12	0.006	Yes	1.46	–	78	1.48×10^{-1}	No
Mean DBP (mmHg)	39	71.33	7.02	70.00	11.00	49.33	No	41	90.71	10.84	90.00	15	117.52	Yes	–	101	–	3.37×10^{-14}	–
Adjusted mean DBP (mmHg)	39	71.33	7.02	70.00	11.00	49.33	No	41	94.61	11.39	93.00	10	129.75	Yes	–	60.5	–	1.26×10^{-16}	–
Mean SBP (mmHg)	39	118.62	11.2	120.00	13.75	125.54	Yes	41	143.18	15.81	140.00	13.5	250.11	Yes	7.98	–	78	1.03×10^{-11}	No
Adjusted mean SBP (mmHg)	39	118.62	11.2	120.00	13.75	125.54	Yes	41	149.04	18.66	145.00	23.5	348.54	Yes	8.88	–	66.05	6.88×10^{-13}	Yes
African ancestry **	39	0.44	0.15	0.49	0.2	0.024	Yes	41	0.46	0.16	0.49	0.24	0.025	No	–	724	–	4.72×10^{-1}	–
European ancestry **	39	0.37	0.16	0.33	0.2	0.026	No	41	0.36	0.17	0.3	0.23	0.029	No	–	748	–	6.25×10^{-1}	–
Native American Ancestry**	39	0.19	0.05	0.2	0.07	0.003	Yes	41	0.19	0.04	0.19	0.05	0.002	Yes	0.89	–	78	3.76×10^{-1}	No

diastolic pressure ($p = 3.37 \times 10^{-14}$), and adjusted diastolic pressure ($p = 1.26 \times 10^{-16}$). Although the p-value for the waist-to-hip ratio was not statistically significant ($p = 1.48 \times 10^{-1}$), its potential physiological relevance warrants further investigation.

Differentially methylated positions (DMPs) between hypertensive and normotensive are not randomly distributed in the genome

A total of 190 DMPs (adjusted p-value ≤ 0.05) were found after comparison of hypertensive and normotensive subjects, with 101 and 89 being more and less methylated, respectively, in hypertensive subjects.

In hypertensive individuals, more DMPs showed lower methylation levels in intergenic regions (IGR) and body regions located in opensea (areas distant from CpG islands) compared to normotensives. In contrast, regions near transcription start sites (TSS1500, TSS200) within CpG islands and shore regions (closer to the islands) exhibited higher methylation levels in hypertensive individuals (Figure 2 and Supplementary Figure S1 and Supplementary Table S2).

Chi-square (χ^2)-independence tests showed significant differences between the distribution of hypermethylated CpGs and hypomethylated CpGs based on their genomic location, both in relation to CpG context ($\chi^2 = 38.74$, $\alpha = 0.05$, and $df = 3$) – island, opensea, shelf, and shore – and gene region ($\chi^2 = 16.96$, $\alpha = 0.05$, and $df = 3$) – 1st Exon, 3'UTR, 5'UTR, body, ExonBnd, IGR, TSS1500, and TSS200 (Figure 2; Supplementary Table S2 and Supplementary Figure S1).

Among the 190 DMPs, 129 are located within 128 genes, with *BMP7* containing two differentially methylated positions (Suplementary Table S3). VarElect was used to assess links between the 128 genes and the term 'hypertension,' revealing that 41 of these genes had a direct connection to the condition. After further analysis and a literature review, 27 of the 128 genes were highlighted in Table 2. Of these, 19 genes were emphasized due to their higher VarElect score, their significance in literature, and/or higher Δβ (Δβ: average β of normotensives minus average β of hypertensives). Additionally, four genes were noted for their higher Δβ values, and four for their association with hypertension in literature (Table 2).

Among the DMPs located in genes with a hypertension link in VarElect, 42 DMPs (from 41 genes) were identified. Of these, 23 showed higher methylation levels and 19 showed lower methylation levels in hypertensive patients. Notably, all DMPs located in CpG islands near promoter regions displayed higher methylation

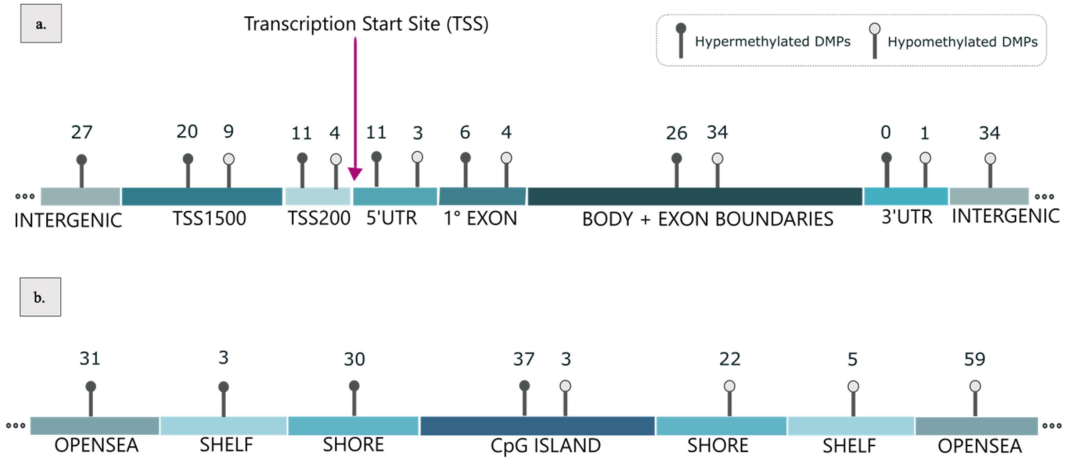

Figure 2. Distribution and classification of 190 DMPs based on their location: a) relative to genes and b) relative to CpG context. Hypermethylated DMPs (indicated by filled symbols) represent increased methylation levels in hypertensive individuals, while hypomethylated DMPs (indicated by empty symbols) represent lower methylation levels in hypertensive individuals. The number above the symbols indicate the total number of hypomethylated or hypomethylated DMPs in each localization. For each category, DMPs showing hypermethylation in hypertensive individuals will be hypomethylated in normotensive individuals, and vice versa. The decision to indicate hypomethylated DMPs on the right and hypermethylated DMPs on the left was random. We did not distinguish upstream from downstream DMPs in intergenic regions or regions in the CpG context (b).

Table 2. Table showing a list of prominent 27 DMPs based on three criteria: mapped to genes strongly associated with the 'hypertension' phenotype according to the VarElect tool and/or association with hypertension by literature search and/or higher Δβ. The data are ordered according to the adjusted p-value. The score provided by VarElect can be used to sort and prioritize the list of genes according to their relevance to the 'hypertension' phenotype as higher scores reflect a stronger association with the phenotype. Δβ: average β of normotensives − average β of hypertensives. Chr: chromosome.

Identification (cgid)	Chr.	Symbol	Description	Localization (gene – CpG island)	Δβ	Adjusted p-value	VarElect Global Rank (Total Genes 8968)	Average Disease-Causing Likelihood (VarElect)	Findings/Publications
cg18743730	17	*CA4*	*Carbonic Anhydrase 4*	TSS1500 - shore	−0.041	0.011	2785	44%	*CA4* inhibition associated with vasodilation [43]
cg03069202	1			IGR – shore	−0.034	0.013	–	–	
cg06357691	11	*ESRRA*	*Estrogen Related Receptor Alpha*	TSS200-island	−0.008	0.013	–	–	Long-term blood pressure regulation [44]
cg19045239	19	*HIF3A*	*Hypoxia Inducible Factor 3 Subunit Alpha*	TSS1500 - shore	−0.049	0.013	6462	14%	Association between DNA methylation at the second *HIF3A* promoter in cord blood and systolic blood pressure [45]
cg04991447	5	*SEMA6A*	*Semaphorin 6A*	1stExon – island	−0.010	0.013	–	–	Related to angiogenesis and cardiovascular development [46]
cg12442809	4	*CRMP1*	*Collapsin Response Mediator Protein 1*	TSS1500 - shore	−0.055	0.013	–	–	
cg08078997	12	*TNS2*	*Tensin 2*	TSS1500 - shore	0.050	0.013	–	–	Association of *TNS2* variants with pre-eclampsia and hypertensive disorders of pregnancy [47]
cg03126904	9			IGR – opensea	0.067	0.013	–	–	
cg03546668	8	*RP1*	*RP1 Axonemal Microtubule Associated*	Body – shore	0.030	0.013	–	–	
cg25677261	15	*PCSK6*	*Proprotein Convertase Subtilisin/Kexin Type 6*	Body – opensea	0.043	0.026	5568	0%	*PCSK6*-mediated Corin activation is essential for normal blood pressure [48]
cg10717739	2	*SERPINE2*	*Serpin Family E Member 2*	5'UTR – island	−0.006	0.026	5575	77%	Serine proteases play a role in the homeostasis of the cardiovascular system, including coagulation, fibrinolysis and tissue remodeling [49]
cg12387699	4	*LINC01094*	*Long Intergenic Non-Protein Coding RNA 1094*	TSS1500 - opensea	0.039	0.026	779	0%	
cg26894820	12	*LUM*	*Lumican*	1stExon – opensea	0.046	0.034	2790	71%	Related with cardiac tissue integrity [50]
cg11574151	20	*BMP7*	*Bone Morphogenetic Protein 7*	Body – opensea	0.018	0.036	742	73%	Increased risk of mortality in patients with pulmonary arterial hypertension [51]
cg20040969	8	*NEFM*	*Neurofilament Medium Chain*	TSS1500 - shore	−0.012	0.038	7896	63%	*NEFM* is a negative regulator of aldosterone production, which may affect blood pressure regulation [52]
cg20636399	14	*SLC24A4*	*Solute Carrier Family 24 Member 4*	Body – opensea	−0.037	0.040	2550	30%	Association with systolic blood pressure by GWAS [53]
cg27120125	6	*TRIM27*	*Tripartite Motif Containing 27*	TSS1500 - shore	0.035	0.040	6940	80%	
cg21513415	7	*FKBP6*	*FKBP Prolyl Isomerase Family Member 6 (Inactive)*	Body – shore	0.033	0.040	646	50%	

(*Continued*)

Table 2. (Continued).

Identification (cgid)	Chr.	Symbol	Description	Localization (gene – CpG island)	Δβ	Adjusted p-value	VarElect Global Rank (Total Genes 8968)	Average Disease-Causing Likelihood (VarElect)	Findings/Publications
cg03691958	11	*DRD2*	*Dopamine Receptor D2*	5′UTR – island	−0.007	0.041	532	63%	Participation in the regulation of blood pressure [54–57]
cg01766943	2	*SPTBN1*	*Spectrin Beta, Non-Erythrocytic 1*	Body – opensea	−0.052	0.042	1467	72%	Association with blood pressure [58]
cg09902250	10	*HPSE2*	*Heparanase 2 (Inactive)*	Body – opensea	0.052	0.042	874	54%	Related to Ochoa urofacial syndrome, which is related to kidney failure leading to hypertension (OMIM #236730)
cg25861340	5	*SLC9A3*	*Solute Carrier Family 9 Member A3*	TSS200 - island	−0.012	0.042	551	48%	Role in sodium absorption in the intestine [59]
cg01803766	11	*NTM*	*Neurotrimin*	Body – island	−0.047	0.045	–	–	Epigenetic association with hypertension (EWAS ATLAS)
cg15851418	20	*BMP7*	*Bone Morphogenetic Protein 7*	Body – shelf	0.067	0.047	742	73%	Increased risk of mortality in patients with pulmonary arterial hypertension [51]
cg20860188	2	*KLHL29*	*Kelch Like Family Member 29*	5′UTR – shelf	0.073	0.048	2977	0%	Association with Hypertension (GWAS catalog [60];
cg27373669	17	*RNF213*	*Ring Finger Protein 213*	Body - opensea	0.036	0.048	816	44%	
cg06086582	8	*PTK2B*	*Protein Tyrosine Kinase 2 Beta*	5′UTR – opensea	−0.010	0.048	2028	55%	Association of polymorphisms in *PTK2B* with hypertension [61]

levels in hypertensive patients (Table 2). In contrast, hypertensive patients showed more methylated positions in promoter regions within CpG islands, while demethylated positions were more frequent in intergenic and body regions (opensea).

A comparison of the 128 genes with a list of 507 genes epigenetically associated with terms such as 'hypertension,' 'blood pressure,' 'systolic blood pressure,' 'diastolic blood pressure,' and/or 'mean arterial pressure,' as per the EWAS Atlas, revealed that only the *NTM* gene was found in common.

Differentially methylated regions (DMRs) and their potential role in hypertension: gene prioritization and enrichment analysis

A total of 46 DMRs (comprising 493 CpG sites) were identified in the comparison between hypertensive and normotensive subjects ($p \leq 0.05$), with 43 of these DMRs mapped to 48 genes (Supplementary Table S4)

Using VarElect, we assessed the potential association between these 48 genes and the phenotype 'hypertension.' Following this analysis and a literature review, 21 genes were highlighted. Sixteen of these genes were selected based on their VarElect scores, considering our genes list (Table 3), although the scores were relatively low (the highest possible score being 200). Another five genes were highlighted due to their association with hypertension found in the literature and/or for presenting the lowest adjusted *p*-values (Table 3).

The DMRs associated with the genes *ABAT, BLCAP, CERS3, EIF4E, FMN1, GABBR1, HLA-DQB2, HOXA5, IL5RA, KCNH2, MIR487B, MIR539, MIR886, MKRN3, NUDT12, PON3, RNF39, RWDD3,* and *TSHBS1* stood out due to their lowest *p*-values, support from the current literature, and/or prioritization by VarElect. Supplementary Figure S2 shows graphically the characterization of the DMR associated with *RNF39* gene as an example.

We also performed enrichment analysis using the EWAS toolkit using Gene Ontology (GO) on the 48 DMR-related genes. The analysis revealed

Table 3. List of 21 highlighted DMRs and their corresponding genes strongly associated with the 'hypertension' phenotype, identified through VarElect and/or literature review, as well as those with the lowest adjusted p-values. The table is sorted by the p-value area, and VarElect scores are included to prioritize genes based on their relevance to hypertension phenotype with higher scores reflect a stronger association with the phenotype. FWER refers to the family-wise error rate. * *MIR539* and *MIR487B* are both in DMR_40, which also includes another gene (*MIR889*) located in TSS1500-opensea region. ***CERS3* is located in DMR_11, which also contains the gene *LASS3*, which is in TSS200-island region. Chr: Chromosome.

Identification (ID)	Chr.	Symbol	Description	Localization (gene – CpG island)	Start	End	Total sites: hyper/ hypo	p-value Area	FWER Area	VarElect Global Rank (Total Genes 8968)	Average Disease-Causing Likelihood (VarElect)	Findings/Publications
DMR_05	6			IGR-opensea	29648161	29649084	22: 22/ 0	0.0007	0.320	–	–	
DMR_02	5	*MIR886*		IGR/TSS1500-shore, Body/ TSS200-island	135415693	135416613	13: 13/ 0	0.00084	0.368	–	–	
DMR_06	6	*RNF39*	*Ring Finger Protein 39*	Body-island/shore	30038975	30039801	19: 19/ 0	0.0012	0.500	–	–	Polymorphism in *RNF39* associated with systolic blood pressure [62,63]
DMR_04	20	*BLCAP*	*BLCAP Apoptosis Inducing Factor*	5'UTR-island	36148604	36149455	31: 31/ 0	0.00172	0.620	–	–	
DMR_03	6			IGR-shore/island	31275148	31276797	23: 21/ 2	0.0024	0.740	–	–	
DMR_07	16	*ABAT*	*4-Aminobutyrate Aminotransferase*	5'UTR-opensea, TSS200-opensea, 1stExon-opensea	8806359	8807308	15: 14/ 1	0.00299	0.812	5641	72%	Connection with GABA neurotransmitter and *GABBR1* gene
DMR_10	7	*PON3*	*Paraoxonase 3*	Body-shore, 1stExon/TSS200-island, TSS1500-island/shore	95025194	95026248	20: 1/ 19	0.0046	0.944	4131	31.92%	Role of *PON3* in the regulation of renal inflammation and fibrosis in the context of hypertensive kidney disease (protective) [64]
DMR_17	7	*HOXA5*	*Homeobox A5*	TSS200/TSS1500/ 5'UTR/1stExon – island	27183133	27183816	13: 13/ 0	0.00919	1.000	2274	81%	
DMR_16	15	*FMN1*	*Formin 1*	1stExon/TSS200-island, TSS1500-shore	33486924	33487681	10: 10/ 0	0.0101	1.000	3928	7.04%	
DMR_08	15	*THBS1*	*Thrombospondin 1*	TSS1500-shore	39871808	39872186	6: 0/6	0.01224	1.000	1019	43%	Association of *THBS1* with endothelial dysfunction [65])
DMR_21	15	*MKRN3*	*Makorin Ring Finger Protein 3*	TSS200/TSS1500/ 5'UTR – opensea	23810163	23810861	9: 0/9	0.0151	1.000	1886	84.36%	
DMR_11	15	*CERS3***	*Ceramide Synthase 3*	TSS1500-island	101084980	101085177	6: 6/0	0.01995	1.000	1993	36%	Positive association between ceramides and blood pressure or vasoconstriction in humans or other animals (review in [66]
DMR_34	6	*HLA-DQB2*	*Major Histocompatibility Complex, Class II, DQ Beta 2*	Body-island	32729465	32729823	11: 0/ 11	0.0268	1.000	3519	34.52%	Association with diastolic blood pressure [67]
DMR_29	5	*NUDT12*	*Nudix Hydrolase 12*	TSS200/TSS1500/ 5'UTR – opensea	102898223	102898733	9: 9/0	0.02888	1.000	7896	62%	Close to promising locus for blood pressure regulation [68]

(*Continued*)

Table 3. (Continued).

Identification (ID)	Chr.	Symbol	Description	Localization (gene – CpG island)	Start	End	Total sites: hyper/ hypo	p-value Area	FWER Area	VarElect Global Rank (Total Genes 8968)	Average Disease-Causing Likelihood (VarElect)	Findings/Publications
DMR_36	6	*GABBR1*	*Gamma-Aminobutyric Acid Type B Receptor Subunit 1*	Body-shore	29599012	29599331	8: 0/8	0.0289	1.000	3928	55.86%	Association of chronic hypertension with upregulation of GABA type B receptor function [69]
DMR_35	7	*KCNH2*	*Potassium Voltage-Gated Channel Subfamily H Member 2*	5′UTR/TSS200-island, 5′UTR/ 1stExon/Body/ TSS1500-shore	150652702	150653449	10: 10/ 0	0.03069	1.000	1449	51%	*KCNH2* polymorphism associated with antihypertensive efficacy [70]
DMR_39	4	*EIF4E*	*Eukaryotic Translation Initiation Factor 4E*	5′UTR-shore	99850885	99851281	8: 0/8	0.0379	1.000	6940	75.36%	
DMR_40	14	*MIR487B**	*MicroRNA 487b*	TSS1500/TSS200-opensea	101512374	101512826	7: 0/7	0.04186	1.000	6018	0%	*MIR487B* connection with vascular remodeling [71]
DMR_40	14	*MIR539**	*MicroRNA 539*	TSS1500-opensea	101512374	101512826	7: 0/7	0.0419	1.000	895	0.00%	*MIR539* connection with angiogenesis [72]
DMR_46	1	*RWDD3*	*RWD Domain Containing 3*	TSS1500-shore	95698827	95699097	6: 0/6	0.04710	1.000	8037	62%	
DMR_47	3	*IL5RA*	*Interleukin 5 Receptor Subunit Alpha*	5′UTR/TSS1500/ TSS200-opensea	3151679	3152530	8: 8/0	0.0493	1.000	3360	46.69%	Related to familial erythrocytosis (MalaCards ID: ERY058) and increased *IL5RA* expression in rat models of hypertension [73]

enrichment for several functional traits, particularly those associated with angiogenesis and blood vessel regulation, such as: 'regulation of the proliferation of endothelial cells of blood vessels involved in germinal angiogenesis,' 'regulation of germinal angiogenesis,' and 'upregulation of endothelial cell migration from blood vessels' (Figure 3). In contrast, applying the same analysis to the 190 DMPs did not show any significant enrichment in terms related to hypertension.

Discriminating hypertensive and normotensive by ROC curve analysis

We performed a ROC analysis to evaluate the discriminatory power of DMPs and DMRs between hypertensive and normotensive groups.

All 190 DMPs were at least moderately effective in distinguishing hypertensive from normotensive individuals, with 34 exhibiting strong discriminatory power ($0.8 \leq AUC < 0.9$). Among these 34, we highlight cg12442809 (AUC = 0.800, *CRMP1*), cg19045239 (AUC = 0.802, *HIF3A*), cg03546668 (AUC = 0.803, *RP1*), cg03126904 (AUC = 0.806), cg10717739 (AUC = 0.808, *SERPINE2*), cg03069202 (AUC = 0.811), cg08078997 (AUC = 0.814, *TNS2*), and cg18743730 (AUC = 0.825, *CA4*) due to their lowest p-values, relevance in the literature, and/or prioritization by VarElect. Supplementary Figure S3 shows eight highlighted DMPs ROC curves.

Regarding the 46 DMRs, nine demonstrated moderate discriminatory power between the groups ($0.7 \leq AUC < 0.8$), namely DMR_08, DMR_16, DMR_18, DMR_19, DMR_23, DMR_27, DMR_31, DMR_35, and DMR_45. Among these, we highlight the DMR_08 (AUC = 0.709), DMR_16 (AUC = 0.727), and DMR_35 (AUC = 0.702) as they are located next to the genes *THBS1*, *FMN1*, and *KCNH2*, respectively, which have been previously associated with hypertension in the literature and/or identified as relevant by VarElect. Supplementary Figure S4 shows the three highlighted DMRs ROC curves.

Correlation analysis

We assessed correlations between DNA methylation levels of DMPs/DMRs and anthropometric traits, including BMI, age, sex, weight, height, waist-to-hip ratio, hypertension status, antihypertensive use, and PAL. Using Spearman's rank correlation test, we identified significant DMP and DMR associations, suggesting epigenetic regulatory roles in blood pressure and metabolism (Supplementary Figure S5 and Supplementary Table S5). Notably, affectation status was the most frequently correlated trait, appearing in 77.24% of the significant associations ($p < 0.05$).

Among the top positively correlated genes ($p < 3.7 \times 10^{-6}$; $0.52 \leq r \leq 0.56$), *ESRRA* regulates metabolic pathways and mitochondrial function,

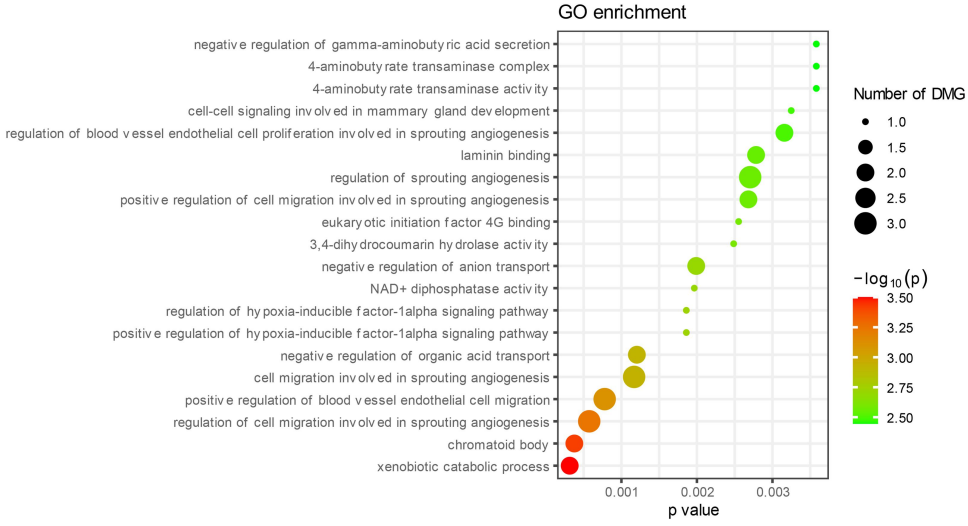

Figure 3. Analysis of functional enrichment of genes with DMRs by Gene Ontology. DMG: number of genes in DMRs differentially methylated for each GO term. Variation in p-values is represented by a color scale, with the highest values shown in green.

influencing blood pressure. *CA4* affects acid–base balance and vascular function, while *NR2E1* regulates the development of the retina and nervous system and *TLX3* controls the development of neurons and other tissues. *SEMA6A* contributes to vascular integrity and *CTNND2* encodes delta-catenin, which plays a role in neuronal development and cell adhesion. *SERPINE2* may play a role in vascular remodeling, whereas *HIF3A* was linked to systolic blood pressure. *CRMP1* regulating cytoskeleton remodeling, neuronal migration, and axon differentiation.

Conversely, the top negatively correlated genes ($p < 1.6 \times 10^{-7}$, $-0.54 \leq r \leq -0.59$) include *SLAIN1* (−0.59), which regulates microtubule dynamics, *BSCL2-GNG3* (−0.55), linked to lipid metabolism and G-protein signaling, and *PALLD* (−0.55), involved in cytoskeletal remodeling. *TNS2* (−0.54) was related with pre-eclampsia and hypertensive disorders of pregnancy, while *NXN* (−0.54) regulates redox homeostasis, cell growth, and differentiation.

Discussion

This study investigated methylation patterns in hypertensive and normotensive individuals from Quilombo populations, focusing on genes associated with hypertension. Differential methylation was observed in various genomic regions.

Several challenges were addressed during this research, particularly due to the complexity of the Quilombo populations. Firstly, the high degree of inbreeding within these populations required careful selection of unrelated individuals within the groups. Secondly, data collection involved concomitant blood pressure measurements and peripheral blood sampling.

The comparison between normotensive and hypertensive individuals identified 190 DMPs (adjusted *p*-value ≤ 0.05). Hypertensive individuals exhibited lower methylation in intergenic and body regions within opensea areas, while regions near transcription start sites with CpG islands exhibited higher methylation compared to normotensives.

A similar pattern was observed with DMPs related to hypertension by VarElect. In hypertensive individuals, 42 VarElect-related DMPs were identified with 23 showing increased methylation and 19 showing decreased methylation. Higher methylation occurred in promoter regions within CpG islands, whereas lower methylation was found in intergenic and body regions (opensea).

We also observed significant differences in the distribution of hypermethylated and hypomethylated DMPs based on their location relative to CpG islands and gene regions. Previous studies have shown a notable reduction in the overall genomic methylation levels (5mC) for the hypertensive group compared to controls, which may even be dependent on hypertension progression [74]. Moreover, both hypermethylation and hypomethylation in gene promoters involved in blood pressure regulation have been associated with hypertension development [75–77]. Our findings are consistent with these reports.

The comparison between normotensive and hypertensive also allowed the identification of 46 DMRs (*p*-value area ≤ 0.05), with enrichment in functional traits associated with angiogenesis and the regulation of blood vessels (Figure 3).

Several prominent DMPs and DMRs associated with hypertension were highlighted. Many genes potentially linked with methylation differences were supported by both VarElect and literature review via PubMed, indicating involvement in blood pressure regulation.

The genes located within or near DMPs and DMRs will be discussed in groups, divided according to their roles in blood pressure regulation.

Genes associated to vascular function

Genes linked to vascular function identified in this study include *CERS3*, *MIR487B*, *MIR539*, *THBS1*, *CA4, LUM, SEMA6A,* and *SERPINE2*. In hypertensive individuals, *CERS3* showed higher methylation, correlating with increased ceramides and blood pressure [66]. Both *MIR487B* and *MIR539* exhibited lower methylation levels, with *MIR487B* influencing *IRS1* in vascular cells and *MIR539* playing a role in angiogenesis [71,72]. *THBS1*, also highlighted in the ROC curve analysis, had lower methylation in hypertensive individuals, potentially linked to higher thrombospondin levels in hypertension [65]. *CA4* displayed higher methylation, with CA inhibition related to vasodilation and potential antihypertensive effects [43]. *CA4*

also stood out by the ROC curve and correlation analysis. In hypertensive individuals, *SEMA6A* showed higher methylation. This gene encodes Semaphorin 6A, which was related with cardiovascular development and angiogenesis [46] and highlighted by the correlation analysis. *LUM*, which is related with cardiac tissue integrity, exhibited lower methylation levels [50]. *SERPINE2*, which exhibited higher methylation at one DMP, plays a critical role in cardiovascular homeostasis, including coagulation, fibrinolysis, and tissue remodeling [49]. This gene was also highlighted by the ROC curve and correlation analysis.

There is substantial evidence in the literature that methylation of promoter regions typically results in gene silencing, whereas hypomethylation is generally linked to gene transcription activation [78]. Consequently, the observed lower methylation levels in the promoter region of *MIR539*, *MIR478B*, and *THBS1* genes in hypertensive individuals align with literature, where increased expression of these genes has been linked to blood pressure regulation [65,71,72]

The relationship between methylation and gene activation is complex, and further research is required to fully elucidate these mechanisms.

Genes related to salt and water retention

NEFM, *PCSK6*, *PTK2B,* and *SLC9A3* are genes involved in regulating salt and water retention or excretion, crucial processes in blood pressure regulation. In hypertensive individuals, the *NEFM* gene showed higher methylation levels at a single DMP. This gene acts as a negative regulator of aldosterone production, potentially influencing blood pressure regulation [52]. *PCSK6*, with lower methylation levels at one DMP, interferes with the excretion of salt and water, indirectly by processing Corin, which also impacts vascular function [48]. *PTK2B*, hypermethylated in hypertensive individuals, has been previously linked to hypertension [61]. It plays roles in cell growth, vascular contraction, inflammatory responses, and the regulation of salt and water retention by activating the angiotensin II type 1 receptor. The *SLC9A3* gene, with exhibited elevated methylation levels in one DMP, is expressed in various tissues such as the small intestine and colon and is key to intestinal sodium absorption. It has been proposed as a target for pharmacological inhibition as a novel approach to hypertension treatment [59].

Genes related to inflammation and kidney diseases

We identified DMRs located near *PON3* and *IL5RA* genes, as well as DMPs in *DRD2, ESRRA, HPSE2*, and *TRIM27* genes all of which are linked to inflammation and/or interference with kidney function. Hypertensive individuals showed reduced methylation levels in specific regions of the *PON3* gene promoter (encodes paraoxonase 3). Decreased *PON3* activity has been linked to increased oxidative stress, contributing to chronic kidney disease development. In *PON3* knockout mice, renal fibrosis and inflammation were exacerbated, emphasizing its role in mitigating hypertensive kidney disease [64].

A DMR in *IL5RA* showed increased methylation levels. In hypertensive rat models on high-fat diets, increased expression of IL5ra suggests its involvement in endothelial dysfunction and kidney injury in response to hypertensive conditions [73].

The *DRD2* gene displayed increased methylation levels at a single DMP, in hypertensive individuals. This gene encodes the dopamine D2 receptor and plays a role in blood pressure regulation. Certain polymorphisms in *DRD2* have been associated with elevated blood pressure and essential hypertension [54–56]. Reduced DRD2 expressions have been shown to increase susceptibility to renal inflammation, contributing to hypertension development in animals [57]. So, the higher methylation level in the *DRD2* gene potentially suggesting reduced gene function appears to influence blood pressure elevation.

The *ESRRA* gene displayed increased methylation levels at a single DMP, in hypertensive individuals and was also highlighted by correlation analysis. It encodes Estrogen-related receptor α, highly expressed in the kidney. This receptor was related as a key pleiotropic regulator of renal blood pressure control, renal Na(+)/K(+) homeostasis, and the renin–angiotensin pathway [44].

We found decreased methylation levels at a single DMP in *HPSE2*, a gene encoding Heparanase 2, associated with Ochoa urofacial syndrome (OMIM #236730), a condition that includes kidney failure leading to hypertension. *TRIM27*, which encodes the Tripartite Motif Containing 27, exhibited decreased methylation at a single DMP. This gene is associated with diabetic kidney disease [79].

The *BMP7* gene was notable for its association with two differentially methylated CpG positions: cg11574151, which exhibited lower methylation levels and cg15851418, which displayed higher methylation levels. *BMP7* encodes bone morphogenetic protein 7 and has been linked to an increased risk of mortality in individuals with pulmonary arterial hypertension, along with the development of comorbidities such as renal fibrosis and spondylolisthesis [51], phenotypes which could be indirectly related to blood pressure.

Gene related to nervous regulation of blood pressure

We identified DMRs in *GABBR1* and *ABAT*, both implicated in nervous system-mediated blood pressure regulation. Hypertensive individuals exhibited lower methylation in *GABBR1*, which encodes a *GABA* receptor subunit involved in baroreflex control. *GABA* receptor activation has been linked to increased arterial pressure, with upregulated *GABBR1* expression in hypertensive rats [69]. *ABAT*, encoding *GABA*-transaminase, showed increased methylation in the TSS200, 5'UTR, and part of the first exon regions. While *ABAT* is primarily associated with neurological functions, its interaction with *GABA* suggests a potential, though less established, role in hypertension.

Genes previously associated to blood pressure regulation by unknown mechanisms

We identified DMRs within or near the *HLA-DQB2*, *KCNH2*, *NUDT12*, and *RNF39* genes, as well as DMPs in *HIF3A*, *KLHL29*, *NTM*, *SLC24A4, TNS2*, and *SPTBN1* genes. These genes have been previously highlighted in genetic or epigenetic studies related to blood pressure, though the mechanisms remain unclear.

In hypertensive individuals, lower methylation levels were observed in HLA-DQB2, which encodes an HLA class II histocompatibility antigen. A genome-wide association meta-analysis of 610,091 individuals linked this gene to diastolic blood pressure, and a locus near HLA-DQB2 was associated with hypertension in Europeans [67].

KCNH2, encoding a potassium channel subunit crucial for cardiac and smooth muscle function, exhibited higher methylation levels and also stood out by ROC curve analysis. Polymorphisms in this gene have been associated with the response to antihypertensive drugs, implicating it in hypertension [70].

The *NUDT12* gene, involved in NAD and nicotinate metabolism, also showed higher methylation levels. SNPs near this gene have been implicated as potential regulators of blood pressure, suggesting its role in hypertension development and drug response [68].

For *RNF39* gene, a gene that encodes the Ring Finger Protein 39, we observed higher methylation levels. Polymorphisms in this gene were associated with systolic blood pressure by an exome-wide association study [62]. Interestingly, a hypomethylated region of RNF39 was previously associated with famine exposure in an epigenome-wide study [63] and increased susceptibility to post-traumatic stress disorder in military servicemen [80], suggesting involvement of this region in mediating epigenetic responses to stress conditions.

In hypertensive individuals, the *HIF3A* gene displayed increased methylation levels at a single DMP (highlighted by ROC curve and correlation analysis). This gene encodes the Hypoxia Inducible Factor 3 Subunit Alpha, with previous studies linking methylation in its second promoter to systolic blood pressure in children [45].

KLHL29 exhibited a higher methylation level at a single DMP. This gene, which encodes the Kelch-like protein 29, is expressed in cardiac tissue and plasma. A GWAS linked it to systolic blood pressure in pre-adolescents of African ancestry [60].

The DMP in the *NTM* gene (Neurotrinin protein), located in the body-island region, displayed higher methylation levels. It has been epigenetically associated with systolic blood pressure, mean

arterial pressure, and preeclampsia in multiple studies cataloged by the EWAS Atlas.

The *SLC24A4* gene featured a DMP with higher methylation levels. This gene encodes the potassium-dependent sodium/calcium exchanger (Solute Carrier Family 24, Member 4) and has been identified as a potential candidate gene for blood pressure regulation in a GWAS study involving 1017 African American individuals [53].

TNS2, encoding Tensin 2, showed lower methylation at a single position and was also highlighted by ROC curve and correlation analysis. Variants in this gene have been associated with preeclampsia and hypertensive disorders of pregnancy [47].

Finally, *SPTBN1*, encoding Beta, Non-Erythrocytic 1, exhibited higher methylation levels. This gene has already been associated to blood pressure regulation [58].

Epigenetics of blood pressure: the Quilombo environment

Previous epidemiological studies have shown associations between certain environmental exposures, particularly undernutrition, and increased susceptibility to hypertension or other complex diseases [81–87]. The Quilombo populations face exposure to various diseases – some prevalent in rural environment (e.g., parasitic infections). Prior studies from our group in these populations indicated a high frequency of anemia and a high seroprevalence rate of toxocariasis among children and adolescents as well as compromised anthropometric parameters [88,89]. These findings led us to hypothesize that the elevated prevalence of hypertension in Quilombo remnants might be influenced by factors like malnutrition and growth delay, potentially leading to epigenetic modifications in the genes under study. Similarly, in several other genes in the EWAS Atlas, differential methylation was linked to various environmental factors, underscoring the potential role of environmental exposures in susceptibility to complex diseases. These findings, along with our study, suggest that the investigation of environmental factors related to methylation is a promising field.

Integration of genetic and epigenetic insights into hypertension etiology

Recent findings from our group have identified distinct genomic loci associated with blood pressure regulation in Quilombo populations, emphasizing the role of admixture in hypertension risk. These genetic insights complement our epigenetic methylation findings, further highlighting the complexity of EH. Notably, our DMRs near *CERS3*, *THBS1*, and *LUM* (Tables 2 and 3) parallel the results of Borges, et al. [90], where *PHGDH* and *RYR2* were implicated in vascular function and oxidative stress pathways, reinforcing the interplay between genetic and epigenetic mechanisms in blood pressure regulation.

Strengths and limitations of the study

The study has strength in providing initial insights into essential hypertension from an epigenetic standpoint in African-descendant Quilombo populations and underscoring the need for further exploration in this vast field. Despite not being investigated here, the observed DNA methylation differences could play a role in EH heritability. Taking the EWAS Atlas of the EWAS Open Platform as an example, only 16 studies were found relating 'blood pressure,' 'diastolic blood pressure,' 'systolic blood pressure,' 'mean arterial pressure,' or 'hypertension.' Among these, only three studies (IDs: ES01623, ES00164, and ES00048) aggregated data on individuals with African American and/or Latin American ancestries, with some cohorts overlap [91,92]. This scarcity of studies exploring the association between methylation and hypertension in admixed populations – those of European, Native-American, and African ancestries – heightens the significance of our findings.

The identification of novel DMPs in genes, such as *NTM*, *RNF39*, and *HIF3A,* suggests previously unexplored epigenetic mechanisms influencing blood pressure regulation. Our study suggests that methylation differences in key blood pressure regulatory genes contribute to the increased prevalence of hypertension in Quilombo populations. These findings could pave the way for more targeted epigenetic therapies or preventative strategies.

Besides, it remains plausible that additional DMPs and/or DMRs showing statistical differences between

the groups play a role in the physiology of blood pressure regulation. Our analysis primarily focused on linking our results with previously reported data on hypertension, yet DMPs and DMRs lacking current literature support may also reveal important insights in the future.

However, our study has certain limitations that must be acknowledged, particularly the limited sample size and the absence of a validation cohort. These constraints stem from the fact that the study was designed as a preliminary, hypothesis-generating investigation, opening new possibilities for follow-up studies. Nonetheless, we intend to address these limitations by expanding our research into future projects conducted by our group. Nevertheless, the sample is characterized by a relative genetic and lifestyle homogeneity, as they come for semi-isolated populations (Quilombos), enhancing their potential to detect phenotype-related differences. Finally, we could not find similar studies to compare data disclosed here, which also indicates that as a novelty study, these are preliminary data.

There were significant differences between the groups in waist circumference, hip circumference, weight, and BMI. We were unable to adjust our analyses for these covariates using the ComBat algorithm in the ChAMP package, as this would result in the loss of biologically relevant variation between groups. Thus, there is a possible influence of body fat composition on our results. This limitation is not unique to our study, as both BMI and waist circumference are well-established predictors of diabetes and hypertension [93]. Furthermore, as suggested by Pan X et al. [94], there may be a complex feedback loop between DNA methylation and BMI, where DNA methylation influences BMI, and BMI, in turn, affects DNA methylation and related diseases, as hypertension [94]. This limitation does not undermine the initial hypothesis that environmental factors may increase susceptibility to hypertension as well as to overweight/obesity occurrence.

Final considerations

Our findings support the hypothesis that differential methylation of genes involved in blood pressure regulation may contribute to the high susceptibility to essential hypertension in Quilombo populations. This study represents one of the few to explore the epigenetic basis of hypertension in African-descendant populations. However, further research is needed to better elucidate the complex interplay between genetics and epigenetics in the development of hypertension and to clarify how these factors interact in diverse populations.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The data that support the findings of this study are available from https://figshare.com/ at https://doi.org/10.6084/m9.figshare.27891285.v1. Access to the data is subject to approval by the corresponding author due to privacy or ethical restrictions.

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