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Original paper

Arylacetamide deacetylase (AADAC) enzyme: presence and inter-individual variability in human lungs

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Presence of arylacetamide deacetylase (AADAC) in human lungs

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Figures: 4

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Abstract: 222 words

Introduction: 456 words

Discussion: 638 words

Abbreviations: AADAC, arylacetamide deacetylase; CES, carboxylesterase; HLM, human liver microsomes; HPLC, high-performance liquid chromatography; Phe, phenacetin.

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ABSTRACT

Human arylacetamide deacetylase (AADAC) is a single microsomal serine esterase involved in the hydrolysis of many acetyl-containing drugs. To date, the presence and activity of the AADAC enzyme in human lungs has been scarcely examined. This study aimed to investigate its gene and protein expression, as well as inter-individual variations in AADAC activities in a large number of human lungs (n=25) using phenacetin as a selective substrate. The kinetic parameters K_m and V_{max} were determined. Our findings highlighted a high inter-individual variability both in AADAC mRNA levels and hydrolysis activities. Furthermore, for the first time, we demonstrated the presence of the AADAC protein in various lung samples by means of immunoblotting analysis. As a comparison, phenacetin hydrolysis was detected in pooled human liver microsomes (HLM). Lung activities were much lower than those found in the liver. However, similar K_m values were found, which suggests that this hydrolysis could be due to the same enzyme. Pulmonary phenacetin hydrolysis proved to be positively correlated with AADAC mRNA (*$p<0.05$) and protein (*$p<0.05$) levels. Moreover, the average values of AADAC activity in smokers was significantly higher than in non-smoker subjects (*$p<0.05$) and this might have an important role in some drug administration. These findings add information to our knowledge of pulmonary enzymes and could be particularly useful in the design and preclinical development of inhaled drugs.

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Significance Statement

This study was aimed at investigating the presence and activity of the AADAC enzyme in several human lungs. Our results highlighted a high inter-individual variability both in AADAC gene and protein expression, as well as in phenacetin hydrolysis activity. These findings add information to our knowledge of pulmonary enzymes and could be particularly useful in the design and preclinical development of inhaled drugs.

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1. Introduction

The human lung is a target organ of numerous inhaled exogenous substances. The lung is frequently exposed to high concentrations of molecules that exert their acute or chronic toxicity within the respiratory tract, where they can be accumulated and/or activated to highly reactive metabolites (Castell et al., 2005). Characterization of drug-metabolizing enzymes, involved in both drug detoxification and pro-drug activation, is required to carry out optimal drug therapy (Shimizu et al., 2014). Therefore, the design and development of potential pro-drugs administered by inhalation would receive benefit from a complete pulmonary enzyme characterization.

Human esterases are important enzymes involved in the catalytic hydrolysis of various therapeutic drugs containing esters, amides, or thioesters moieties (Shimizu et al., 2014; Yoshida et al., 2018). Among the esterases, carboxylesterases (CES) and arylacetamide deacetylase (AADAC) are the key liver enzymes catalyzing the hydrolysis of many clinical drugs (Shimizu et al., 2014).

In a previous study, we demonstrated the presence and inter-individual variation of the major human carboxylesterases (CES1 e CES2) in a large number of human lungs (Gabriele et al., 2018). To date, the presence and contribution of arylacetamide deacetylase to pulmonary drug hydrolysis has been scarcely investigated (Watanabe et al. 2009; Kobayashi et al 2012).

Recent studies have demonstrated an overlapping tissue distribution of AADAC and CES enzymes, as well as a quite similar substrate preference (Yoshida et al., 2018). For example, AADAC favors substrates with smaller acyl moieties than CES2 (Fukami et al., 2015).

AADAC is a single serine esterase expressed in the human liver and intestinal tract and to a less extent in the pancreas and adrenal gland (Fukami and Yokoy, 2012; Yoshida et al., 2018; Quiroga and Lehner, 2018). This enzyme is principally involved in the hydrolysis of several drugs containing the acetyl group, such as phenacetin, flutamide, rifamycins, prasugrel, ketoconazole, and indiplon (Watanabe et al., 2009; Watanabe et al., 2010; Nakajima et al., 2011; Kurokawa et al., 2016; Fukami et al., 2016; Shimizu et al., 2014). In a previous study, Watanabe et al. (2010)

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demonstrated that AADAC is the principal enzyme involved in phenacetin hydrolysis with respect to CES enzyme contribution. These results were also strengthened by the inhibitory effect observed in the presence of eserine, a strong inhibitor of the AADAC enzyme (Watanabe et al., 2010). Furthermore, AADAC may hydrolyze N-acetylarylamine drugs producing arylamine, which could cause toxic effects (Fukami and Yokoy, 2012). Similarly to the CES enzymes, also AADAC may be relevant in the preclinical development of inhaled drugs.

Therefore, this study was aimed at investigating the presence and inter-individual variation of AADAC in several human lungs by measuring the hydrolase activity of phenacetin, used as a selective substrate of the AADAC enzyme, as well as the expression level of the AADAC transcript and protein.

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2. Materials and Methods

2.1 Chemicals and reagents

Phenacetin and p-phenetidine were purchased from Sigma-Aldrich (St. Louis, MO). Human liver microsomes (HLM), male pool of 10 (H1000/Lot No. 0710494), were obtained from XenoTech (Kansas City, USA). LiverPool™ Human Cryopreserved Hepatocytes (10-donor, mixed gender, No X008801) was obtained from BioreclamationIVT (Brussels, Belgium). All chemicals and reagents were of analytical grade and were obtained from common commercial sources.

2.2 Microsomes and cytosol preparation

Human lung specimens (10 females and 15 males) were obtained following lung cancer reduction surgery and stored at -80°C until use. The selected healthy sample was at least 6 cm away from the neoplastic tissue, a distance sufficient to not affect the workout analysis for the patient's follow-up and future treatment. Patients gave informed consent for using lung specimens that are otherwise discarded. Each lung specimen was weighed, washed in ice-cold KCl 1.15% to remove the blood, finely minced, and homogenized with 4 volumes of homogenization buffer (100 mM K_2HPO_4, 1.15% KCl, 1 mM EDTA, pH 7.4) with a LabX Kinematica Polytron PT MR 2100 homogenizer (Midland, ON, Canada) under ice-cold conditions. The homogenates were centrifuged 10000 x*g* for 25 min at 4°C to give the supernatant fraction, which was further centrifuged at 105000 x*g* for 70 min at 4°C to separate the microsomes from the cytosol. The cytosol was immediately aliquoted, while the microsomal pellet was resuspended with 1 volume of resuspension buffer (100 mM K_2HPO_4, 1 mM EDTA, pH 7.4). The cytosolic and microsomal fractions were stored at -80°C until use. Protein content was determined by the method of Lowry et al. (1951), using bovine serum albumin (100 μg/ml) as the standard.

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2.3 Phenacetin hydrolase activity

The AADAC activity was measured as hydrolysis of phenacetin using the HPLC method described by Watanabe et al. (2010) with some modifications. The reaction, containing 1 mg/ml of lung microsomal or cytosolic proteins in 100 mM potassium phosphate buffer, pH 7.4, in a final volume of 500 µl, was started by addition of 6 mM phenacetin following 10 min at 37°C. The reaction was terminated by the addition of 25 µl of ice-cold 60% perchloric acid after 90 min of incubation at 37°C. A 30 µl portion of the filtered supernatant (Thermo Scientific™ Nalgene™ 4mm Syringe Filters, 0.2 µm pore size) was subjected to HPLC after removal of proteins by centrifugation at 9.500 x*g* for 5 min. The HPLC analysis was performed using the Waters 2487 HPLC Absorbance UV-Vis Detector (Marshall Scientific, Hampton, NH) equipped with a reverse-phase Luna® column (5 µm C18(2) 100 Å, LC Column 150 x 4.6 mm) in isocratic conditions and a water solution containing 25 mM KH_2PO_4 and 10% acetonitrile, as the mobile phase. The eluent was monitored at 232 nm and the flow rate was set at 1.5 ml/min. Spontaneous hydrolysis of phenacetin was measured under the above-mentioned conditions in the presence of denaturated proteins. A standard curve of p-phenetidine (1-80 µM) in the presence of denaturated proteins was used for the quantification of the product by comparing the HPLC peak height of the standard with that of analyzed samples. The rate of each hydrolysis was linear with time and protein concentration.

2.4 Gene expression of human AADAC

Total RNA was isolated from the human lung specimens (N=20) and from LiverPool™ Human Cryopreserved Hepatocytes using the E.Z.N.A.® Total RNA Kit I (OMEGA bio-tek, Norcross, GA, USA) and reverse-transcribed using the iScriptTM cDNA Synthesis Kit (Bio-Rad, CA). Housekeeping gene primers were designed using Beacon Designer Software (Premier Biosoft International, USA), synthesized by Sigma (St. Louis, MO, USA), and shown in Table 1.

Pre-developed PrimePCR™ SYBR® Green Assay (Bio-Rad, CA, USA) was used for gene expression analysis of the human AADAC (qHsaCID0015655). Quantitative Real-Time PCR was

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performed using the SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad, CA) in a CFX Connect Real-Time PCR Detection System (Bio-Rad, CA, USA). The AADAC gene was assayed in triplicate and its expression was calculated using a comparative critical threshold (Ct) method.

2.5 Immunoblot analysis

Protein samples were denatured for 10 min at 95°C prior to SDS-polyacrylamide gel electrophoresis and immunoblot analysis performed according to Laemmli (1970). Enzyme sources (45 μg) were separated on 10% Mini-Protean® TGX Stain-Free TM Gels (Biorad, CA, USA) and electrophoretically transferred onto nitrocellulose membranes (Amersham™-Hybond™-ECL membranes, GE Healthcare, Chicago, USA). The membranes were incubated with rabbit polyclonal anti-human AADAC (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and anti-GAPDH (Abcam, Cambridge, UK) primary antibodies at 4°C overnight followed by 3 washes with 1X PBST (5 min/each wash). Subsequently, the membranes were incubated with a goat anti-rabbit IgG peroxidase-conjugated secondary antibody (Sigma, St. Louis, MO) at room temperature for 1 hour. After washings, immunoreactive proteins were visualized with a chemiluminescence reaction kit (Clarity™ Western ECL substrates, Biorad, CA, USA) and protein band intensities were analyzed using the ImageJ software.

2.6 Data analysis

Statistical analysis was performed using GraphPad Prism, version 5.00 for Windows (GraphPad software, San Diego, CA). Assays were carried out in triplicate and results were expressed as mean values ± standard error (SE). Data were analyzed by Pearson-correlation and the Student's *t*-test. *P* values <0.05 were considered as statistically significant.

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3. Results

3.1 Phenacetin hydrolase activity in human lungs

To examine the pulmonary catalytic hydrolysis of phenacetin, using HPLC we determined the kinetic parameters (K_m and V_{max}) in microsomes and cytosol of 5-6 human lung samples. Preliminary experiments (data not shown) enabled us to establish optimal hydrolysis conditions in which there was linear product formation with respect to protein concentration and time incubation, displaying a typical Michaelis-Menten saturation curve.

The Michaelis-Menten and Lineweaver-Burk plots relative to phenacetin hydrolysis in individual lung microsomal fractions are shown in Figure 1 and the K_m and V_{max} values were 3.49 ± 4.06 mM and 0.16 ± 0.07 nmol/min/mg protein, respectively. Unfortunately, due to the high spontaneous hydrolysis of the substrate, lung activities were not well detectable below 2 mM phenacetin, making uncertainty around the accuracy of the K_m value. By using cytosol samples no product formation was observed.

As a comparison, the kinetic of phenacetin hydrolase was determined in pooled HLM (1 mg/ml), under the same conditions, showing a K_m of 5.43 ± 1.1 mM and a V_{max} of 6.10 ± 0.64 nmol/min/mg protein. These data are comparable to the K_m of 3.30 ± 0.16 mM and the V_{max} of 3.58 ± 0.20 nmol/min/mg protein reported by Watanabe et al. (2010). As expected, the liver activity was found to be much higher than that measured in the lung, whereas the K_m values were similar to each other. This suggests that the hydrolysis in both liver and lung could be due to the same enzyme.

Furthermore, the phenacetin hydrolase activity was extended to a larger number of human lung samples (n=25), using 1 mg/ml of microsomal proteins for each tube and a phenacetin concentration of 6 mM, corresponding to the maximum enzymatic rate (Fig. 2). The average activities in all lung microsomes were 0.047 ± 0.035 nmol/min/mg protein, which proved to be about 70-fold lower than that found in the liver.

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3.2 Gene and protein expression of AADAC in human lungs

In this study, we analyzed the expression level of AADAC mRNA in 20 different human lung specimens. The expression level of AADAC mRNA, normalized by using three different housekeeping genes (β-actin, TBP and HPRT-1, see Table 1), was analyzed by quantitative Real-Time PCR. The presence of AADAC mRNA was detected at different levels in all tested human lung samples (Fig. 3). In agreement with AADAC activities, we found a high inter-individual variability in AADAC mRNA levels (from 1.25 to 51.7 fold) in the examined lungs.

In addition, we analyzed the AADAC protein level in the liver and lung microsomes by means of Western Blot, using a rabbit polyclonal anti-human AADAC antibody. The protein expression levels of AADAC were determined in six representative human lung microsomes showing the highest phenacetin hydrolase activities and in a pooled HLM. Figure 4 shows a representative photograph of immunoblot analysis of AADAC (panel a) and GAPDH (panel b) in HLM (lane 1-3) and lung (lane 4-9) samples. The liver microsomes were loaded at three different protein concentrations (15, 30 and 45 μg) and the relative band intensities at 45 kDa, the molecular mass of the AADAC protein, reflect the loaded protein concentration. Similarly, AADAC bands were detected at lower intensities than liver microsomes in the six lung samples loaded at 45 μg of proteins/lane. Among AADAC pulmonary bands, a higher intensity was observed in correspondence with microsomes loaded in lanes 6-9, which showed the highest phenacetin hydrolase activity (samples number 19, 23, 24, and 12 see Fig. 2).

3.3 Statistical analyses

A Pearson-correlation analysis between microsomal AADAC activities, AADAC gene and protein expression, and anthropometric measurements (ages, weight, and body mass index-BMI) was performed. As shown in Table 2, the phenacetin hydrolase activities of the human lung microsomes appeared to be positively correlated with the expression level of AADAC mRNA (p=0.0439, r:

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0.2302) and protein (p=0.0187, r: 0.7850). Moreover, as expected, a positive correlation was observed between weight and BMI (p=0.0023, r: 0.3950).

An unpaired Student's *t*-test was used to compare the mean values of hydrolysis activities and AADAC gene expression between smokers and non-smokers, as well as between male and females. This comparison highlighted a significant difference in hydrolysis activities detected in smokers and non-smoker subjects (*p<0.05), suggesting smoking as a positive modulator of AADAC activity. Conversely, no difference was found in the AADAC mRNA level between smokers and non-smokers and there was no difference relative to gender in hydrolysis activity or in AADAC gene expression.

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4. Discussion

Human arylacetamide deacetylase (AADAC) is a microsomal serine esterase of about 45kDa that is involved in the hydrolysis of many clinical drugs containing the acetyl group such as phenacetin. To date, AADAC pulmonary expression has been seldom investigated. Our study was aimed at investigating the presence and activity of the AADAC enzyme in several human lungs.

The activity of AADAC enzyme was determined as phenacetin hydrolase by HPLC analysis in both microsomal and cytosol lung fractions.

First, to characterize the AADAC enzyme, a kinetic analysis was performed using microsomes of 5-6 human lung samples and, for the sake of comparison, a pool of human liver microsomes. The hydrolysis of phenacetin with pulmonary and liver microsomes showed a simple Michaelis-Menten curve with similar K_m values, suggesting that the same enzyme could be involved in catalyzing this reaction. In the lung cytosol, no activity was found. This result is not surprising since AADAC is a type II membrane protein anchored to the endoplasmic reticulum lumen through an N-terminal signal anchor sequence (Fukami and Yokoi, 2012).

Furthermore, our results demonstrated in a large number of human lung microsomal specimens (n=25) the presence of AADAC activity, as well as a high inter-individual variability of phenacetin hydrolysis, with values ranging from a minimum of 0.008 to a maximum of 0.153 nmol/min/mg protein. These lung activities were lower than that detected in human pulmonary microsomes (single donor) by Kobayashi et al. (2012), showing a V_{max} value of 0.8 nmol/min/mg protein.

The wide variability of AADAC activity observed in human lungs was similar to that found in the human liver by Fukami and Yokoi (2012), and it appears to be linked to genetic polymorphisms, diet, drug administration and environmental factors.

When the gene expression of the AADAC enzyme was analyzed in many lung samples a general presence of AADAC transcript was demonstrated with a high inter-individual variability, in agreement with the activity data. Previously, Watanabe et al. (2009) reported that AADAC mRNA was detected at a very low level in the human lung (single donor) and kidney, whereas it was highly

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expressed in the liver, colon, small intestine and to a less extent in the stomach, adrenal gland, and bladder. Conversely, the AADAC transcript was not detected by Kobayashi et al. (2012) in a single human lung sample.

For the first time, we also demonstrated using immunoblotting analysis that there is a variable presence of AADAC protein in various human lung samples. On the contrary, no band was detected by Watanabe et al. (2009) and Kobayashi et al. (2012) in human pulmonary microsomes from a single donor. The lack of immune detection of the AADAC protein by these authors may be due to the single lung analyzed and/or to the low specificity, sensitivity, or affinity of the employed primary antibody.

Taken together, AADAC gene and protein expression, as well as the phenacetin hydrolase activity measured in human lungs, were much lower than those found in the liver.

The analyzed hydrolysis activities toward phenacetin proved to be significantly correlated with AADAC mRNA and protein levels, as well as significantly different between smoker and non-smoker subjects. Indeed, the average values of AADAC activity in smokers were significantly higher than in non-smoker subjects. These results suggest smoking as a positive modulator of AADAC activity. Since AADAC enzyme may contribute to the formation of toxic substances, i.e. arylamine from N-acetylarylamine drugs hydrolysis, the smoking habit associated with the use of these drugs might exacerbate their toxic effects. The positive association between pulmonary AADAC activities and smoking, although resulting from a small number of human lungs, might be relevant in the administration of some drugs. These results are preliminary and warrant further and more in-depth investigation.

These findings add information to our knowledge of pulmonary enzymes and could be particularly useful in the design and preclinical development of inhaled drugs.

Author contributions

Participated in research design: Gabriele, Gervasi, and Longo.

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Conducted experiments and performed data analysis: Gabriele

Provided human specimens: Lucchi and Aprile

Wrote or contributed to the writing of the manuscript: Gabriele, Puccini, Gervasi, and Longo.

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Footnote

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Figure legends

Figure 1. Michaelis-Menten and Lineweaver-Burk plots relative to the human lung microsomal hydrolysis of phenacetin (Phe). Data are shown as mean ± SD of 5-6 individual samples.

Figure 2. Hydrolysis activity of 6 mM phenacetin (Phe) by microsomal fractions from human lungs (N = 25). Data are expressed as pmol/min/mg protein.

Figure 3. AADAC gene expression in human lung samples (n=20). Data are expressed as mean value of relative normalized expression.

Figure 4. SDS-PAGE and Western Blot of AADAC (panel a) and GAPDH (panel b) in a pooled HLM (lane 1-3) and in six human lung microsomes (lane 4-9). Semi-quantitative measurement of AADAC band intensity in the lungs relative to the liver (panel c). Lung microsomes were loaded at 45 μg proteins/lane whereas liver microsomes were loaded at three different concentrations corresponding to 15, 30 and 45 μg proteins/lane.

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Table 1. Housekeeping gene primers: β-actin; TBP= TATA-box binding protein; HPRT-1= Hypoxanthine Phosphoribosyltransferase 1.

Gene	Forward 5'-3'	Reverse 5'-3'
β-actin	GAGATGCGTTGTTACAGGAAG	TGGACTTGGGAGAGGACT
TBP	GTGGTGTTGTGAGAAGATGGATGT	GCGGTGGGCACTTACAGAA
HPRT-1	GCCTAAGATGAGAGTTCAAGTT	GCTCTACTAAGCAGATGGC

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Table 2. Linear correlation coefficients (r) between AADAC hydrolase activities in microsomes of human lungs, AADAC gene and protein expression, and anthropometric data. All correlation results, with the exclusion of AADAC protein levels (N=6), were derived from 18 lung samples.

	Correlations					
	1.	2.	3.	4.	5.	6.
1. AADAC activity	1					
2. AADAC gene expression	0.2302*	1				
3. AADAC protein level	0.7850*	0.0001	1			
4. Ages	0.0583	0.0341	0.0479	1		
5. Weight	0.0012	0.0046	0.3461	0.0309	1	
6. BMI	0.0033	0.0182	0.0887	0.0000	0.3950**	1

*Pearson correlation, two-tailed: *p<0.05; **p<0.01.*

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Figure 1

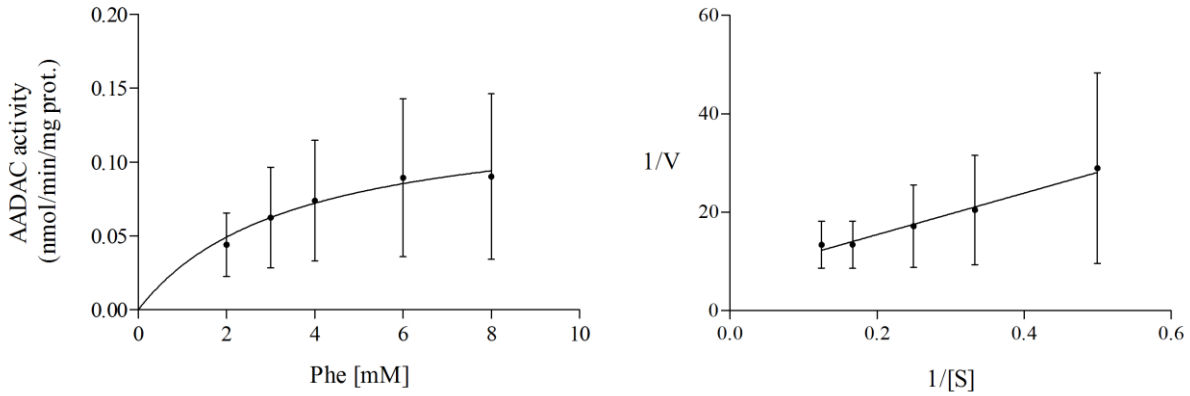

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Figure 2

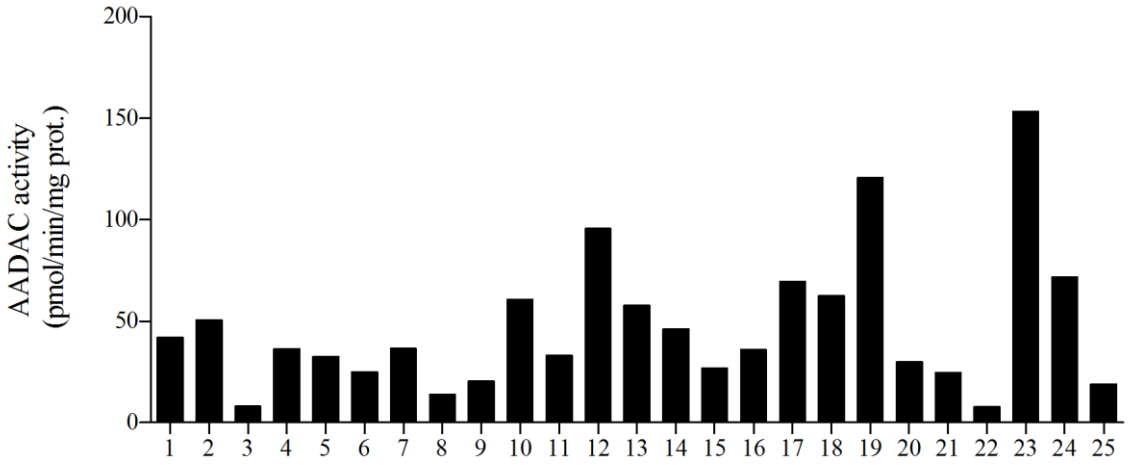

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Figure 3

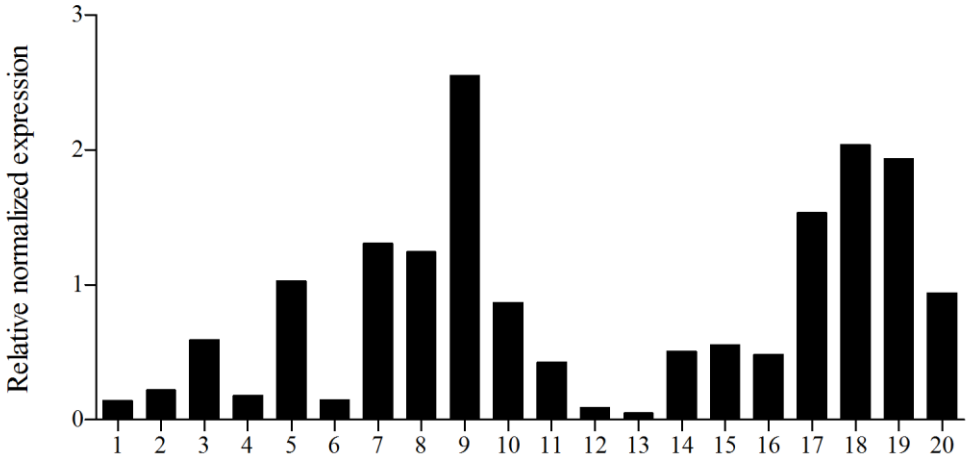

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Figure 4