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Epigenetic Landscape of Pain in Diabetic Neuropathy

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List of abbreviations

Abbreviation	Meaning
BMI	Body Mass Index
BOPs	Blocks Of Probes
CAN	Cardiovascular Autonomic Neuropathy
DM	Diabetes Mellitus
DMRs	Differentially Methylated Regions
DN	Diabetic Neuropathy
DPN	Diabetic Polyneuropathy
HbA1c	Glycosylated Haemoglobin
HDL	High-Density Lipoprotein
IENF	Intra-Epidermal Nerve Fibers
NP	Neuropathic Pain
NRS	Numeric Rating Scale
PDN	Painful Diabetic Neuropathy
PLDN	Painless Diabetic Neuropathy
RRBS	Reduced Representation Bisulfite Sequencing
T2DM	Type 2 Diabetes Mellitus

1. Introduction

Phenomena of human environmental-genetic mismatch led to epidemic of chronic non-infectious degenerative pathologies known as civilization diseases with the Diabetes Mellitus (DM) among the most widespread (Kopp, 2019). They were rather unobserved in hunter-gatherers but they became commonness with continuous intensification and expansion of urban environments, and consequent drastic switch of the lifestyle. They cause important health complications and serious consequences for the modern economies, and moreover according to the estimates the problem will keep growing with the increasing disease prevalences during next decades (https://www.who.int/diabetes/global-report/en/ Accessed August 12, 2020).

1.1. Neuropathies and Neuropathic Pain in DM

One of the most frequent complications of diabetes – observed in 60% of cases - is neuropathy (England, 2013), particularly in it's polyneuropathic form (Boulton & Malik, 1998; Rodica Pop-Busui et al., 2017). Diabetic Polyneuropathy (DPN) is described as an axonal length-dependent nerve damage with loss of distal sensation, and with characteristic sensory loss progression in the arms and legs called as a "glove and stocking" mode (England, 2013). It was reported to reach around 16% – 20% of diabetic populations in Europe (Davies et al., 2006; Sadosky et al., 2008; Veves et al., 2008), 20% - 37% in Western Asia (Al-Mahroos & Al-Roomi, 2007; D. D. Wang et al., 2014) and around 50% in US countries (45% of non-insulin-dependent DM and 54% of insulin-dependent DM) (Dyck et al., 1993). The diagnosis of neuropathy in patients is standardly done through sensory and motor nerve conduction study, quantitative sensory testing, confocal corneal microscopy or skin biopsy (Solomon Tesfaye et al., 2010). The latter one allows monitoring of the state of degeneration of intra-epidermal nerve fibers (IENF) which simultaneously correlates with clinical phenotype and with outcomes of other neurophysiological examinations (Devigili et al., 2008). Skin biopsy assessment is though a significant instrument providing important information on the pathogenesis, character and progression of neuropathy.

Majority of DPN patients may continue without manifesting any of the symptoms that could impede their everyday life (asymptomatic diabetic neuropathy) but still around 1/3 of them develop disabling neuropathic pain (NP) – the main cause of morbidity in DM. In UK the prevalence of painful form of diabetic neuropathy among diabetic individuals was estimated for 21% (Abbott et al., 2011). In the same population 34% of studied DM patients had painful neuropathy symptoms in the absence of clinical diagnosis of diabetic neuropathy (DN) (Abbott et al., 2011). Another studies

conducted always on UK diabetic patients estimated prevalence specifically of pure neuropathic pain to 19%, of chronic form painful peripheral neuropathy to 16.2% and of mixed pain to 7.4% (Daousi et al., 2004; Davies et al., 2006). According to meta-analysis results, between 5.8–34% of diabetic patients from European countries suffered from NP (Alleman et al., 2015). The estimates for Korea (Kim et al., 2014) and Japan (Tsuji et al., 2013) were comparable reaching respectively 14.4% and 22.1% of DM cohorts, while the prevalence assessed in patients from Saudi Arabia attained 65.3% (Halawa et al., 2010).

Neuropathic pain is distinguished from other types of pain due to its particular character – simultaneous sensory loss and pain, with or without symptoms of sensory hypersensitivity as allodynia or hyperalgesia (Feldman et al., 2017). The sensory alterations may be observed as a response to one or more different modalities of stimuli: touch, pressure, pinprick, cold or heat (Jensen & Finnerup, 2014). NP in DN can reach various intensity levels and can have different forms usually described as “burning”, “electric”, “sharp”, “shooting”, “aching” (Galer et al., 2000). Thus generally it has serious adverse effects on performance of quotidian routines, on sleep and on general well-being of individual (Alleman et al., 2015). Furthermore, the available treatments for NP are highly inefficient – in around 50% of cases the achieved pain relief is at best 50% (Attal et al., 2010) and often they cause deleterious side effects (Rodica Pop-Busui et al., 2017).

Overall, why some of the DN patients develop painful form of neuropathy while others (with similar or even more severe degree of a nerve damage) – painless, it remains unclear and continues intriguing specialists of the field. Another fascinating question is how to predict and identify the individuals that are in higher risk to develope NP comparing to standard population exposed to hazards of similar levels (Lauria et al., 2014).

1.2. Risk factors of neuropathic pain in DPN

Glycemic control and duration of diabetes are main risk factors for the development of DPN (Franklin et al., 1990; Maser et al., 1989; S. Tesfaye et al., 1996). This is valid for both types of diabetes, though type 2 DM (T2DM) increases the probability of DPN onset (Van Acker et al., 2009). Additional favorable conditions that have been described up-to-date include hypertension, obesity, high levels of triglycerides and smoking (Andersen et al., 2018; Callaghan et al., 2018; R Pop-Busui et al., 2013).

On the other hand, the risk factors specific for neuropathic pain in DM are still not well identified and they are largely overlapping with the elements associated with DM and/or DN. Demographics,

clinical phenotype, lifestyle with habitat and genetics may substantially contribute to increased risk of NP (Hébert et al., 2017). Among the demographic characteristics, higher age (Halawa et al., 2010; Jambart et al., 2011; Van Acker et al., 2009; Ziegler, Rathmann, Dickhaus, et al., 2009) (particularly above 50 years) and female sex (Abbott et al., 2011; Erbas et al., 2011; Halawa et al., 2010; Jambart et al., 2011; Pai et al., 2018; Raputova et al., 2017) were identified as risk factors for neuropathic pain. The commonly observed greater prevalence of painful manifestations among women may be the reflection of possible joined sex-related nuances of biologic and psychosocial nature, and interaction between them (Hébert et al., 2017). Even though the prevalence of painful-DPN differs among the countries (as referred above, see 1.1 Neuropathies and Neuropathic Pain in DM) no evidence on association between the ethnicity and NP in diabetic patients was found (Halawa et al., 2010; Harris et al., 1993; Sorensen et al., 2002; Themistocleous et al., 2016).

Regarding the clinical factors, according to available studies it is not clear if susceptibility to develop painful-DPN could be associated with the type of DM (Abbott et al., 2011; Jambart et al., 2011) but it was shown to increase with the duration of diabetes (Jambart et al., 2011; Sorensen et al., 2002; Van Acker et al., 2009). Poor glycaemic control (Algeffari, 2018; Harris et al., 1993; Themistocleous et al., 2016), elevated glycosylated haemoglobin (HbA1c) levels (Algeffari, 2018; Themistocleous et al., 2016), low high-density lipoprotein (HDL) cholesterol (Van Acker et al., 2009), high triglyceride levels (Van Acker et al., 2009), coexisting cardiovascular diseases (Jambart et al., 2011; Ziegler, Rathmann, Dickhaus, et al., 2009), hypertension (Harris et al., 1993; Jambart et al., 2011), nephropathy (Van Acker et al., 2009), glycosuria (Harris et al., 1993) were also identified as clinical factors contributing to painful-DPN. Nevertheless, since those are complications closely related to diabetes, it is not clear if they directly rise the NP risk or rather they are tightly coexisting conditions.

Some of the components of the lifestyle – the term that broadly embraces one's diet and alimentary habits, physical activity, nature of daily executed profession, living conditions and environment - also were found to contribute to painful-DPN. Particularly body mass index (BMI) - especially reaching levels of obesity above 30 kg/m^2, weight and waist circumference were shown to significantly increase the risk of NP onset in diabetic patients (Van Acker et al., 2009; Ziegler et al., 2018; Ziegler, Rathmann, Meisinger, et al., 2009). Psychosocial profile including depression, anxiety, quality of life and social interactions, seems to be also related to neuropathic pain (D'Amato et al., 2016; Gore et al., 2005; Raputova et al., 2017). However, those factors must be interpreted with caution since the temporal relationship between them and painful manifestations in

majority of the cases is not determined. Instead, there was found no evidence that smoking and alcohol consumption could be associated with NP (Abbott et al., 2011; Halawa et al., 2010; Harris et al., 1993; Sorensen et al., 2002; Spallone et al., 2011; Ziegler, Rathmann, Meisinger, et al., 2009). Study by Jambart et al (Jambart et al., 2011) indicated that also habitat could be one of the risk factors for painful-DPN since the corresponding odds ratios of the pathology differed significantly between Egypt and two other Middle East countries: Gulf States and Lebanon, reaching respectively 0.44 and 0.66.

The first evidence on involvement of genetics in the development of painful-DPN came from a prospective survey study carried on a group of 105 patients (mostly Caucasians – 93%) with painful diabetic neuropathy (Galer et al., 2000). 56% of cases reported having relatives of first or second degree with the same pathology. To date, two genome-wide association studies were published, both involving the same diabetic cohort but applying different phenotype definitions (W. Meng et al., 2015; Weihua Meng et al., 2015). Thus, in total three chromosome loci emerged and were proposed as NP risk factors: i) sex-independent 8p21.3 cluster, located next to the gene GFRA2; ii) female-specific 1p35.1 cluster, with the ZSCAN20/TLR12P gene; iii) male-specific 8p23.1 cluster, positioned next to HMGB1P46 gene. Moreover, according to two recent studies using candidate-gene approach, there is a significant association between painful experience in DM and SCN9A (Q. S. Li et al., 2015) and OPRM1 (Cheng et al., 2010) variants.

1.3. DNA methylation and DN

While one's genetic heritage is at this point considered as immutable, epigenetics is a powerful mechanism that is able to control and alter the expression of genes. Through DNA methylation, histone acetylation and non-coding RNAs interference, epigenetics responds to external stimuli and eventually may trigger or prevent the onset of certain diseases to which one is genetically predisposed as for example T2DM and its complications (Agardh et al., 2015; Bansal & Pinney, 2017; Liu et al., 2014).

Epigenetics and particularly DNA methylation in diabetic neuropathy only very recently has been studied in humans. Although limited in sample sizes, published works are concordant in providing the evidence on altered DNA methylation patterns observed among DN patients and in proposing it as a new risk factor for this disease.

Zhang and colleagues (Zhang et al., 2019) assessed DNA methylome of white blood cells of 186 T2DM patients among who 100 and 86 were positive and negative for DPN diagnosis respectively.

Methylation levels were measured using high-performance liquid chromatography-tandem mass spectrometry. According to published results, leukocytic genomic DNA was hypomethylated in the DPN cases comparing to non-DPN controls. Additionally it was observed that the degree of DNA methylation decreased with the increasing severity of the disease, with duration of diabetes and with BMI of the individuals. Authors suggest that this can be related to both alterations in the expressions of the methyltransferases DNMT3a and DNMT3b and to the reduction of one-carbon unit (vitamin B12 and folic acid) due to T2DM.

Another evidence on contribution of epigenetic mechanisms in onset and development of diabetic neuropathy came from study involving DPN patients with T2DM from Guo and colleagues (Guo et al., 2019). Genome-wide DNA methylation was evaluated in sural nerve biopsies with reduced representation bisulfite sequencing (RRBS) technique. Two extreme DPN phenotypes were compared: six degenerators and six regenerators, identified according to the two subsequent outcomes of biopsies separated in time by 52 weeks. They represent respectively patients with significantly reducing or improving nerve fibre counts. In total there were 3,460 CpG sites (55% hypo- and 45% hyper-methylated) that were found differentially methylated between the phenotypic groups and which mapped to 2,835 unique genes. Two of three selected targets (within *DYSPL2* and *miR3138* genes) were successfully validated using loci-specific pyrosequencing methylation analyses. Nervous system development, axon guidance, glycerophospholipid metabolism and MAPK signalling were the main biological pathways that emerged from the enrichment analysis, indicating that sural nerve methylation state could be involved in modulation of regenerative capacity of the fibres in DPN.

The same research team investigated methylome in T2DM patients with DPN, comparing two extreme phenotypes: 21 and 32 individuals respectively with the highest (more severe health condition) and the lowest HbA1c levels (Guo et al., 2020). As previously they studied DNA methylation in sural nerve biopsies using RRBS approach. 2,066 CpG sites (mapping to 1489 unique genes with valid official symbols) were detected as differentially methylated, of which 57% were hypo- and 43% hyper-methylated in group with high HbA1c levels relative to group with low levels. Beside of modified methylation patterns, 71 of unique genes (4,7%) were found to have also altered expression, indicating at the same time the contribution of genetic-epigenetic interplay to DPN progress. The pathways that emerged from network analysis performed on shared differentially methylated and expressed genes were involved in immune response – known to be closely related to neuropathies (Rodica Pop-Busui et al., 2016).

Although peripheral neuropathy is the most common class of neuropathy in diabetes mellitus, there are also other forms of neurological complications that often remain under-diagnosed but still lead to very severe health consequences. Cardiovascular Autonomic Neuropathy (CAN) is one of such cases, reaching the prevalence of around 60% among diabetic patients (Pan et al., 2019). Recently, whole-genome DNA methylation analysis with Infinium Methylation EPIC BeadChip array was used to assess and compare methylome in peripheral blood cells of 24 patients with and 25 without CAN in DM type 1 (Gastoł et al., 2020). Three genomic regions were identified as differentially methylated, of which two (*BRSK2* (intron 1) and *CLDN4* (5'UTR)) were hypo-methylated, and one (*NINJ2* (intron 1)) was hypermethylated among CAN cases. Additionally, epigenetic modifications observed in *CLDN4* and *NINJ2* was successfully confirmed to have biological significance since they showed decreased expression in CAN positive individuals.

One of the genes that is the most extensively described among methylation studies of pain-related phenotypes is transient receptor potential ankyrin 1 (*TRPA1*). This gene was firstly associated with a heat sensitivity by Bell and colleagues (Bell et al., 2014). They found that *TRPA1* promoter was hypermethylated in twins that had lower heat pain suprathresholds comparing to their co-twins. In another study, similar hypermethylation pattern was discovered in subjects that were more sensitive to pressure and mechanical pain (Gombert et al., 2017). The primary evidence regarding the epigenetics of *TRPA1* particularly in neuropathic pain came from work of Sukenaga and colleagues (Sukenaga et al., 2016), who examining 12 chronic pain patients showed positive correlation between hypermethylation of this gene and number of NP symptoms. The observation was reproduced in another study involving subjects with neuropathic pain (n = 18), with non-neuropathic pain (n = 8) and without pain (n = 22), recruited from group of chronic pain patients and of lung cancer patients scheduled for thoracic surgery (Takenaka et al., 2020). According to published results, five of six evaluated CpG sites were significantly hypermethylated in neuropathic pain subjects when compared to two other phenotypic groups (with non-neuropathic pain and without pain).

To our knowledge, this is a pioneering genome-wide DNA methylation study exploring epigenetic signature of neuropathic pain in unique populations of T2DM patients that received diagnosis of diabetic neuropathy basing on evaluation of skin biopsy.

2. Aims

The aim of European-funded project PAIN-Net "Molecule-to-man pain network" is to better understand the experience of pain and individual susceptibility to it through the collective effort of multidisciplinary scientific board. Particularly, the comprehensive characterization of painful neuropathy and cancer pain profiles, (encompassing clinical, genetic, neurophysiologic, skin biopsy and the epigenetic assessment), and the integration of multiomic data produced and shared within the consortium, intended to allow identification of patients that are at higher risk to develop chronic painful manifestations and to recognize individual responsiveness to analgesics. In the framework of PAIN-NET, the aim of the present thesis was to describe the DNA methylation patterns in painful and painless type 2 diabetic neuropathy patients and to identify a set of candidate genes potentially associated with the experience of pain. Identified short list of most promising genes would serve for further validation of epigenetic signature in independent cohorts that include individuals presenting pain-related phenotypes.

Furthermore we tested the hypothesis that neuropathic pain in DN contributes to acceleration of biological age estimated with DNA methylation.

3. Study Design

The design of the present DNA methylation analysis of NP in T2DM is aimed at determining a set of potential candidate genes with different methylation patterns in painful and painless patients. Identified short list of most promising loci was validated in independent pain-related whole-genome methylation dataset available at UNIBO and produced previously in a frame of a study on migraine (Terlizzi et al., 2018). The selection of interesting candidates will be also used in future validation of epigenetic signals in independent cohorts of individuals presenting various painfull phenotypes. This study was complemented with evaluation of biological age expressed by different prediction models based on DNA methylation data.

Two independent cohorts were considered in the experimental design: PROPGER and PROPENG. Both cohorts were provided by the partners of PAIN-Net ITN Consortium and involved diabetic neuropathy patients. PROPGER cohort was provided by University of Maastricht (NL) and consisted of painful and painless DN individuals recruited in German Diabetes Center in Düsseldorf (Germany). Those individuals represented principally German population (93%). PROPENG cohort consisted of DN subjects provided by Fondazione I.R.C.C.S. Istituto Neurologico Carlo Besta in Milan (Italy) and recruited at Manchester University (UK) predominantly from English population.

A consistent clinical assessment and characterization of phenotypes were a crucial factor guaranteeing successful accomplishment of this study. Therefore, within PAIN-Net consortium uniform diagnostic approach was defined, accepted and applied by all the members. According to this agreement, neuropathy was determined basing on the result of skin biopsy and patients were considered as “painful” if they experienced neuropathic pain for more than 1 year and if their pain intensity reached score 4 or higher on the Numeric Rating Scale (NRS).

The study design foresaw assessment of the genome-wide DNA methylation using Illumina Infinium MethylationEPIC BeadChip technology. The adopted strategy allowed the identification of pain-associated regions through validated pipeline developed and originally implemented at UNIBO, in charge of this analysis within PAIN-Net project.

3.1. Sample Selection

The populational genomic background is heteregenous since it is continously shaped by demographic history, neutral and adaptive evolution and the complex interplay between them (Sazzini et al., 2016). Genetic variations are closely linked to DNA methylation and can easily

influence the epigenetic patterns, introducing in this way a confounding variability in the data that should not be neglected (Gutierrez-Arcelus et al., 2013). Therefore, in order to reduce the bias coming from populational genetics it was opted to include exclusively individuals of European ethnicity and to analyze the cohorts from German and English populations separately.

Diabetes mellitus of type 1 and of type 2 involve distinct biological mechanisms and have different genetic predisposition factors (Grant et al., 2010), and as such they could be a source of additional variability in studied phenotypes. Thus, only DN patients diagnosed with T2DM were selected and epigenetically assessed.

Whenever possible, it was attempted to match painful and painless samples regarding the chronological age and sex since both factors were shown to alter the DNA methylation levels (Bacalini et al., 2015; Davegårdh et al., 2019).

3.2. Profiles of Study Cohorts

PROPGER cohort involved 72 Painful Diabetic Neuropathy (PDN, mean age 68.8 years; age range 48 - 83) and 67 Painless Diabetic Neuropathy (PLDN, 69.7 mean age; age range 49 - 84) patients (Table 1). The phenotypic groups did not differ significantly in mean age (p-values obtained from two-sided T-test = 0.554).

The duration of T2DM was indifferent between painful and painless group, ranging between 0 – 46 and 0 – 44 years respectively and reaching in average 13.6 years. Typically DN individuals were diagnosed with neuropathy since 6 years, with PDN subjects starting suffering from the pathology slightly earlier than PLDN, however the difference of 1.4 year did not reach the level of statistical significance (p-values from two-sided T-test = 0.202). In majority of the patients (68%) diabetes was medically recognized prior to neuropathy. 27% of individuals received as primary the diagnosis of neuropathy.

Table 1 Size, sex, age, duration of T2DM and of neuropathy for each phenotypic group within PROPGER cohort are reported. PDN: Painful Diabetic Neuropathy; PLDN: Painless Diabetic Neuropathy; T2DM: Type 2 Diabetes Mellitus; SD: Standard Deviation.

	PDN	**PLDN**
Number of samples (%)	72 (52%)	67 (48%)
Sex (Females / Males)	15 / 57	14 / 53
Age Average ± SD (years)	68.8 ± 9.0	69.7 ± 9.1
T2DM Duration (years)	13.5 ± 10.3	13.6 ± 9.2
Neuropathy Duration (years)	6.9 ± 6.1	5.5 ± 6.5

In the PROPENG cohort 27 painful (PDN, mean age 62.1 years; age range 46.2 – 78.1) and 65 painless (PLDN, mean age 65.0 years; age range 40.9 – 78.1) DN patients were included (Table 2). There were no significant differences in mean age between the groups (p-values obtained from two-sided T-test = 0.169). On average, painful subjects suffered from T2DM for the last 13.4 years (duration range 0.5 – 38.8 years) while painless individuals - for 9 years (duration range 0.2 – 28.0 years). Mean span of T2DM among PDN patients was about four years longer than within PLDN group (Welch two-sided T-test p-value = 0.041).

Table 2 Size, sex, age and T2DM duration for each phenotypic group within PROPENG cohort are reported. PDN: Painful Diabetic Neuropathy; PLDN: Painless Diabetic Neuropathy; T2DM: Type 2 Diabetes Mellitus; SD: Standard Deviation.

	PDN	**PLDN**
Number of samples (%)	27 (15%)	65 (37%)
Sex (Females / Males)	9 / 18	19 / 46
Age Average ± SD (years)	62.1 ± 9.1	65.0 ± 9.0
T2DM Duration (years)	13.4 ± 9.8	9.0 ± 7.0

3.3. DNA Methylation Study

DNA extraction and experiment preparation

DNA was extracted from whole blood specimens of DN patients using Puregene Blood Quiagen kit following the steps of corresponding protocol. All the samples were quantified with Qubit dsDNA BR (Broad Range) Assay kit on Qubit Fluorometer by Thermo Fisher Scientific and verified to contain ≥ 1000ng of DNA, *i.e.* the amount required for a methylation assay. 1000 ng of genomic DNA were normalized in 50µL of H2O and were used in bisulfite conversion with EZ DNA Methylation Kit (Zymo Research) following the manufacturer's instructions. DNA methylation experiment was performed with Infinium HumanMethylationEPIC BeadChip (Illumina) according

to original protocol. Within each array the samples and phenotypic groups were accurately randomized.

Preprocessing of raw data

All the handling and manipulation of files were performed in Linux environment. Output raw data files (*.idat* format) from the two experiments were separately preprocessed using *minfi* package within R software (version 3.6.1). As summarized on Figure 1, the applied pipeline conduced the files through normalization, cleansing step, quality control and filtering according to the recommendations of Maksimovic et al. (Maksimovic et al., 2016). Poor quality samples with mean detection p-value < 0.05 were removed from the analysis. The raw values of intensities in green and red channels were normalized using functional approach removing undesired variation with a regression model of explained variability based on the control probes included in the array (using *preprocessQuantile* function from *minfi* package). Probes that failed in at least one of the samples (*i.e.* presented detection p-value > 0.01), those located on sex chromosomes and those mapping to loci with SNPs were eliminated. Additional filtering steps were implemented according to the recently published recommendations regarding the Illumina arrays therefore consequently were removed: i) probes with potential hybridization issues since aligning to the human genome hg19 in multiple sites (Benton et al., 2015); ii) non-specific and cross-reactive HM450K probes (Chen et al., 2013); iii) cross-reactive and variant containing EPIC-specific probes (Pidsley et al., 2016); iv) probes suggested to be masked from mapping according to Zhou W et al. (Zhou et al., 2017) due to non-unique 3'-subsequences, presence of INDELs or color-channel-switching SNPs etc. Finally, beta values estimating the methylation levels as a ratio of methylated to unmethylated alleles intensities, ranging between 0 (totally unmethylated) and 1 (totally methylated), were retrieved from processed *GenomicRatioSet* and used in subsequent identification of differentially methylated regions (DMRs).

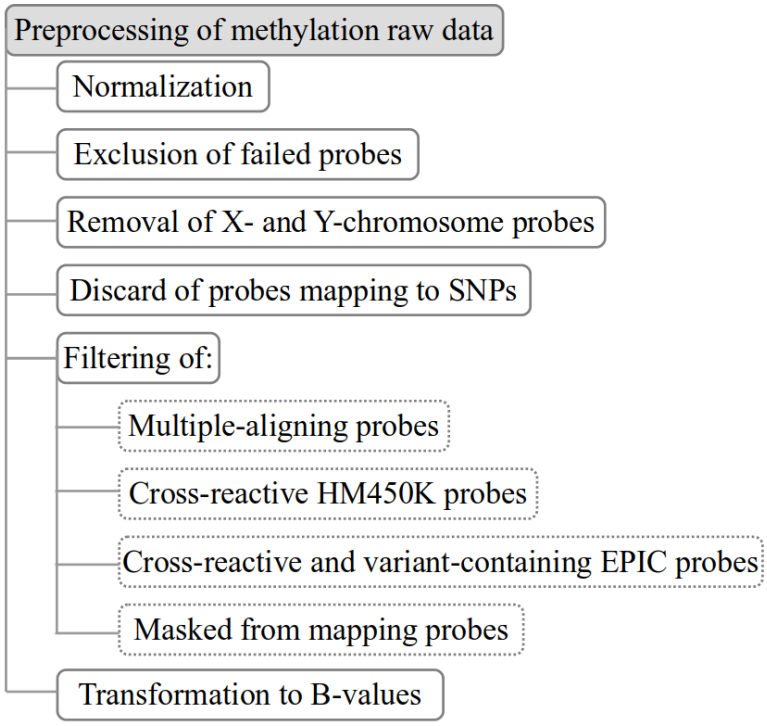

Figure 1 Summary of preprocessing pipeline of the raw methylation data inlcuding a sequence of normalisation, cleansing, quality control and filtering steps.

Applying the filtering criteria described above, all the samples from PROPGER and PROPENG cohorts were retained, while in total 211,458 and 214,251 probes were removed from respective datasets (Table 3).

Table 3 Number of probes removed in filtering steps during processing EPIC methylation results from PROPGER and PROPENG experiments.

Probes:	**PROPGER**	**PROPENG**
with detection p-value > 0.01	18310	21021
located on X- and Y-chromosomes	18298	18263
mapping to loci with SNPs	27442	27479
multi-mapping and/or non-specific cross-reactive on HM450K array	32436	32462
cross-reactive and variant-containing on EPIC array	95011	95156
additionally masked	19961	19870
In total	**211458**	**214251**

Differential analysis and identification of candidate genes

The epigenetic landscape of pain in PROPGER and PROPENG cohorts was delineated implementing an already validated pipeline developed by Bacalini et al. (Bacalini et al., 2015).

Figure 2 summarizes the applied workflow which passes through 3 stages: 1) classification of probes, 2) their grouping and eventually 3) statistical analysis. During classification step each probe was assigned to one of four groups (A-D), depending on its genomic localization (*i.e.* CpG-rich regions and genic DNA sequences) as visualized on Figure 3.

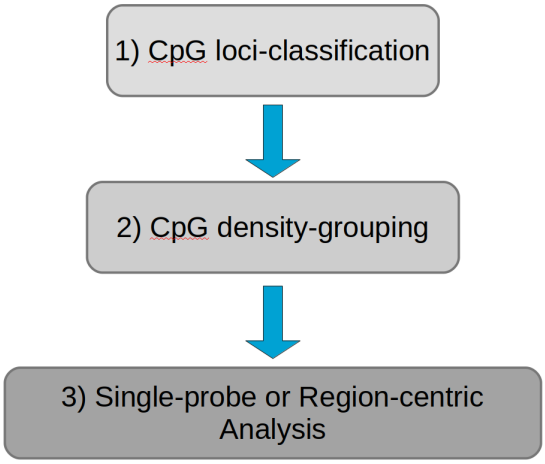

Figure 2 Graphical representation of the analytical pipeline to select differentially methylated regions, proposed by Bacalini et al. (Bacalini et al., 2015)

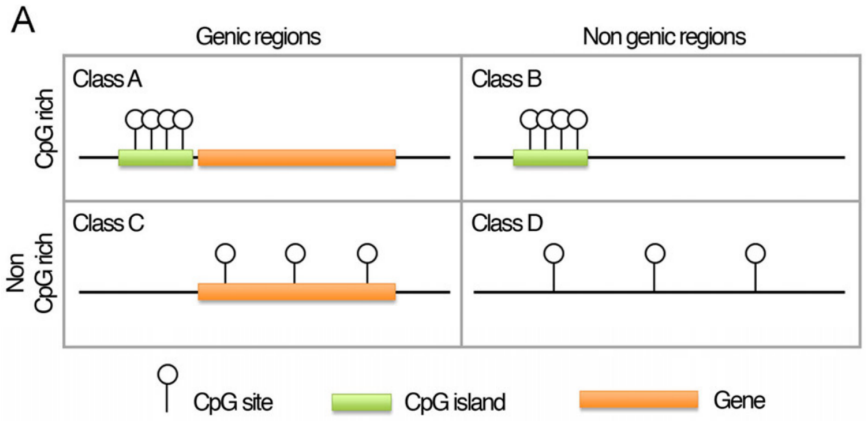

Figure 3 Summary of the annotation of probes to separate classes according to their genomic localization. (Figure from (Bacalini et al., 2015))

Subsequently probes were annotated and grouped into blocks of probes (BOPs), according to their proximity along the genome. The CpG island is a genomic region with CG/GC content ratio above 0.6 (Cross & Bird, 1995) and it is formed of central island body extended with shelfs and shores located respectively on it's both (N- and S-) extremities as presented in Figure 4. These CpG units constitute individual BOPs covered by the probes of classes A and B. The principle of this grouping is the experimental evidence of correlation of DNA methylation levels of neighboring CpGs within a span of 250 – 500 bp (Y. Li et al., 2010), suggesting that this concordant alteration of DNA methylation level likely may have a biological effect. Applied pipeline initially assumes that CpG-rich regions (class A and B) exhibit condensed distribution of methylation sites and this statement is

further verified and corrected during the statistical analysis. In a case of class C - non CpG-rich genic regions – BOPs are delimited by genic sequences, presupposing that methylation status of CpGs within a single gene shall be directed to reach the same biological effect. Since probes of class D are disperse along non-genic sequence, they remain ungrouped as single players. Described assignment of BOPs is summarized in Figure 5.

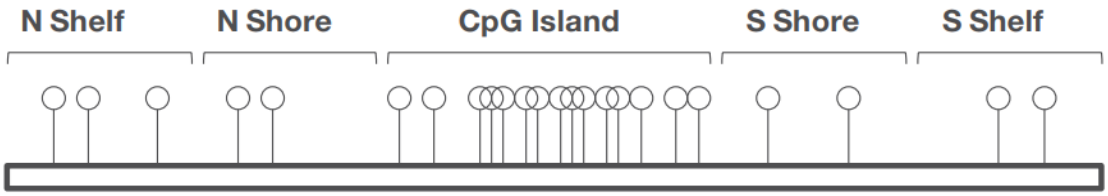

Figure 4 Sketch of general architecture of CpG island, with its specific regions. (Retrieved from (Bibikova et al., 2011))

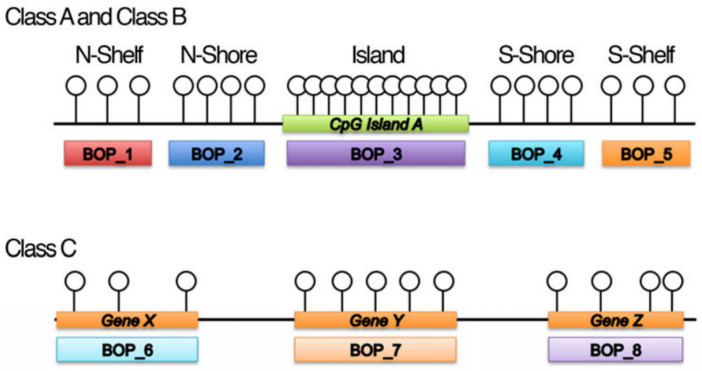

Figure 5 Representation of organization and assignment of BOPs. (Retrieved from (Bacalini et al., 2015))

Afterwards, for each identified BOP the average base-pair distance of adjacent CpGs was calculated. The statement on epigenetic correlation within 250 – 500 bp genomic intervals was verified and served as a threshold to determine the statistical approach. If computed distances were below 500 bp, the probes within this particular BOP were analyzed with region-centric approach using sliding window MANOVA. Contrary, if the base-pair intervals were longer, or there were only 2 or less probes within the single BOP, they were considered as a singletons and were analyzed applying ANOVA (Figure 6). Eventually all estimated p-values were corrected for multiple tests with Benjamini–Hochberg (BH) procedure.

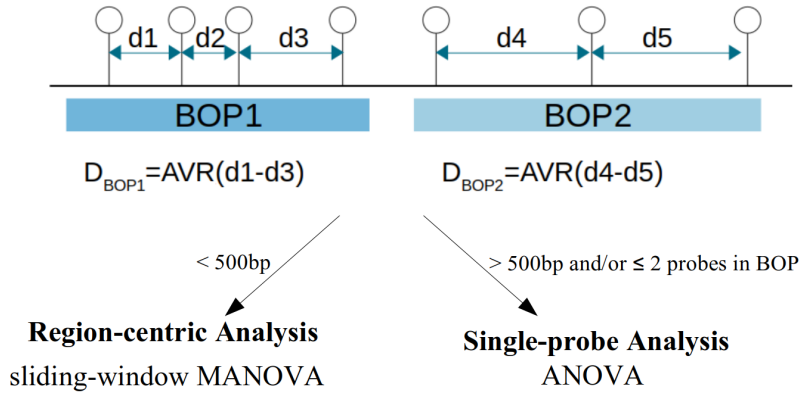

Figure 6 BOP-specific identification of statistical approach that shall be applied and proper statistical analysis to be applied.

In order to identify the promising pain-related genes and select the candidates for further validations, the most reliable epigenetic signals were searched. For this purpose, CpG sites were filtered according to following criteria:

a) significant nominal p-value from ANOVA test in PROPGER cohort;

b) absolute value of methylation change in PROPGER > 1%;

c) significant nominal p-value from ANOVA test in PROPENG cohort;

d) concordant direction of methylation change observed in both cohorts PROPGER and PROPENG.

In this analysis, consistently the statistical significance level of 0.01 was used.

To further explore obtained results, additional analyses were performed:

- inspection of independent public methylation datasets related to pain-related phanotypes (heat pain sensitivity (Bell et al., 2014); fibromyalgia (Ciampi de Andrade et al., 2017));

- inspection of methylation dataset already available from previous studies (migraine (Terlizzi et al., 2018));

- methylation standard deviation distribution analysis;

- multidimensional scaling (MDS).

In order to verify if found epigenetic patterns could be reproduced in other pain-related phenotypes, DNA methylation levels in selected candidate genes were reviewed in three independent DNA methylation datasets: i) heat pain sensitivity (Bell et al., 2014); ii) fibromyalgia (Ciampi de Andrade et al., 2017); iii) headache syndromes (Terlizzi et al., 2018). First of the datasets came from a study assessing 20 monozygotic female twin pairs discordant for heat pain suprathreshold. The second investigated DNA methylation in 24 female cases of fibromyalgia and 20 sex- and age-matched

controls. Both datasets were generated using Infinium Human Methylation 450K BeadChip. The migraine dataset was a prospective, observational study that included three subgroups of individuals: patients with chronic migraine and medication overuse headache (MOH, 25), episodic migraines (EM, 18) and healthy controls (HC, 13). The methylation data was produced with Infinium Methylation EPIC platform.

Methylation standard deviation distribution analysis was performed in order to assess the epigenetic drift in studied cohorts and to evaluate if it could be related to the experience of neuropathic pain. Methylation drift concept embraces naturally occurring age-related alterations within the methylome that are environmental and random effects (Jones et al., 2015). This phenomenon manifests across population as continuously increasing dysregulation of methylation patterns with the age (Heyn et al., 2012; Talens et al., 2012). Thus, changes in epigenetic variance were assessed through computation of standard deviation for each of methylation probes of EPIC platform and the differences between PDN and PLDN samples were evaluated with two-sample Kolmogorov-Smirnov (KS) test (statistical significance level at 1%). The results were presented as density plots visualizing the distribution of standard deviation values after logarithmic transformation.

Multidimensional scaling approach was applied to examine the dissimilarity between painful and painless phenotypes and assess if selected set of CpG sites brought out the most discriminating epigenetic signal that could help to clusterize the subjects according to clinical phenotypes or eventually could indicate presence of even more homogeneous phenotypic sub-groups. The classical method known as principal coordinates analysis was implemented (Gower, 2005). Visual representation of MDS results was created projecting the distances between the samples in 2-dimensional space.

Biological age estimation

DNA Methylation Age Calculator (https://dnamage.genetics.ucla.edu/) developed by Horvath (Horvath, 2013) was used to assess epigenetic clocks for painful and painless DN patients. These clocks directly correlate with chronological age, can predict lifespan and relate to many age-related health conditions. They are based on DNA methylation values and through algorithms involving beta values measured at specific CpG sites estimate the biological age of samples. Among propsed epigenetic clocks there are: standard Horvath's "pan-tissue" clock (Horvath, 2013), Hannum's blood-specific clock (Hannum et al., 2013), skin and blood clock that was developed for fibroblasts and other cell types used in *ex vivo* studies (Horvath et al., 2018), the PhenoAge which is enriched

in component of clinical measures of phenotypic age reflecting differences in lifespan and healtspan (Levine et al., 2018), and the GrimAge (incorporating estimates of seven different proteins and hormones, along with predicted number of smoked cigarette packs per year) claimed to have outstanding predictive potential regarding mortality and number of serious health complications (Lu, Quach, et al., 2019).

Preprocessed and normalized PROPGER and PROPENG methylation datasets were independently submitted to online tool. The results returned included a set of estimates characterizing a biological state of each individual and giving a broad view on biological functions (that change with age in standard population) and their eventual alterations in studied phenotypes. Detailed description of all calculated DNAm-based variables is provided in Table 6 in annexes.

Prior to subsequent analysis the eventual outliers were removed, *i.e.* samples for which DNAmAge estimates were below Q1 – 1.5IQR or above Q3 + 1.5IQR, where Q1 – first quartile, Q3 - third quartile, IQR - interquartile range. Thus, four and one samples were excluded from PROPGER and PROPENG cohorts respectively. The distributions of all epigenetic measures were examined and it was verified that the assumption of normal distribution could be applied to all analyzed variables. Finally, in order to examine the impact of neuropathic pain on biological age, DNAm-based estimates were used in multiple linear regression approach to explore biological age acceleration. Specifically, the differences in each DNAm estimation between painful and painless samples were assessed through linear regression model correcting for chronological age and sex: *lm(DNAm-based_variable ~ Phenotypic_group + Age + Sex)*.

4. Results

4.1. Differential Analysis

The analysis involved in total 139 samples from PROPGER cohort (72 painful and 67 painless) and 92 from PROPENG cohort (27 painful and 65 painless). The list of interesting candidates was generated by selecting CpGs that reached statistical significance level (nominal ANOVA p-value < 0.01) and presented methylation change above 1% within PROPGER cohort, and which in the same time resulted significant also in PROPENG and showed the same direction of methylation change.

In total there were 27 sites that passed established filters of which 8 were non-genic. Resulting list of 19 unique genes was further inspected in details as summarized in Table 4. 12 of the selected candidates were found hypo-methylated in painful patients while 7 were hyper-methylated. 12 (63%) of probes belonged to class C hence being located in non CpG-rich regions, and other 7 were from class A falling in CpG islands (4) and shores (3). Regarding the architecture of genes, observed alterations encompassed predominantly body of the genes (15) and 5' untranslated regions 5'URT (7) overlapping with Exon-1 or transcriptional start site TSS respectively in 3 and 1 cases. For majority of those genes (13) a link to neurological mechanisms and/or complications was found in scientific literature and databases. Presented candidates require further investigation as well as validation in independent cohorts and in broader spectrum of pain-related phenotypes. Example of methylation plots for five most promising candidates are presented on Figures 7 - 11.

Table 4 List of promising candidates that could be associated with neuropathic pain in type 2 diabetes mellitus.

Significant CpGs	Chriomosomal coordinates (hg38)	SNP	Gene symbol	Gene part	CpG Class	Direction of methylation change in PDN	Evidence on association with neurological conditions
cg16513468	chr1: 154476659	rs542970632	TDRD10	5URT	ClassA / S_Shore	Hypo-methylated	-
cg05848965	chr2: 174136765	rs139759067 rs528947674 rs547112254 rs558874572 rs116606348 rs551647325	MLK-AS1	Body	ClassC	Hypo-methylated	-
cg21147282	chr7: 18868877	rs115552810 rs138291818	HDAC9	Body	ClassC	Hypo-methylated	Involved in regulation of neuronal functions and in neurodegenerative processes (Majdzadeh et al., 2008)
cg00722325	chr7: 70044770	rs187873092 rs79912831	AUTS2	Body	ClassC	Hypo-methylated	Impicated in neuronal migration underling pathology of schizophrenia, ASD, epilepsy etc. (Hori & Hoshino, 2017)
cg26777475	chr7: 100199952	rs537022088	PCOLCE PCOLCE-AS1	5URT 1stExon Body	ClassA / N_Shore	Hypo-methylated	Contribute to muscle degeneration in oculopharyngeal muscular dystrophy (Raz et al., 2013)
cg03808984	chr7: 102631307		FBXL13	Body	ClassC	Hypo-methylated	Associated with spinocerebellar ataxia (GeneCards: FBXL13)
cg05119480	chr12: 104850745	rs143021773	CHST11	5URT 1stExon	ClassA / Island	Hypo-methylated	Shown to be related to cerebral arteriovenous malformation in mouse (MGI: Chst11)
cg04543407	chr12: 110990222		PPTC7	Body	ClassC	Hypo-methylated	-
cg08985029	chr12: 114843296	rs149450392	TBX5	5URT	ClassA / Island	Hypo-methylated	-
cg13600488	chr13: 43385844	rs568188522	LINC01050	Body	ClassC	Hypo-methylated	-
cg03533123	chr14: 55368924	rs534139027	GCH1	Body	ClassA / Island	Hypo-methylated	Associated with dopa-responsive dystonia and Parkinson's disease (Yoshino et al., 2018)
cg09630123	chr18: 76930760	rs145846343	ATP9B	Body	ClassC	Hypo-methylated	May contribute to Alzheimer's Disease risk (Barber et al., 2015)
cg16254946	chr1: 54058616	rs192034032 rs530486384	GLIS1	Body	ClassC	Hyper-methylated	May be associated with epilepsy (GeneCards: GLIS1)
cg25157472	chr3: 12996362	rs373678394	IQSEC1	Body	ClassC	Hyper-methylated	Associated with intellectual disability and epilepsy (Zerem et al., 2016)
cg23828851	chr3: 142296843		ATR	Body	ClassA / N_Shore	Hyper-methylated	Related to neurodevelopmental disorders (Lee et al., 2012)
cg23928123	chr10: 91063132	rs369032186 rs562956726	IFIT2	Body	ClassC	Hyper-methylated	-
cg11908751	chr11: 66790671	rs563475094	SYT12	TSS200 5URT	ClassA / Island	Hyper-methylated	Linked to global parkinsonism (Tasaki et al., 2019)
cg00364287	chr12: 121570752	rs575837631 rs201308543	P2RX7	5URT 1stExon Body	ClassC	Hyper-methylated	Related to abnormal pain threshold in mouse (Sorge et al., 2012)
cg20640742	chr14: 58880253		TIMM9	Body 5URT	ClassC	Hyper-methylated	Associated to myoclonic epilepsy and spinocerebellar ataxia (GeneCards:TIMM9)

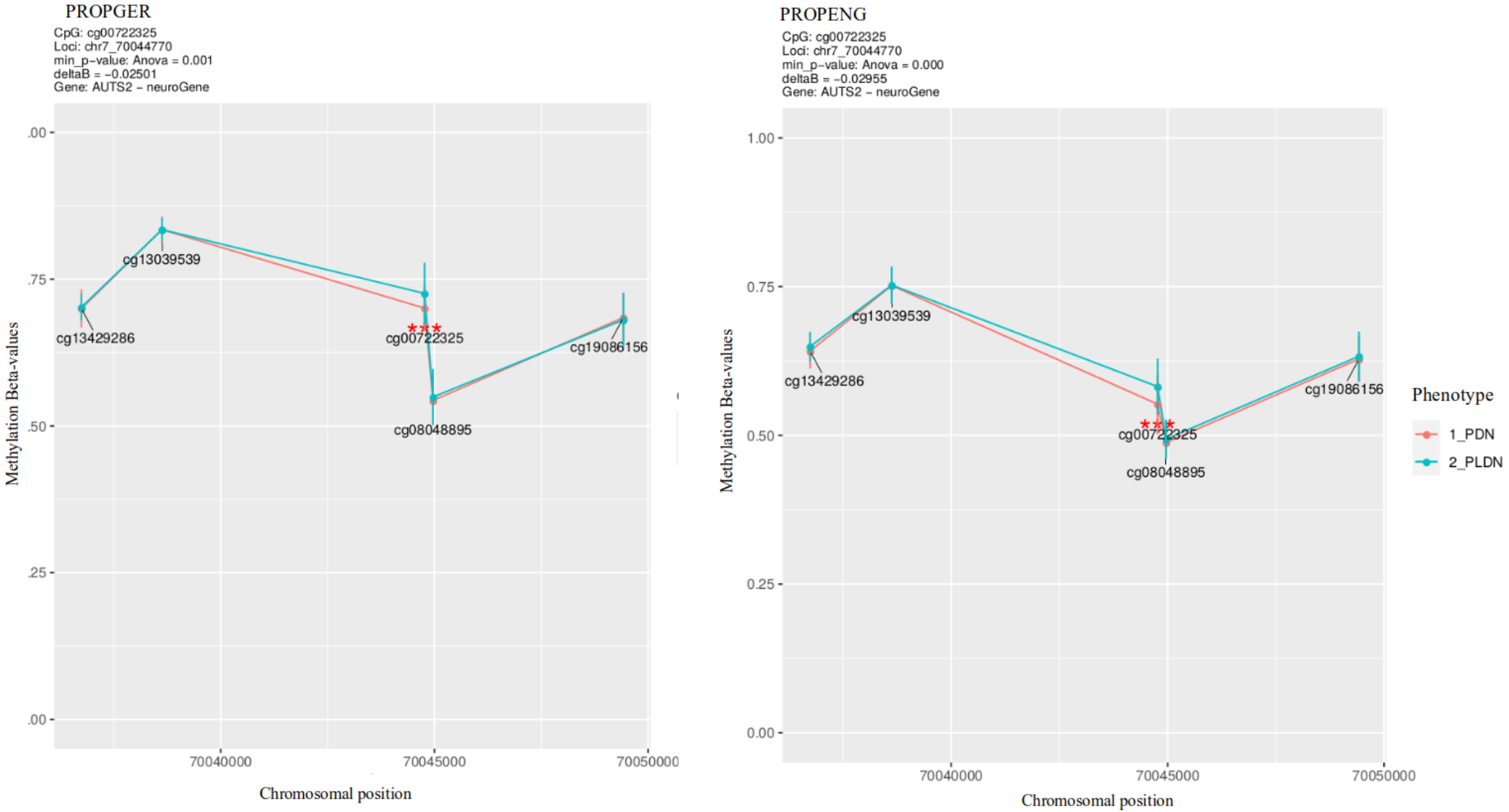

Figure 7 Methylation loci plot of cg00722325 CpG site within *AUTS2* gene. The probe reached statistical significance level of 0.01 and presented concordant direction of methylation change in both PROPGER and PROPENG cohorts – hypo-methylation among PDN subjects.

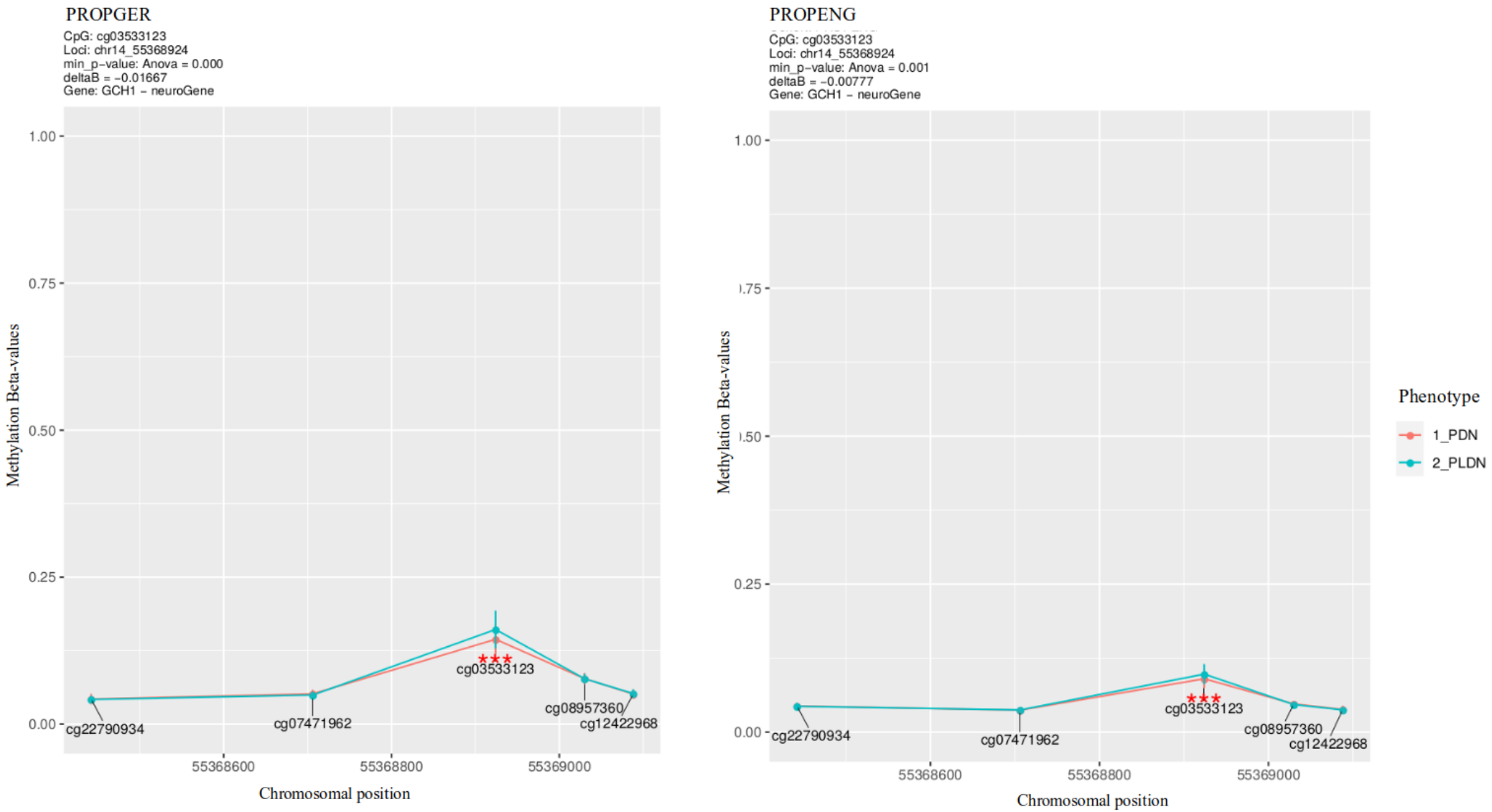

Figure 8 Methylation loci plot of cg03533123 CpG site within *GCH1* gene. The probe reached statistical significance level of 0.01 and presented concordant direction of methylation change in both PROPGER and PROPENG cohorts – hypo-methylation among PDN subjects.

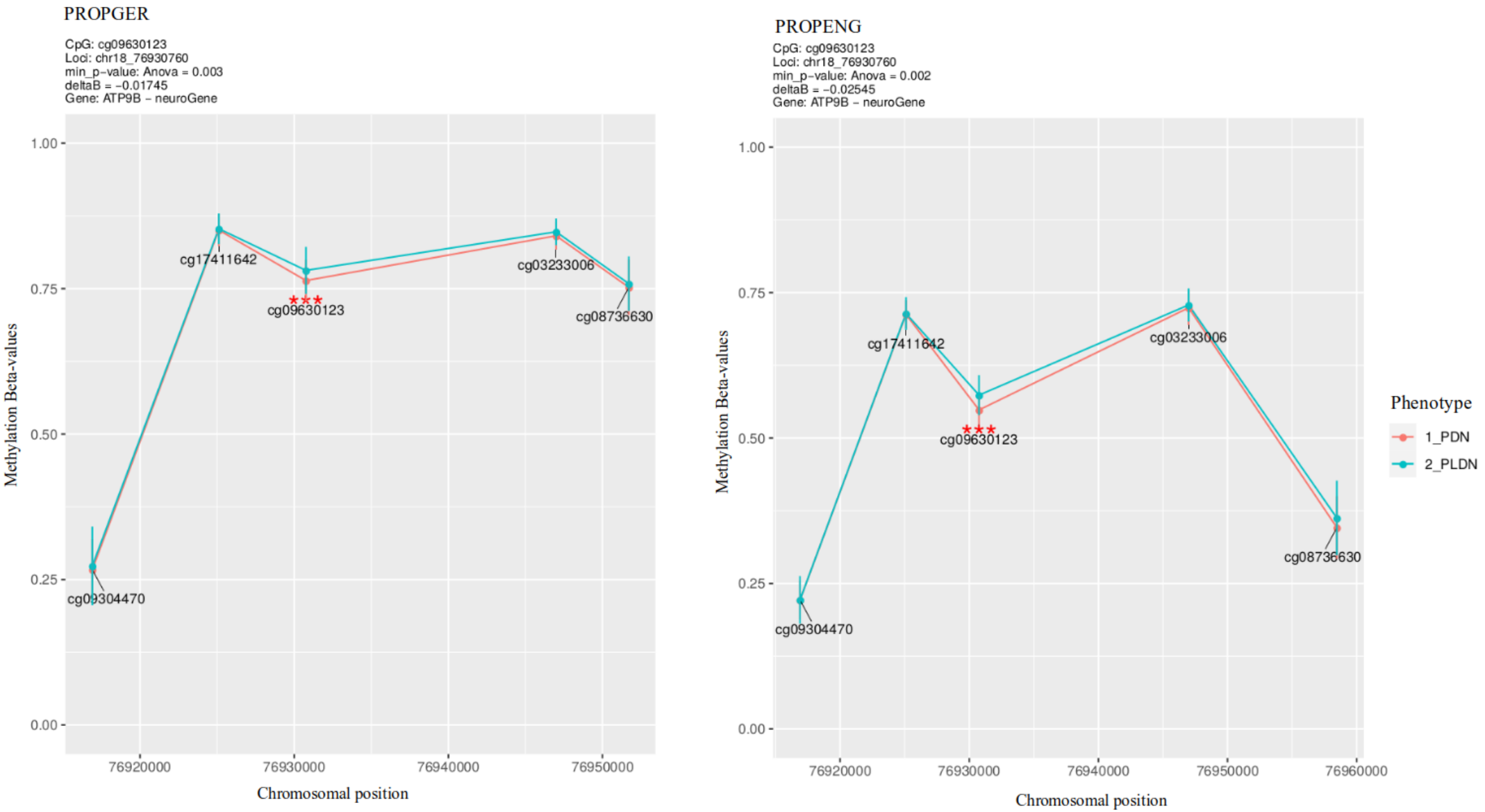

Figure 9 Methylation loci plot of cg09630123 CpG sites within *ATP9B* gene. The probe reached statistical significance level of 0.01 and presented concordant direction of methylation change in both PROPGER and PROPENG cohorts – hypo-methylation among PDN subjects.

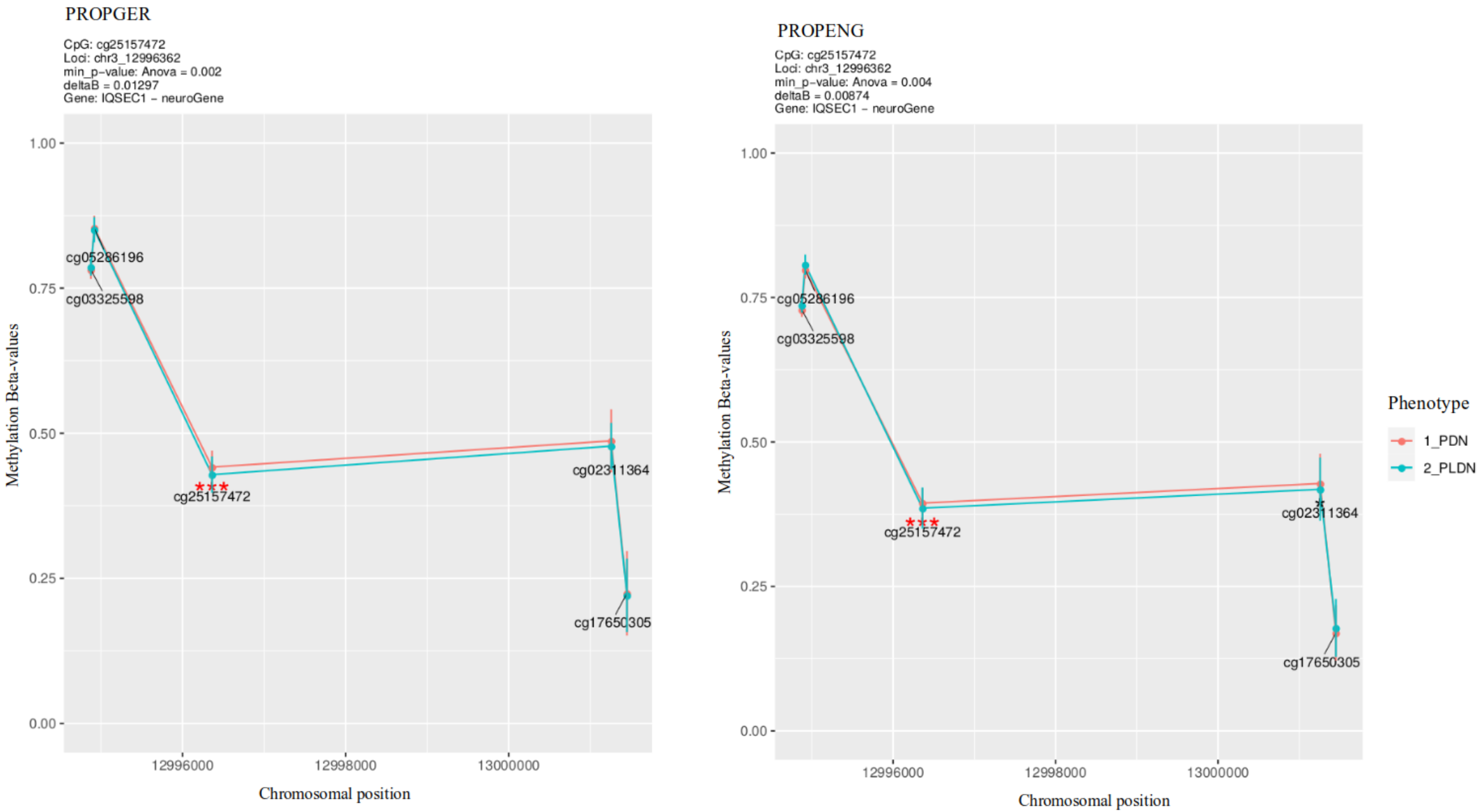

Figure 10 Methylation loci plot of cg25157472 CpG site within *IQSEC1* gene. The probe reached statistical significance level of 0.01 and presented concordant direction of methylation change in both PROPGER and PROPENG cohorts – hyper-methylation among PDN subjects.

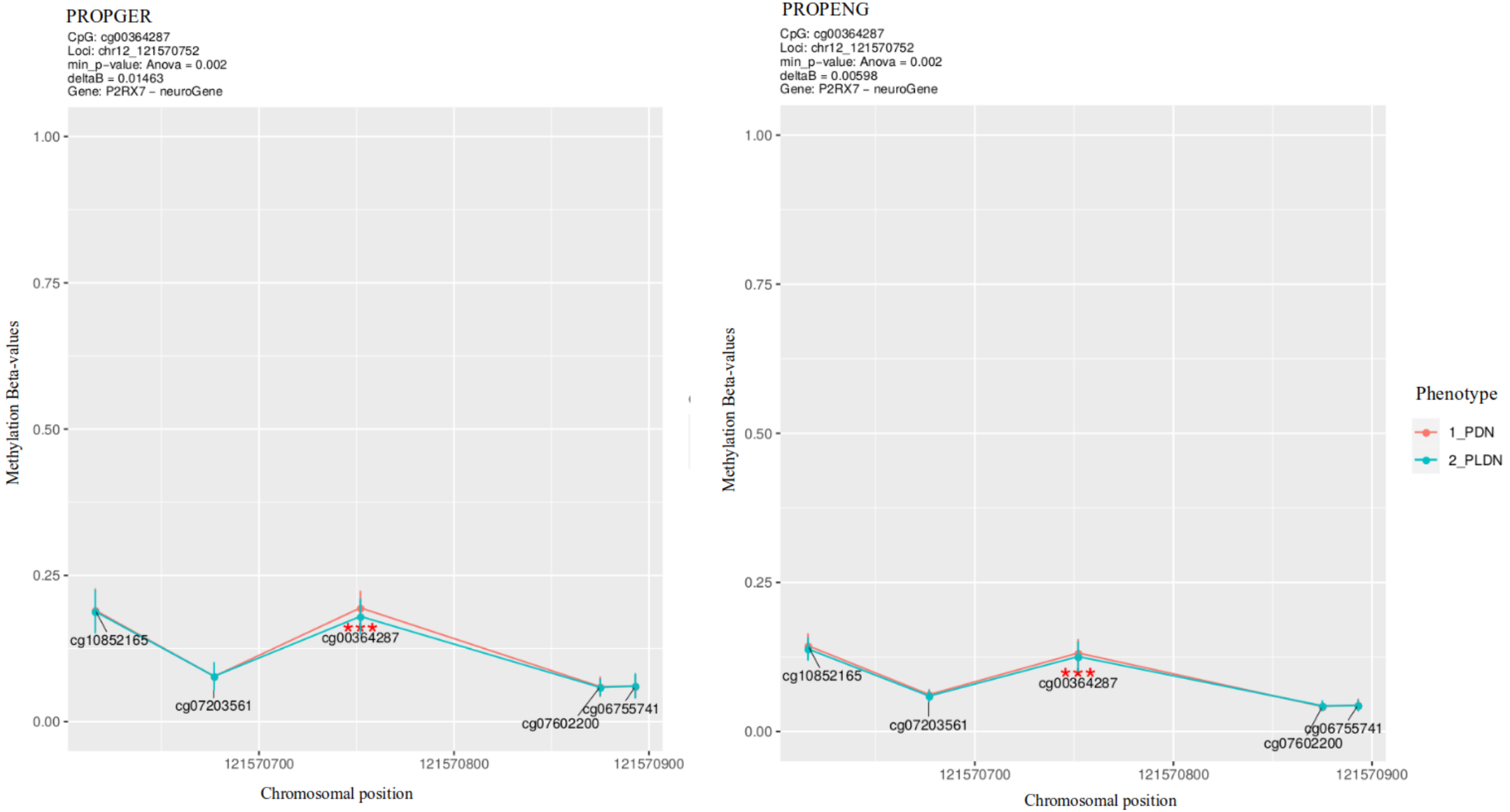

Figure 11 Methylation loci plot of cg00364287 CpG site within *P2RX7* gene. The probe reached statistical significance level of 0.01 and presented concordant direction of methylation change in both PROPGER and PROPENG cohorts – hyper-methylation among PDN subjects.

Produced epigenome-wide data allowed to have additionally a closer look to transient receptor potential cation channel A1 (*TRPA1*) that was confirmed in number of methylation studies as a pain-related gene. However, the results on our cohorts did not provide any evidence on its' contribution to biological mechanisms linked to neuropathic pain in studied DN cohorts. As reported in Table 5, none of CpGs located in the *TRPA1* region reached the statistical significance level when PDN and PLDN from the two considered cohorts were compared and the methylation signals were inconsistent between PROPGER and PROPENG (Figure 12). Also in independent dataset on migraine no evidence on differential methylation within *TRPA1* gene was found.

Table 5 List of CpG sites in a region of a gene *TRPA1* and nominal p-values obtained in ANOVA test comparing painful DN individuals with painless patients in PROPGER and PROPENG cohorts, and comparing painful MOH, EM cases to healthy controls in headache cohort. Statistical significance level was defined at 1%. *probes assessed in the study of Sukenaga et al (Sukenaga et al., 2016); ** probes assessed in the study of Takenaka et al (Takenaka et al., 2020).

CpG sites within *TRPA1*	Chromosomal coordinates (hg37)	SNP	Gene part	ANOVA nominal p-value			
				PROPGER	PROPENG	Headache MOH	Headache EM
cg25229089*	chr8: 72984967	rs112195358 rs543136088	Body	0.298	0.662	0.844	0.436
cg01414726	chr8: 72987055	rs16937981 rs530829519	Body	0.532	0.468	0.757	0.916
cg11052780*	chr8: 72987499	rs201980871 rs200099674 rs559544590	Body	0.077	0.815	0.374	0.045
cg19439706*/**	chr8: 72987872	rs568799632 rs73687869	TSS200	-	-	0.918	0.371
cg14703605*/**	chr8: 72987916		TSS200	0.411	0.067	0.171	0.612
cg27528660*/**	chr8: 72987924		TSS200	0.407	0.835	0.432	0.862
cg09514401	chr8: 72988022	rs532783684	TSS1500	0.675	0.606	0.860	0.791
cg17352610	chr8: 72988263	rs78841650 rs16937983 rs187283846 rs76988688 rs560281697	TSS1500	0.719	0.289	0.737	0.597
cg27619291*	chr8: 72988280	rs187283846 rs76988688 rs560281697 rs148285677	TSS1500	0.286	0.798	0.900	0.235

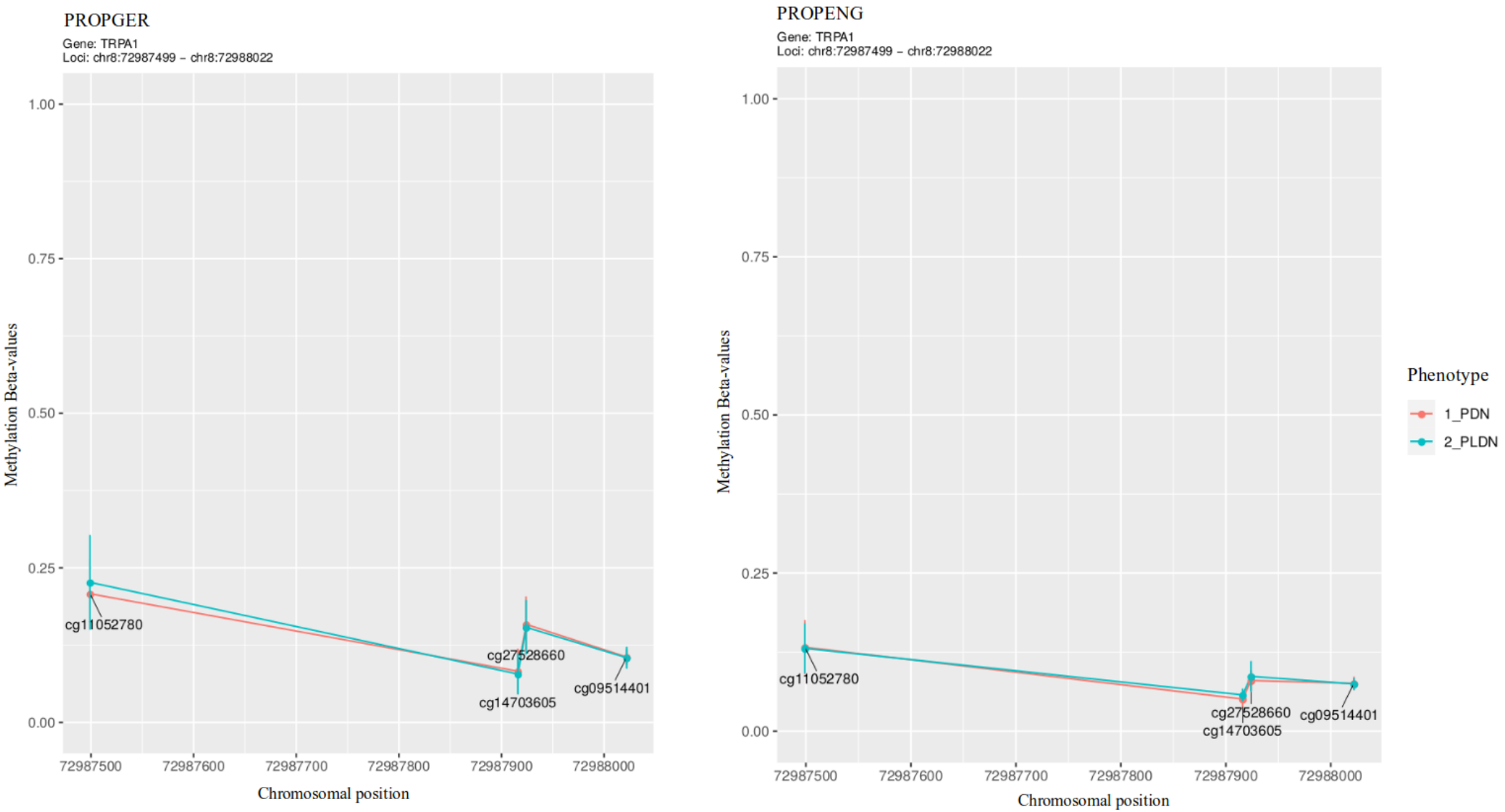

Figure 12 Methylation loci plot of CpGs located in a region of *TRPA1* gene. None of the probes reached statistical significance level of 0.01 in any of the studied cohorts.

Interestingly, identified promising genes were further verified in three independent DNA methylation datasets on pain-related conditions. In heat pain sensitiviy dataset 4 of 19 candidates were found with at least 1 CpG site that reached the significance level at 1%. Remaining 13 genes were not significant and 2 genes were excluded during preprocessing steps. In fibromyalgia dataset already 14 candidates had at least 1 significant CpG, 3 genes did not reached significance level and 2 genes were filtered out in preprocessing pipeline. While in migraine dataset all 19 candidate genes were present and for 9 at least 1 probe reached significance level.

Identified set of 27 CpG sites was used in multidimensional scaling that was applied to obtain a visual representation of distances and dissimilarities between PDN and PLDN phenotypes specifically observed in selected regions of the genome. In the analysis normalized beta values were used as input. The results obtained for PROPGER and PROPENG cohorts are presented on Figure 13. Although the perfect separation of PDN and PLDN was not obtained, according to visualizations there was observed a trend for groups to separate in different directions indicating that the selected CpG sites are able to highlight the presence of dissimilarities between two phenotypes.

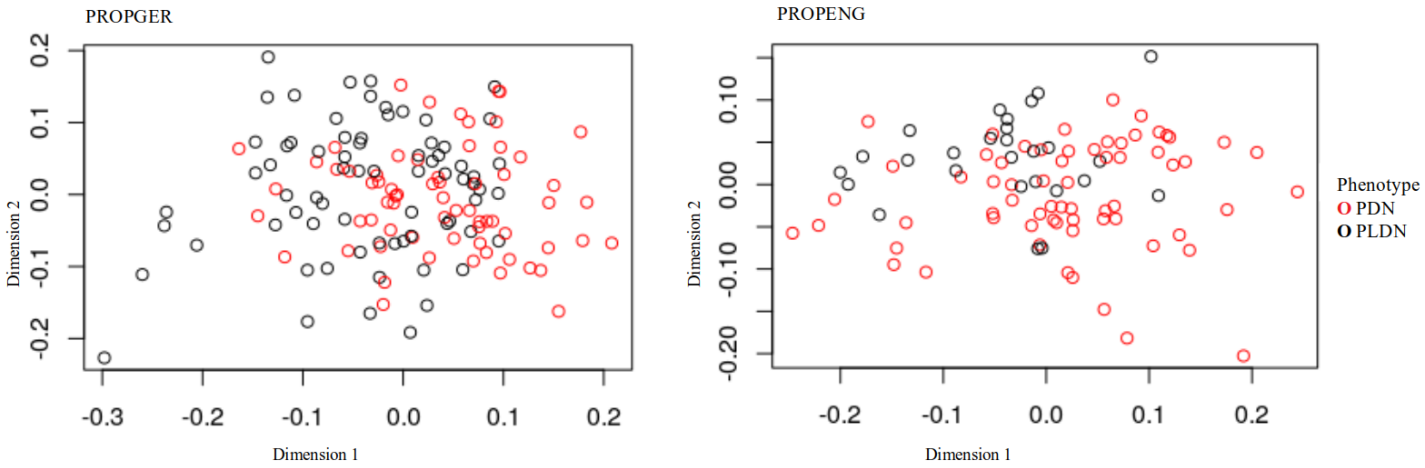

Figure 13 Results of principal component analysis carried out on normalized beta values within selected 27 CpG sites.

To further explore obtained results, analysis of methylation standard deviation distribution was performed. According to KS test the spread of normalized and filtered beta values did differ between painful and painless patients in both PROPGER and PROPENG cohorts (p-value < 0.01). In PROPENG dataset, the drift in standard deviation distribution was particularly notable in PLDN group presenting significantly increased loss of coherence in methylation patterns in respect to PDN individuals and showing shifted density curve (Figure 14).

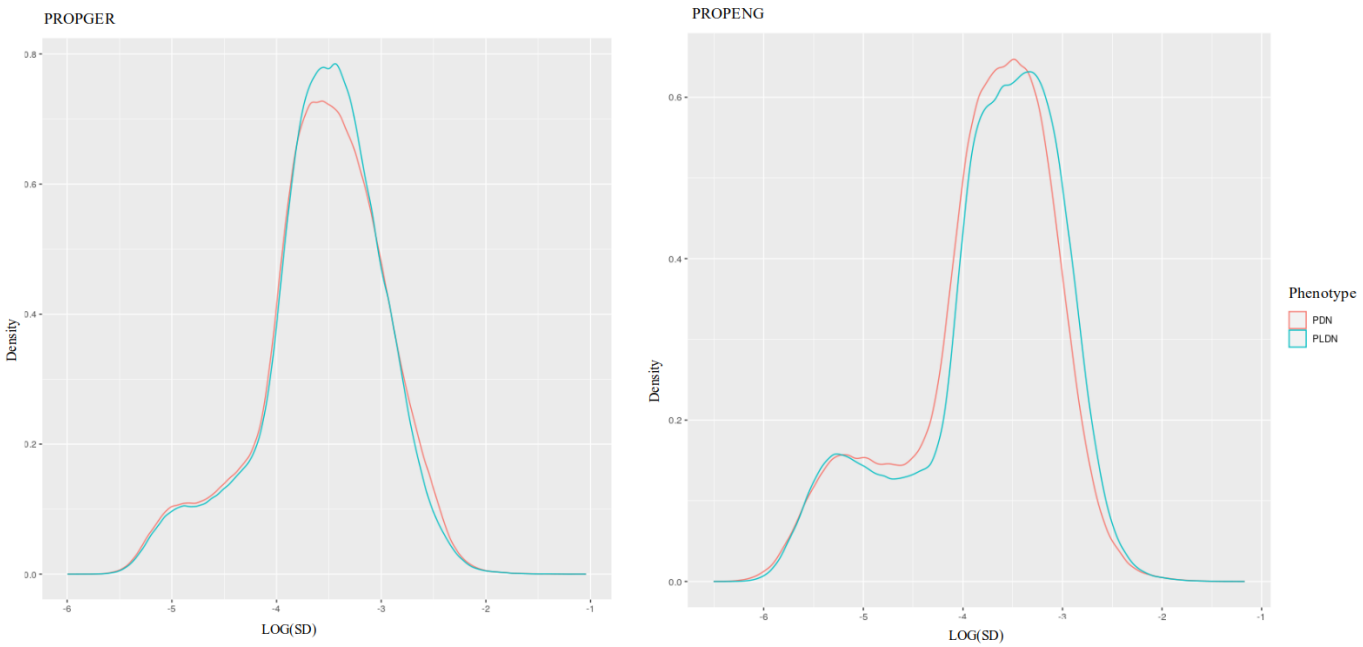

Figure 14 Density of distribution of standard deviation after logarithmic transformation Log(SD) of normalized methylation beta values within PROPGER and PROPENG cohorts.

4.2. Evaluation of Biological Age

The performed analysis involved two cohorts of T2DM patients diagnosed with painful or painless form of diabetic neuropathy: more numerous, selected from German population PROPGER cohort and inferior in number, recruited from English population PROPENG cohort. Additional details on both studied groups were provided respectively in Table 1 and Table 2. For each of the two cohorts a battery of estimations of DNAm-based variables provided by Horvath's epigenetic age calculator were analyzed: i) epigenetic age measures (DNAmAge, DNAmAgeHannum, DNAmAgeSkinBloodClock, DNAmPhenoAge, GrimAge); ii) telomere length estimation (DNAmTL); iii) surrogates of protein levels in plasma (DNAmADM, DNAmB2M, DNAmCystatinC, DNAmGDF15, DNAmLeptin, DNAmPAI1, DNAmTIMP1); iv) prediction of number of cigarette packs smoked in one year (DNAmPACKYRS); v) blood cell counts (CD8T, CD4T, CD8.naive, CD4.naive, CD8pCD28nCD45RAn, NK, Bcell, Mono, Gran, PlasmaBlast). The differences in the parameters among phenotypic groups within PROPGER and PROPENG cohorts were examined with multiple linear regression approach correcting for chronological age and sex.

PROPGER cohort

DNAm-based estimates were analyzed in 70 painful and 65 painless age- and sex-matched DN subjects from German population. Multiple linear regression results obtained for PROPGER cohort

are presented in Table 7 in annexes. No significant differences in epigenetic age acceleration were identified between painful and painless patients. However, considering nominal p-values and the statistical significance level of 0.05, there were found the differences between PDN and PLDN in estimates of telomere length (DNAmTL nominal p-value = 0.038). In painful group shorter telomere length was observed compared to painless subjects as visualized on Figure 15. The observed difference was not significant after BH correction for multiple tests.

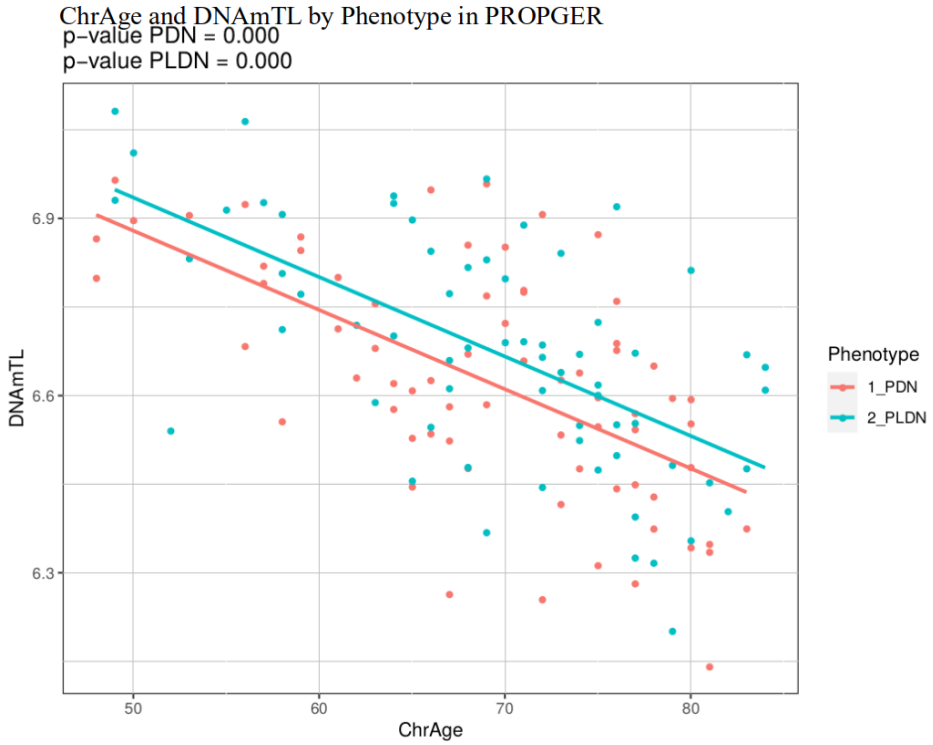

Figure 15 Association between chronological age and DNAm-based prediction of telomere length DNAmTL in painful and painless DN in PROPGER cohort. P-values of linear regressions for PDN and PLDN are reported.

PROPENG cohort

27 PDN and 64 PLDN subjects from English population were compared regarding the differences in DNAm-based estimates. The results from multiple linear regression analysis for all variables are provided in Table 8 in annexes. There was found a significant difference (nominal p-value = 0.038) in epigenetic age acceleration calculated with GrimAge model when painful and painless patients were compared (Figure 16). PDN showed increased estimates of GrimAge regarding the PLDN subjects. The differences between phenotype groups were observed also in DNAmTL and DNAmPAI1- one of the DNAmGrimAge components. The predicted telomere length was significantly reduced in painful individuals comparing to painless (DNAmTL nominal p-value = 0.024) while PAI1 plasma levels were increased (DNAmPAI1 nominal p-value = 0.020). The

visualization of associations between chronological age and both significant DNAm-based estimates in phenotypic groups of PROPENG cohort are provided respectively on Figures 17 and 18. None of the detected difference remained significant after multiple testing with BH procedure.

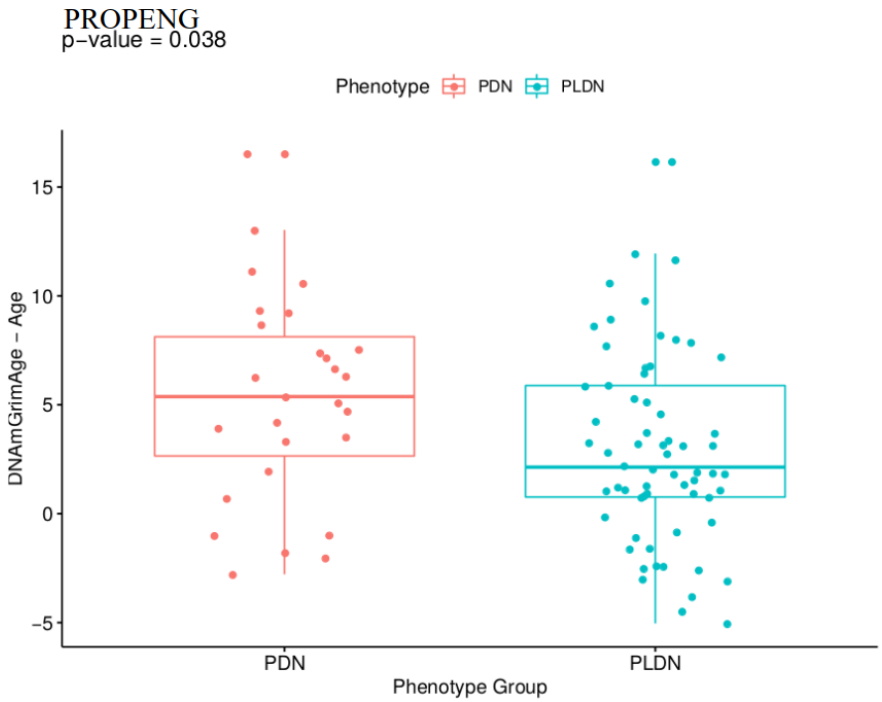

Figure 16 Epigenetic age difference (DNAmGrimAge – chronological age) adjusted for chronological age and sex in the painful and painless groups in PROPENG cohort. Nominal p-values from multiple linear regression analysis are reported.

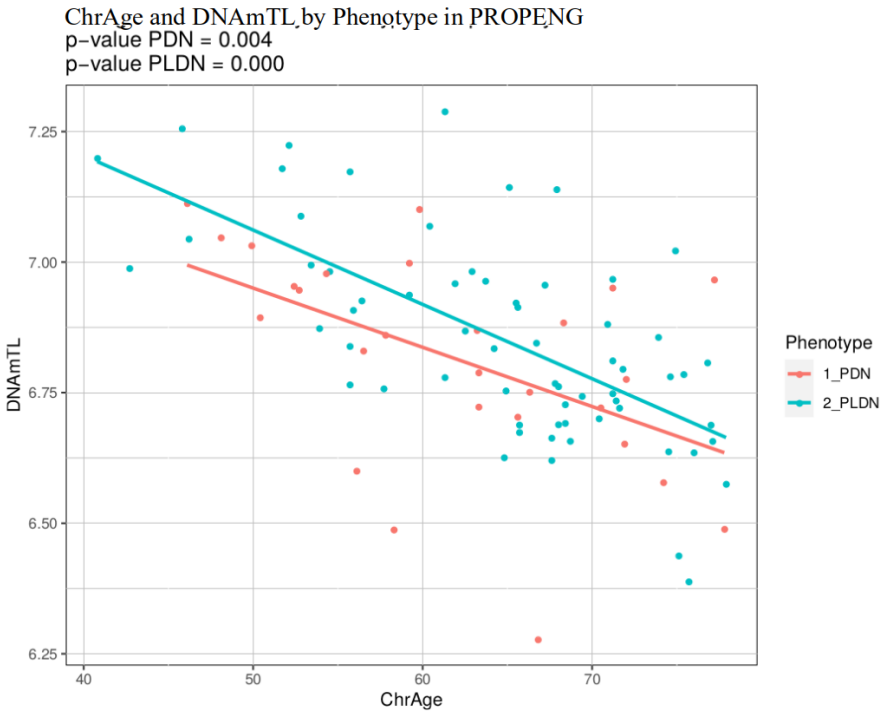

Figure 17 Association between chronological age and DNAm-based prediction of telomere length DNAmTL in painful and painless DN in PROPENG cohort. P-values of linear regressions for PDN and PLDN are reported.

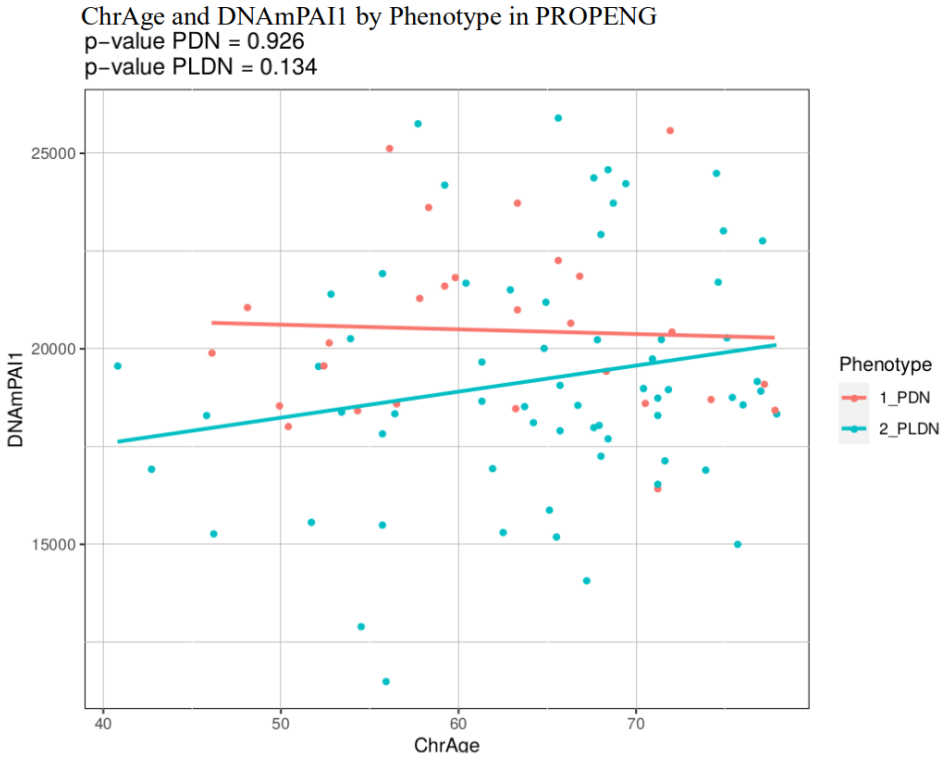

Figure 18 Association between chronological age and DNAm-based prediction of plasma levels of PAI1 in painful and painless DN in PROPENG cohort. P-values of linear regressions for PDN and PLDN are reported.

5. Discussion

The main cause of morbidity in DM is neuropathic pain, affecting performance of everyday routines, significantly reducing quality of sleep and life, or even impeding continuing normal functioning and accomplishing usual responsibilities. The available pain treatments are still unsatisfactory – they cope with low efficiency and serious side effects. Thus, the identification of patients that are at risk of developing NP and to adjust the therapy according to the individual responsiveness to analgesic, could improve the prevention, management and quality of life of suffering patients.

To our knowledge, this is a pioneering study evaluating whole-genome DNA methylation in unique populations of T2DM patients that were histopalogically diagnosed with diabetic neuropathy, with or without neuropathic pain. We provided an evidence on the significant differences in methylation patterns between painful and painless phenotypes.

Epigenomes of patients from two independent cohorts – PROPGER and PROPENG, were assessed in whole blood samples with Infinium Methylation EPIC BeadChip. Produced whole-genome DNA methylation data allowed to perform differential analysis and to identify epigenetic signals that highlight the presence of dissimilarities between painful and painless subjects. We expanded our study estimating epigenetic age of the patients from both cohorts and evaluating eventual acceleration of biological age in PDN and PLDN phenotypes.

Regarding the differential analysis, probes with altered methylation levels were identified using validated pipeline that takes into consideration genomic context of CpG sites and their distribution along the chromosomes. In order to select the most promising methylation signals that could be plausibly associated with experience of NP, the filtering steps were applied. Thus, it was required that short list of CpGs captures the regions reaching the significant methylation differences between PDN and PLDN, presenting the methylation change > 1% in PROPGER and having the direction of methylation change concordant between the two cohorts. With this approach 27 sites passed the established filters and 19 of them were genic, resulting in a list of 19 unique genes (Table 4). Multidimensional scaling analysis confirmed the potential of generated set of CpG sites to separate painful and painless subjects and to highlight the dissimilarities between two phenotypes.

Those differential epigenetic signals tended to be extended along genic regions and predominantly involved genes of broadly considered neurological relevance. Among the most interesting genes, *AUTS2* gene was demonstrated to be involved in control of neuronal migration – a mechanism

plausibly contributing to psychiatric disorders as schizophrenia or autism spectrum disorder (Hori & Hoshino, 2017). Mutations in another interesting candidate gene – *GCH1*, were reported as increasing risk of dopa-responsive dystonia and Parkinson's disease (Yoshino et al., 2018), while genetic variants within *ATP9B* could be associated with Alzheimer's Disease (Barber et al., 2015). A number of pathogenic variants within IQSEC2 was described and linked to disorders including epilepsy and epileptic encephalopathy (Zerem et al., 2016). Yet interestingly, gene encoding *P2X7* receptor (*P2X7R*) was shown to regulate chronic pain sensitivity through molecular alterations (Sorge et al., 2012).

Methylation level of *TRPA1* gene was found different in independent studies (Sukenaga et al., 2016; Takenaka et al., 2020) but this result was not confirmed in our cohort. This may be due to the distinct clinical phenotypes of studied cohorts. Indeed, both the papers considered 12 and 48 subjects respectively with age comparable to that of our cohort, with NP diagnosis but based on either Douleur Neuropathique 4 questionnaire or the short-form McGill Pain questionnaire and not on evaluation of histological biopsies, and without T2DM. Although methological approach used by Sukenaga and Takenaka was different from our as they used Illumina HumanMethylation450 BeadChip instead of Infinium Methylation EPIC BeadChip, this factor apparently was not a source of major discrepancies between assessed CpG sites within *TRPA1* - there was only a signle methylation probe assessed in work by Sukenaga that was not present in EPIC platform (chr8:72988608 (hg19/GRCh37)). However, due to the different implemented preprocessing procedures, some of the probes present in cited studies were lacking in PROPGER and PROPENG datasets since they were lost in the filtration steps of our pipeline. These explanation could be also valid for missing reproducibility of the identified epigenetic pattern encompassing CpG sites within *TRPA1* gene in independent migraine-related methylation dataset from UNIBO.

Performed methylation standard deviation distribution analysis confirmed presence of epigenetic drift and showed significantly increased loss of coherence in methylation patterns. However, in PROPENG cohort the observed shift was contrary to our expectations since painless group tended to present higher state of dysregulation. The very reasonable explanation of this outcome is a fact that PLDN group in PROPENG is significantly more advanced in chronological age comparing to PDN. The increasing discordance of methylome is an individual, non cell-specific, widespread phenomenon, very closely related to age (Jones et al., 2015). The evidence on this strong association came from range of the studies involving embryonic stem cells, cloned mice, monozygotic twin pairs or centenarians (Fraga et al., 2005; Heyn et al., 2012; Humpherys et al.,

2001; Talens et al., 2012). Epiegentic drift was also associated with age-related disorders as Alzheimer's diseas and geriatric pathologies (S.-C. Wang et al., 2008). Thus, the notable drift visualized on Figure 14 plausibly reflects the difference in chronological age between PDN and PLDN groups.

Regarding evaluation of biological aging, according to obtained results, there was no association between painful phenotype and acceleration of biological age expressed by any of the assessed epigenetic clocks. This outcome is coherent with a recent study investigating DNAm-based estimates in three different pain-related phenotypes: heat pain sensitivity, fibromyalgia and headache (Kwiatkowska et al., 2020). Whereas, DNA methylation analysis involving limited group of adults in advanced age provided evidence on accelerated biological age estimated with Horvath's clock in subjects suffering from chronic pain (Cruz-Almeida et al., 2019).

DNA methylation based prediction of telomere length (DNAmTL) was found to vary between PDN and PLDN groups and this result was uniform among both studied cohorts. Particularly, calculated telomere lengths were significantly shorter among painful subjects. Telomere length and epigenetic alterations are two main features of aging and they are closely interacting players (Lee et al., 2019). The length of telomers was shown to be associated with chronological age and with degenerative diseases such as idiopathic pulmonary fibrosis, bone marrow failure, and cryptogenic liver cirrhosis (Armanios, 2013). Obtained result is in line with a study supporting a relationship between experience of chronic pain and reduced telomere length in women with fibromyalgia comparing to healthy controls (Hassett et al., 2012). Another work related to fibromyalgia showed negative correlation between evaluative dimension of pain measured in patients with McGill Pain Questionnaire and DNAmTL (Kwiatkowska et al., 2020). Also in two other studies patients with chronic knee osteoarthritis pain presented significantly shorter telomeres than individuals without or with low pain levels (Sibille et al., 2012, 2017).

In PROPENG cohort significant difference in GrimAge – *i.e.* a measure commonly used as a predictor of lifespan, between painful and painless subjects was detected. This observation plausibly reflected the biological alterations occurred due to progressing diabetes since PDN group suffered from diabetes from significantly longer time. The results identified also plasminogen activator inhibitor 1 (DNAmPAI1) as a plausible main actor of driving mechanism involved in progression of T2DM and contributing to observed epigenetic differences. Similarly, recently published study investigating epigenetic clocks in DN cohorts of patients with type 1 diabetes provided evidence on significant acceleration of biological age expressed as GrimAge (Roshandel et al., 2020).

As a consideration for future, we recognize that including in the analysis a control group free from neuropathic pain, without diabetic neuropathy neither diabetes, would add an important insight and would allow to obtain a more complete picture of this complex phenotype.

In conclusion, obtained results confirmed the presence of epigenetic differences between painful and painless diabetic neuropathy patients. Promising genes were identified that may be linked to NP through DNA methylation mechanisms. Selected candidates require further validation in independent DN cohorts, and in other populations of patients with pain-related phenotypes.

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7. Annexes

Table 6 List of DNAm-based variables returned by the New DNA Methylation Age Calculator available online at https://dnamage.genetics.ucla.edu/. (Retrived from (Kwiatkowska et al., 2020))

Variable name	Variable description
DNAmAge	DNAm age estimate based on methylation of 353 CpG sites described by Horvath et al. (Horvath, 2013)
DNAmAgeHannum	DNAm age estimate based on methylation of 71 CpG sites described by Hannum et al. (Hannum et al., 2013)
DNAmAgeSkinBloodClock	DNAm age estimate (based on methylation of 391 CpG sites) for human fibroblasts, keratinocytes, buccal cells, endothelial cells, lymphoblastoidcells, skin, blood, and saliva samples; devolped by (Horvath, 2013)
DNAmPhenoAge	DNAm based estimate of phenotypic age (Levine et al., 2018)
DNAmGrimAge	DNA methylation age model build on eight DNAm based measures (DNAmADM, DNAmB2M, DNAmCystatinC, DNAmGDF15, DNAmLeptin, DNAmPACKYRS, DNAmPAI1, DNAmTIMP1), chronological age and sex (Lu, Quach, et al., 2019)
DNAmTL	DNAm based estimate of telomere length (Lu, Seeboth, et al., 2019)
DNAmADM	DNAm based prediction of plasma levels of adrenomedullin - a vasodilator peptide hormone (Lu, Quach, et al., 2019)
DNAmB2M	DNAm based prediction of plasma levels of beta-2 microglobulin - a component of major histocompatibility complex class 1 (MHC I) molecular (Lu, Quach, et al., 2019)
DNAmCystatinC	DNAm based prediction of plasma levels of cystatin C or (cystatin 3) – formerly called gamma trace, post-gamma-globulin, or neuroendocrine basic polypeptide (Lu, Quach, et al., 2019)
DNAmGDF15	DNAm based prediction of plasma levels of GDF-15 - growth differentiation factor 15 (Lu, Quach, et al., 2019)
DNAmLeptin	DNAm based prediction of plasma levels of leptin - a hormone predominantly present in adipose cells (Lu, Quach, et al., 2019)
DNAmPAI1	DNAm based prediction of plasma levels of plasminogen activator inhibitor antigen type 1 (PAI-1) - the major inhibitor of tissue-type plasminogen activator and unokinase plasminogen activator (Lu, Quach, et al., 2019)
DNAmTIMP1	DNAm based prediction of plasma levels of TIMP-1 or TIMP metallopeptidase inhibitor 1 - a tissue inhibitor of metallo-proteinases (Lu, Quach, et al., 2019)
DNAmPACKYRS	DNAm based prediction of a number of pack of cigarettes during year (Lu, Quach, et al., 2019)
CD8T	DNAm based estimate of CD8 T cells, expressed as ordinal abundance measures (Houseman et al., 2012)
CD4T	DNAm based estimate of CD4 T cells, expressed as ordinal abundance measures (Houseman et al., 2012)
CD8.naive	DNAm based estimate of naive CD8 T cells, expressed as ordinal abundance measures (Horvath et al., 2016; Horvath &

	Levine, 2015)
CD4.naive	DNAm based estimate of naive CD4 T cells, expressed as ordinal abundance measures (Horvath et al., 2016; Horvath & Levine, 2015)
CD8pCD28nCD45RAn	DNAm based estimate of exhausted cytotoxic T defined as CD8+, CD28-, and CD45R- cells, expressed as ordinal abundance measures (Horvath et al., 2016; Horvath & Levine, 2015)
NK	DNAm based estimate of natural killer cells, expressed as ordinal abundance measures (Houseman et al., 2012)
Bcell	DNAm based estimate of B cells, expressed as ordinal abundance measures (Houseman et al., 2012)
Mono	DNAm based estimate of monocytes, expressed as ordinal abundance measures (Houseman et al., 2012)
Gran	DNAm based estimate of granulocytes, expressed as ordinal abundance measures (Houseman et al., 2012)
PlasmaBlast	DNAm based estimate of plasma blasts, expressed as ordinal abundance measures (Horvath et al., 2016; Horvath & Levine, 2015)

Table 7 Results of statistical hypothesis testing comparing painful and painless DN patients from PROPGER cohort with multiple linear regression approach correcting for chronological age and sex. Linear model coefficient and corresponding p-values - nominal and multiple tests corrected - are reported. P-values reaching significance level of 0.05 are reported in bold.

Epigenetic Variable	Coefficient	P-value_nominal	P-value_AdjBH
DNAmAge	-1.376	0.156	0.758
DNAmAgeHannum	-1.517	0.191	0.758
DNAmAgeSkinBloodClock	-0.661	0.295	0.758
DNAmPhenoAge	-0.672	0.512	0.945
DNAmGrimAge	0.067	0.919	0.997
DNAmTL	0.053	**0.038**	0.651
DNAmADM	0.976	0.714	0.997
DNAmB2M	-14609.644	0.277	0.758
DNAmCystatinC	2929.745	0.379	0.758
DNAmGDF15	27.554	0.356	0.758
DNAmLeptin	-875.269	0.279	0.758
DNAmPAI1	-50.469	0.918	0.997
DNAmTIMP1	-35.417	0.713	0.997
DNAmPACKYRS	-0.424	0.819	0.997
CD8T	0.007	0.311	0.758
CD4T	-0.010	0.254	0.758
CD8.naive	9.980	0.054	0.651
CD4.naive	-3.884	0.794	0.997
CD8pCD28nCD45RAn	0.002	0.997	0.997
NK	0.000	0.955	0.997
Bcell	0.004	0.329	0.758
Mono	0.003	0.552	0.946
Gran	-0.002	0.855	0.997
PlasmaBlast	-0.004	0.868	0.997

Table 8 Results of statistical hypothesis testing comparing painful and painless DN patients from PROPENG cohort with multiple linear regression approach correcting for chronological age and sex. Linear model coefficient and corresponding p-values - nominal and multiple tests corrected - are reported. P-values reaching significance level of 0.05 are reported in bold.

Epigenetic Variable	Coefficient	P-value_nominal	P-value_AdjBH
DNAmAge	0.652	0.433	0.612
DNAmAgeHannum	-0.529	0.565	0.678
DNAmAgeSkinBloodClock	-0.419	0.525	0.678
DNAmPhenoAge	-1.872	0.140	0.372
DNAmGrimAge	-1.827	**0.038**	0.304
DNAmTL	0.078	**0.024**	0.284
DNAmADM	-3.736	0.171	0.372
DNAmB2M	5194.480	0.765	0.798
DNAmCystatinC	-4000.534	0.339	0.612
DNAmGDF15	-24.393	0.414	0.612
DNAmLeptin	-1123.137	0.156	0.372
DNAmPAI1	-1455.249	**0.020**	0.284
DNAmTIMP1	-64.820	0.619	0.688
DNAmPACKYRS	-4.322	0.106	0.372
CD8T	0.016	0.070	0.338
CD4T	-0.009	0.420	0.612
CD8.naive	3.851	0.550	0.678
CD4.naive	8.775	0.631	0.688
CD8pCD28nCD45RAn	1.602	0.057	0.338
NK	0.015	0.158	0.372
Bcell	0.004	0.319	0.612
Mono	-0.001	0.820	0.820
Gran	-0.029	0.167	0.372
PlasmaBlast	-0.027	0.406	0.612