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HUMAN TRANSCRIPTOMIC STUDIES IN MIGRAINE

Ph.D thesis

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List of Abbreviations

MO – migraine without aura

CGRP – calcitonin gene-related peptide

HPA – hypothalamic-pituitary-adrenal

ACTH – adrenocorticotropic hormone

mRNA – messenger RNA

RNA-seq – RNA sequencing

LEGs – leading edge genes

MIDAS B – Migraine Disability Assessment (MIDAS), Section B: headache severity

SDS – Zung Self-Rating Depression Scale

STAI – State-Trait Anxiety Inventory

MINI – Mini International Neuropsychiatric Interview

DiffCitalopram – Difference in cortisol levels (post- vs. pre-infusion) after citalopram administration

DiffPlacebo – Difference in cortisol levels (post- vs. pre-infusion) after placebo administration

DiffofDiffs – Difference between DiffCitalopram and DiffPlacebo (net pharmacological cortisol response)

FDR – False Discovery Rate

GSEA – Gene Set Enrichment Analysis

MsigDB – Molecular Signatures Database

GO – Gene Ontology

GOCC – Gene Ontology Cellular Component

GOBP – Gene Ontology Biological Process

GOMF – Gene Ontology Molecular Function

miRNA – microRNA

Log_2FC – Logarithmic (base 2) fold change

LEG5 – Top 5 consistently downregulated leading edge genes (with Log2FC ≤ –2)

LEG69 – Remaining 69 leading edge genes with Log2FC ≤ –0.5

SNP – Single nucleotide polymorphisms

atRA – all-trans retinoic acid

ANP – Atrial natriuretic peptide

BNP – Brain natriuretic peptide

NO – Nitric oxide

EMSA – Electro Mobility Shift Assay

HPLC – High-Performance Liquid Chromatography

PBMC – Peripheral Blood Mononuclear Cell

8-OHdG – 8-Oxo-2'-deoxyguanosine

GSH – Glutathione

GSSG – Oxidized form of GSH

1. Introduction

1.1. Migraine and its subtype without aura (MO)

Migraine is one of the most disabling and prevalent neurological disorders worldwide. With a global prevalence of 14–15%, migraine presents a considerable personal and socioeconomic challenge due to its recurrent and debilitating characteristics (1).

A migraine attack typically lasts between 4 and 72 hours and is characterised by a throbbing headache of moderate to severe intensity, usually affecting one side of the head (unilateral). Individuals suffering from migraines frequently develop a heightened sensitivity to light (photophobia) and sound (phonophobia), leading them to look for comfort in a dark, silent setting. Beyond those symptoms, nausea and vomiting are among the common gastrointestinal symptoms, which can add to the overall discomfort of migraine patients (2).

Migraine is principally classified into two major subtypes: migraine with aura and migraine without aura. Approximately 30% of individuals with migraine experience aura, which is defined by transient neurological symptoms, such as visual disturbances, sensory alterations, or speech impairments, that typically precede the headache phase. The more prevalent form, migraine without aura (MO) is distinguished by the absence of these preceding neurological phenomena (2).

The condition was traditionally viewed as a vascular disorder, but it is now recognised as a neurovascular disorder that results in hypersensitivity and pain from the convergence of dysfunctions in both the peripheral and central nervous systems (3,4). Migraine attacks involve the release of vasoactive neuropeptides like calcitonin gene-related peptide (CGRP), which play a key role in triggering neurogenic inflammation (5). This inflammatory response is thought to contribute to both peripheral and central sensitisation in the nervous system (6). Additional mechanisms, like 1) dysfunctions in ion channels (e.g. TRP, CaV, NaV, KV channels) increasing trigeminal neuron excitability (7,8), 2) trigeminovascular system sensitization (9), 3) deficient descending pain inhibition from brainstem nuclei (10), 4) oxidative stress and mitochondrial oxidative

phosphorylation deficits (11), and 5) impaired cerebral glucose metabolism (12). However, even after decades of research, the exact mechanisms underlying migraine remain unclear (2).

1.2. Stress Sensitivity and the Migraine Brain

Many individuals with migraine report stress a major trigger for their attacks. Early studies with small sample sizes have shown a clear link between the intensity or frequency of stress and the occurrence of migraine attacks, with around 84% of migraine patients identifying stress as a factor that worsens or initiates their symptoms (13).

Stress can be broadly defined as the way an organism perceives and responds to a perceived stressor and includes physiological and psychosocial reactions to both internal and external challenges. The response encompasses intricate regulatory systems, particularly the autonomic nervous system and the hypothalamic-pituitary-adrenal (HPA) axis. These systems trigger neurobiological changes designed to adapt to physical or psychological demands. Any stressor, whether mental or physical, can trigger a physiological response that leads to significant sympathetic outflow and the release of stress hormones like epinephrine, norepinephrine, and cortisol (14).

The neuronal and hormonal changes linked to stress, along with the perceived psychological stress response, have various connections to migraine. Stress can trigger the emergence of migraine (incidence), serve as a risk factor for migraine attacks, exacerbate migraine-related disability and/or burden. Beyond that it might play a role in the onset of chronic migraine. Additionally, migraine attacks and the ensuing disability can serve as stressors, leading to a harmful feedback loop (15).

This connection is not confined to high stress levels. A reduction in perceived stress has been linked to the onset of migraine attacks in the following 6, 12, and 18 hours, supporting the “let-down” hypothesis that suggests a sudden decrease in stress can trigger migraines (16). However, a larger and more recent study involving 351 patients found that among 2,115 reported valid migraine episodes, “let-down” attacks constituted the smallest proportion (16).

Overall, current research underscores the important role of stress in migraine, acting either as an immediate trigger or as a contributing factor with delayed onset. This has led to the hypothesis that maladaptive stress responses play a key role in migraine pathophysiology. Although some studies have attempted to link physiological stress-related processes to migraine, there remains a lack of systematic research investigating the precise molecular mechanisms underlying the stress–migraine connection in humans.

1.3. Neuroendocrine Challenge as a Model for Stress Reactivity

To examine the biological foundations of altered stress responses in migraine an experimental approach that allows for a controlled and reproducible induction of stress is crucial. The neuroendocrine challenge fulfils this criterion by offering a time-locked and well-tolerated method for activating stress-related pathways in humans.

Citalopram works primarily through inhibition of serotonin transporter, while serotonin is also playing a role in vascular tone, nociception, our work reflects a design to focus on stress-related effects from broader serotonergic actions. An acute citalopram challenge can trigger arousal and activate a neuroendocrine stress response by stimulating the HPA, which in turn leads to the release of hormones such as prolactin, adrenocorticotropic hormone (ACTH), and cortisol (17,18).

This model has shown its capability to reveal changes in tryptophan and kynurenine metabolism and to trigger increased neuroendocrine sensitivity in those with MO, which makes it especially appropriate for investigating migraine-associated phenomena.

Using citalopram is considered safe due to its minimal side effects, supporting its application as an experimental tool in migraine patients for testing the stress axis (19). Few serotonergic substances can be administered intravenously, however citalopram's bioavailability and excellent tolerability compared to other substances make it a favored choice. Furthermore, the use of citalopram for a neuroendocrine challenge has been established in several experiments (18,19).

The neuroendocrine challenge can mimic stress to determine whether patients with MO exhibit distinct responses compared to healthy controls. Responses to citalopram and placebo infusions could be compared to identify the genes and biological pathways that are associated with maladaptive stress reactivity, a mechanism that may contribute to migraine pathophysiology.

1.4. The Field of Transcriptomics

In the field of transcriptomics the complete set of RNA agents is studied, collectively named as transcriptome, which is produced by the genome in specific states or biological samples. This approach offers a snapshot of gene-expression activity enabling to gain knowledge about transcriptional regulation, cellular states, and the molecular mechanisms underlying physiological and pathological processes (20–22).

RNA-species such as messenger RNA (mRNA), non-coding RNAs, and small RNAs can be measured. The most frequently investigated type remains mRNA, since it reflects protein-coding genes allowing inference of protein levels too. It enables to investigate gene regulation and identify biologically relevant changes in gene expression across different conditions or sample groups (20).

Currently, RNA sequencing (RNA-Seq) is a widely used high-throughput method, a technology introduced as an alternative to microarrays (20,23). Bulk RNA-Seq is used, where RNA is extracted from tissues or blood and further converted to complementary DNA, giving the opportunity to quantify transcripts abundance across the genome, as well as the detection of features such as alternative splicing, fusion transcripts, and previously unannotated RNAs (20,22).

Compared to earlier methods, RNA-Seq offers a greater dynamic range, higher sensitivity for low-expressed genes, and the ability to identify novel transcripts without prior knowledge of their existence (20,24). It also allows to test for differential gene expression comparing biological conditions. While genetic studies were able to identify risk loci for migraine, they offer limited insight about the biological mechanisms that underlie the disorder. Transcriptomics was chosen because it captures active gene expression states and serves as a focused strategy to bridge the gap between genetic

predisposition and subsequent biological impacts. That makes it suitable for a systems-level approach in a multifactorial disorder such as MO. This was all the more pressing, as no replicable transcriptomic studies in MO had been conducted yet. Transcriptomics also offered to investigate the direction of gene expression changes and pathway-level alterations in both stress-free and stress-related responses, and thus allowing the characterization of dynamic biological mechanisms underlying MO.

1.5. Transcriptomics Approaches in Migraine

Despite extensive study of migraine pathophysiology through animal models and targeted interventions, systematic research into the transcriptomic mechanisms involved in humans has resulted in only a small number of genes. Differential gene expression studies comparing migraine patients with controls mostly failed to identify replicable gene-level results after correction for multiple testing (25–30). Comparing migraine patients with and without aura, few pathway-level signals were found such as ribonucleoprotein complex and no discernible differences in gene-level expression could be observed (25). Gene expression changes during spontaneous attacks vs post-treatment included metabolic, ion-transport and immune-related pathways (26). In another study, disease-specific pathway alterations using a combination of transcriptomic and metabolomic analyses were observed, yet, again lacking robustly replicated gene markers (27). Gerring et al. found in migraine showed a downregulation of immune-related pathways (28). In chronic migraine, elevated inflammatory gene transcripts in the periosteum were found (29). In another study, biomarkers specifically in migraine with aura were explored (30). Interestingly, only one study used RNA-Seq to specifically target MO (25), and even after correcting for multiple testing, it was unable to find any significant genes. The inconsistency and lack of solid findings in the above studies can probably be attributed to the methodological diversity of patient samples, joint investigations of migraine subtypes, influence of comorbid conditions and medication usage.

To address this, it has been hypothesised that more rigorous study designs, based on clinically confirmed MO cases, strict exclusion criteria and appropriate correction for phenotypic variability, may allow for the identification of replicable transcriptomic signatures in MO. A robust transcriptomic map could in turn enhance understanding of

the molecular underpinnings of MO, support the integration of genetic and transcriptomic data, promote drug development, and generate clinically testable hypotheses for future intervention studies.

2. Objectives

This work aimed to investigate the transcriptomic profile of migraine in both the interictal state without intervention and under an acute neuroendocrine challenge, with the goal of elucidating molecular mechanisms that may contribute to the pathophysiology of the disorder and the influence of stressors. In accordance, the answers for the following questions were sought for:

1. Are there significantly different and consistently replicated genes and -pathways in migraineurs without aura compared to healthy controls in a well-controlled population without serious co-morbidities and medication intake?

2. Are there migraine-associated drugs or potential drug candidates capable to bind migraine-relevant genes or act on pathways with the potential to advance targeted migraine therapy?

3. Can a stress-mimicking neuroendocrine challenge in interictal MO patients reveal an altered stress response and associated genes and -pathways highlighting stress-dependent mechanisms behind MO?

To investigate these hypotheses, two RNA-seq studies were performed: the first one interictally at two different time points without intervention and the second one using a double-blind, placebo-controlled neuroendocrine stress challenge with acute citalopram infusion.

3. Methods

3.1. Participants

The research adhered to the ethical standards specified in the Declaration of Helsinki and was approved by the Hungarian Medical Research Council (Approval Numbers: 23609-1/2011-EKU, dated December 19, 2011; as well as 23421-1/2015-EKU from May 12, 2015). Before their enrolment, all participants gave their written informed consent.

Participants were recruited in Hungary through advertisements posted at universities, headache clinics, online platforms, and in local newspapers. The study population consisted of patients diagnosed with episodic MO and healthy control subjects. Expert neurologists diagnosed episodic MO in accordance with the International Classification of Headache Disorders, third edition (31).

In the MO group, additional data were collected on age at migraine onset, monthly headache frequency, headache severity (MIDAS B), and attack triggers using validated questionnaires and headache diaries. Psychological characteristics were assessed using the Zung Self-Rating Depression Scale (SDS) and the State-Trait Anxiety Inventory (STAI), Mental health was further evaluated using the Mini International Neuropsychiatric Interview (MINI) (32,33).

Individuals were excluded based on strict criteria: those with neurological or psychiatric conditions (excluding MO), serious acute or chronic diseases (except for non-active allergies), or regular medication use (other than contraceptives) were not included. Pregnancy and breastfeeding served as additional exclusion criteria. In both studies, participants with a migraine attack within 24 hours before or after the infusion were excluded from sampling. In the general interictal transcriptomic study involving the same cohort, post-infusion exclusion was not implemented to keep statistical power and due to its replicative nature. For healthy controls, it was necessary that they had not experienced headaches for 24 hours leading up to sampling. All participants verified that they had refrained from using analgesics for 48 hours and caffeine for at least 4 hours before blood collection.

3.2. Study design

Data collection followed a placebo-controlled, randomized, double-blind, crossover design as visible on Figure 1. The cohort comprised 52 individuals in total: 22 patients with MO (5 men, 17 women) and 30 healthy controls (14 men, 16 women). Due to the onset of a migraine attack after the neuroendocrine challenge, one additional MO patient was excluded during data analysis in the stress challenge analysis. This participant experienced gradually worsening symptoms and vomiting following the citalopram infusion and was treated with intravenous metoclopramide and metamizole after blood sampling.

3.2.1. General Interictal Transcriptomic Study

In the general interictal study, samples were taken at events (1) and (5) (see Figure 1), reflecting blood samples collected 10 minutes prior to the infusion (0 minutes) for RNA-Seq at two time points with an average difference of 4 weeks. Samples represented the condition without any pharmacological intervention.

3.2.2.Neuroendocrine Challenge Study

The neuroendocrine challenge was conducted in two sessions in a placebo-controlled, randomized, double-blind, crossover design. It contained a washout period of minimum 12 days. In one of the test days, the participants received an intravenous infusion of 7.5 mg citalopram (event 2 or 6); the other day they received an equivalent volume of saline placebo (event 2 or 6) (see Figure 1).

Blood samples were collected at three time points during each session:

I. 10 minutes before infusion (events 1 and 5)

II. 30 minutes post-infusion (events 3 and 7)

III. 70 minutes post-infusion (events 4 and 8)

In this experiment, RNA-Seq samples from events (1) and (5) were used as pre-infusion samples for the neuroendocrine challenge. Events (4) and (8) were used for post-infusion RNA-Seq. This allowed the assessment of transcriptomic changes induced by

citalopram or placebo. Cortisol concentrations were measured at all three time points in each session (events 1, 3, 5 and 5, 7, 8, respectively) (see Figure 1).

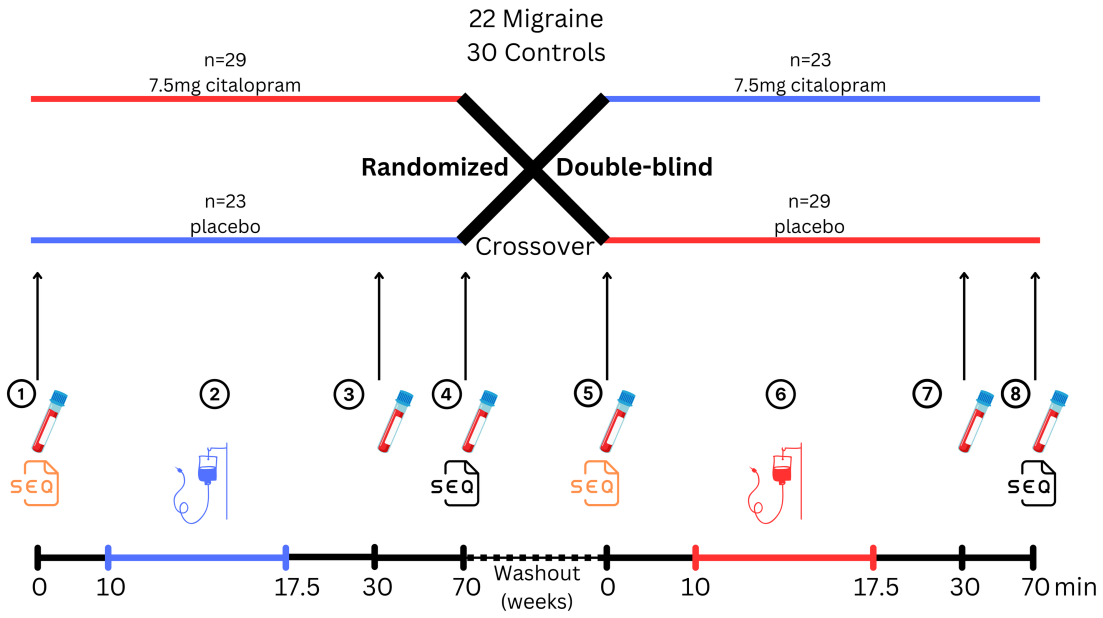

Figure 1: Study design of general interictal experiment and neuroendocrine challenge

52 participants were involved in a crossover design that was placebo-controlled, randomized, and double-blind, using either a placebo or citalopram challenge. On the challenge days, three blood samples were collected for cortisol measurement and two for RNA-seq analysis: the first collection occurred 10 minutes prior to the challenge (at 0 minutes); subsequently, they received either a 7.5-mg citalopram infusion or saline solution for 7.5 minutes, with the assignment being random; participants provided second and third blood samples 20 and 60 minutes after the challenge began (30 and 70 minutes in relation to the first sample). Before the participants repeated the process with the opposite substance, a washout period of varying duration, lasting at least 12 days, took place.

3.3. Cortisol Concentration Measurement

We previously reported the cortisol measurement protocol in detail (19). In short, to assess the stress response provoked by the neuroendocrine challenge, cortisol levels were

measured. Blood samples were collected in K3EDTA tubes and immediately centrifuged for 10 minutes at 2000 RPM. The plasma derived from these samples was kept at −80°C until further examination. Concentrations of cortisol in plasma were measured with ELISA kits from NovaTec Immunodiagnostica GmbH (Dietzenbach, Germany).

To correlate the evaluation of cortisol with transcriptomic measurements, the difference in cortisol levels between post-challenge and pre-challenge was computed for both the citalopram (DiffCitalopram) and placebo (DiffPlacebo) conditions, separately. A difference-of-differences value (DiffOfDiffs) was computed by subtracting the placebo-induced cortisol change from the citalopram-induced change in each participant. These DiffOfDiffs values were then compared between MO and healthy controls using the nonparametric Mann–Whitney U test.

In the analysis, only individuals with valid cortisol measurements were included, leading to a final sample of 19 patients with MO and 29 healthy controls. The threshold for statistical significance was established at $p \leq 0.05$. Further analyses were carried out to evaluate potential baseline and placebo-induced effects on cortisol levels. These were modelled using a linear model: lm(Cortisol ~ Group + Sex + Age + Allergy + Smoking).

3.4. Blood Sample Processing and RNA Sequencing

Whole blood was collected in PAXgene Blood RNA tubes (Qiagen, Venlo, The Netherlands) and stored at −80°C. RNA extraction was conducted using the PAXgene Blood mRNA Kit (Qiagen) on a QIAcube instrument at King's College London's Institute of Psychiatry, Psychology & Neuroscience (UK), in accordance with the manufacturer's instructions.

Library preparation was conducted using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs, Cat# E7420S/L), and all subsequent library and sequencing procedures were carried out at GenomeScan (Leiden, The Netherlands). Sequencing was performed on an Illumina NextSeq 500 platform using 75 bp single-end reads, with a sequencing depth of approximately 20 million reads per sample. All steps adhered to the standard operating procedures of Illumina and GenomeScan and were conducted in accordance with ISO quality control standards.

Adapter sequences were removed using Trimmomatic (v 0.39) (34), and read quality was assessed using FastQC (v0.11.8) (35). All reads achieved Phred quality scores above Q30, indicating 99.9% base call accuracy. Hence, no samples were excluded. Cleaned reads were aligned to the Ensembl GRCh38.96 human reference genome using HISAT2 (v2.2.1) (36) with default settings and spliced alignment enabled. Alignment rates ranged from approximately 91–96%, with one sample exhibiting a slightly lower rate of 84%. Transcriptome assembly and quantification were carried out using StringTie (v2.1.4) (37), utilizing the quasi-likelihood F-test, gene level analysis was performed in R (v4.0.3) (38) with the Bioconductor (v3.11) (39) package edgeR (v3.30.3) (40) to identify genes that were differentially expressed, applying the quasi-likelihood F-test. P-values were adjusted for multiple testing using the Benjamini-Hochberg false discovery rate (FDR) (41). Genes were considered significant when a gene was reaching at both measurements an FDR equal or ≤ 0.05. Due to the expected higher variability in response to stress, in the neuroendocrine challenge study gene-level results with a p-value ≤ 0.005 have been marked as suggestive significant and were discussed as well, albeit significant genes still had to have and FDR ≤ 0.05.

All analyses included covariates: age and sex, or age alone for sex-stratified models. In the neuroendocrine challenge we also considered a correction to avoid sampling day effects and corrected for whether citalopram has been infused in the first or second challenge. EdgeR allows inclusion of covariates via generalized linear models (GLMs) built on negative binomial distributions. Covariates sex and age were added as design covariates in the design matrix, which allowed adjustments for their potential confounding effects on gene expression. EdgeR controlled for the effect of sex and age when it estimated the log fold change between groups. To ensure that group differences were not solely due to age or sex distributions, the edgeR GLM framework adjusted the log-likelihood estimates of gene expression based on known covariates, thereby accounting for confounders at the modeling stage.

3.5. Gene Set Enrichment Analyses and Gene-Level analysis

Gene set enrichment analyses (GSEA) (42,43) were conducted using the Bioconductor package fgsea (44). The analysis was performed with default settings and a gene set size

ranging from 15 to 500. The gene sets used in this analysis were sourced from the Molecular Signatures Database (MSigDB) (utilizing v.7.4 for the general interictal experiment and 2023v1 in the neuroendocrine challenge, due to updates in the meantime for the databases). Both experiments investigated C5 gene-sets which contain Gene Ontology (GO) Cellular Constituent (CC), Biological Pathway (BP) and GO Molecular Function (MF), sets. GO CC results were difficult to evaluate with respect to MO. The neuroendocrine experiment also investigated C3 sets containing transcription factor target genes and microRNAs (miRNAs), because of the reasonable expectation of stress inducing transcription level regulation. C2 sets were included for validation in the neuroendocrine challenge study, containing curated gene sets, derived from various sources like PubMed, and domain experts. A pathway was deemed significant if the FDR was ≤ 0.05. In the general interictal study this requirement must have been met at both time points separately.

3.5.1. Leading Edge Gene Identification

LEGs are the genes that have the greatest impact on pathway enrichment; they are identified as those that appear in the ranked gene list prior to the point at which the running sum peaks.

LEGs were identified by the fgsea software and were presented if their mean absolute logarithmic 2-fold change (Log_2FC) was greater than 2 for the top 5 LEGs (LEG5) and greater than 0.5 for the remaining LEGs (LEG69). These thresholds were chosen based on existing literature and the general conventions in transcriptomic analysis, where a Log_2FC of ≥ 2 is typically regarded as representing substantial gene alteration (45).

3.6. Binding predictions using AutoDock Vina

To investigate potential drug-protein interactions, a set of 64 migraine medications was selected from DrugBank (46). Antiemetic drugs and non-small molecule drugs (such as

antibodies and botulinum toxin) were excluded from the analysis, leaving a final selection of 54 small molecule drugs for further evaluation.

The 3D structures of these small molecule drugs were obtained from PubChem, and docking simulations were performed using AutoDock Vina (47,48). For the docking analysis, protein structures were predicted using AlphaFold2 and downloaded from UniProt (49). These predicted protein structures were prepared for docking simulations using the "prepare_receptor" command, with the exception of adding hydrogens to the structures via the "-A" flag. Ligands (the selected drugs) were prepared using the "ml_prepare-ligand" command.

The basic docking simulations were set up with grid parameters centred at (0,0,0) and a grid box size of 126x126x126, with the Vina forcefield and an exhaustiveness level set to 32 (47). Preliminary docking runs were conducted with the default maximum number of binding modes. From the binding modes generated, the one with the lowest score (indicating the strongest binding) was selected as the most representative of the drug-protein interaction.

To ensure the accuracy and reliability of the binding predictions, normalization was performed by randomly sampling 100 proteins for each drug (50). Z-scores were calculated for the binding affinities of the drugs using the mean and standard deviation of the randomly sampled binding energies. One-sided p-values were derived from these Z-scores to assess the statistical significance of the binding interactions, with energetic favourability being the primary criterion. Significance was determined after Bonferroni multiple hypothesis correction, with p-values $\leq 1.3617 \times 10^{-5}$ considered statistically significant.

3.7. Drug-protein binding prediction with machine learning

A machine learning-based approach was developed to predict drug-protein binding interactions relevant to migraine treatment, integrating gene expression data and drug information. Genes from the significant pathways identified in the previous analyses, along with all drugs from DrugBank (46) were tested in the prediction model. Given the lack of a deployable comprehensive drug-binding prediction pipeline for genes, a novel

pipeline was designed. In migraine, traditional one drug–one target approaches to migraine drug development have been criticised for their limited ability to find therapeutically relevant candidates (51).

All genes were extracted to construct a comprehensive gene set, and their corresponding protein structures were obtained from the UniProt database (49). For drug-binding prediction, HyperAttentionDTI, a convolutional neural network model with an attention block, was employed. This model processes both the drug's SMILES strings and the amino acid sequences of proteins to predict binding affinities. HyperAttentionDTI enables the prediction of drug-binding scores for proteins associated with selected genes (52).

The key hyperparameter settings included a learning rate of $1x10^{-4}$, a weight decay of $1x10^{-4}$, and input embeddings of size 64. The CNN blocks comprised three stacked 1D-CNN layers with 32, 64, and 96 filters, with window sizes of 4, 6, and 8 for drugs, and 4, 6, and 12 for proteins. The output block contained four layers with 1024, 1024, 512, and 2 neurons, respectively. A dropout rate of 0.1 and a batch size of 32 were applied, and the predictions were run on a 24GB Nvidia RTX A5000 GPU.

The model generated drug-protein binding predictions for 11,064 drugs in DrugBank. For each drug, the maximum binding score across all proteins associated with a gene was selected as the binding score for that drug-gene pair. To assess the specificity of the binding profiles, the frequency with which each drug appeared in the top 10 most binding drugs for each of the 9,657 genes was calculated. Drugs appearing more than 10 times in the top 10 for any gene were excluded, as their widespread binding profiles were considered less suitable for potential drug candidates.

Normalization was performed using the mean and standard deviation of the predicted binding scores across all proteins. Right-sided p-values were calculated for the binding predictions, and Bonferroni correction for multiple hypothesis testing was applied. FDR values were calculated for each drug based on its binding to the LEGs of the enriched pathways.

To further refine the drug selection process, a pathway-level drug prediction pipeline was developed to assess multi-target drugs. The drug-gene prediction scores were multiplied by the mean absolute Log_2FC values obtained from the allergy-corrected migraine versus control comparison, provided both drug-binding predictions and gene expression data were available. This approach enabled the aggregation of gene expression and binding affinity data, offering a comprehensive evaluation of the effects of drugs on entire pathways.

To calculate the pathway-level effects of each drug, the binding scores for genes within a given pathway were aggregated using the standard L2-norm, which provides a weighted sum of the binding scores. This method accounts for both the binding affinity of the drug and the gene expression changes within the pathway. Empirical p-values for pathway-gene pairs were calculated using Monte Carlo integration of the ranked p-values list, with a significance threshold set at ≤ 0.0005. Pathway-gene pairs with p-values below this threshold were considered significant (Figure 2).

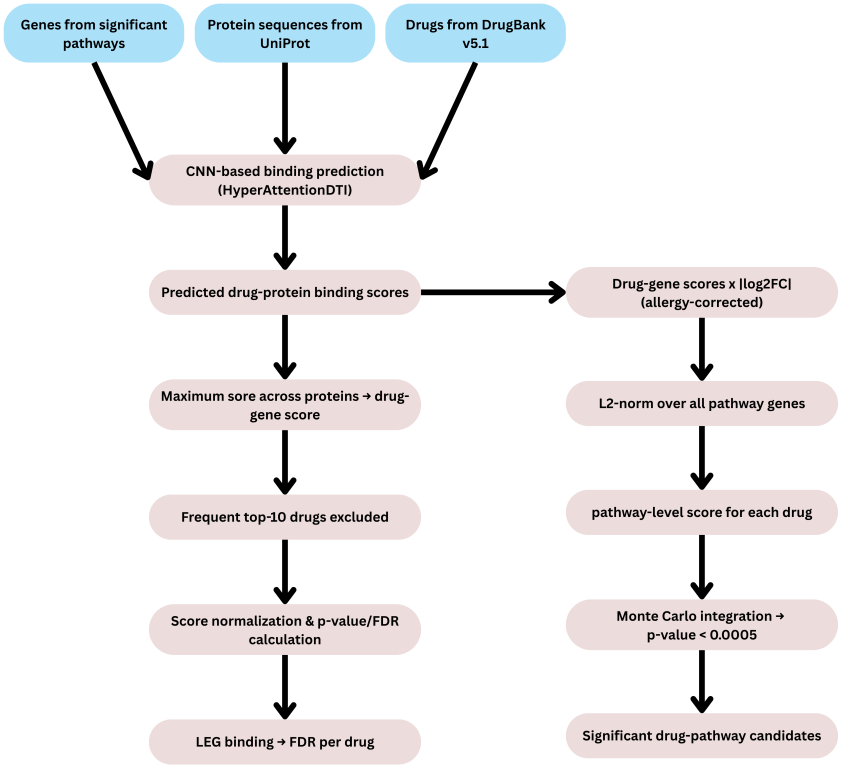

Figure 2: Schematic workflow of the machine learning-based drug–protein and pathway-level prediction pipeline.

4. Results

4.1. Descriptive statistics

The study population consisted of 22 migraine patients (17 females, 5 men), and 30 healthy controls (16 females, 14 males). Due to the onset of a migraine attack during the neuroendocrine challenge, one additional MO patient was excluded from analyses after neuroendocrine challenge. All participants underwent phenotyping and were screened for exclusion criteria including neurological comorbidities (except MO in the patient group), regular medication use, and recent migraine attacks or headaches within 24 hours of sampling. Additional information was collected regarding smoking status, history of allergies, and mental health using validated instruments such as the MINI, SDS, and STAI. See Table 1 for population descriptors for the general interictal data and Table 2 for the neuroendocrine experiment.

Table 1: Population descriptors of the cohort from the general interictal experiment

		Con(MO)					X² test statistic		p-value	
a	**Male**	14(5)					2.1895		0.139	
	Female	16(17)								
b			**Mean**	**Median**			**Range**	**SD**	**U**	**p-value**
	S1	Mig	26.8	25.6			20-37	5.13	324	0.92
		Con	26.4	25.7			21-37	4.02		
	S2	Mig	26.9	25.7			20-38	5.17	319	0.847
		Con	26.6	26			21-37	3.99		
c			**Counts-Con (MO)**	**Pollen, weed, dust**			**Drug**	**Food**	**X²**	**p-value**
	Allergy	Male	4(2)	4(7)			3(2)	1(0)	0.61	0.44
		Female	3(7)							
d			**Smokers - Con(MO)**	**1**	**2**	**3**	**4**	**5**	**U**	**p-value**
	Smoking	Male	5(5)	23(0)	4(14)	0(4)	2(4)	1(0)	90	1.98E-06
		Female	2(17)							

This table describes in a) & c) absolute numbers of sex and history of allergy using chi-square test. In b) age difference between MO and Con was tested using Mann-Whitney test at both time points. In d) differences for smoking status were utilized with the Mann-Whitney U-test.

Con – controls, MO – migraine without aura, χ – Chi-squared (χ^2) distribution, SD – Standard deviation, U – Mann-Whitney U test statistic, S1 – Time point 1, S1 – Time point 2, 1 – never, 2 – occasionally, 3 – a few stems/day, 4 – half box/day, 5 – one or more than one box/day(53)

Table 2: Population descriptors of the cohort from the neuroendocrine challenge experiment

		Con (MO)						**χ**	**p-value**
a	**Male**	14(5)						1.87	0.1715
	Female	16(16)							
b	**Allergy**	**Counts Con (MO)**	**Pollen, weed, dust**			**Drug**	**Food**		
		8 (9)	4(7)			3(2)	1(0)	0.82	0.3653
c	**Age**	**Mean Con (MO)**	**Median Con (MO)**			**SD Con (MO)**		**U**	
		26.38 (26.91)	25.75 (25.63)			4 (5.2)		315	1
d	**Smoking**	Smoker Con (MO)	1	2	3	4	5		
		7 (21)	23(0)	4(13)	0(4)	2(4)	1(0)	556.5	8.75E-08
e	**MO onset age**	Mean onset age (years)	14.33						
		SD (years)	5.45						
f	**MIDAS B scores**	Score	5.5						
		SD	1.6						
g	**Trigger for migraine attack**	Stress	Yes	12					
			No	9					
h	**Psychological assessment**			**t-Value**	**df**	**p-Value**	**95% CI**	**Mean MO**	**Mean Con**
		Basis	ZUNG	2,02	72,05	0.046	0.0663, 8.8923	37,73	33,25
			STAI I	2,36	82,91	0.026	0.6272, 7.3343	41,57	37,59
			STAI S	3,38	77,01	0.001	1.3286, 5.1276	37,09	33,86
		Day 1 and Day 2 adjusted from basis	ZUNG	0,06	91,94	0,95	-1.2670, 1.3495	-2,01	-2,05
			STAI I	-0,3	93,88	0,76	-2.3385, 1.7200	-2,46	-2,15
			STAI S	-0,5	88,25	0,62	-4.8331, 2.9042	-2,47	-1,51

The following are displayed in the table: a) Sex distribution, which displays the absolute numbers of male and female controls and MO patients along with the corresponding Chi-Square test statistics; b) History of allergy in MO and controls is shown with regard to pollen, weed, dust, drug, and food as potential allergens and the corresponding Chi-Square test statistics; c) Age differences among MO patients and controls on the citalopram administration day and the corresponding Mann-Whitney U-test statistics; d) Smoking status metrics and corresponding Mann-Whitney U-test statistics; e) MO onset age in years; f) MIDAS B scores in MO; g) Migraine attack triggers, particular stress, with incidences of "Yes" and "No" answers h) Psychological tests, such as ZUNG, STAI-I, and STAI-S scores, along with the statistics from the Welch's t-test; Evaluations were carried out: Comparing MO patients to controls is the foundation. Results are deducted from the basis scores for day 1 and day 2 before blood sampling.

Con – Control; MO – Migraine without aura; χ – Chi-squared (χ^2) distribution; SD – standard deviation; Numbers regarding smoking: 1 – never, 2 – occasionally, 3 – a few cigarettes/day, 4 – half box/day, 5 – one or more than one box/day; t-Value – test statistic from Welch's t-test; df – degrees of freedom; CI – confidence interval. (54)

4.2. General Interictal Transcriptomic Differences Between MO Patients and Controls

4.2.1. No FDR-corrected Differentially Expressed Genes were Identified in Allergy-Corrected Analysis

At both measurement points, no genes were identified as significantly and consistently differentially expressed between the MO and control groups. However, *CYP26B1* exhibited a significant and consistently replicated downregulation, characterized by a mean $\log_2$ fold change (Log_2FC) of –5.860 and uncorrected p-values ≤ 0.0001 at both time points. Nonetheless, the FDR remained above the threshold of statistical significance (FDR = 0.2000 and 0.0600). The ten genes with the highest positive and negative change of expression, determined by their average $\log_2FC$, are shown in Table 3. The genes were among those with the most significant downregulation. although they exhibited

substantial changes in expression, none of them remained FDR-significant at both timepoints.

Table 3: Top 10 positively and top 10 negatively enriched genes based Log_2FC.

Symbol	Pval S1	FDR S1	Pval S2	FDR S2	Mean log_2FC
Upregulation					
RIMBP2	0.0061	0.3400	0.0047	0.5100	5.7554
MYO18B	0.0091	0.8010	0.0012	0.2743	4.9803
PRSS22	0.0002	0.2410	0.0067	0.5597	4.9248
LINC01271	0.9999	1.0000	0.0048	0.5059	4.8222
OR52N1	0.0003	0.2567	0.0724	0.8574	4.1222
KRT79	0.0127	0.8329	0.0060	0.5255	3.9668
GSDMA	0.0006	0.3385	0.0025	0.4235	3.9225
SMARCA1	0.0037	0.7220	0.0478	0.8325	3.9210
ETV3L	0.0214	0.8655	0.0211	0.7722	3.8825
RFPL4A	0.0670	0.8813	0.0409	0.8218	3.7772
Downregulation					
CYP26B1	0.0001	0.2000	0.0001	0.0600	-5.8600
TTK	0.0011	0.4437	0.0010	0.2664	-4.8779
CORIN	0.0015	0.5275	0.0004	0.1426	-4.1653
SCGB1C1	0.0351	0.8734	0.0087	0.6305	-3.1269
LRRC43	0.0172	0.8642	0.0218	0.7775	-3.0739
PRTN3	0.0005	0.2991	0.0029	0.4484	-3.0534
CCL23	0.0386	0.8735	0.0458	0.8297	-2.7878
CTSG	0.0004	0.2936	0.0009	0.2410	-2.6687
OLAH	0.0105	0.8064	0.0115	0.6671	-2.5815
HRASLS2	0.2878	0.8973	0.0173	0.7417	-2.1917

None of the genes reached statistical significance after multiple hypothesis correction. Abbreviations: Log_2FC – Log_2 fold change; FDR – False discovery rate; Pval S1/FDR S1 and Pval S2/FDR S2 – p-values and FDR at two different time points S1 and S2.

4.2.2. Identification of Consistently Negatively Enriched Pathways

A total of 88 pathways were found to be significantly and consistently enriched in MO patients when compared to healthy controls. Every enriched pathway exhibited negative NES values, indicating a downregulation in MO. The results were manually categorized into seven functionally relevant groups by leveraging expertise in migraine pathophysiology, with a focus on the biological systems most affected in MO (Table 4).

Table 4: Overview of significantly negatively enriched pathways observed in MO compared to healthy controls.

Category			Pathway
Immune System	Innate immune system		GOBP Activation of immune response GOBP Activation of innate immune response GOBP Innate immune response activating signal transduction GOBP Innate immune response GOBP Regulations of innate immune response
	Adaptive immune system		GOBP Antigen processing and presentation GOBP Antigen processing and presentation of exogenous peptide antigen vie MHC class I GOBP Antigen processing and presentation of peptide antigen GOMF MHC protein binding
	General		GOBP Cell activation involved in immune response GOBP Phagocytosis HP Abnormal myeloid leukocyte morphology GOBP Leukocyte migration GOBP Endocytosis GOBP Myeloid leukocyte activation GOBP Myeloid leukocyte differentiation GOBP Myeloid leukocyte-mediated immunity GOBP Inflammatory response
	Signaling Mediatiors	Cytokine-related	GOBP Cytokine-mediated signaling pathway GOBP Fcε-receptor signaling pathway GOBP Fc-receptor signaling pathways GOBP Interleukin 1-mediated signaling pathway
		Interferon-related	GOBP Response to interferon gamma
Cardiovascular			HP Abnormality of cardiovascular system electrophysiology

Oxidative stress		GOBP Regulation of superoxide anion generation GOBP Regulation of transcription from RNA-polymerase II promoter in response to hypoxia GOBP Response to oxygen levels GOBP Regulation of DNA-templated transcription in response to stress GOBP Reactive oxygen species metabolic process GOBP ATP metabolic process GOBP Cellular response to external stimulus GOBP Cellular response to nitrogen compound GOBP Cellular response to oxygen levels
Metabolism	Carbohydrate metabolism	GOBP Carbohydrate catabolic process GOBP Carbohydrate metabolic process GOBP Cellular ketone metabolic process GOBP Cellular response to insulin stimulus GOBP Pyruvate metabolic process GOBP Response to carbohydrate GOBP Response to insulin GOBP Response to monosaccharide GOBP Monocarboxylic acid metabolic process
	Aminoacid metabolism	GOBP Cellular response to peptide GOBP Response to peptide GOBP Regulation of cellular amino acid metabolic process GOBP Amine metabolic process GOBP Regulation of cellular amine metabolic process
	Lipid metabolism	GOBP Response to lipid
Hormone response		GOBP Response to hormone GOBP Cellular response to hormone stimulus GOBP Response to estrogen GOBP Response to peptide hormone GOBP Cellular response to peptide hormone stimulus GOBP Response to peptide hormone

Additional pathways	HP Abnormality of urine homeostasis HP Abnormal urine metabolite level HP Skin ulcer GOBP Bone cell development GOBP Cell killing GOBP Positive regulation of cell death GOBP Regulation of apoptotic signaling pathway

MO – Migraine without Aura; GO – Gene Ontology; BP – Biological Pathways; MF – Molecular Function; HP - Human Phenotype Ontology.

4.2.3. Leading-Edge Genes Driving Pathway-Level Differences

LEG analysis was performed on the 88 pathways identified as significantly enriched through gene set enrichment analysis. Five genes (CYP26B1, CORIN, PRTN3, CCL23, and CTSG, henceforth LEG5) showed consistent and significant downregulation at both time points with large mean log_2FC ($Log_2FC \leq -2$) (Figure 3), reflecting the topmost downregulated genes from our analysis. Additionally, 69 (LEG69) had a mean log_2FC of ≤ -0.5. No LEGs exhibited a positive log_2FC. As shown in Figure 3, there was a notable and consistent downregulation of all five LEG5 genes. The expression of CYP26B1 changed most, with a mean log_2 fold change of approximately –5.86 at both time points. Although none of the LEG5 genes showed significance after FDR correction, their nominal p-values varied from ≤ 0.05 to ≤ 0.001, indicating a consistent pattern of differential expression across replicates.

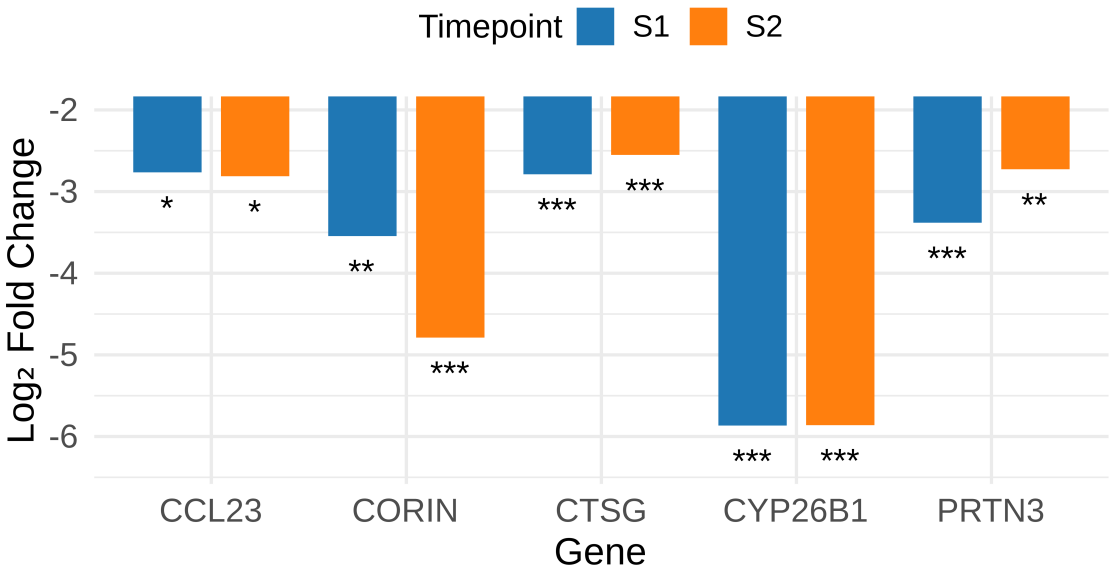

Figure 3: Log₂ Fold Change of LEG5 Genes at two time points.

The bars represent mean log₂FC values from allergy-corrected analyses for five consistently downregulated genes. None of the genes remained significant after correction for multiple testing, however nominal significance could be observed indicated with asterisks. Nominal p-values: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

4.2.4. Stability of Pathway-Level Findings Across Sex and Allergy-Corrected Models

To evaluate the robustness of pathway-level transcriptomic alterations in MO results from the core model were compared with those from alternative analytic conditions, including allergy-uncorrected and sex-specific analyses. The fully adjusted core model, incorporating age, sex, smoking status, and allergy as covariates, found 88 pathways significantly enriched when comparing MO patients to healthy controls (Table 5, comparison a). After the allergy covariate was removed, 298 enriched pathways were identified (comparison b), of which 82 overlapped with the core set.

A direct comparison between female and male MO patients (comparison c) revealed 33 significantly enriched pathways, yet only 13 of these were directionally consistent with the core set. The other 22 pathways either showed contrary directions of enrichment or were not statistically significant.

In comparison d, which involved female MO patients and female controls, 20 pathways were found to be significantly enriched, with 17 of these coinciding with the core set. Conversely, the comparison of male MO patients with male controls (comparison e) produced a larger set of 62 enriched pathways, of which only 29 overlapped with the core set.

Table 5: Overlap and directionality of significant pathway enrichments across sex- and allergy-corrected analyses

	Comparison	**Significant Pathways**	**Overlap with Core Set**	**% Overlap**	**Notes**
a	Migraine vs Control (allergy- and sex-corrected)	88	-	-	Core pathway set
b	Migraine vs Control (allergy-uncorrected)	298	82	93,2%	6 pathways lost compared to Core Set
c	Female vs Male (migraine only, sex-specific comparison)	33	13 (same NES direction)	-	13 pathways had same direction; 22 showed opposite or non-significant
d	Female migraine vs female controls	20	17	85%	Most overlap with Core Set
e	Male migraine vs male controls	62	29	46,8%	Less overlap with Core Set

In addition to the comparisons at the pathway level, analyses were conducted to assess gene-level stability for two important leading-edge genes, *CYP26B1* and *CORIN*, across sex-specific and allergy-uncorrected analyses.

In both female and male patients with MO, *CYP26B1* showed significant and consistent downregulation compared to sex-matched controls. In females, $\log_2$ fold changes of –3.91 and –4.20 were recorded at the two respective time points, with nominal p-values of 0.01 and 0.007. The differences in expression among males were even more marked (–7.82 and –7.52), with p-values of 0.0026 and 0.003. The allergy-uncorrected comparison of MO patients versus controls, showed a significant and reproducible downregulation of *CYP26B1*, with $\log_2$ fold changes of –6.79 and –6.72. This identified

CYP26B1 as the only gene that reached FDR significance in the allergy-uncorrected analysis (Table 6).

Across all models, *CORIN* also showed consistent downregulation. For females, the $\log_2$ fold changes were –3.57 and –2.75, and the corresponding p-values were 0.001 and 0.02. In males, CORIN expression decreased by –3.51 and –6.83 at the two time points, with nominal p-values again ≤ 0.05. The $\log_2$ fold changes from the allergy-uncorrected comparison were –3.35 and –4.69, with p-values of 0.0019 and 0.0004, respectively. In all instances, however, *CORIN* did not achieve significance after FDR correction.

Table 6: CYP26B1 and CORIN across sex-specific and allergy-uncorrected analysis

Gene	Comparison	$\log_2$FC (S1)	p-value (S1)	FDR (S1)	$\log_2$FC (S2)	p-value (S2)	FDR (S2)
CYP26B1	Female MO vs Female Controls	-3.91	0.01	0.57	-4.2	0.007	0,75
	Male MO vs Male Controls	-7.82	0.0026	1.0	-7.52	0.003	0.46
	MO vs Control (allergy-uncorrected)	-6.79	0.0000109	0.04	-6.72	0.000017	0.008
CORIN	Female MO vs Female Controls	-3.57	0.001	0.28	-2.75	0.02	0.98
	Male MO vs Male Controls	-3.51	0.04	1.0	-6.83	0.0029	0.46
	MO vs Control (allergy-uncorrected)	-3.35	0.0019	0.56	-4.69	0.0004	0.13

Summary of $\log_2$FC, nominal p-values, and FDR values is given. Log_2FC - $\log_2$ fold changes; FDR – False discovery rate.

4.3. Drug Target and Binding Predictions for MO-Associated Genes

4.3.1. Limited Binding of Current Migraine Drugs to Identified DEGs and LEGs

Molecular docking simulations using AutoDock Vina were performed to assess whether existing migraine treatments can directly target the proteins encoded by the LEG69s identified through transcriptomic analysis. Binding interactions were examined, as a required characteristic for pharmacological efficacy. None of the migraine-associated compounds curated from DrugBank demonstrated statistically significant binding to any LEG69-encoded proteins after correction for multiple testing (with a one-sided p-value ≤ 1.3617x10-5).

4.3.2. Identification of Four Candidate Compounds with Predicted Binding to CORIN

Whilst standard migraine treatments did not show a significant binding affinity to transcriptomically altered gene products in MO (see 4.3.1), further analyses using machine learning–based drug-protein binding predictions of 11,164 Drug-Bank compounds revealed several new candidates (Figure 4). Probucol, Fasoracetam, Ginsenosides, and Rovafovir showed a statistically significant predicted binding to *CORIN* with FDR ≤ of 0.1. *CORIN* is a serine protease, that has been discovered as one of the LEGs in MO patients.

Probucol demonstrated the highest predicted binding affinity to CORIN, with a Z-score of 2.570 and FDR = 0.070. Fasoracetam followed with a Z-score of 2.557 and FDR = 0.073. Ginsenosides exhibited a Z-score of 2.454 with FDR = 0.081, while rovafovir showed an identical Z-score of 2.454 and an FDR = 0.098. It must be noted that these are in silico findings and experimental validation is pending.

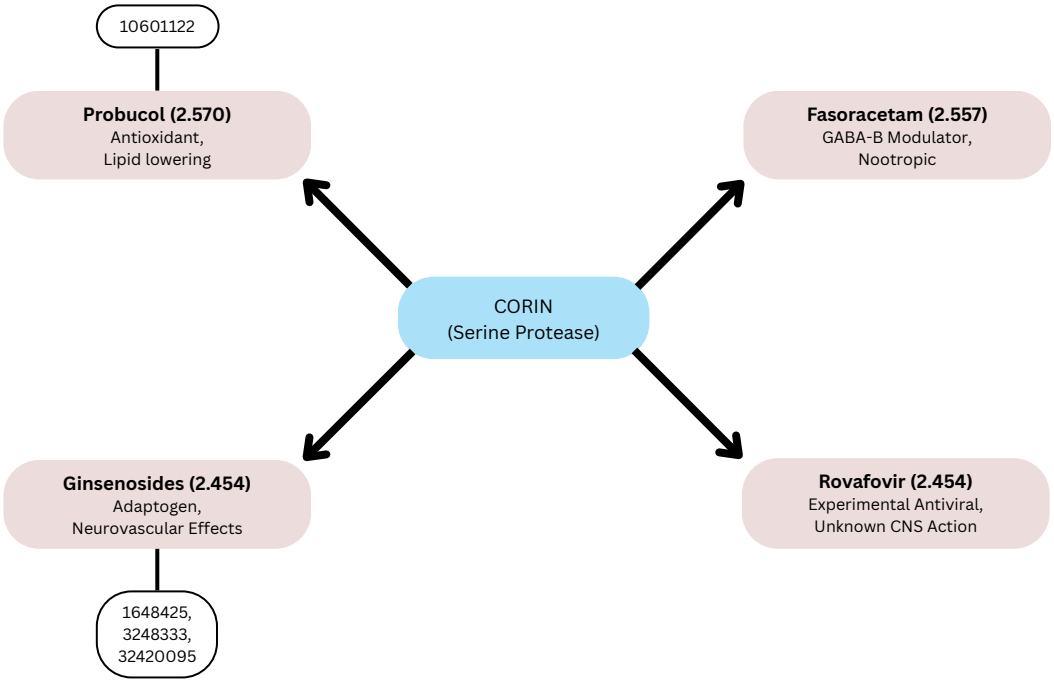

Figure 4: Predicted four drug candidates showing computational binding affinity to CORIN

The potential drug candidates probucol, fasoracetam, ginsenosides, and rovafovir were identified, based on predicted binding to CORIN, where utilization of machine learning-based in silico screening of 11,164 DrugBank compounds was applied. Candidates displayed predicted binding with an FDR ≤ 0.1. Z-scores, literature references (PubMed IDs) for their investigated use, both are shown in the figure. FDR – False discovery rate.

4.3.3. Drug-Pathway Binding Predictions Reveal Additional Therapeutic Candidates

Based on binding predictions at the pathway level advised by machine learning, several other compounds with possible therapeutic significance in migraine were identified. The predictions were grounded in drug-pathway binding scores that took into account predicted drug-protein affinities and gene expression changes across significantly enriched pathways from the MO versus control analysis.

Five migraine-associated drugs showed a statistically significant predicted binding ($p \leq 0.0005$) to at least one replicated and functionally enriched pathway. The

pathways encompassed positive regulation of cell death, response to lipid, and transmembrane receptor protein tyrosine kinase signalling (Figure 5). These are all mechanistically connected to known facets of migraine pathophysiology.

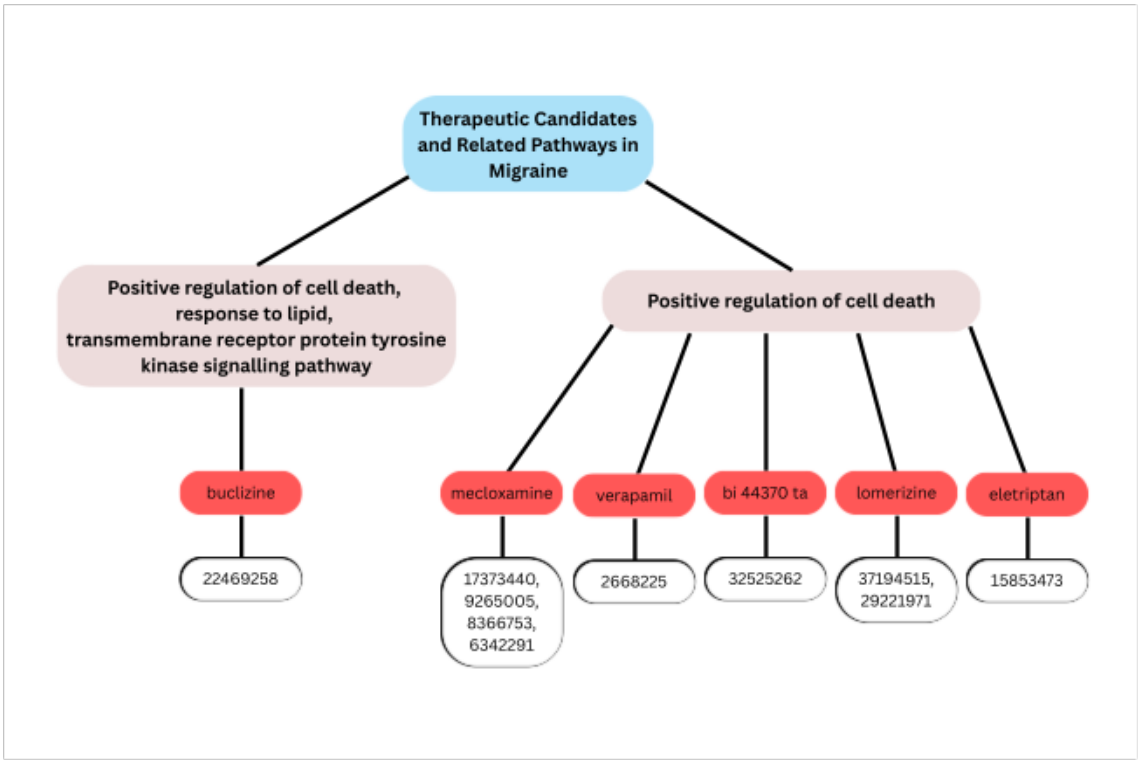

Figure 5: Therapeutic candidates and related pathways in migraine

Weighted drug-pathway binding predictions identified migraine-associated drugs and additional potential drug candidates. Mentioned drugs show five migraine-associated drugs having significant predicted binding ($p \leq 0.0005$) to minimum one significantly replicated pathway from the MO vs control comparison. Associated pathways include positive regulation of cell death, response to lipid and transmembrane receptor protein tyrosine kinase signaling. PubMed IDs are highlighted, indicating literature references for each drug's known or studied mechanism.

4.4. Transcriptomic and Hormonal Response to Neuroendocrine Stress Challenge

4.4.1. Altered Cortisol Response Following Citalopram Infusion in MO Patients

In MO patients, a notably different cortisol response was noted compared to controls, featuring a delayed increase at 70 minutes after the infusion (Figure 6). The difference in plasma cortisol concentration from pre- to post-challenge for both the citalopram and placebo conditions was used to calculate cortisol reactivity. The net pharmacological effect of citalopram was calculated by subtracting the change induced by the placebo from the change induced by citalopram (ΔCitalopram – ΔPlacebo). At 0 and 30 minutes after infusion, the differences between groups were not statistically significant (0 min: $P = 0.13$, $U = 203$; 30 min: $P = 0.1355$, $U = 347$).

At the 70-minute measurement point, a trend-level difference ($p \leq 0.1$) was identified: MO patients showed a larger increase in cortisol than controls ($P = 0.07292$, $U = 361$). It is worth mentioning that the migraine group exhibited greater inter-individual variability, which reflects a heterogeneity in neuroendocrine stress sensitivity. In MO patients, the negative mean value recorded at 0 minutes indicates that baseline cortisol concentrations were elevated before the administration of placebo (mean = –24.40 ng/mL, SD = 47.41). See Table 7 for Mann–Whitney U test results and exact P-values.

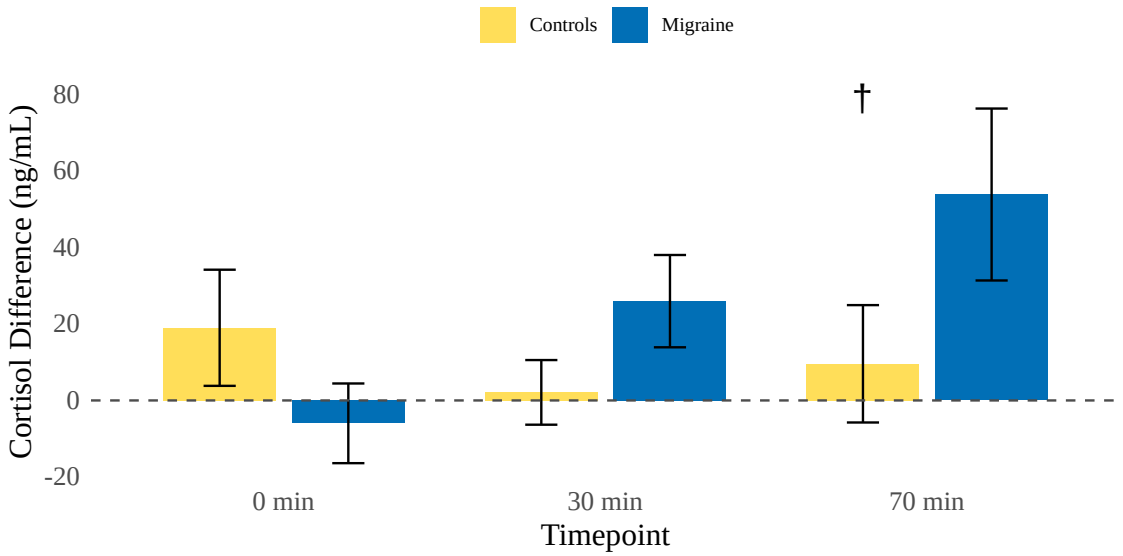

Figure 6: Relative cortisol changes in response to neuroendocrine challenge with citalopram

Relative cortisol concentrations reflecting the net effect (Δcitalopram – Δplacebo). The whiskers are representing the whiskers of the standard error of the mean. Results indicate a trend-level significance at 70min post-infusion.

† - Trend-level P-value ≤ 0.1; MO - migraine without aura.

Table 7: Mann–Whitney U test results for cortisol concentration changes following citalopram neuroendocrine challenge

t (min)	p-value	Mann–Whitney U
0	0.13	203
30	0.1355	347
70	0.07292	361

This tables reflects the p-values and U statistics of cortisol levels between MO and controls at all three timepoints t=0min (pre-challenge), t=30min and 70min post-infusion. Trend-level significant difference ($p \leq 0.1$) was observed at 70 minutes.

4.4.2. Suggestive Gene-Level Differences Post-Challenge implicate IKBKGP1 and NKRF & Stress-Induced Pathway-Level Alterations in MO Patients

Following citalopram-induced neuroendocrine stress, a gene-level transcriptomic analysis identified ten genes with suggestive significance ($p \leq 0.005$) in comparisons between MO and healthy controls, including placebo-controlled data which allowed to account for any changes attributable to the infusion procedure itself or baseline fluctuations, allowing us to isolate citalopram-induced neuroendocrine stress effects.

Even though none of these genes met significance criteria after FDR correction, their expression patterns suggest biologically significant trends that could indicate early molecular effects of a modified stress response in MO patients (Figure 7).

Pathway enrichment analysis revealed upregulation in several functional domains, including immune-related processes (such as B cell proliferation), protein biosynthesis (including ribonucleoprotein complex biogenesis and cytoplasmic translation), and carbohydrate metabolism. Phototransduction was the only mechanism that was identified as being downregulated. Changes are indicated by arrows: upward arrows (↑) signify upregulation, and downward arrows (↓) signify downregulation, based on the sign of the log_2FC for genes and the normalized enrichment scores for pathways. The colours of the boxes correspond to the regulatory direction: green indicates upregulated features, while red denotes downregulated ones (Figure 7).

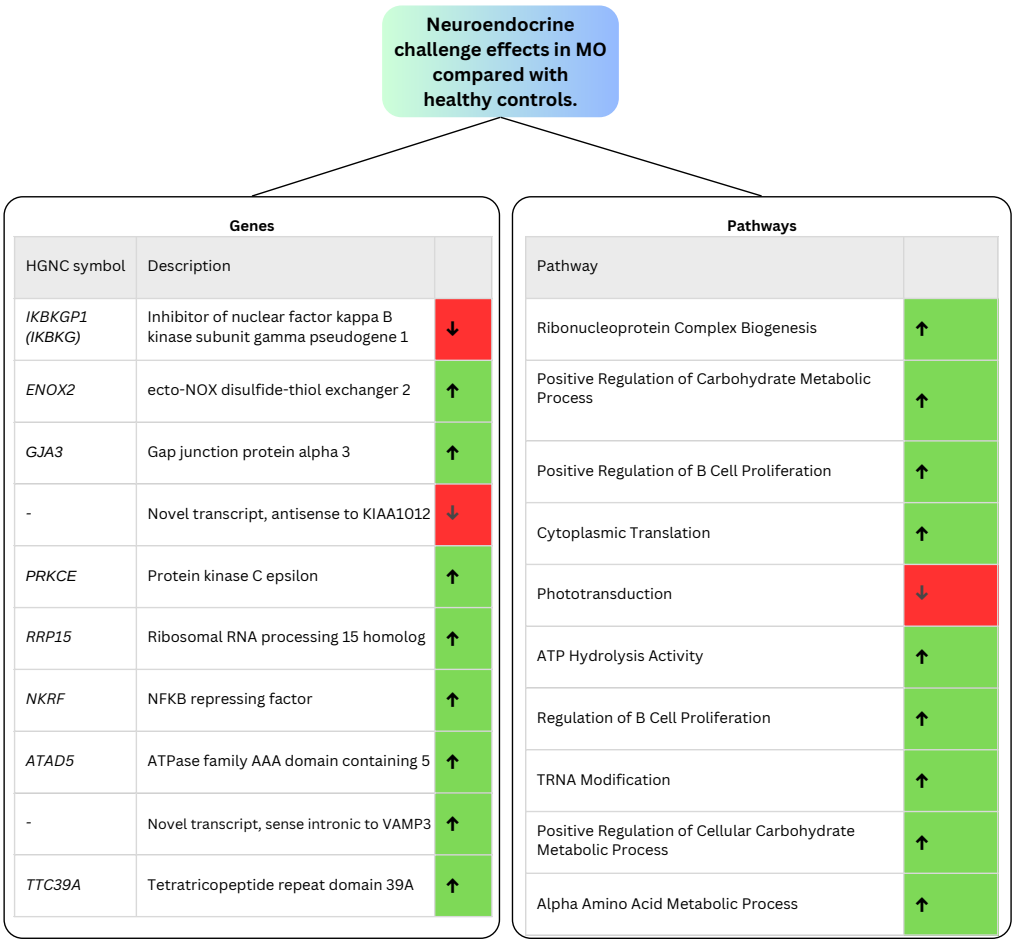

Figure 7: Suggestively significant genes and enriched pathways following a neuroendocrine challenge in MO compared to healthy controls.

4.4.3. Opposite Patterns Between MO Patients and Controls

Separate gene set enrichment analyses for MO patients and healthy controls showed largely opposite directional effects across numerous biological pathways after the neuroendocrine challenge (Figure 8). Although not every result at the pathway level achieved nominal statistical significance ($p \leq 0.05$), a less stringent threshold of $p \leq 0.10$ was used to pinpoint trends of biological relevance.

Among the differentially regulated pathways, those related to glycosylation stood out. Specifically, glycosylation related processes were consistently upregulated in MO patients. These pathways reached suggestive significance when including the migraine attack patient and nominal significance in a subgroup of 21 MO patients, when excluding the patient who experienced the migraine attack during the neuroendocrine challenge. This suggests that glycoprotein biosynthesis may be robustly involved in the stress response of migraineurs. In MO patients, there was a consistent upregulation of B cell proliferation, ATP hydrolysis activity, and carbohydrate metabolic process regulation, while these were downregulated in healthy controls. In contrast, mechanisms like the formation of a skin barrier, dampening of epithelial cell differentiation, and modulation of DNA recombination were downregulated in MO but upregulated in controls. This indicates differing transcriptional responses to the stressor. The upregulation of carbohydrate metabolic pathways in MO patients was notably replicated in this subgroup analysis, reinforcing its status as a consistent finding throughout the study.

Pathway	Mig	Con
Atp Hydrolysis Activity	↑	↓
Regulation of B Cell Proliferation	↑	↓
Positive Regulation of B Cell Proliferation	↑	↓
TRNA Methyltransferase Activity	↑	↓
Establishment of Skin Barrier	↓	↑
Positive Regulation of Carbohydrate Metabolic Process	↑	↓
Negative Regulation of DNA Recombination	↑	↓
Regulation of DNA Recombination	↑	↓
Regulation of Mitotic Spindle Assembly	↑	↓
Negative Regulation of Epithelial Cell Differentiation	↓	↑
Positive Regulation of Cellular Carbohydrate Metabolic Process	↑	↓

Figure 8: Opposing pathway enrichment patterns in MO and controls following neuroendocrine challenge

5. Discussion

This dissertation investigated transcriptomic changes in MO using RNA-Seq interictally without and with a neuroendocrine challenge elicited by citalopram.

5.1. Significantly Different and Consistently Replicated Genes and Pathways in MO Patients Compared to Healthy Controls

A finding of this thesis is that, despite rigorous design and strict correction for confounders, no individual gene reached statistical significance after multiple testing correction at the genome-wide level. We must note, that at individual time point S1, we could identify FDR significant genes, which, however, could not be replicated. At the same time, pathway-level analysis revealed 88 consistently and significantly replicated biological pathways in MO patients compared to controls. These pathways spanned downregulated immune, metabolic, cardiovascular, and hormonal regulatory functions and reflect the complex, multifactorial nature of migraine, where system-level dysregulation rather than single gene mutations is paramount (25,27,55–58). Furthermore, these replicable pathway-level results aligned to the existing literature, downregulated and altered amino acid-, lipid- and carbohydrate metabolism (59–62), immunologic processes (55,63), cardiovascular comorbidities (64,65), and hormonal influences (66) were previously implicated in migraine. The convergence of the effects to that measured with many previously proposed attack mechanisms from the literature (23,62–69) even suggest that these are not only interictal characteristics but may be more general in migraine. Our results could also highlight gene-level results, *CORIN* and *CYP26B1*, behind the top 5 most downregulated pathways. 1) They were identified as key transcriptomic markers due to the LEG analysis that was only performed in significantly replicated pathways. 2) Only five genes met the criterion of $|\log_2 FC| \geq 2$ at both timepoints in the allergy-corrected analysis 3) These two LEGs remained stable in terms of their downregulation and nominal significance in sex specific and allergy-uncorrected analysis. Investigation of genetic polymorphisms, within the LEGs could not show direct

single nucleotide polymorphisms (SNP)-level associations for the *CYP26B1*, which supports its role as a transcriptomic marker of underlying processes rather than a primary, genetically driven causal factor (53). The suspected mechanism possibly involves diminished retinoic acid availability due to *LRP1* gene disease-associated genetic polymorphisms, which is involved in retinoid metabolism and vitamin A transport (67–73). *CYP26B1* downregulation and reduced all-trans-retinoic acid (atRA) levels, are mechanistically associated with *LRP1* dysfunction that results in defects in retinol uptake and signaling (67,70). Investigation of SNPs, which was not a part of this thesis (59) identified migraine-associated *LRP1* polymorphisms, possibly contributing to impaired retinoid metabolism in MO. This downregulation is supported by vitamin A-associated metabolic disturbances found in migraine (72,73). RXR/RAR receptor association to leading-edge genes in our dataset further reinforced a potential disturbance. In addition, recent genomic investigations also point to the involvement of retinoic acid pathway, while carotenoids, known proforms of vitamin A showed strong association with migraine in the NHANES cohort (74,75).

Expression of CORIN, a synthesis enzyme of atrial and brain derived natriuretic peptide (ANP and BNP, respectively), was also decreased indicating a complementary mechanism. BNP levels were shown to be altered in migraineurs (76), and a deficiency in BNP is modulating trigeminal sensitivity through mechanisms involving P2X3 receptor (77,78).

Beyond the findings on *CYP26B1* and *CORIN*, SNPs analysis of LEGs emerging from LEG69, revealed nominally significant associations in genes such as *EFNB2*, *CBR3*, *TEK*, *KCNMA1*, *HTR7* with migraine or related traits in previous studies (79–83). Thus, these genes may represent genetically driven transcriptional alteration. Notably, *SGCD*, a gene that that has not been previously linked to migraine, yielded several nominally significant hits.

As an example, we highlight two downregulated pathways from Table 4, which we discuss in detail: 1) GOBP Cytokine-mediated signaling pathway: The pathway cytokine-mediated signaling pathway is a key regulator of immune and inflammatory processes, which includes a cascade of cellular responses to cytokines. Inflammation in

the periphery and centrally is suspected to contribute to the pathophysiology of migraine attacks. In support of that, studies revealed increased levels of proinflammatory cytokines in migraine, such as IL-1β, IL-6, and TNF-α (84), which are known to sensitize trigeminovascular neurons, improve pain transmission, and facilitate CGRP release (85), which is a hallmark of migraine neuroinflammation. The general interictal transcriptomic data showed negatively enriched cytokine-related pathways, suggesting a basal suppression of the immune response, reflecting a maladaptive compensatory mechanism that suppresses the immune tone impairing immune flexibility. Elevated cortisol during acute stress further suppresses cytokine signaling, potentially setting up a rebound effect, followed by a possible surge in inflammatory cytokine activity when cortisol levels are normalised. This phenomenon is implicated in post-stress neuroinflammation. The blood–brain barrier integrity may also be related to that pathway, since pro-inflammatory cytokines can increase endothelial permeability and thereby enable peripheral immune activation affecting central nociceptive circuits (86). 2) GOBP Carbohydrate Metabolic Process: This pathway contains numerous biological processes, including glucose homeostasis, glycolysis, and glycogen metabolism, which are part of carbohydrate metabolic processes essential for the energy supply of neurons. In the general interictal state, negative enrichment of this pathway could possibly give a good foundation for hypometabolism or decreased metabolic flexibility. This also supports a more general theory that migraine is caused by mitochondrial dysfunction, which impairs energy production even between attacks. This pathway was upregulated after a neuroendocrine challenge, which suggests a robust glycolytic response to stress. This sudden increase could be the result of a metabolic overload in a weakened system, which could cause lactate accumulation, oxidative stress, and depletion of ATP. Trigeminal neurons are known to become sensitised by all of these, which could serve as a catalyst for migraine attacks (11). In conclusion, chronic energy deficiencies and stress-induced energy mismanagement are both reflected in the dysregulation of carbohydrate metabolism, potentially increasing migraine susceptibility.

Taken together, no gene could be identified at transcriptome-wide significance after correction, however, consistent negative enrichment of pathways supports a systems-level dysregulation in MO, involving immune, metabolic, cardiovascular, and

hormonal functions. LEGs *CYP26B1* and *CORIN* potentially reflected altered retinoid metabolism and natriuretic peptide signaling. No SNP-level associations could be shown for *CYP26B1*, however its downregulation aligns with known vitamin A-related disturbances and migraine-associated *LRP1* polymorphisms. Other LEGs showed nominally significant SNP associations, possibly influencing their gene expression.

5.2. Identification of Potential Drug Candidates for Migraine Therapy

Another important aspect is the pharmacological relevance of these transcriptomic changes (46,52). Using molecular docking simulations, it was shown that current migraine medications do not significantly bind to the proteins encoded by LEGs. A limited binding of existing migraine treatments to corresponding proteins may reflect their constrained effectiveness observed in clinical practice (77). In contrast, we could demonstrate through machine learning that several novel compounds such as probucol, fasoracetam, ginsenosides, and rovafovir had a high binding affinity to *CORIN*. Compounds like probucol (an antioxidant lipid-lowering drug) and ginsenosides were tested for migraine or related symptoms (Figure 4) and can possibly serve as scaffolds for the development of future migraine treatments. In addition to gene-level binding, pathway-level analyses revealed several drugs showing a significant weighed binding to positive regulation of cell death, response to lipid and transmembrane receptor protein tyrosine kinase signalling pathways (Figure 5). Some of the compounds like probucol and ginsenosides interacted with the identified LEGs using computational predictions and dysregulated pathways based on molecular docking and database mining (46,48). Gene and pathway-level findings underscore the gap between current therapies and the underlying molecular pathology of MO and highlight potential new avenues for mechanism-based, targeted treatment approaches.

5.3. Transcriptomic and Neuroendocrine Response to Acute Neuroendocrine Stress Challenge

In the neuroendocrine challenge we could demonstrate a difference between MO and controls in their cortisol response induced by citalopram infusion. Observed trend-level cortisol elevation is consistent with literature describing altered HPA axis reactivity in migraine (88,89). The transcriptomic data which is associated with the elevated stress response pinpoints a reduction in NFκB regulation, initiation of cellular protein synthesis, and an upregulation in carbohydrate metabolism-related pathways. Taken together these findings possibly point to early molecular mechanisms that lead to a migraine attack after stress-induction in MO and demonstrate an altered stress reaction in patients. The genes *IKBKGP1* and *NKRF* have been associated with NFκB signaling. *IKBKG* (the parent gene of *IKBKGP1*) is considered as regulatory element that is capable to induce canonical NFκB activation, while NKRF is a transcriptional silencer known to repress NFκB target genes (90,91). Observed downregulation *IKBKGP1* and upregulation of NKRF pinpoint a possible diminished NFκB activation and transcriptional repression of NFκB targets in MO. It is in line with prior findings that cortisol can antagonize NFκB signaling, potentially via glucocorticoid receptor-dependent mechanisms (92,93). The pathway-level results, nonetheless, did not reach significance for NFκB-related terms, suggesting rather a gene-level regulation than a pathway-level one. Indications of a starting cellular protein synthesis in MO were supported by upregulation of protein synthesis pathways, cytoplasmic translation and ribonucleoprotein complex biogenesis and elevated ENOX2 expression. The changes may reflect the initiation of transcriptional/translational processes responding from increased cortisol levels (90,92,94). When including the patient experiencing an attack following the neuroendocrine challenge, pathway-analyses showed significant positive enrichment of glycosylation and glycoprotein biosynthesis related pathways. These findings could come from glucocorticoid-mediated preservation of the endothelial glycocalyx, a structure composed of glycoproteins and proteoglycans modulating vascular permeability and protecting against inflammation (95,97). An increased production of components of glycoproteins and glycocalyx may enhance nitric oxide (NO)-mediated vasodilation, a known process involved in migraine

pathophysiology (98-100). Such connection between stress-induced cortisol responses and NO signalling may explain at least in part how stress contributes to migraine attack generation.

5.4. Integrated Interpretation of General Interictal and Stress Response Transcriptomics

The convergence of findings from these two studies allows us to construct a mechanistic model: the general interictal molecular landscape in migraine “primes” the patient for an abnormal response to stress, which in turn can trigger an attack. Several interconnected angles emerge.

5.4.1. Immune/NF-κB Pathway Suppression

In the general interictal experiment, MO showed downregulated immune and inflammatory pathways, potentially reflecting chronically lower NF-κB activity or cytokine production. Retinoic acid (we observed impaired signaling in MO) normally helps to keep immune responses balanced and can limit NF-κB activation (98) and vitamin A deficiency heightens NF-κB activity in immune cells (102). In MO, this pro-inflammatory potential may not be realised at rest because the immune system as a whole seems to be chronically suppressed, which is probably a result of long-term RA signalling deficiency.-The immune system suppression observed in general interictal state may be further maintained or worsened by repeated or prolonged glucocorticoid exposure which counteract NF-κB activity through glucocorticoid receptor signaling (100). Not all stressors trigger attacks, suggesting e.g. dysregulated glucocorticoid signalling or surpassed thresholds, which might lead to a rebound activation of NF-κB, contributing to neuroinflammation which can potentially trigger migraine. This suggests that cortisol may push an already suppressed system deeper into an anti-inflammatory condition. Elevated cortisol may transiently suppress immune activity, due to acute stress, a subsequent rebound phase may lead to NF-κB hyperactivation, especially if regulatory buffers, like retinoic acid signalling, are impaired. It might trigger a transient inflammatory flare, which contributes to neuroinflammation and sensitization in the trigeminovascular system, and this might be a possible mechanism of migraine attacks

since they have been associated with elevations of inflammatory cytokines (IL-1β, TNF-α) and activation of immune cells (104).

5.4.2. Metabolic Downregulation and Stress-Induced Energy Burst

In the general interictal experiment we could see downregulation of metabolic pathways suggests that migraineurs' cells may be operating below their full metabolic capacity at rest. We assume this could be due to mitochondrial dysfunction, which is consistent with clinical findings of reduced mitochondrial respiration and low energy metabolites in migraine (105), or it can be a protective mechanism to reduce oxidative stress in the brain. When stress hits, catecholamines and cortisol demand a rapid mobilization of energy stores (gluconeogenesis, glycogenolysis) and upregulation of glycolysis and protein synthesis for the fight-or-flight response. In post-challenge migraine patients, we observed a sharper upregulation of carbohydrate metabolism and protein synthesis pathways than in controls. If their general baseline is suppressed, the relative transcriptional surge during stress becomes exaggerated, potentially overwhelming mitochondrial capacity and exacerbating inefficiencies in energy metabolism. This can have several consequences. First, a sudden glycolytic burst in someone with suboptimal mitochondrial function may lead to inefficient pyruvate utilization, lactate accumulation, and oxidative stress (106). In support, oxidative stress markers and lactate have been elevated in migraineurs (105). At the same time, oxidative stress can activate trigeminal pain pathways and cause meningeal inflammation (107). Second, the imbalance between energy demand and supply could lead to a localized ATP depletion in the brain (108), which can potentially trigger spreading depolarization or other migraine-initiating events. In support, metabolic changes (like hypoglycemia or intense exercise) possibly induce migraines, effectively acting as stress tests on energy metabolism (105). Thus, the stress-induced metabolic overdrive in a system which is metabolically fragile might be a key step that predisposes to an attack. There may be therapies that improve baseline metabolic capacity (e.g. coenzyme Q10 or riboflavin supporting mitochondrial enzymes) which raise the threshold for this surge (11,109,110).

5.4.3. Stress-Induced Glycosylation Shifts and Vascular-Immune Vulnerability

An intriguing finding of the neuroendocrine challenge in MO is the upregulation of pathways related to glycosylation. In glycosylation sugar moieties are enzymatically added to proteins, which is essential for appropriate folding and function of many immune and endothelial proteins such as adhesion molecules, cytokine receptors, and immunoglobulins (111). During inflammation cytokines are released which can alter glycosylation patterns, significantly impacting immune cell trafficking and endothelial interactions, by altering N-glycan branching on molecules like ICAM/VCAM or integrins (111). In MO, pre-dysregulations in the general interictal study might lack the flexibility to adapt to sudden glycosylation changes. We speculate that the acute stress-induced glycosylation pattern shifts, possibly in immune cell receptors or endothelial glycocalyx composition, potentially exacerbating endothelial leakiness or triggering misdirected immune activation. Imbalance of the glycosylation state potentially reduces the protective anti-inflammatory glycan properties on IgG or cell surfaces, favouring a more pro-inflammatory state. In essence, glycosylation perturbations that are triggered by acute stress add another level of vulnerability, endothelial cells and leukocytes in MO could respond differently to stress, leading to an intensified sterile inflammation or blood-brain barrier alterations during a migraine attack.

5.4.4. Targeting CORIN and CYP26B1: Emerging Therapeutic Directions

Identification of new molecular targets for migraine intervention were revealed by the synthesis of the general interictal and neuroendocrine challenge data. We found *CYP26B1* and *CORIN* (the atrial natriuretic peptide convertase) to be downregulated in the general interictal dataset. ANP/BNP promote vasodilatation and fluid balance. We speculate *CORIN* downregulation in MO may affect natriuretic peptide activation, possibly reducing cerebrovascular stress-buffering, possible mechanisms that are essential for glycocalyx maintenance under stress, suggesting that restoring CORIN-ANP activity could improve endothelial resilience to stress. Our recent analysis could indeed demonstrate compounds binding or modulating *CORIN*. Probucol (an antioxidant lipid-lowering drug) was highlighted as a good potential candidate. Restoring the CORIN

function through modulation of probucol rescuing endothelial functions and thereby attenuating stress responses could attenuate stress-induced vascular dysfunction.

Taken together, negative enrichment of pathways related to immune system (via NF-κB inhibition) and metabolism chronically in general interictal patients creates a vulnerable baseline, which predisposes them to an abnormal response to acute stress. The impairment in the retinoid signalling at general interictal state may further compromise immune regulation, making it more susceptible to cortisol-induced inflammatory rebound during stress exposure. At the same time, downregulations in general interictal patients set the stage for exaggerated stress-induced shifts in glycolysis, may lead potentially to oxidative stress and mitochondrial dysfunction, conditions known to trigger an attack. Furthermore, we speculate that these acute changes due to stress-induced glycosylation might further destabilize endothelial and immune cell function, possibly increasing inflammation and exacerbating blood-brain barrier disturbances. In addition, the drug-binding simulation provided valuable insights, identifying *CORIN* as potential candidate for pharmacological modulation to stabilize vascular and immune homeostasis in MO. These results collectively provide support to a theory that suggest, that these chronic and acute disruptions lower the threshold for migraine attack initiation in synergy and raise the possibility of a combined approach correcting for both altered retinoid signalling and *CORIN* downregulation (77,78).

5.5. Limitations and Future Directions

This thesis presented a large transcriptomic dataset of MO and stress-related responses, however, limitations must be acknowledged. 1) The whole blood samples have limited insights into brain-specific expression patterns, meaning that they can partially reflect neurological processes. Further studies may identify changes in brain cells. 2) Single-cell RNA-seq would have given us detailed results, however the aim of the studies was not to identify cell-type specific alterations. 3) Our population consisted of a small sample size and protein-level validation was not conducted, which may affect the generalizability of the findings, furthermore due to the small size we might have failed to capture smaller gene expression changes. 4) Any alterations caused by contraceptives could not be excluded due to our inability to correct for the effects due to a broad range of available

active substances. 5) Lifestyle-related factors like nutrition, exercise and sleep may have influenced results, but we had no deep phenotyped data regarding these. 6) Due to missing protein-level validation of *CYP26B1*, the relationship between *LRP1* and *CYP26B1* remains hypothetical. 7) MO and migraine with aura differ from each other, they may represent a different general interictal and stress-induced response. 8) The citalopram-induced reproducible neuroendocrine stress response does not completely replicate the complexity of real-world psychosocial stress. 9) In MO, the role of the glycocalyx remains hypothetical. Further investigations are necessary to determine such a relationship. 9) Direct measurement of NF-κB activity and retinoic acid signaling in MO patients, interictal and post-stress, is lacking, so the immunological rebound is speculative, even though cortisol-induced NF-κB suppression and the ensuing inflammatory rebound are plausible mechanisms.

Despite these limitations, the work opens several avenues for further research. 1) Tissue-specific investigations would overcome peripheral tissue limitations, such as analyzing brain-derived extracellular vesicles or applying single-cell RNA-seq. Investigating brain-derived extracellular vesicles (e.g., neuron- or glia-derived extracellular vesicles) originating from plasma serves as "liquid biopsy" possibly uncovering neural transcriptomic changes that are not detected by whole-blood analysis (112). 2) Functional and proteomic validation of the transcriptomic data might give us more insights. Downregulation of *CORIN* reduces activation of natriuretic peptides, a follow-up in cell or in an animal model could test if its loss increases neuronal excitability via P2X3 sensitization. Similarly, the observed downregulation of *CYP26B1* suggested impaired retinoic acid signaling. We suspect a manipulation of retinoic acid levels in immune or neural cells may clarify its role in MO pathophysiology. 3) The glucocorticoid receptor-NF-κB axis may serve as a therapeutic target. General interictal immune pathway suppression pinpoints a maladaptive stress response, which was amplified by cortisol-induced NF-κB inhibition. This may possibly lead to a rebound effect that contributes to neuroinflammation. Following a citalopram infusion, measurement of NF-κB pathway activity in peripheral blood mononuclear cells utilizing single-cell RNA-seq or chromatin immunoprecipitation might identify NF-κB occupancy and histone changes. Furthermore, modulation of glucocorticoid receptor sensitivity or NF-κB activity (e.g.

through NF-κB decoy oligonucleotides or safer glucocorticoid analogues) could possibly interrupt the stress-induced cascade, thereby lowering the threshold for migraine attacks. In fact, NF-κB inhibition demonstrated a promising effect in models of steroid-withdrawal (113). 4) Measuring NF-κB activity might offer insights, since it could confirm functional engagement of inflammatory pathways observed, that can be assayed by electro mobility shift assay (EMSA) (114). Retinoic acid level measurements allow us to test whether the observed downregulation of CYP26B1 and the retinoid pathways observed translate into altered circulating retinoid concentrations using high-performance liquid chromatography (HPLC) (115). Glycocalyx integrity impairment would demonstrate neurovascular inflammation and endothelial dysfunction, that could be done by Syndecan-1 measurement which is used as a marker for endothelial glycocalyx. Injury (116). Such further studies might be very promising and could give a great impact on the understanding of migraine pathophysiology.

Clinical application of the maladaptive stress responses in MO can be targeted with different approaches by combining experimental, longitudinal, and biomarker-based investigations: 1) Experimental Stress Paradigms: Longitudinal cohorts of MO and healthy controls can be used for further, controlled neuroendocrine stress tests to confirm causality and reproducibility of these responses. An assessment of repeated exposures over time should give more insights into the within-subject variability and trajectory of stress response. Methods like RNA-Seq might further assist to validate pathways and gene-expression changes. 2) Molecular Biomarker Measurement: Since we found valuable possible biomarkers, systematic measurements at multiple time points pre- and post-stress might be helpful, reflecting the transcriptomic state and possibly guiding diagnosis or patient stratification. These stress-responsive and pathway-relevant markers include a) NF-κB pathway activity using RNA-Seq and proteomics to find surrogate readouts in peripheral blood mononuclear cells (PBMCs). b) Oxidative stress markers such as I) F2-isoprostanes, a gold marker of lipid peroxidation which reflects systemic oxidative damage to cell membranes and high levels of these indicate stress-related metabolic surges leading to ROS accumulation (117). II) 8-Oxo-2'-deoxyguanosine (8-OHdG) indicates oxidative damage to DNA, allowing us to assess whether transcriptomic changes extend to nuclear or mitochondrial genome instability (118). III) Glutathione

(GSH) ratios (GSH/GSSG, with GSSG being the oxidized form of glutathione) give a real-time snapshot of the antioxidant system capacity, a diminished ratio suggests compromised redox homeostasis and cellular susceptibility (119). c) Measurement of serum levels of CGRP, inflammatory cytokines (IL-1β, TNF-α), and retinoic acid. d) Circulating endothelial glycocalyx markers, such as syndecan-1 or hyaluronic acid fragments (120). 3) Stratification by retinoid and CORIN-related Markers: It may prompt personalised medicine by matching patients to pathophysiological subtypes. By measuring CORIN levels or ANP/BNP indicating vascular susceptibility, since reduced levels of CORIN possibly explain decreased ANP levels in blood of migraineurs (121) and increased precursors of BNP in migraine patients (76). Furthermore, expression levels of CYP26B1 and levels of retinoids in serum may be utilized to stratify patients based on baseline immune resilience. 4) Pharmacological Probing: The causality and therapeutic potential of identified transcriptomic targets can be assessed. Trials in, e.g. animals could be conducted to detect whether the predicted CORIN or pathway-level binding substances have an impact on migraine, testing causality and their therapeutic relevance.

6. Conclusion

The aim of this thesis was to investigate the transcriptomic mechanisms underlying MO by analysing gene expression profiles in general interictal conditions and in response to a pharmacological neuroendocrine stress challenge. Three core objectives guided the investigation: (1) determining whether MO is associated with consistent and replicable differences in gene and pathway expression; (2) assessing whether migraine-relevant or novel compounds show binding affinity to molecular targets identified through transcriptomic analysis; and (3) evaluating whether a controlled neuroendocrine stress challenge elicits distinct hormonal and transcriptomic responses in patients with MO compared to healthy individuals.

No individual genes reached genome-wide significance after multiple testing correction. However, pathway-level analyses identified 88 biological processes that were significantly downregulated in MO patients across two independent time points. These processes included metabolic, cardiovascular, immune and hormonal signalling. The results were influenced by leading-edge genes such as *CYP26B1* and *CORIN*, both of which showed significant and replicated downregulation. In silico binding analyses revealed limited interaction between existing migraine treatments and dysregulated gene products. By contrast, compounds such as probucol exhibited a high predicted affinity for *CORIN* and other targets were identified for further LEGs.

In comparison to the control group, patients with MO showed a tendency for an increased cortisol response to the acute citalopram challenge. At the transcriptomic level, suggestive downregulation of *IKBKGP1* and *NKRF* was observed, consistent with suppression of NF-κB–mediated immune signalling through the elevated cortisol levels. Furthermore, pathway-level analyses revealed significant upregulation of carbohydrate metabolism, protein synthesis and glycosylation in MO patients, as opposed to downregulation in the control group.

Taken together, when combining general interictal and stress-induced transcriptomic signatures a chronic baseline of suppressed immune and metabolic pathways has been observed, making patients more susceptible to acute stressors. The combined data point

to a model in which altered glycosylation responses, upregulated glycolysis, and glucocorticoid-induced inhibition of NF-κB act synergistically to disrupt homeostasis and trigger migraine attacks. Findings also support novel avenues for mechanism-based interventions. Based on our approach, MO can be seen as a systems-level vulnerability state, in which a chronic dysregulation is priming the system for maladaptive responses to stress.

7. Summary

Migraine without aura (MO) is among the most common and debilitating neurological disorders globally, with stress as a significant trigger for attacks. Molecular mechanisms that underlie MO are still not understood. The role of stress sensitivity and transcriptional dysregulation in migraine pathophysiology has become an area of interest, but transcriptomic studies are still limited and inconsistent. Our work aimed to systematically study changes in gene expression related to MO 1) under general interictal conditions and 2) after acute neuroendocrine stress from a citalopram challenge. Under general interictal conditions, no individual genes reached our genome-wide significance thresholds. Gene set enrichment analysis revealed 88 significantly downregulated, replicated biological processes in patients including pathways related to metabolism, immune function, cardiovascular regulation, response to oxidative stress, and hormone signalling. Leading-edge genes such as *CYP26B1* and *CORIN* exhibited substantial and consistent downregulation across time points. In silico binding simulations and machine learning based drug-target prediction models highlighted minimal binding affinity for existing migraine treatments to dysregulated gene products. In contrast, compounds such as probucol and ginsenosides exhibited strong predicted binding to *CORIN* and additional candidate drugs with potential to modulate key molecular pathways. A pharmacological stress model involving an intravenous administration of citalopram revealed altered neuroendocrine and transcriptional responses in MO patients in parallel to an increase in HPA axis activity at 70 minutes post-infusion. Transcriptomic analysis showed accompanying downregulation of the immune-related genes *IKBKGP1* and *NKRF* and upregulation of carbohydrate metabolism, protein biosynthesis and glycosylation. All in all, molecular patterns support the existence of a maladaptive molecular stress response in migraine and provide a molecular framework for understanding MO as an impaired allostatic regulation disorder. Further investigation based on our findings is warranted to guide future research into mechanism-based therapeutic strategies in the disorder.

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9. Bibliography of the candidate's publications

9.1. Publications related to the PhD thesis

- Petschner P, **Kumar S**, Nguyen DA, Torok D, Gal Z, Baksa D, et al. The interictal transcriptomic map of migraine without aura. The Journal of Headache and Pain. 2025 May 12;26(1). IF: 7.9
- **Kumar S**, Petschner P, Kinga Gecse, Torok D, Juhasz G. Acute neuroendocrine challenge elicits enhanced cortisol response and parallel transcriptomic changes in patients with migraine. PAIN Reports. 2025 10(3):e1254–4. IF: 4.8
- **Kumar S**, Gal Z, Gonda X, Huse RJ, Juhasz G, Bagdy G, et al. Transcriptomic changes following chronic administration of selective serotonin reuptake inhibitors: a review of animal studies. Neuropsychopharmacologia Hungarica: A Magyar Pszichofarmakologiai Egyesulet Lapja = Official Journal of the Hungarian Association of Psychopharmacology. 2019 Mar 1;21(1):26–35.

9.2. Publications not related to the PhD thesis

- Petschner P, Balogh N, Csaba Adori, Tamasi V, **Kumar S**, Juhasz G, et al. Downregulation of the Vitamin D Receptor Regulated Gene Set in the Hippocampus After MDMA Treatment. Frontiers in Pharmacology. 2018 Dec 3;9.2. IF: 3.845
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- Baksa D, Gecse K, **Kumar S**, Toth Z, Gal Z, Gonda X, et al. Circadian Variation of Migraine Attack Onset: A Review of Clinical Studies. BioMed Research International. 2019 Aug 25;2019:1–9. IF: 2.276

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