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PROF. DILDAR KONUKOGLU
Address: Maslak Mah. AOS 55. Sok. No: 2, 42 Maslak A Blok Daire: 231 Sarıyer, İstanbul-Türkiye
Phone: +90 212 241 26 53
Fax: +90 212 241 26 54
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- Epub ahead of print Milbury CA, Li J, Makrigiorgos GM. PCR-based methods for the enrichment of minority alleles and mutations. [Epub ahead of print] Clin Chem February 6, 2009 as doi:10.1373/clinchem.2008.113035.
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Research Article

25-hydroxyvitamin-D3 analysis with high-performance liquid chromatography in terms of total analytical error and measurement uncertainty

Muhammed Seyithanoglu

Department of Biochemistry, Kahramanmaras Sutcu İmam University Faculty of Medicine, Kahramanmaras, Türkiye

Abstract

Objectives: Ensuring the accuracy and consistency of data obtained in the biochemistry laboratory is essential for obtaining reliable and comparable results. This study aims to calculate total analytical error (TAE) and measurement uncertainty (MU) values to assess the analytical performance of the 25-hydroxyvitamin D3 (25-OH vitD3) analyte measured using high-performance liquid chromatography (HPLC) in our laboratory.

Methods: In our study, the internal quality control (IQC) results, which were analyzed at two levels daily between 01–01.2022–31.12.2022, and the data of the external quality control (EQC) program, which was performed at two levels per period for four periods per year, were retrospectively examined for the MU and TAE calculations of the 25-OH vitD3 analyte. TAE was calculated by the formula TAE% = Bias% + (1.65×CV%). MU has been calculated adhering to the Nordtest guideline.

Results: In our study, while TAE values and U value calculated using EQC data for bias and u(bias) calculation were found to be higher than the analytical performance goals we used in our study, TAE values and U value calculated using IQC data for bias and RMSbias calculation were found to be lower than the analytical performance goals we used in our study.

Conclusion: Clinical laboratories should evaluate analytical performance at regular intervals using appropriate methods. In cases where the number of participants using the same method and device in the EQC program is low, we recommend that EQC data not be used in the calculation of the bias component when evaluating analytical performance with TAE or MU.

Keywords: Allowable total error, analytical quality management, clinical chemistry, measurement uncertainty, total analytical error

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Clinical laboratories play a crucial role in diagnosing, treating, and monitoring diseases. Ensuring the accuracy and consistency of data obtained in the biochemistry laboratory is essential for obtaining reliable and comparable results.

Scientifically, all measurement results, including those from clinical laboratories, inherently contain some degree of error. To evaluate analytical performance characteristics, clinical laboratories utilize parameters such as total analytical error (TAE), bias, coefficient of variation (CV), and uncertainty of measurement (MU) [1].

The concept of total analytical error was introduced by Westgard et al. [2] in 1974, defining error in clinical laboratory results. TAE combines imprecision and bias in a test result, encompassing both random and systematic errors [3]. Tradi-

Address for correspondence: Muhammed Seyithanoglu, MD. Department of Biochemistry, Kahramanmaraş Sütçü İmam University Faculty of Medicine, Kahramanmaraş, Türkiye
Phone: +90 553 388 88 56 **E-mail:** dr.muh.seyit@gmail.com **ORCID:** 0000-0002-8027-7549
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tionally, bias and imprecision (CV) are linearly added in TAE detection, represented as TAE=bias+z.CV, where the value establishes the range around the "true" value of the measured analytical results with a specified probability, often 95% [4].

The Guide to the Expression of Uncertainty in Measurement (GUM), published in 1995, introduced the measurement uncertainty concept [5]. ISO standards, such as ISO 15189, mandate laboratories to determine uncertainty for each test [6]. Measurement uncertainty can be assessed through bottom-up or top-down approaches. The bottom-up approach involves identifying all uncertainty components for subsequent calculation, while the top-down approach utilizes internal and external quality control data for practical uncertainty estimation [7]. The Nordtest approach provides a practical and understandable method for uncertainty estimation [8].

Quality, defined as conformity to requirements, is evaluated by comparing measured performance with intended use requirements [9]. Models like the Clinical Laboratories Improvement Amendments (CLIA) and data from organizations like the College of Pathologists of Australasia (RCPA) and the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) are instrumental in setting quality targets [10, 11].

It is known that vitamin D plays an important role in various physiological processes, especially bone metabolism, and its deficiency is associated with many diseases in humans [12]. Vitamin D is transported in the circulation mainly as vitamin D3 (cholecalciferol) and vitamin D2 (ergocalciferol) by binding to vitamin D binding-protein. 25-hydroxyvitamin-D3 (25-OH-D3), which accounts for more than 95% of measurable vitamin D in serum with its relatively low biological variation and long half-life, is considered the analyte of choice in the assessment of vitamin D status [13]. Nowadays, 25-OH-D3 measurement depends on different measurement techniques such as high-performance liquid chromatography (HPLC), liquid chromatography-tandem mass spectrometry (LC-MS/MS), or immunosorbent (ELISA), chemiluminescence (CLIA), electrochemiluminescence (ECLIA). While chromatographic methods are acknowledged as the gold standard for measuring low concentrations of analytes in serum, the time-consuming sample pretreatment procedures associated with chromatography to measure 25(OH)D metabolites have led to the widespread use of electrochemiluminescence methods. Despite drawbacks like cross-reactivity between the D2 and D3 forms of antibodies, these electrochemiluminescence methods are favored due to their efficient and rapid workflow [14].

This study aims to calculate TAE and MU values to assess the analytical performance of the 25-hydroxyvitamin D3 (25-OH vitD3) analyte measured using high-performance liquid chromatography (HPLC) in our laboratory. The results will be compared to the desirable biological variation database specification for total allowable error (TEa) from EFLM [15] and the desirable analytical performance specification for standard measurement uncertainty according to Braga et al.'s [16] article.

Materials and Methods

25-OH vitD3 analyses were carried out on an HPLC device (Thermo Ultimate 3000, ABD) using the Vitamin D ClinRep HPLC kit (Recipe Chemicals & Instruments, Munich, Germany) at the biochemistry laboratory of Training and Research Hospital. In our study, the internal quality control (IQC) results, which were analyzed at two levels daily between 01–01–2022 and 31–12–2022, and the data of the external quality control (EQC) program (Instand, Düsseldorf, Germany), which was performed at two levels per period for four periods per year, were retrospectively examined for the MU and TAE calculations of the 25-OH vitD3 analyte. Instand External Quality Assessment Scheme distributes human serum samples four times per year to between 272–337 participants worldwide for the determination of total serum 25-OH-D3 with 8 participants in peer group. Each level of IQC material had the same lot number throughout the study. EQC results were within acceptable limits in all periods, and no exclusions were made. No human or animal biological material was used in the study.

Analytical performance characteristics

Calculation of total analytical error (TAE): Total analytical error was calculated separately for both levels of IQC material using the following formula:

TAE%=Bias% + (1.65×CV%)

The CV% of IQC samples analyzed at two levels on each analysis day was calculated separately for both levels of IQC material using the following formula (a). In our study, three separate bias calculations were made using IQC and EQC data. In addition to the bias calculation using the peer group EQC data using the formula (b) below, a separate bias calculation was made using the formula (c) below with the EQC data of all participants due to the low number of peer group participants. The absolute values of the calculated biases were summed and divided by the total number of EQC analyses, and the bias value used in the total analytical error formula was obtained. In addition, the bias was calculated separately for both levels of IQC materials using the following formula (d) with the results obtained from the IQC samples. The mean value declared by the manufacturer was used as the target value in the bias calculation from the IQC material.

a. CV% = (Standard Deviation/mean)×100
b. Bias% = [(laboratory result – mean of peer group)/mean of peer group]×100
c. Bias% = [(laboratory result – mean of all participants)/mean of all participants]×100
d. Bias% = [(mean of IQC results – target value)/target value] ×100

Calculation of measurement uncertainty: The Nordtest guide, which is a more useful and practical method for clinical laboratories in calculating uncertainty, recommends using both internal and external quality control results. In our study, the calculation model defined in the Nordtest guide was used to calculate measurement uncertainty[8]. Calculations were made step by step.

Step 1: Calculation of the intra-laboratory repeatability (Rw) component of uncertainty: To express the uncertainty coming from the analytical process, arithmetic mean, SD, and CV% values were calculated separately for each level (L1, L2) using daily internal quality control data. The obtained CV% values were substituted into the formula below, and the repeatability uncertainty component (Rw) was calculated. The standard uncertainty u(Rw) value was obtained by dividing the Rw value by two.

$Rw = \sqrt{[(CVL1)^2 + (CVL2)^2]} / n$

$u(Rw) = Rw/2$

Since each level of IQC materials had the same lot number throughout the study, a total of two CV% values were obtained, one for each level (n=2).

Step 2: Calculation of the bias component of the uncertainty u(bias): It indicates the standard uncertainty of the bias value and is divided into two: laboratory (RMSbias) and material (u(Cref)) bias.

$u(Bias) = \sqrt{(RMSbias)^2 + u(Cref)^2}$

Calculation of RMSbias (Laboratory bias): Three different RMSbias calculations were made with three different bias values calculated using the IQC data and the EQC data of all participants and the peer group. When using IQC data, the average of the absolute values of the biases calculated for both levels of IQC materials was used as the RMSbias value. The following formula was used to calculate RMSbias with the results obtained from EQC samples.

$RMSBias = \sqrt{\Sigma(bias)^2} / n$

The "n" value in the formula refers to the number of biases in the year obtained from the EQC evaluation results.

Calculation of u(Cref) (Material-induced bias): u(Cref) is defined as the uncertainty component obtained from certified reference material or by calculating the actual or expected value from external quality control results. Three different u(Cref) values were obtained using the uncertainty data obtained from the calibration and the EQC data of all participants and the peer group.

The following formula was used to calculate the u(Cref) value from EQC data. When the u(Cref) value obtained with this formula was used, u(Bias) calculations were made with the RMSbias value calculated from EQC data.

$u(Cref) = CV_{mean}/\sqrt{nLab}$

In the EQC report, bias and CV% values are reported as calculated to contribute to measurement uncertainty calculations. CV% values obtained from EQC reports were used. For each period and level, the CV% values of peer or all group in the EQC report were summed and divided by the number of CV% obtained. CV_{mean} was obtained. The number of participants of peer or all group was summed and divided by the number of periods in the EQC program. nLab value was obtained.

In addition to the u(Cref) value calculated from EQC data, the uncertainty data from calibration was also used for the u(Cref) value. In this case, the RMSbias value calculated from IQC data was used in u(Bias) calculations. A single level calibrator is used in the analysis of 25-OH vitD3. For the uncertainty value from the calibration, the information in the calibrator package insert was used: The uncertainty of the mean value is ±2.3% at a 95% confidence level.

Step 3: Calculation of the combined standard uncertainty (uc): Using u(Rw) and u(bias), the combined standard uncertainty (uc) was calculated using the formula below.

$uc = \sqrt{[u(bias)^2 + u(Rw)^2]}$

Step 4: Calculation of the expanded uncertainty value (U): The expanded uncertainty value was calculated by multiplying the combined standard uncertainty value by the k factor. The value 1.96 was taken for k, which represents the 95% confidence interval.

$U = k \times uc$

All calculations were made using Microsoft Office Excel (Microsoft Corporation, Redmond, WA, USA).

Analytical Performance Goals

Total allowable error (TEa): As the TEa value, the desirable biological variation database EFLM specification for TEa was used [15]. Permissible limits of measurement uncertainty

In our article, the desirable analytical performance specifications for standard measurement uncertainty in the article by Braga et al. [16] were used. The expanded uncertainty limit was determined by multiplying this standard uncertainty value with the value of 1.96, which represents the 95% confidence interval.

Results

The determined total analytical error (TAE) values, obtained by utilizing EQC data from a peer group for bias calculation, were identified as 23.89 and 20.36 for L1 and L2, respectively. Similarly, when TAE values were calculated using EQC data from all participants for bias calculation, the results were 27.71 and 24.18 for L1 and L2, respectively. In contrast, TAE values derived from IQC data for bias calculation were 12.30 and 8.73 for L1 and L2, respectively. Our study employed a TAE analytical performance goal of 12.40, as outlined in Table 1-3.

The determined uncertainty (U) value, calculated by using EQC data from a peer group for the calculation of u(bias), was identified as 32.58. Similarly, when the U value was calculated using EQC data from all participants for the computation of u(bias), the result was 35.77. Furthermore, the U value, calculated using IQC data for RMSbias calculation and incorporating the uncertainty value from calibration for u(Cref), was established at 6.31. In our study, the analytical performance goal for U was set at 19.60, as indicated in Table 1-3.

Discussion

The assessment of analytical performance holds significant importance in the realm of quality management for clinical labora-

Table 1. Analytical performance characteristics and analytical performance goals (bias and u(bias) values were calculated using EQC data of peer group)

CV% (IQC L1)	CV% (IQC L2)	Mean of Bias% (EQC)	TAE (IQC L1)	TAE (IQC L2)	U	Desirable specification for TEa*	Permissible limits of U
7.42	5.28	11.65	23.89	20.36	32.58	12.40	19.60

*: Desirable biological variation database specification for TEa. EQC: External quality control; CV(%): Coefficient of variations; IQC: Internal quality control; L1: level 1; L2: level 2; TAE: total analytical error; U: expanded uncertainty; TEa: Allowable total error.

Table 2. Analytical performance characteristics and analytical performance goals (bias and u(bias) values were calculated using EQC data of all participitants)

CV% (IQC L1)	CV% (IQC L2)	Mean of Bias% (EQC)	TAE (IQC L1)	TAE (IQC L2)	U	Desirable specification for TEa*	Permissible limits of U
7.42	5.28	15.47	27.71	24.18	35.77	12.40	19.60

*: Desirable biological variation database specification for TEa.

Table 3. Analytical performance characteristics and analytical performance goals (Bias and RMSbias values were calculated using IQC data and u(Cref) value was calculated using the uncertainty value from calibration)

CV% (IQC L1)	CV% (IQC L2)	Bias% (IQC L1)	Bias% (IQC L2)	TAE (IQC L1)	TAE (IQC L2)	U	Desirable specification for TEa*	Permissible limits of U
7.42	5.28	0.06	0.02	12.30	8.73	6.31	12.40	19.6

*: Desirable biological variation database specification for TEa.

tories. Total Analytical Error (TAE) has been widely employed in clinical laboratories globally due to its practicality and straightforward mathematical model. Its simplicity and ease of calculation have notably influenced clinical chemistry, particularly in the United States, where the US Food and Drug Administration cites guidance from the Clinical and Laboratory Standards Institute based on the TAE approach for clinical laboratory testing [17]. While total error methods have firmly established themselves in laboratory medicine, other fields of metrology have shifted towards measurement uncertainty methods. It's worth noting that unlike measurement uncertainty, the concept of Total Analytical Error is not part of the International Vocabulary of Metrology (VIM) [18] or the Guide to the Expression of Uncertainty in Measurement (GUM) [5]. ISO 15189 specifically mandates the use of Measurement Uncertainty (MU) in calculations, although the standard doesn't provide explicit guidance on practical determination [6]. In response, ISO/TS 20914 has been published as a practical guide for estimating MU in clinical laboratories [19]. This technical standard takes a more practical "top-down" approach, in contrast to GUM's intricate mathematical models rooted in a "bottom-up" methodology.

There is controversy about the pros and cons of TAE and MU. The similarity between both models is that they both express the reliability of the test result from a different perspective. Regardless of the controversy on the pros and cons of TAE and MU, in our study we evaluated the analytical performance characteristics of the 25-OH vitD3 analyte in terms of TAE and MU. When calculating TAE in our study, we used different data sources for the bias component: IQC and EQC data. TAE results calculated with the bias obtained from EQC data were higher than TAE results calculated with the bias obtained from IQC data. In addition, while the TAE results calculated with the bias obtained from EQC data were higher than our analytical performance targets, the TAE results calculated with the bias obtained from IQC data were lower than our analytical performance targets. While our EQC results were within acceptable limits in all periods, we examined the root reason why the TAE results calculated with the bias obtained from EQC data were higher than the analytical performance targets: It was seen that the number of participants using the same method and device as us in the EQC evaluation program of which we are a member was 8. We think that the small number of participants is not suitable for both external quality assessment and analytical performance evaluation with TAE. Furthermore, given the absence of standardization in vitamin D measurement, we believe it would be inappropriate to compare our vitamin D results with those obtained through different methods in external quality assessment. Because of this, calculating TAE using the bias result derived from EQC data from all participants will not give accurate outcomes. Similar to the TAE calculation, we used different data sources for the RMSbias component in our MU calculation. Findings similar to our TAE results were

also present in the MU results. In the study conducted by Demir et al. [20] using the chemiluminescence immunometry method, they found the expanded measurement uncertainty for 25-OH vitD3 measurement to be 24%. Cavalier et al. [21], in their study using low, medium, and high serum pools on the Roche Elecsys device for measurement uncertainty of 25-OH vitD3, found the relative uncertainty values to be 22.4%, 20.9%, 14.8%, respectively. Basat et al. [22], in their study where they evaluated the measurement uncertainty of 25-OH vitD3 analyte with Liquid Chromatography-Tandem Mass Spectrometry, found the expanded measurement uncertainty to be 34.64%. In this study, the RMSbias component was calculated with EQC data. In our study, when the RMSbias component was calculated with EQC data, the expanded measurement uncertainty was found to be 32.58%. However, while the number of EQC program participants in our study was 8, the number of EQC program participants in Basat et al.'s [22] study was between 152–157.

To determine whether the measured performance aligns with quality standards, it must be compared to an analytical performance specification (APS) or target. EFLM has recommended a straightforward approach for determining the APS of an analyte. According to this approach, there are three models: model 1—clinical outcomes, model 2—biological variation, and model 3—state-of-the-art performance [23]. In general, it is favored to apply Model 2, where APS is based on BV [24]. As the TEa value, the desirable biological variation database EFLM specification for TEa was used in our study [15]. Quite recently, Cavalier et al. [25] conducted an investigation into Model 2, generally regarded as the most comprehensive approach, and Model 1 over the course of one week and three months. The objective was to evaluate the APS for MU necessary for detecting a significant or highly significant change in 25(OH)D3 concentration. Cavalier et al. [25] asserted that the conventional method of generating and applying Biological Variation (BV) data is inappropriate for 25(OH)D, given the seasonal fluctuations in analyte levels. They suggested an alternative approach. Considering the physiological changes in 25(OH)D3 concentrations over time, they proposed the APS for MU as follows: 9.6% to detect a difference at $p<0.01$ (defined as 'desirable' MU). Braga et al. [16], in their article where they presented the performance characteristics of measurement uncertainty of common biochemical measurements according to Milan models, stated that the desirable specification for MU according to model 1 in vitamin D measurement was 10%. In our article, the desirable analytical performance specifications for standard measurement uncertainty in the article by Braga et al. [16] were used.

Variations in vitamin D measurement methods and the absence of standardization in calibrators pose challenges in establishing a single cut-off point, leading to discrepancies among laboratories. Given that the diagnosis of 25(OH)D3 deficiency relies heavily on the measurement outcome in conjunction with the clinical condition, achieving comparable results across laboratories is currently unattainable due to substantial disparities in measurement methods and the absence of measurement uncertainty incorporated into the results [26]. Consequently, when reporting the 25(OH)D3 measurement outcome, it is essential to also provide the measured or calculated measurement uncertainty [27]. The inclusion or exclusion of the measurement uncertainty value can result in new values, potentially altering the diagnostic categorization of patients from severe deficiency to a sufficient level [22].

Conclusion

As a conclusion, clinical laboratories should evaluate analytical performance at regular intervals using appropriate methods and produce solutions to error sources in line with the findings they obtain. In line with our results in this study, we would like to emphasize that laboratories should pay attention to the number of participants using the same method and device during the membership phase of the external quality control program. In cases where the number of participants using the same method and device in the EQC program is low, we recommend using IQC data to calculate the bias component when evaluating analytical performance with TAE or MU.

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Research Article

Bioinformatic assessment of the relationship between breast cancer and autophagy-related protein Ambra1 mutation

Umut Karabay[1], Durmus Ayan[2,3]

[1]Department of Internal Disease, Gulhane Research and Training Hospital, Ankara, Türkiye
[2]Department of Medical Biochemistry, Nigde Omer Halisdemir University Faculty of Medicine, Nigde, Türkiye
[3]Department of Medical Biochemistry, Nigde Ömer Halidemir University Reserarch and Training Hospital, Nigde, Türkiye

Abstract

Objectives: Autophagy protein 1, regulated by Beclin 1 (Ambra1), promotes tumor formation and development by modulating autophagy. Therefore, in situ intervention in autophagy is a promising new strategy for tumor therapy. We aimed to evaluate the possible effects of changes in the Ambra1 gene on breast cancer (BC) treatment in the BRCA cohort.

Methods: The gene profile of a total of 996 patients with BC was examined using data obtained from the Cancer Genome Atlas database via cBioPortal. The effects of mutations on proteins were examined by scoring the Polymorphism Phenotyping v2, Mutation Assessor, and Sorting Intolerant from Tolerant databases. The association of genes with other genes was determined with the STRING database. Kaplan-Meier Plot database was used by evaluating the overall survival (OS). The promoter methylation was evaluated by the UALCAN database.

Results: Eleven mutations were detected. Four of these mutations were truncated proteins. Ambra1 tissue expression levels were upregulated compared to healthy tissue in the BRCA cohort; this was not statistically significant ($p>0.05$). Decreased Ambra1 expression levels were associated with a shorter OS ($p=0.038$). Ambra1 promoter region hypermethylation was significant in the BRCA cohort compared to healthy tissue ($p<0.001$).

Conclusion: To our best knowledge, our study is the first to examine the relationship between BC and Ambra1 using bioinformatic tools. Ambra1 may be a candidate target molecule within the treatment strategy due to the mutations evaluated in the BRCA cohort, hypermethylation status, and the association of Ambra1 with shorter OS. However, these situations need to be confirmed by further studies.

Keywords: Apoptosis, autophagy, breast cancer, breast medicine, genetics-cancer genetics, genetics-carcinogenesis

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Breast cancer (BC) stands as the predominant malignancy affecting women globally, holding the position of the second most prevalent cause of cancer-related fatalities in the female population [1]. BC manifests as a heterogeneous and multifaceted disease influenced by diverse pathogenic factors. The prognosis of BC significantly improves with early-stage detection, evident in the notable enhancement of the 5-year survival rate. However, 15% of BC patients still have a poor prognosis due to being diagnosed at an advanced stage [2]. Furthermore, BC is characterized by distinct molecular subtypes and inherent biological properties, necessitating diverse therapeutic strategies tailored to each subtype, resulting in subtype-specific clinical outcomes. It is a global health problem due to the lack of effective treatment strategies that can be used for all disease subtypes [3]. Targeted therapies improve patient overall survival and reduce healthcare costs [3].

The regulatory protein Beclin1-regulated protein 1 (Ambra1) serves as an inherently dysregulated molecular protein exerting control over the viability and apoptosis of cancer cells

Address for correspondence: Durmus Ayan, MD, PhD. Department of Medical Biochemistry, Nigde Omer Halisdemir University Faculty of Medicine, Nigde, Türkiye; Department of Medical Biochemistry, Nigde Ömer Halidemir University Reserarch and Training Hospital, Nigde, Türkiye
Phone: +90 553 633 81 85 **E-mail:** durmusayan@hotmail.com **ORCID:** 0000-0003-2615-8474
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through the modulation of autophagic processes [4, 5]. Ambra1 protein has high plasticity. With this feature, it adapts very well to conformational changes. Current research reveals that Ambra1 is involved in multiple complex pathological processes. Therefore, Ambra1 protein becomes a molecule with high research potential. While Ambra1 protein plays a role as a tumor suppressor in the regulation of cell proliferation and tumor formation, it also functions as an oncogene in the regulation of tumor invasion and metastasis. This means that Ambra1 may play different roles in different genetic changes and different microenvironments [5].

Despite the contentious interpretations of Ambra1's involvement in cancer, the deliberate suppression of autophagy in specific circumstances emerges as a potentially beneficial foundational approach for cancer therapy [5]. Recent findings indicate that Ambra1 may exert inhibitory effects on the initiation, safeguarding, and advancement of cancer through the modulation of c-MYC and cyclins, which are commonly overexpressed in human cancer cells. Furthermore, Ambra1 exhibits elevated expression levels across diverse cancer types and demonstrates a significant association with the prognostic outcomes of patients. Consequently, the multifaceted roles played by Ambra1 endure potential implications for clinical oncology, particularly in the contexts of tumorigenesis and cancer progression [5].

Autophagy has been extensively studied in BC cells to understand its functions and mechanisms. Despite this, there is limited work to understand the specific role of a protein called Ambra1 in BC. Ambra1 plays a crucial role in autophagy induction, thus it may increase resistance or sensitivity to chemotherapeutic agents in cancer treatment. Studies report that Ambra1 inhibits paclitaxel-induced apoptosis and chemosensitivity via the AKT–FOXO1–BIM pathway in MCF-7 and MDA-MB-231 breast cancer cells. Additionally, Ambra1 is tightly associated with chemoresistance. During chemotherapy, cancer cells can reduce the cytotoxicity of chemotherapeutic agents through autophagy, thus promoting cancer survival. Therefore, autophagy inhibition by targeting Ambra1 may enhance the therapeutic target-achieving effect of agents [4].

In light of the above information, we evaluated whether Ambra1 gene mutations, promoter region methylation status, and overall survival (OS) could contribute to the possible future treatment strategy in our BRCA cohort.

Materials and Methods

Determination of the study group

The BRCA cohort to be evaluated in the study was obtained through the cBioPortal (https://www.cbioportal.org/) database. When accessing this database from the given internet address, breast tissue was selected as the tissue option. Then, the Breast Invasive Carcinoma (TCGA, PanCancer Atlas) option was selected. Research was conducted in the BRCA cohort by selecting the relevant gene via the "Query By Gene" option.

Our BRCA cohort consists of a total of 996 cases. The BRCA cohort includes Breast Invasive Ductal Carcinoma (BIDC), Breast Invasive Lobular Carcinoma (BILC), Breast Invasive Carcinoma (BIC), and Invasive Breast Carcinoma (IBC) as cancer types. The results of these cases were obtained using the cBioPortal database (https://www.cbioportal.org/). The data were accessed on September 13, 2023, from The Cancer Genome Atlas (TCGA). The data used in this study were obtained from the public database TCGA; therefore, ethical approval was not required.

Analysis of gene mutations

The cBioPortal database (https://www.cbioportal.org/) was used to evaluate the mutations in Ambra1. Thanks to this database, the amino acid in which the mutation occurred, the cancer subtype, and clinical information about the cancer were accessed. It was determined whether there was a somatic mutation or not thanks to the COSMIC (https://cancer.sanger.ac.uk/cosmic) database.

Survival prognosis of Ambra1 gene

In conducting prognosis analysis for overall survival (OS), the Kaplan-Meier Plotter (KM) tool, accessible at https://kmplot.com/analysis/, was employed. This tool systematically examines the associations between gene expressions and corresponding cancer survival rates, providing valuable insights into overall survival outcomes [6]. Moreover, the application of this tool facilitated an in-depth comprehension of the prognostic significance associated with the expression levels of the Ambra1 gene in BC patients. The KM plotter can evaluate the correlation between the expression of Ambra1 (mRNA) and survival in BC. The KM plotter uses Cox proportional hazards regression and the computation of the False Discovery Rate.

Gene-gene interaction

The STRING database (https://string-db.org/) systematically compiles and integrates information on protein-protein interactions, encompassing both physical associations and functional relationships. The dataset is derived from various sources, including automated text mining of scientific literature, computational predictions based on co-expression and conserved genomic context, information from interaction experiments databases, and established complexes/pathways sourced from curated references. Rigorous assessment and scoring of these interactions are performed [7].

Gene expression

Gene Expression Profiling Interactive Analysis, version 2 (GEPIA2.0), was used to evaluate the expression of the Ambra1 gene between the tumor tissues and the adjacent normal tissues. GEPIA2.0 uses TCGA database and genotype--tissue expression dataset (GTEx) samples to perform this analysis. The screening criteria used in GEPIA2.0 were $p<0.05$

and |Log2FC| the cutoff point was 0.1. These criteria were used to filter out genes that were not significantly differentially expressed between the two datasets [8]. In the dataset used for gene expression, the number of breast cancer tissue samples is 1085, and the number of adjacent healthy tissue samples that do not contain cancerous tissue is 291, provided from the GEPIA2.0 database.

Pathogenicity of mutations

We used Polymorphism Phenotyping v2 (PolyPhen-2), Mutation Assessor (MA), and Sorting Intolerant from Tolerant (SIFT) tools' algorithms on cBioPortal to investigate the possible pathogenicity and clinical effects of mutations detected in the Ambra1 gene. PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) uses a machine learning approach to classify genetic variants as benign or harmful [9]. This tool is automated, meaning it can analyze large datasets of rare genetic variants quickly and efficiently. Predictions made by PolyPhen-2 are essential for interpreting the impact of rare genetic variants on human health and disease. The output of PolyPhen-2 can classify the substitution effect as benign (score≤0.5), possibly damaging (0.5<score≤0.9), or possibly damaging (score>0.9) [10]. Mutation Assessor (http://mutationassessor.org/r3/) functions as a server/tool that predicts the functional impact of amino acid substitutions in proteins. This tool is particularly useful for identifying mutations discovered in cancer or missense polymorphisms. The functional impact of these mutations is evaluated based on the evolutionary conservation of the affected amino acid in protein homologues, that is, how similar the amino acid is between different species and how important it is for the function of the protein. The set used for validation contains 60,000 variants associated with diseases listed in the Online Mendelian Inheritance in Humans (OMIM) database. The evaluation results are given as low, medium, high, and neutral.

Sorting Intolerant from Tolerant (SIFT) (https://sift.bii.a-star.edu.sg/) is a computational tool used to predict the potential impact of amino acid substitutions on protein function. These substitutions can have varying effects on protein function, ranging from no effect to complete loss of function. SIFT uses a combination of sequence and structural information to predict the effect of these substitutions on protein function. The algorithm compares the amino acid sequence of the protein in question against a database of known protein sequences. Subsequently, SIFT assigns a score to each substitution indicating its likelihood of having a significant effect on protein function.

Promoter methylation status

UALCAN is an interactive open-access webpage for OMICS data analysis (http://ualcan.path.uab.edu/index.html). This database is built on PERL-CGI and can be used to analyze approximately 6000 gene methylation levels [11]. In this study, the promoter methylation level of Ambra1 in the BRCA dataset was examined.

Results

The cBioPortal web tool was used to analyze changes in the Ambra1 protein in BC patients. Among 996 cases, 11 cases (1.1%) of BC patients had genetic changes in the Ambra1 gene. The types of mutations encountered in BC in the Ambra1 gene are shown in Table 1. In the BRCA cohort, the most detected mutation type in the Ambra1 gene was a missense mutation (4 mutations, 36.3%), while the other mutations were 1 fusion gene mutation (9.1%), 2 frame shift deletions (18.2%), 2 splice region mutations (18.2%), 1 frame shift insertion (9.1%), and 1 nonsense mutation (9.1%). All mutations were confirmed to be of somatic origin. The Ambra1 gene contains the WD40 domain, which is 100% conserved throughout evolution, and three different types of motifs: two PxP (aa 275–281 and 1206–1212), two TQT (aa 1104–1106 and 1116–1118), and one LIR (aa 1043–1052). Ambra1 is cleaved by caspases in the D482 region. While no mutations were detected before the WD40 domain and the D482 region, 7 different mutations were found in the regions covering PxP (aa1206–1212) and other motifs. Additionally, in the BRCA cohort, there are 4 mutations in the Ambra1 gene that can cause truncated protein (p. I1256Ffs17, p. D1287Tfs78, p. E523*, p. S629Lfs*11). Additionally, there were recurrent hotspot (statistically significant) mutations along with these mutations in the Ambra1 gene. All recurrent hotspot mutations accompanying other Ambra1 mutations are shown in Table 2.

The OS results we obtained from the KM plotter analysis are shown in Figure 1. According to the analysis results, we recognized that decreased expression levels of Ambra1 were associated with shorter survival (p=0.038).

The examination of gene-gene interactions was conducted through the utilization of the STRING database software program. The outcomes of this analysis are visually represented in Figure 2, encapsulating the data derived from our investigation. The other genes with which the Ambra1 gene interacts most frequently are ATG14, BECN1, CUL4A, CUL4B, DDA1, DDB1, PIK3C3, PIK3R4, TRAF6, and UVRAG, respectively, according to the relationship scoring, and it consists of a total of 10 nodes. Apart from this, according to the gene relationships examined in different publications, it was also found to be associated with the ANXA5, GABARAPL2, SQSTM1, ATG5, CASP9, BCL2L11, FOXO1, AKT1, and ATG12 genes (Fig. 2).

Ambra1 gene expression levels of BC (n=1085) patients were higher than the healthy control group (n=291), but this increase was not statistically significant (p>0.05) (Fig. 3).

The scores of PolyPhen-2, MA, and SIFT are shown in Table 2. Based on the outcomes of the analysis and scoring metrics, the mutation identified in the Ambra1 gene (p. L1090F) was ascertained as the most impactful missense mutation, inducing substantial alterations in both protein structure and function. This mutation was consequently deemed the most pathogenic among the identified variants.

Table 1. Ambra1 Gene Mutation Analysis and treatment option in cases

No	Gen	Nucleotit change	Somatic status	Type of cancer	Subtype	American joint committee on cancer metastasis stage code	Neoplasm disease lymph node stage American joint committee on cancer code	Neoplasm disease stage American joint committee on cancer code	Diagnosed age	Overall survival (months)	Treatment option
M-1	Ambra1	NA	Confirmed somatic	BIDC	Luminal A	M0	N1	T2	47	50.0	Doxorubicin, Cyclophosphamide, Paclitaxel, Radiation, Tamoxifen, Anastrazole
M-2	Ambra1	c.3765del	Confirmed somatic	BIDC	Luminal B	M0	N1A	T2	42	17.0	Epirubicin, Cyclophosphamide, Docetaxel, Zoledronic Acid, Radiation, Tamoxifen
M-3	Ambra1	c.3859del	Confirmed somatic	BILC	Luminal B	M0	N0	T3	83	0	NA
M-4	Ambra1	c.2822-2A>G	Confirmed somatic	BILC	Luminal B	M0	N0	T3	83	0	NA
M-5	Ambra1	c.1648A>C	Confirmed somatic	IBC	Basal-like	M0	N0 (I-)	T1C	82	23.0	NA
M-6	Ambra1	c.3691G>C	Confirmed somatic	BIDC	Basal-like	M0	N0	T2	62	7.5	NA
M-7	Ambra1	c.3268C>T	Confirmed somatic	BIDC	Luminal A	M0	N2	T2	67	0.4	NA
M-8	Ambra1	c.3608C>A	Confirmed somatic	BIDC	Basal-like	M0	N1	T2	51	125.1	NA
M-9	Ambra1	c.1567G>T	Confirmed somatic	BIDC	Luminal A	M0	N0	T1	62	71.0	Radiation, Trastuzumab, Anastrazole
M-10	Ambra1	c.1884dup	Confirmed somatic	BIDC	Luminal A	M0	N1	T2	31	74.7	NA
M-11	Ambra1	c.2976+1del	Confirmed somatic	BIC (NOS)	Luminal A	MX	N3A	T3	39	25.9	Docetaxel, Doxorubicin, Cyclophosphamide, Tamoxifen, Goserelin, Radiation

BIDC: Breast Invasive Ductal Carcinoma; BILC: Breast Invasive Lobular Carcinoma; IBC: Invasive Breast Carcinoma; BIC: Breast Invasive Carcinoma; NA: not applicable

Table 2. Mutation profile of Ambra1 gene and recurrent hotspot mutations

No	Gen	Nucleotit change	Protein change	Variation type	Mutation assesor score	SIFT score	PolPhen-2 score	Recurrent hotspot mutations (statistically significant)
M-1	Ambra1	NA	*PEX16-AMBRA1 Fusion*	Fusion	–	–	–	PIK3CA (p. H1047R missense)
M-2	Ambra1	c.3765del	*I1256Ffs*17*	FS del	–	–	–	PIK3CA (p.E545K ve p.E453K missense), TP53 (p.X187_splice site mutation)
M-3	Ambra1	c.3859del	*D1287Tfs*78*	FS del	–	–	–	–
M-4	Ambra1	c.2822-2A>G	*X941_splice*	Splice	–	–	–	–
M-5	Ambra1	c.1648A>C	*T550P*	Missense	0.60 (neut.)	0.12 (tole.)	0.00 (benign)	TP53 (p. S241C missense), IL6ST (p. S279C missense)
M-6	Ambra1	c.3691G>C	*A1231P*	Missense	1.10 (low)	0.01 (delet.)	0.19 (benign)	FGFR2 (p. *N549K missense*, *TP53 (p. X225_splice)*, NCKAP1L (p. *R21H missense*), PACSIN1 (p. *R389H missense*)
M-7	Ambra1	c.3268C>T	*L1090F*	Missense	2.21 (med.)	0.00 (delet.)	1.00 (Prob.D.)	BAX (p. *I133M missense*)
M-8	Ambra1	c.3608C>A	*T1203N*	Missense	0.00 (neut.)	0.02 (tole.)	0.25 (benign)	–
M-9	Ambra1	c.1567G>T	*E523**	Nonsense	–	–	–	PIK3CA (p.N345K missense) PGR (p.E723K missense)
M-10	Ambra1	c.1884dup	*S629Lfs*11*	FS ins	–	–	–	PIK3CA (p.K111E missense) ERBB2 (p.D769Y missense) TP3 (p.N247I missense)
M-11	Ambra1	c.2976+1del	*X992_splice*	Splice	–	–	–	PIK3CA (p.H1047R missense)

SIFT: Sorting Intolerant from Tolerant; FS del: Framashift Deletion; FS ins: Frameshift Insertion; Neut: Neutral; Delet: Deleterious; med: Medium; Prob.D: Probably Damage; NA: Not applicable

Ambra1 promoter region methylation status was found to be statistically hypermethylated in BRCA samples compared to healthy tissue ($p<0.001$) (Fig. 4).

Discussion

Ambra1 has been shown to increase metastasis in BC mouse models [12]. Although the results need to be confirmed, upregulation of Ambra1 is thought to be associated with tumorigenesis and BC progression by affecting some vital pathways [2]. The sensitivity of cells to chemotherapeutic agents and the regulation of chemosensitivity are quite important in BC patients. Ambra1 is a key protein controlling the switch between autophagy and apoptosis and has been shown to modulate paclitaxel-induced apoptosis in BC cells via the BIM/mitochondrial pathway [13]. Studies have argued that Ambra1 regulates BIM expression at the transcriptional level through the Akt-FoxO1 pathway, and this regulation could be considered a potential target for BC treatment [13, 14].

Post-translational modifications in the Ambra1 gene generally focus on phosphorylation and ubiquitylation. It is stated that these modifications are closely related to autophagy [15]. Two important autophagy-related kinases, unc-51 like autophagy activating kinase 1 (ULK1) and mTOR complex 1, are activated through the phosphorylation of Ambra1. As a result of phosphorylation at the Ser52 and Ser465/Ser635 regions of Ambra1, it becomes a substrate for mTORC1 and ULK1, respectively [16]. In our BRCA cohort, unstable small polypeptide chains may occur as a result of the E523* nonsense mutation. In this case, the polypeptide chain may terminate at this point, and the Ser 635 region will not be formed. Thus, Ser635 region phosphorylation of Ambra1 may not occur. This phosphorylation is required for the association of ULK1 with Ambra1, as well as for regulating the dissociation of AmbRa1-Vps34-beclin-1 from the dynein complex [17]. When autophagy is induced, activated ULK1 phosphorylates Ambra1 at Ser465 and Ser635 sites. If these reactions do not occur, autophagy may be disrupted.

In the BRCA cohort, four truncated protein-forming mutations were found in the Ambra1 gene. These mutations do not include the W40 region, which is a 100% conserved domain, and the D482 region, which is cleaved by cas-

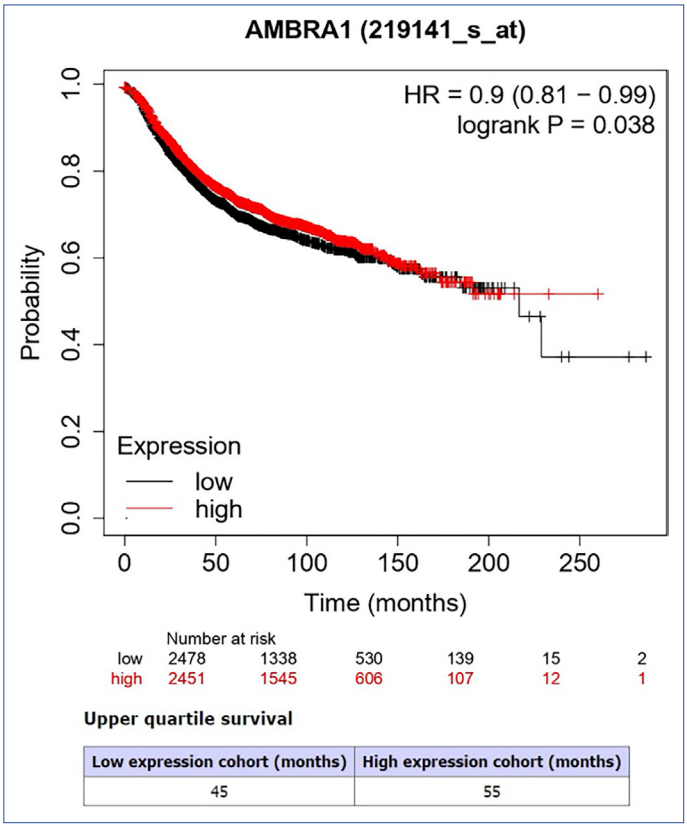

Figure 1. Different expressions of Ambra1 in BC patients in the overall survival (OS) curve (using the Kaplan-Meier plotter). The red line represents the survival rate curve of patients with BC who expressed the gene, and the black line represents the survival rate curve of BC patients who did not express the gene.

HR: Hazard ratio; BC: Breast cancer.

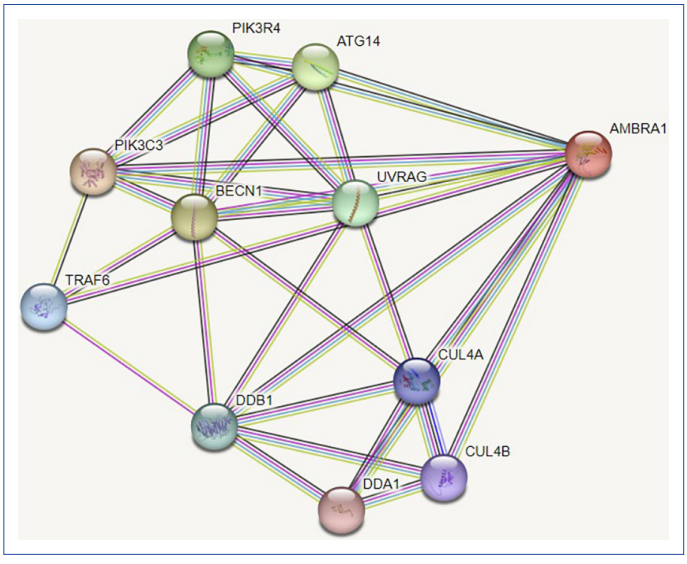

Figure 2. Schematic representation of known and predicted protein-protein interactions with the *Ambra1* gene. Each line has features. (Red line-indicates the presence of fusion evidence; Green line-neighborhood evidence; Blue line- cooccurrence evidence; Purple lineexperimental evidence; Yellow line- textmining evidence; Light blue line-database evidence; Black line-coexpression evidence.).

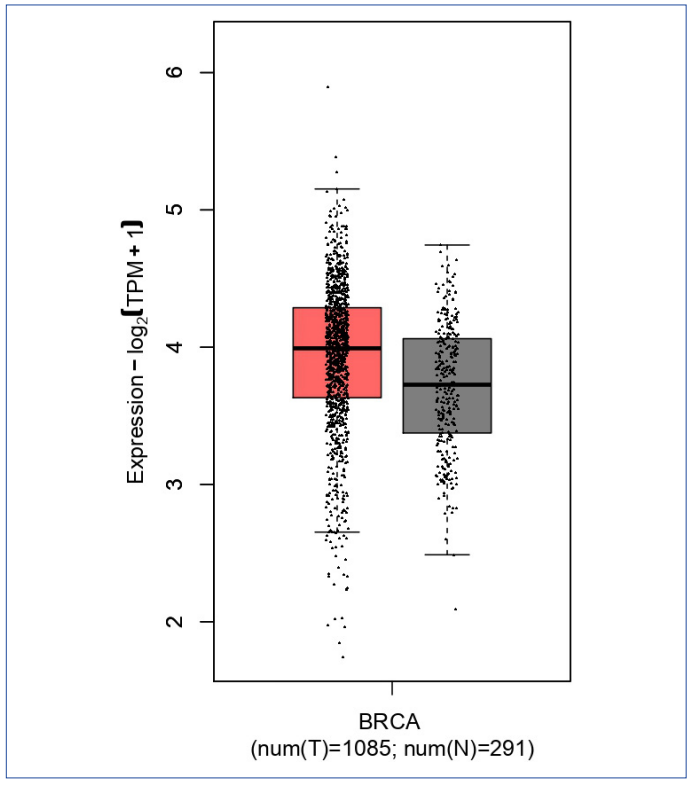

Figure 3. mRNA expression of Ambra1 in BC (red) and normal breast tissues (gray).

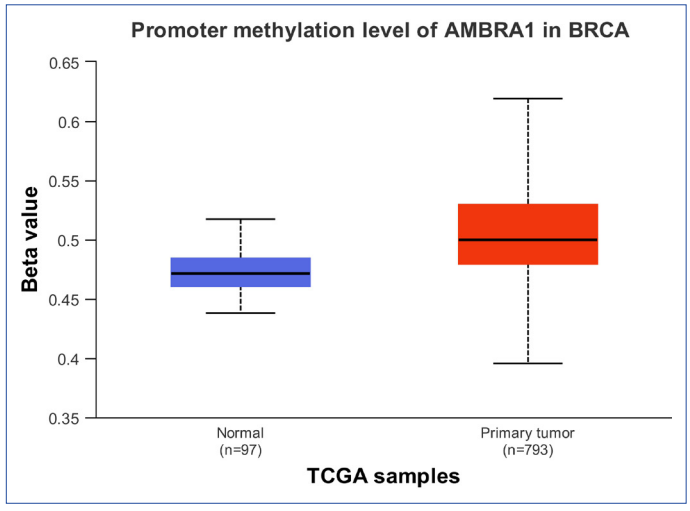

Figure 4. Promoter methylation level analysis of Ambra1.

TCGA: The Cancer Genome Atlas.

pases during apoptosis. However, they may affect functional motifs located after the splicing site. The remaining part after cleaving the D482 region is also called the BH3-like domain. The pro-apoptotic segment within Ambra1 is designated as the BH3-like domain. Specifically, the PxP motifs situated at amino acid positions 275–281 and 1177–1183 within Ambra1, which reside within this pro-apoptotic region, play a crucial role in binding to the catalytic subunit of protein phosphatase 2A. This binding interaction serves to regulate the activity of c-MYC [18].

The interaction between Ambra1 and dynein light chain 1 is facilitated through the mediation of TQT motifs located within the C-terminal sequence of Ambra1. Furthermore, they have a role in binding Ambra1 to the dynein motor complex in the absence of autophagy induction [19]. Ultimately, the binding interaction between Ambra1 and autophagy-associated protein 8 family proteins, specifically light chain 3 beta, is contingent upon the critical involvement of the LIR motif situated in the C-terminal region of Ambra1 [20]. The p.I1256Ffs17, p.D1287Tfs78, p.E523*, and p.S629Lfs*11 truncated mutations occurring in this region in our BRCA cohort may cause early termination of the polypeptide and the formation of a dysfunctional protein. As a result, loss of function in the BH3-like domain may occur. In this case, the balance between autophagy and apoptosis may be disrupted, and negative consequences may occur for cancer pathogenesis.

In addition, cases with these truncated changes (except p.D1287Tfs*78) are accompanied by mutations in the PIK3CA gene as recurrent hotspot changes. These mutations frequently occur in BC patients and have an oncogenic character. Additionally, mutations in this gene often appear to be an important genetic change that promotes cancer growth and may play a role in resistance to treatment [21]. In our BRCA cohort, the four proteins most associated with the Ambra1 protein are PIK3C3, PIK3R4, BECN1, and ATG14. PIK3C3 and PIK3R4 are involved in the maturation, initiation, and endocytosis processes of autophagosomes. Ambra1 interacts with these proteins at the initiation stage of autophagy, and autophagy is induced [22].

Beclin 1-associated autophagy-related key regulator (ATG14 gene) is required for both basal and inducible autophagy. It determines the localization of the autophagy-specific PI3-kinase complex PI3KC3-C1. ATG14 plays a role in autophagosome formation and MAP1LC3/LC3 conjugation to phosphatidylethanolamine [22]. It promotes BECN1 translocation from the trans-Golgi network to autophagosomes and enhances PIK3C3 activity in a BECN1-dependent manner [23]. ATG14 is essential for the autophagy-dependent phosphorylation of BECN1. It stimulates the phosphorylation of BECN1, but suppresses the phosphorylation of PIK3C3 by AMPK [24]. After the autophagy initiation phase, nucleation and phagophore formation are very important for the regulation of autophagy. With the formation of the ULK1 complex, the nucleation phase of autophagic membranes begins. The molecules that play a key role in the nucleation stage are Beclin 1 (BECN1) and Bcl-2. Autophagy is suppressed by the binding of Bcl-2 to BECN1. On the other hand, interaction of BECN1 with the lipid kinase vacuolar sorting 34 protein (VPS34) promotes membrane nucleation. The ULK1 complex phosphorylates the class III phosphatidylinositol-3-kinase (PtdIns3K) complex, enabling its activation. The PtdIns3K complex includes BECN1, NRBF2, ATG14, PIK3C3, PIK3R4, and Ambra1. Phagophores are formed by the fusion of membranes formed by nucleation from ER membranes. The ATG9 system, including ATG9, ATG2A/B, and WDR45, also plays an important role in phagophore formation [25]. In this case, loss of function in Ambra1 as a result of mutations in the Ambra1 protein may prevent the induction of autophagy as it will disrupt the above protein-protein interaction mechanisms. In the treatment options related to these proteins, PIK3C3 inhibitors (Lapatinib) are used to inhibit proliferation in BC [26].

Extant literature indicates a reported association of BECN1 and ATG14 with potential implications in cancer progression or resistance to chemotherapeutic interventions. BECN1 plays a pivotal role as a key regulator within the PIK3C3 complex, influencing autophagosome nucleation and participating in endocytic trafficking processes. ATG14, as an additional regulatory subunit within the PIK3C3-C1 complex, participates in the process of autophagosome nucleation and facilitates the fusion of autophagosomes with lysosomes [27]. ATG14 demonstrates the capacity to modulate the responsiveness to targeted therapeutic agents, such as gemcitabine, cisplatin, and sorafenib, through its influence on the expression profiles of microRNAs (miRNAs) in pancreatic, ovarian, colorectal, and hepatic cancers [28–30].

It is argued that the Ambra1 gene, like ATG14 and BECN1, may be involved in chemoresistance and may support cancer survival by reducing the cytotoxicity of chemotherapeutic agents through autophagy during chemotherapy. It is considered that when autophagy inhibition is performed by targeting Ambra1, the therapeutic effectiveness of the agents used in treatment may increase [4].

Studies conducted on different cancer types have tried to reveal the relationship between Ambra1 expression levels and OS [31]. A study conducted in patients with gastric cancer stated that high Ambra1 expression levels were an independent factor in predicting poor OS in patients [31]. In addition, two studies have demonstrated that high expression levels of Ambra1 correlate with poorer survival in pancreatic ductal adenocarcinoma and cholangiocarcinoma patients [32, 33]. While this is the case in other types of cancer, *in vivo*, Ambra1 has been demonstrated to be an important protein that determines whether epirubicin-treated BC cells undergo apoptosis or autophagy [5]. Although Ambra1 tissue expression levels were upregulated compared to healthy tissue in our BRCA cohort, this was not statistically significant. However, decreased Ambra1 expression levels were associated with poor OS.

Epigenetic mutation changes gene expression in a heritable manner, without any change in the DNA sequence, and is as effective as genetic changes in cancer formation. In cancer cells, hypermethylation in CpG islands in promoter regions is observed along with widespread hypomethylation in the genome. Promoter region hypermethylation causes subsequent gene silencing, which is especially important in inactivating tumor suppressor genes. DNA methylation is considered a potential marker for early detection of cancer. Promoter hypermethylation has been identified as a poten-

tial marker and has been shown to be able to detect established BC. Changes in promoter methylation status are common events that occur in the early stages of tumorigenesis and can be detected with minimally invasive measures. A number of cancer-associated genes have been found to be frequently methylated in BC. These markers are promising in distinguishing between malignant disease and benign disease or normal tissue and may inform the detection of lobular carcinomas [34].

When we examined the Ambra1 gene in the BRCA cohort, the promoter region methylation level of Ambra1 was hypermethylated in BC patients compared to healthy controls. Although this situation is thought to lead to gene silencing and decreased expression levels, it is contrary to the upregulation of the Ambra1 gene in our BRCA cohort.

Normally, promoter sequence methylation typically results in chromatin becoming more densely packed. As a result, transcription is negatively affected. In this process, the methyl-CpG (mCpG)-binding domain specifically binds to the methylated sequences of proteins. It can be explained by the classical model that it recruits repressor complexes such as the histone deacetylase complex. Histone deacetylation causes chromatin to become condensed, thereby inhibiting transcription. However, this model does not account for how promoter hypermethylation could lead to increased expression rather than the expected reduction.

In the first scenario, recent findings propose a competitive mechanism for methylation-dependent transcription regulation, wherein methylated sequences might also attract transcription factors (TFs) that specifically recognize methylated binding motifs. This process could then lead to the initiation of transcription [35]. In the second scenario, this hypermethylation situation may be caused by post-translational modification. These may explain the increased expression levels of Ambra1 in our BRCA cohort.

Considering the above possible first scenario, the increase in expression occurring with hypermethylation in the Ambra1 gene may put a different perspective on treatment options targeting the Ambra1 molecule.

Limitation: There were some limitations in our study. The most important of these limitations is the promoter region methylation status. Significant differences in normal and tumor tissue sample sizes in data obtained from UALCAN databases may cause bias in the results. For this reason, we believe that prospective studies are needed in groups with homogeneously distributed sample sizes in order to obtain more meaningful information about promoter region methylation. The same situation occurs in the data obtained from GEPIA2, where Ambra1 gene expression is evaluated. Therefore, more reliable results can be obtained by reducing the serious difference between sample sizes when evaluating data obtained from databases and making statistical comparisons.

Conclusion

To our best knowledge, our study is the first to examine the relationship between BC and Ambra1 using bioinformatic tools. In summary, changes in autophagy-related genes can be used as potential treatment targets. It is not clear how promoter region hypermethylation occurs in the autophagy-related Ambra1 gene in the BRCA cohort. Once this situation is clarified, it may be investigated to determine whether there is a potential treatment target. However, in order for Ambra1 to be evaluated as a potential treatment target, mutations occurring in the Ambra1 gene need to be clarified in population-based prospective studies both in breast cancer and other types of cancer. For this, geneticists and clinical biochemists will need to collaborate and carefully evaluate possible changes in the Ambra1 gene.

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Research Article

Relationship between atherogenic index of plasma, asprosin, and metrnl levels in hemodialysis patients

Meltem Yardim[1], Levent Deniz[2], Mehmet Akif Saltabas[3], Damla Kayalp[4], Ramazan Fazil Akkoc[5], Tuncay Kuloglu[6]

[1]Department of Medical Biochemistry, Yerkoy State Hospital, Yozgat, Türkiye
[2]Department of Medical Biochemistry, University of Health Sciences, Bagcılar Training and Research Hospital, Istanbul, Türkiye
[3]Department of Internal Medicine, Yerkoy State Hospital, Yozgat, Türkiye
[4]Department of Medical Biochemistry, Yozgat City Hospital, Yozgat, Türkiye
[5]Department of Anatomy, Faculty of Medicine, Firat University, Elazig, Türkiye
[6] Department of Histology and Embryology, Faculty of Medicine, Firat University, Elazig, Türkiye

Abstract

Objectives: This study aimed to evaluate the relationship between the atherogenic index of plasma (AIP) and plasma asprosin and metrnl levels in hemodialysis (HD) patients.

Methods: Forty-eight patients receiving HD treatment with a diagnosis of end-stage renal disease (ESRD) were included. The control group comprised 34 age-, sex-, and body mass index-matched healthy volunteers without a history of renal disease. ESRD patients were divided into two groups: high-risk (AIP≥0.24) and low-moderate risk (AIP<0.24). Asprosin and metrnl levels in the plasma of blood samples taken just before dialysis were studied by enzyme-linked immunosorbent assay.

Results: A significant difference was found between the control group [23.3(19.9-27.7 ng/mL)], the low-moderate risk group [39.3(34.9-40.8 ng/mL)], and the high-risk group [48.1(44.5-49.9 ng/mL)] in terms of asprosin levels (for each p<0.001). Asprosin values of both low-moderate risk and high-risk groups were significantly higher than the controls. In the high-risk group, plasma asprosin levels were higher than in the low-moderate risk group (p=0.012). Metrnl levels of the high-risk group were found to be lower than both the control and low-risk groups (p<0.001 and p=0.003, respectively). AIP showed a positive relation to asprosin and a negative relation to metrnl.

Conclusion: Logistic regression analysis has revealed important insights into the independent relationships between metrnl, asprosin, and high AIP values in HD patients. These findings support the anti-atherogenic potential of metrnl and suggest the potential atherogenic effects of asprosin, highlighting the complex interplay between adipokines and cardiovascular risk in this patient population.

Keywords: Adipokine, asprosin, atherogenic index of plasma, hemodialysis, meteorin-like protein

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Hemodialysis (HD) patients are more vulnerable to the risk of cardiovascular and metabolic disorders, depending on the underlying disease causing chronic kidney disease and the adverse effects of HD. In HD patients, changes in the visceral and subcutaneous fat tissue, first in the form of adiposity and then in the form of lipodystrophy, may lead to insulin metabolism disorders, which in turn may lead to changes in adipokine synthesis and release [1]. Fat cells try to control the risk of cardio-metabolic diseases by reregulating the synthesis of many different adipokines to prevent adiposity-related adverse effects. Meteorin-like protein (metrnl) and asprosin are two important adipokines involved in insulin resistance, hepatic glucose release, and inflammation regulation [2, 3].

Address for correspondence: Meltem Yardim, MD. Department of Medical Biochemistry, Yerkoy State Hospital, Yozgat, Türkiye
Phone: +90 505 906 49 62 **E-mail:** meltem_yardim@hotmail.com **ORCID:** 0000-0002-0348-6312
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Stimulation of appetite during fasting and the release of hepatic glucose into the circulation are vital and evolutionary processes regulated by the coordination of the brain and peripheral organs. Metrnl, a peptide homologous to neurotrophin, is an adipokine involved in lipid and glucose metabolism, energy expenditure, insulin sensitivity, and browning of white adipose tissue [4, 5]. In experimental models, metrnl has been shown to reverse hyperglycemia-induced cardiac and renal fibrosis, apoptosis, and oxidative stress [2]. Asprosin is a protein of ~30 kDa and 140 amino acids encoded by fibrillin-1, secreted from adipose tissue during fasting, increasing appetite and stimulating hepatic glucose release [3, 6]. Asprosin, whose circulating levels increase during fasting, decreases to normal levels again with refeeding. In fibrillin gene mutations, decreased circulating levels of asprosin cause loss of fat mass and a lipodystrophic appearance of the individual, while allowing the continuation of insulin sensitivity and the cell's survival [3, 6]. It is known that circulating asprosin levels are increased in type 2 diabetes mellitus (T2DM) and diabetic kidney diseases [7, 8]. Reducing lipid accumulation in macrophages is evidence of the possible protective role of asprosin in atherosclerosis and cardiovascular diseases (CVD) [9].

There are no sufficient clinical studies investigating serum metrnl and asprosin levels and their relationship with metabolic and cardiovascular risk factors in hemodialysis patients. In end-stage renal disease (ESRD) patients at risk of CVD, adipose tissue attempts to balance cardiometabolic risk factors by reregulating the production of adipokines such as asprosin [8]. Metrnl may play a role in contributing to cardiac function and providing protection by exerting anti-inflammatory effects and influencing processes such as vascular function and cardiac remodeling. Metrnl is expressed intensely, especially in the left ventricle and myeloid cells. In experimental infarct models, it has been reported that macrophage-derived metrnl prevents the growth of the infarct area by stimulating post-infarction angiogenesis. Metrnl also acts as a ligand for stem cells to stimulate receptor tyrosine kinase in heart muscle. In metrnl deficiency, the risk of heart failure increases because tyrosine kinase activation cannot be achieved [10]. The atherogenic index of plasma (AIP) has been identified as a predictive biomarker for cardiovascular illnesses, particularly in the context of atherosclerosis and coronary artery disease [11]. Since AIP is a valuable marker in evaluating cardiovascular risk and atherogenicity in various diseases, it is preferred as a cost-effective, fast, and reliable method to reveal early-stage CVD risk [12].

We aimed to assess the association between AIP and plasma asprosin and metrnl levels in HD patients. In addition, we aimed to analyze plasma metrnl and asprosin level changes according to the AIP index and the relationship between these two adipokines and metabolic and demographic parameters.

Materials and Methods

This study was carried out from July 2023 to October 2023, after the permission of Fırat University Non-Invasive Research Ethics Committee (Date and Number: 08.06.2023-2023/08-31). Forty-eight patients receiving HD treatment with a diagnosis of ESRD in Yerköy State Hospital Hemodialysis Unit were included. The control group comprised 34 age-, sex-, and body mass index (BMI)-matched healthy volunteers without a history of T2DM, hypertension, or cardiovascular disease. The study adhered to the principles outlined in the Declaration of Helsinki. All participants were informed, and their consent was obtained.

Patients with active local or systemic infection, malignancy, neurodegenerative disease, or chronic anti-inflammatory and steroid therapy were excluded from this study. ESRD patients were categorized into two groups: high-risk (AIP value ≥0.24) and low-moderate risk (AIP value <0.24). The age, gender, height, and weight of the individuals included in the study were recorded. Lipid parameters and other routine biochemical tests of the participants were performed on 12-hour fasting blood. Fasting plasma glucose, total cholesterol, triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), Hemoglobin A1c (HbA1c), serum sodium, potassium, calcium (Ca), phosphorus (P), uric acid, urea, total protein, albumin, creatinine, alkaline phosphatase (ALP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels were measured with an AU680 (Beckman Coulter, Inc., Brea, CA, USA) device. AIP was obtained by taking the logarithm of the ratio of plasma TG to HDL-C level [log10 (TG/HDL-C)]. An AIP≥0.24 was considered high-risk [13]. Fasting insulin, parathyroid hormone (PTH), and ferritin levels were measured by the chemiluminescence method with the Snibe Maglumi 4000 Plus (Snibe Diagnostics, Shenzhen, CHINA) immunoassay analyzer. Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) = Fasting glucose (mg/dL) × Fasting insulin (uIU/mL) / 405 [14].

Asprosin and metrnl levels in the plasma of blood samples taken just before dialysis were studied. After the blood samples in the aprotinin tubes were centrifuged at 4000 rpm for 10 minutes, the obtained plasma was transferred to Eppendorf tubes and stored at −20°C until analyzed. Asprosin and metrnl were measured with Human ELISA (Catalog no: E4095Hu, E3941Hu, Bioassay Technology Laboratory, Shanghai, CHINA) kits. The concentration values of the samples were calculated in ng/mL according to the standard curve. The measurement range for asprosin was 0.5–100 ng/mL, and the minimum measurable level (sensitivity) was 0.23 ng/mL. The measurement range for metrnl was 0.05–15 ng/mL, and the minimum measurable level (sensitivity) was 0.023 ng/mL. The intra-assay CV values for both were <8%, and the inter-assay CV values were <10%.

Statistical analysis

Statistical analyses were carried out via SPSS v. 26 (IBM Corp., Armonk, NY). Graphs were created using Graphpad Prism 8.0 (GraphPad Software, San Diego, California, USA). The normality of the data was assessed using the Shapiro-Wilk test. Normally distributed data were presented as mean±standard deviation. Comparisons between groups were made with the one-way ANOVA test. Data that were not normally distributed were pre-

Table 1. Comparison of demographic and laboratory findings among controls and hemodialysis patients with AIP<0.24 and hemodialysis patients with AIP≥0.24

Parameter	Control group (n=34)	Low-moderate risk (n=24)	High-risk (n=24)	p
Age (years)	58 (46–65)	55 (52–67)	59 (47–67)	0.750**
Gender (n, %)				0.905***
Male	15 (44)	12 (50)	11 (46)	
Female	19 (56)	12 (50)	13 (54)	
Body mass index (kg/m^2)	24.2 (22.6–25.9)	24.1 (23.4–25.5)	24.9 (24.0–29.0)	0.063**
Metrnl (ng/mL)	6.80 (5.86–7.71)	3.00 (2.88–3.67)[a1]	1.88 (1.50–2.26)[a1, b2]	**<0.001****
Asprosin (ng/mL)	23.3 (19.9–27.7)	39.3 (34.9–40.8)[a1]	48.1 (44.5–49.9)[a1,b2]	**<0.001****
Urea (mg/dL)	26.4±6.30	140±35.9[a1]	117±25.1[a1]	**<0.001***
Creatinine (mg/dL)	0.68±0.15	7.21±1.77[a1]	7.52±2.16[a1]	**<0.001***
Total protein (g/L)	74.71±3.99	64.0±3.21[a1]	67.58±5.87[a1]	**<0.001***
Albumin (g/L)	43.9 (42.3–46.6)	35.7 (32.8–36.4)[a1]	38.1 (33.9–38.8)[a1]	**<0.001****
Insulin (mIU/L)	9.9 (7.7–11.8)	18.1(11.6–22.2)[a1]	26.6 (22.4–42.9)[a1, b2]	**<0.001****
Glucose (mg/dL)	89.5 (85–92.3)	99.5 (84–136)	143.5 (100.5–201.3)[a1]	**<0.001****
HOMA-IR	2.10 (1.70–2.50)	4.30 (2.45–7.50)[a1]	11.4 (5.75–18.8)[a1, b2]	**<0.001****
HbA1c (%)	5.30 (5.14–5.51)	5.55 (5.44–5.88)[a2]	6.37 (5.68–8.85)[a1]	**<0.001****
AST (U/L)	20.0 (15.8–23.0)	14.0 (11.0–16.8)[a1]	11.0 (7.00–14.0)[a1]	**<0.001****
ALT (U/L)	16.0 (13.0–19.0)	11.0 (10.0–15.5)[a1]	9.00 (5.00–11.0)[a1]	**<0.001****
ALP (U/L)	71.0 (64.0–78.0)	102 (74.5–147)[a1]	147 (106–193)[a1]	**<0.001****
AIP (U/L)	–0.03 (–0.20–0.07)	–0.06 (–0.19–0.11)	0.44 (0.35–0.52)[a1, b1]	**<0.001****
TC (mg/dL)	174 (154–191)	160 (125–176)	150 (121–186)	0.047**
HDL-C (mg/dL)	51.5 (43.0–60.0)	49.5 (39.0–59.0)	30.0 (28.0–33.0)[a1, b1]	**<0.001****
LDL-C (mg/dL)	104 (82.0–112)	92.0 (63.0–98.0)	85.0 (55.0–120)	0.112**
Triglyceride (mg/dL)	112 (73.0–138)	100 (63.0–122)	193 (153–228)[a1,b1]	**<0.001****
Uric acid (mg/dL)	4.45 (4.10–5.20)	5.95 (5.20–7.00)[a1]	6.10 (5.40–6.40)[a1]	**<0.001****
Ca (mg/dL)	9.88±0.39	8.43±0.94[a1]	8.60±1.04[a1]	**<0.001***
P (mg/dL)	3.55±0.44	4.56±1.22[a2]	4.92±5.40[a1]	**<0.001***
Ferritin (µg/L)	62.6 (34.8–105)	454 (89.0–777)[a1]	420 (167–561)[a1]	**<0.001****
PTH (ng/L)	48.5 (32.4–57.8)	602 (453–1376)[a1]	697 (286–1115)[a1]	**<0.001****

*: One-way ANOVA test; **: Kruskal-Wallis test; ***: Chi-Square test. [a]: Comparison with control, [a1]: <0.001; [a2]: <0.017; [b]: Comparison with low-moderate risk group, [b1]: <0.001; [b2]: <0.017. AIP: Atherogenic index of plasma; Metrnl: Meteorin-like protein; HOMA-IR: Homeostasis Model Assessment of Insulin Resistance; HbA1c: Hemoglobin A1c; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; TC: total cholesterol; HDL-C: High-Density Lipoprotein Cholesterol; LDL-C: Low-density Lipoprotein Cholesterol; Ca: Calcium; P: Phosphorus; PTH: parathyroid hormone.

sented as median (1st-3rd quartile), and comparisons were analyzed with the Kruskal-Wallis test. Pairwise comparisons were made with the Bonferroni correction. The statistical significance level for Bonferroni correction was accepted as <0.017. *Post-hoc* Bonferroni or Tamhane T2 tests were used for pairwise comparisons of groups in the one-way ANOVA test. Categorical variables were expressed as numbers and percentages, and comparisons between groups were analyzed with the Chi-Square test. Spearman correlation analysis was employed to examine relationships between variables. Binary logistic regression analysis was performed to assess the association between asprosin or metrnl and high-risk (AIP ≥0.24) in HD patients. A p-value <0.05 was considered statistically significant.

Results

A total of 82 patients, including 48 hemodialysis patients with a mean age of 58±11 and 34 healthy controls with a mean age of 55±12, were included in the study. The hemodialysis group consisted of 25 women (52.8%) and 23 men (47.2%), and the control group consisted of 19 women (56%) and 15 men (44%). HD patients with AIP <0.24 were defined as the low-moderate risk group (n=24), and HD patients with AIP ≥0.24 were defined as the high-risk group (n=24). There was no significant difference between the groups regarding age, gender, BMI, total cholesterol, and LDL-C. In the low-moderate and high-risk groups, urea, creatinine, insulin, HOMA-IR, HbA1c, ALP, uric acid, P, ferritin, and PTH levels were significantly higher than those in the control group. Nevertheless, no significant difference was observed between the high-risk and low-moderate risk groups for these parameters, except for insulin and HOMA-IR. Total protein, albumin, AST, ALT, and Ca levels in both low-moderate and high-risk groups were significantly lower compared to the control group (p<0.001); these parameters were similar in the low-moderate and high-

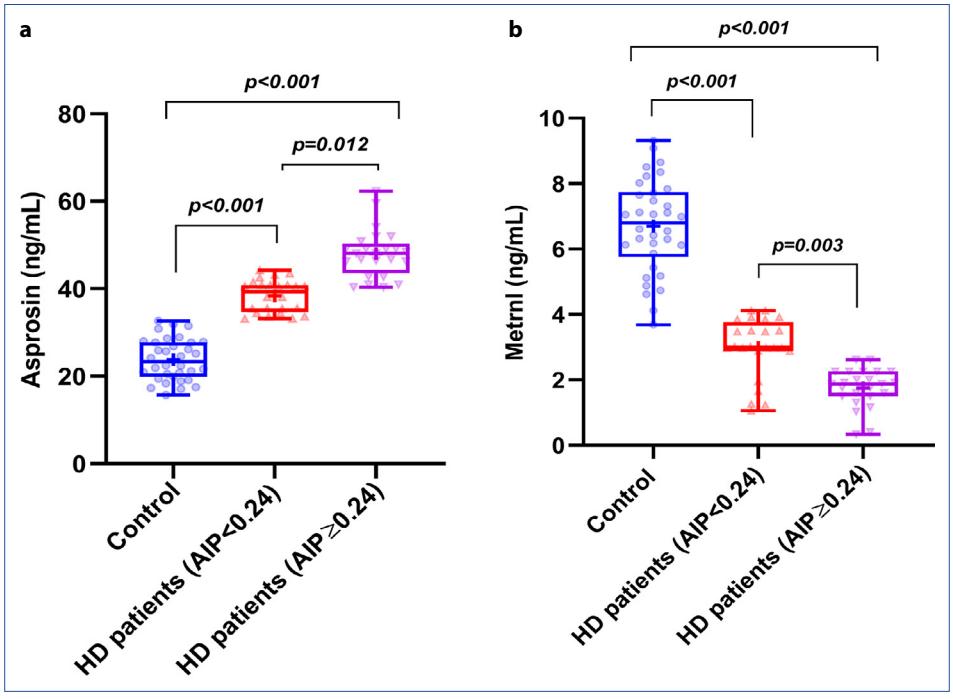

Figure 1. Graphical representation of plasma asprosin (a) and metrnl levels (b) according to AIP values of HD patients.

HD: Hemodialysis; AIP: Atherogenic index of plasma.

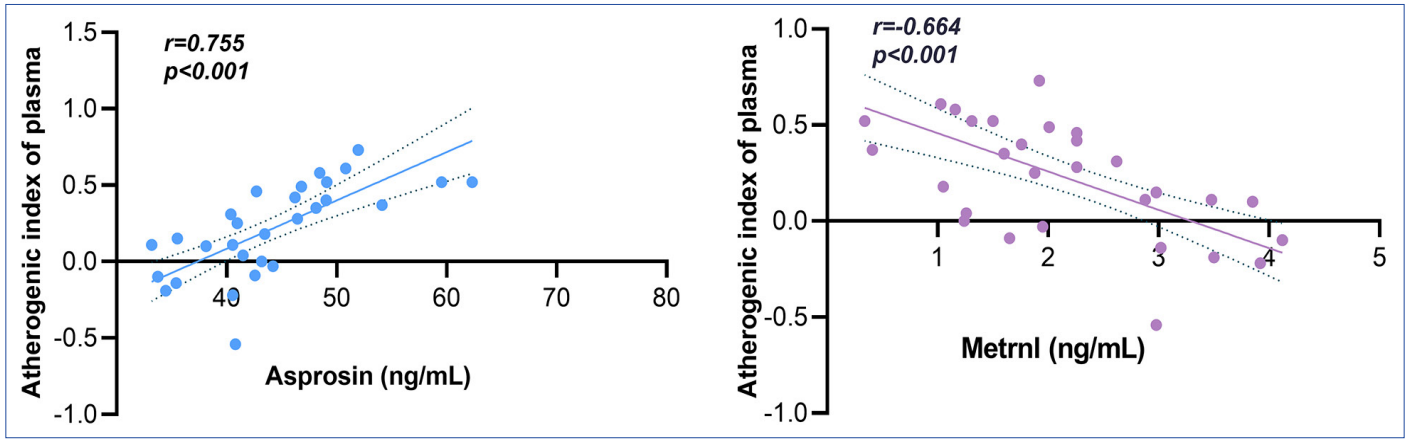

Figure 2. Graphical representation of the relationship between asprosin, metrnl and AIP index in HD patients.

-risk groups. Glucose values were significantly higher in the high-risk group than in the control group (p<0.001) (Table 1).

A significant difference was found between the control group [23.3 (19.9–27.7 ng/mL)], the low-moderate risk group [39.3 (34.9–40.8 ng/mL)] and the high-risk group [48.1 (44.5–49.9)] in terms of asprosin levels (for each p<0.001), (Fig. 1a). Asprosin values of both low- moderate risk and high-risk groups were significantly higher than the controls. Plasma asprosin levels were significantly higher in the high-risk group than in the low-moderate risk group (p=0.012). Metrnl level of the high-risk group was found to be lower than both the control and low-risk groups (p<0.001 and p=0.003, respectively), (Fig. 1b).

AIP showed a positive association with asprosin and a negative association with metrnl (Fig. 2). Metrnl demonstrated a negative correlation with BMI, glucose, TG, HbA1c, HOMA-IR, insulin, and a positive correlation with HDL-C and ferritin (Table 2). Although there was a positive correlation between asprosin and BMI, glucose, TG, P, HbA1c, HOMA-IR, and insulin, a negative correlation was found between asprosin and HDL-C, ferritin, and metrnl (Table 3).

It was observed that asprosin had an independent positive predictive value for high-risk AIP in HD patients when independent variables such as age, BMI, DM, and hypertension were included in the model (OR: 4.662, 95% CI: 1.272 to 17.09; p=0.020). It was observed that metrnl had an independent negative predictive value for high-risk AIP in HD patients

Table 2. Significant correlations between metrnl and the other variables in hemodialysis patients

Parameter	Meteorin-like protein (ng/mL)	
	r	p
AIP	–0.664	**<0.001**
Body mass index (kg/m²)	–0.457	**0.001**
Asprosin (ng/mL)	–0.845	**<0.001**
TG (mg/dL)	–0.568	**<0.001**
HDL-C (mg/dL)	0.575	**<0.001**
HOMA-IR	–0.638	**<0.001**
HbA1c (%)	–0.569	**<0.001**
Glucose (mg/dL)	–0.528	**<0.001**
Insulin (mIU/L)	–0.568	**<0.001**
Ferritin (µg/L)	0.315	**0.029**

r: Spearman correlation coefficient; AIP: Atherogenic index of plasma; TG: Triglyceride; HDL-C: High-Density Lipoprotein Cholesterol; HOMA-IR: Homeostasis Model Assessment of Insulin Resistance; HbA1c: Hemoglobin A1c.

Table 3. Significant correlations between asprosin and the other variables in hemodialysis patients

Parameter	Asprosin (ng/mL)	
	r	p
AIP	0.755	**<0.001**
Body Mass Index (kg/m²)	0.517	**<0.001**
TG (mg/dL)	0.666	**<0.001**
HDL-C (mg/dL)	–0.676	**<0.001**
HOMA-IR	0.752	**<0.001**
HbA1c (%)	0.672	**<0.001**
Ferritin (µg/L)	–0.403	**0.004**
P (mg/dL)	0.334	**0.020**
Glucose (mg/dL)	0.633	**<0.001**
Insulin (mIU/L)	0.661	**<0.001**

r: Spearman correlation coefficient; AIP: Atherogenic index of plasma; TG: Triglyceride; HDL-C: High-Density Lipoprotein Cholesterol; HOMA-IR: Homeostasis Model Assessment of Insulin Resistance; HbA1c: Hemoglobin A1c; P: Phosphorus.

Table 4. Binary logistic regression analysis for the association between asprosin or metrnl and high-risk (AIP≥ 0.24)

Univariate regression				Multivariate Regression			
Parameter	**OR**	**95% CI**	**p**	**Parameter**	**OR[a]**	**95% CI**	**p**
Metrnl (ng/mL)	0.143	0.049–0.414	**<0.001**	Metrnl (ng/mL)	0.112	0.028–0.441	**0.002**
Asprosin (ng/mL)	1.818	1.265–2.613	**0.001**	Asprosin (ng/mL)	4.662	1.272–17.09	**0.020**

[a]: Odds ratio adjusted for age, body mass index, diabetes mellitus and hypertension. AIP: Atherogenic index of plasma; OR: Odds ratio; CI: Confidence interval; Metrnl: Meteorin-like protein.

when independent variables such as age, BMI, DM, and hypertension were included in the model (OR: 0.112, 95% CI: 0.028 to 0.441; p=0.002), (Table 4).

Discussion

In this study, for the first time to our knowledge, we analyzed the levels of asprosin and metrnl, two important adipokines, in the plasma of HD patients who were divided into two groups as high (AIP≥0.24) and low-moderate risk (AIP<0.24) according to their AIP values. Plasma asprosin values in both low and high-risk groups in terms of AIP were higher than the controls. Moreover, plasma asprosin levels were significantly higher in the high-risk compared to the low-moderate risk. Since there is no difference between high and low-risk groups in terms of serum urea and creatine values, accompanying metabolic disease and CVD, we can suggest that hemodynamic and metabolic changes due to the HD process may be responsible for the increase in asprosin levels. The positive correlation between asprosin and AIP, BMI, HOMA-IR, HbA1c suggests that the patient's nutritional status and HD-related metabolic disorders could increase asprosin synthesis and release. The fact that plasma metrnl levels of patients in the high-risk group are lower than those in the low-risk group and the inverse correlation between metrnl and AIP values suggests that this adipokine may be anti-atherogenic.

Approximately one-third of patients undergoing hemodialysis for ESRD face increased morbidity and mortality due to non-renal pathologies such as nutritional, metabolic, or atherosclerotic coronary artery disease [15]. Sometimes, as a combined effect of more than one pathology, deterioration in the patient's general condition and even sudden cardiac death may occur [16]. Lipodystrophic changes occur due to fluid-electrolyte imbalance, vascular bed volume changes, and nutritional problems in HD patients who are in the risk group for cardiovascular disease [17]. Adipokines play a role in regulating metabolic pathologies and cardiac risk factors [6, 8, 10]. The reported L- or H-shaped relationship between type 2 diabetes, insulin resistance, and AIP supports a gradual association of AIP with underlying ESRD or other metabolic diseases [18].

Consistent with our results, higher asprosin levels have been reported in diabetic nephropathy. The same study reported a positive correlation between asprosin, BMI, and insulin, while glomerular filtration showed an inverse correlation with asprosin [8]. Zou et al. [19] reported that HD patients with metabolic syndrome had higher plasma asprosin levels than those without. However, we did not classify HD patients according to whether they had metabolic syndrome or not. On the other hand, most of our patients exhibited all or some of the metabolic syndrome criteria. Metabolic syndrome criteria were less

frequent in participants in the control group. When our findings and literature data are evaluated together, increased asprosin levels in HD patients can potentiate cardio-metabolic risks.

The inverse correlation between asprosin and HDL-C is critical as it indicates the atherogenic potential of asprosin. The positive correlation between plasma asprosin and API value is important evidence of our concern that high asprosin levels stimulate atherogenic risk factors in HD patients. In logistic regression analysis, there is an independent association between asprosin and high AIP values, which is a finding indicating the atherogenic potential of asprosin in HD patients. The increase in AIP value by asprosin in HD patients, even after adjustment for age, BMI, diabetes, and history of hypertension, supports that this adipokine is an atherogenic molecule independent of other parameters. Clinical approaches to the regulation of asprosin metabolism may be promising in the treatment of HD-related nutritional pathologies and lipodystrophy [7, 8]. On the other hand, the results of studies analyzing the relationships between asprosin levels and CVD are controversial. In addition to studies reporting that asprosin is cardioprotective, it has also been reported that it is ineffective in cardiac pathologies. The fact that asprosin ameliorates cardiac endothelial damage and corrects dilated cardiomyopathy in diabetic mice supports its cardioprotective nature [20–22]. However, we observed that, paradoxically, asprosin levels increased at a high AIP value.

It has been shown that metrnl is directly associated with obesity in experimental obesity models and in individuals with high BMI. Consistent with this, it has been reported that metrnl levels of mice fed a fat-rich diet are up-regulated [23]. Similarly, it has been shown that children with low BMI have lower Metrnl expression than those with high BMI [24]. The fact that metrnl has a negative correlation with BMI, HOMA-IR, and insulin, and a positive correlation with HDL-C suggests that metrnl is released into the systemic circulation from adipose tissue to prevent cardiac and metabolic pathologies in HD patients. In experimental models, metrnl administration reverses cardiac and renal fibrosis by regulating apoptosis and oxidative stress, which indicates this adipokine's cardioprotective effect [2]. Reporting that circulating and myocardial metrnl levels are downregulated in streptozotocin-induced diabetic mice supports that decreased levels of this protein in metabolic pathologies increase the risk of CVD. Increased expression of metrnl in heart muscle suggests that it could be protective against diabetes-related cardiac damage. Metrnl plays a significant role in improving complications caused by insulin resistance, diabetes, and metabolic syndrome [25]. The logistic regression analysis revealed a negative independent relationship between high AIP values and metrnl, supporting the anti-atherogenic potential of this adipokine. This finding indicated that metrnl could protect against CVD, independent of other confounders. When our results and literature data are evaluated together [25], we can clearly argue that the critical role of metrnl in cardiovascular diseases, obesity, and diabetes is also demonstrated in hemodialysis patients.

The study has several limitations, including a low sample size, a single-center cross-sectional study design, and the inclusion of only end-stage patients with chronic renal failure. Although asprosin and metrnl were recommended as potential metabolic syndrome biomarkers, the grouping of HD patients was not made according to metabolic syndrome criteria [25, 26]. Since all HD patients have ESRD and more than one metabolic risk factor, we thought it would be more appropriate to classify the patients according to AIPs. Not grouping patients according to metabolic syndrome criteria is also a limitation.

Conclusion

Our study is of clinical importance as it shows that adipose tissue-derived metrnl and asprosin secretion changes in HD patients and the relationships between these two adipokines and atherogenic potential. Logistic regression analysis has revealed important insights into the independent relationships between metrnl, asprosin, and high AIP values in HD patients. These findings support the anti-atherogenic potential of metrnl and suggest the potential atherogenic effects of asprosin, highlighting the complex interplay between adipokines and cardiovascular risk in this patient population. It will be possible to reach a more precise conclusion regarding the effects of adipokines on cardiovascular events with prospective cohort studies involving more HD patients.

Ethics Committee Approval: The study was approved by The Fırat University Non-Invasive Research Ethics Committee (No: 2023/08-31, Date: 08/06/2023).

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Research Article

Evaluation of drug abuse test analysis: One year experience

Gamze Gok[1], Turan Turhan[1,2]

[1]Medical Biochemistry Laboratory, Ankara Bilkent City Hospital, Ankara, Türkiye
[2]Department of Medical Biochemistry, University of Health Sciences, Ankara Bilkent City Hospital Health Application and Research Center, Ankara, Türkiye

Abstract

Objectives: Drug abuse is a major problem for public health and it has negative impacts on people's health. Drug analysis methods used in our country vary, but the results show that drug abuse is an increasing problem in our country compared to previous years. In our hospital, drug urine screen tests are performed to analyze amphetamines, benzodiazepines, cannabinoids, cocaine, and their metabolites, opiates, and synthetic cannabinoids. Our research aimed to evaluate the frequency of drug use according to age and gender.

Methods: A total of 2172 amphetamine, 2172 benzodiazepine, 2172 cannabinoids, 2169 cocaine and cocaine metabolites, 2168 opiates, and 1906 synthetic cannabinoid analysis results were included in our study. Analyses were performed by Advia® (Siemens Healthineers, Erlangen, Germany) autoanalyzer with a homogeneous immunoassay method that enables qualitative or semi-quantitative analysis of analytes. At every stage of our study, we worked in accordance with the Declaration of Helsinki.

Results: The results were as follows: 1989 (91.6%) were negative, 183 (8.4%) were positive for amphetamine, 1924 (88.6%) were negative, and 248 (11.4%) were positive for benzodiazepine; 2057 (94.7%) were negative and 115 (5.3%) were positive for cannabinoids; 2157 (99.4%) were negative and 12 (0.6%) were positive for cocaine and its metabolites; and 1865 (97.8%) were negative and 41 (2.2%) were positive for synthetic cannabinoids. Males' positive results for amphetamine ($p<0.001$), cannabinoid ($p<0.001$), and opiates ($p=0.026$) were statistically significant when compared according to gender. The adult group's positive results for amphetamine ($p<0.001$), cannabinoid ($p<0.001$), and synthetic cannabinoids ($p=0.046$) were statistically significant when compared according to age.

Conclusion: Sharing drug positivity situations in different age groups and genders may help to draw attention to this problem and maybe preventive. More studies including more than one year of results may be beneficial.

Keywords: Amphetamine, cannabinoids, drug addiction, DSM-5, opiates

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Addiction is defined as continuing to use a substance despite it causing mental, physical, or social problems, not being able to give up despite the desire to quit, and not being able to stop the desire to take the substance [1]. The word "addiction" originates from the Latin word "addicere," which means "to be a slave to someone" or "to devote oneself to something or someone else" [2]. When using drugs for the first time, the individual takes drugs voluntarily, using his or her own will. But as time progresses, the changes occurring in the brain affect the individual's self-control, and the individual's resistance to intense urges to take drugs is prevented [3]. Stimulant substances such as cocaine and amphetamine cause increased arousal and feelings of well-being and euphoria. The analgesic properties of opiates such as morphine and codeine allow them to be used in clinical settings. Taking high doses of these substances makes the user feel good and may lead to abuse of these substances [4]. The emergence and persistence of substance addiction in a person depends on both genetic and environmental characteristics. People who use substances may experience social, economic, personal, and professional problems. Tolerance develops in individuals, and this developing situation leads to the use of increasingly larger amounts of substances. People who use substances may have more psychological and physical problems com-

Address for correspondence: Gamze Gok, MD. Medical Biochemistry Laboratory, Ankara Bilkent City Hospital, Ankara, Türkiye
Phone: +90 505 378 67 03 **E-mail:** gamze_gok@outlook.com **ORCID:** 0000-0002-2804-5548
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pared to other people [5]. In general, substance addiction ranks fourth after occasional use, recreational use, and regular use, and it also causes a chronic condition. Substance use is an important problem for society, so substance use prevention and treatment studies are carried out. In the diagnosis of substance use, the World Health Organization (WHO) and American ICD (International Classification of Diseases) and DSM (Diagnostic and Statistical Manual of Mental Disorders) classifications published by the Psychiatric Association (APA) are used. Addiction definition from DSM-I (1952) to DSM-V has been made. The current classification today is DSM-V (2013). The DSM-V is considered the gold standard for the names, symptoms, and diagnostic features of mental illness. As indicated in the DSM-5, clinically, the situation of those diagnosed with substance use disorder (SUD) is not the same as that of discretionary substance use. According to DSM-5, to be diagnosed with SUD, graded as mild, moderate, or severe, depending on the type of substance used, criteria must be met. Ten substance classes are defined in DSM-V: Alcohol, caffeine, cannabis (hemp), hallucinogens (including phencyclidine and other hallucinogens), volatile substances (inhalants), opiates, sedative-hypnotics, stimulants, tobacco, and others (or unspecified) [6]. Drug use is prohibited in Türkiye under the provision of Article 191 of the Turkish Penal Code No. 5237 [7]. It has been stated that the rate of substance use in Türkiye is increasing day by day [8]. In our hospital, drug urine screen tests are performed to analyze amphetamines, benzodiazepines, cannabinoids, cocaine, and their metabolites, opiates, and synthetic cannabinoids. The aim of our research is to evaluate the frequency of drug use according to age and gender, to draw attention to substance addiction, and to contribute to the literature by sharing the results obtained.

Materials and Methods

In this retrospective study, the results of patients whose urines were analyzed for amphetamines, benzodiazepines, cannabinoids, cocaine and their metabolites, opiates, and synthetic cannabinoids at Ankara Bilkent City Hospital between 01.05.2022 and 31.05.2023 were included. Test results, age, and gender information of individuals who underwent urine drug test analysis were accessed from the hospital information system. The present study involves only the first result of patients who underwent more than one urine drug test analysis between the specified dates. Analyses were performed by Advia® (Siemens Healthineers, Erlangen, Germany) autoanalyzer with a homogeneous immunoassay method that enables qualitative or semi-quantitative analysis of analytes. The measurement is based on competition for antibody binding sites between the substance and the enzyme glucose 6 phosphate dehydrogenase (G6PDH). The enzyme activity decreases after binding to the antibody, thus making it possible to measure the concentration of the substance in the sample as enzyme activity. In the presence of Glucose 6 phosphate (G6P), the active enzyme converts nicotinamide adenine dinucleotide (NAD) to reduced nicotinamide adenine dinucleotide (NADH), causing an absorbance change that can be measured spectrophotometrically at 340/410 nm. At every stage of our study, we worked in accordance with the Declaration of Helsinki.

Statistical analyses

Descriptive statistics data were expressed as mean, standard deviation, and numerically, and categorical variables were expressed as percentages. A chi-square test was performed for comparing categorical data among the groups. A p-value <0.05 was accepted as statistically significant. Statistical analyses were performed by IBM SPSS Statistics for Windows, Version 27.0 (IBM Corp., Armonk, NY, USA).

Results

A total of 2172 amphetamine, 2172 benzodiazepine, 2172 cannabinoids, 2169 cocaine and cocaine metabolites, 2168 opiates, and 1906 synthetic cannabinoid analysis results were included in our study. The data obtained in the study were evaluated separately according to gender and age groups: pediatric (0–18 years), adult (18–64 years), and geriatric (65 years and over) for each test. The mean and standard deviations of the pediatric, adult, and geriatric age groups in the analyses of amphetamine, benzodiazepine, cannabinoids, cocaine and cocaine metabolites, and opiates were the same. They were as follows: 15 (±3.81), 33.9 (±11.2), 74.1 (±7.51), respectively.

The mean and standard deviations of the pediatric, adult, and geriatric age groups of Synthetic cannabinoids were as follows: 15 (±3.6), 34 (±11.2), and 73.9 (±7.49) respectively. The minimum substance level values expressed as minimum drug levels as threshold values to be considered drug-positive used in the analysis were 500 µg/L for amphetamine, 300 µg/L for benzodiazepine, 50 µg/L for cannabinoids, 150 µg/L for cocaine and its metabolites, 2000 µg/L for opioids, and 5 µg/L for synthetic cannabinoids. Drug Abuse Test Analysis Data is presented (Table 1).

The positive results of amphetamine 35 (19.1%), benzodiazepine 170 (68.5%), cannabinoid 98 (85.2%), cocaine and its metabolites 11 (91.7%), opiates 31 (83.8%), synthetic cannabinoids 32 (78%) were all higher in males. Males' positive results of amphetamine ($p<0.001$), cannabinoid ($p<0.001$), and opiates ($p=0.026$) were statistically significant (Table 2). There were no positive results for the pediatric group of synthetic cannabinoids and the geriatric groups of amphetamine, cannabinoids, cocaine and its metabolites, and opiates.

The positive results of amphetamine 178 (97.3%), benzodiazepine 199 (80.2%), cannabinoid 113 (98.3%), cocaine and its metabolites 10 (83.3%), opiates 36 (97.3%), synthetic cannabinoids 40 (97.6%) were all higher in each test's adult groups and the positive results of amphetamine ($p<0.001$), cannabinoid ($p<0.001$), and synthetic cannabinoids ($p=0.046$) were statistically significant (Table 3).

Table 1. Drug abuse test analysis data

Analytes	Negative		Positive	
	n	%	n	%
Amphetamine (µg/L)	1989	91.6	183	8.4
Benzodiazepine (µg/L)	1924	88.6	248	11.4
Cannabinoid (µg/L)	2057	94.7	115	5.3
Cocaine and its metabolites (µg/L)	2157	99.4	12	0.6
Opiates (µg/L)	2131	98.3	37	1.7
Synthetic cannabinoids (µg/L)	1865	97.8	41	2.2

Table 2. Drug abuse test analysis data among genders

	Female		Male		p*
	n	%	n	%	
Amphetamine (µg/L)					
Negative	689	34.6	1300	65.4	<0.001
Positive	35	19.1	148	80.9	
Benzodiazepine (µg/L)					
Negative	648	33.7	1276	66.3	=0.484
Positive	78	31.5	170	68.5	
Cannabinoid (µg/L)					
Negative	708	34.4	1349	65.6	<0.001
Positive	17	14.8	98	85.2	
Cocaine and its metabolites (µg/L)					
Negative	721	33.4	1436	66.6	0.072
Positive	1	8.3	11	91.7	
Opiates (µg/L)					
Negative	715	33.6	1416	66.4	0.026
Positive	6	16.2	31	83.8	
Synthetic cannabinoids (µg/L)					
Negative	618	33.1	1247	66.9	0.132
Positive	9	22	32	78	

*: Chi-square Test.

Discussion

Drug abuse including opiates, amphetamine derivatives, and cannabis is a major problem for public health [9]. According to the results of the research conducted by the Turkish Drug and Drug Addiction Monitoring Center in 2011; the rate of trying any illegal addictive substance at least once in the population for individuals between the ages of 15–64 was 2.7% (3.1% for males, 2.2% for females), it was 1.5% for individuals between the ages of 15–16 (2.3% for males, 0.7% for females) [10]. Each test in our research was evaluated in 3 groups according to age: pediatric (0–18 years), adult (18–64 years), and geriatric (65 years and over). The use of amphetamine first began in the 1930s, and its use in different areas of medicine was investigated in the following years [11]. Amphetamine, which has a strong stimulating effect on the central nervous system, is used for the treatment of central nervous system disorders such as attention deficit hyperactivity disorder and narcolepsy [12]. Amphetamines have appetite suppressant effects and, due to this effect, they can also be used for weight loss purposes [13]. Amphetamines, also known as "speed," are synthetic psychoactive substances that provide pleasure [14]. They can be easily synthesized from cheap and readily available chemicals, which plays a role in the spread of amphetamine addiction and abuse [15]. In our study, amphetamine was the second most positive drug and it was found to be higher in men (p<0.001) when compared according to gender, and in the adult group (p<0.001) when compared according to age groups. Karakükçü et al. [16] stated that illicit drug use was higher in men than in women and that the highest use was between the ages of 20 and 29, in their study.

A face-to-face study indicated that men's prevalence of substance use is higher than women's and the frequency of substance use is higher in the 15–24 age group than in those over 25 years of age [17]. Our results were consistent with all these studies [13, 14].

Benzodiazepines are known as the most commonly prescribed drugs in the world, such as anesthetics, tranquilizers, hypnotics, anticonvulsants, or muscle relaxants to treat depression, anxiety, insomnia, and epilepsy [18]. In our study, the most positive results were benzodiazepines. This may be due to its medical use and/or its illicit use. In an epidemiological study conducted

Table 3. Drug abuse test analysis data among age groups

	Pediatric group		Adult group		Geriatric group		p*
	n	%	n	%	n	%	
Amphetamine (µg/L)							
Negative	255	12.8	1634	82.3	97	4.9	<0.001
Positive	5	2.7	178	97.3	0	0	
Benzodiazepine (µg/L)							
Negative	227	11.8	1615	83.9	82	4.3	=0.273
Positive	34	13.7	199	80.2	15	6	
Cannabinoid (µg/L)							
Negative	259	12.6	1701	82.7	97	4.7	<0.001
Positive	2	1.7	113	98.3	0	0	
Cocaine and its metabolites (µg/L)							
Negative	258	12	1802	83.5	97	4.5	=0.684
Positive	2	16.7	10	83.3	0	0	
Opiates (µg/L)							
Negative	259	12.2	1775	83.3	97	4.6	=0.073
Positive	1	2.7	36	97.3	0	0	
Synthetic cannabinoids (µg/L)							
Negative	215	11.5	1559	83.6	91	4.9	=0.046
Positive	0	0	40	97.6	1	2.4	

*: Chi-square Test.

in Germany, the prevalence of benzodiazepine dependence was 1.3% among women and 1.4% among men according to the DSM-IV criteria [19]. In our study, positive benzodiazepine results were higher in men and the adult group but statistically, there was no difference among the genders and the age groups. For this reason, our results may not be accepted as in line with the previous studies [16, 17, 19].

Indian hemp 'Cannabis sativa', which has been used for relaxing purposes for approximately 4000 years, has antiemetic, analgesic, anticonvulsant, and intraocular pressure-lowering effects and is today used in many diseases such as Huntington's, multiple sclerosis, epilepsy, Alzheimer's, and Parkinson's [20]. The common name for cannabinoids is marijuana, and it is one of the most illegal substances that are widely produced and consumed for malicious purposes [21]. Cannabinoids are divided into three groups: natural, endogenous, and synthetic. Delta 9-tetrahydrocannabinol is the best-known and most abundant natural cannabinoid and is also the main component of marijuana. Anandamide, arachidonylglycerol, noladinether, virodamine, and N-arachidonyldopamine are endogenous cannabinoids [20]. Synthetic cannabinoids are produced in laboratories and their numbers are increasing daily. Marijuana and synthetic cannabinoids both act on the same receptors, but synthetic cannabinoids are much more potent than marijuana. Long-term use of synthetic cannabinoids may play a role in the emergence of severe psychological and physical symptoms in people [22]. Many studies show that men use drugs more than women [16, 17, 23, 24]. Our study was in line with the previous studies presenting higher positive cannabinoid (p<0.001) results for males [16, 17, 23, 24]. Gökler and Koçak stated in their article that gender and age factors are effective in substance use, that the 12–17 age period is the most dangerous period in drug use, and that the number of addicts increases in the 17–25 age period [24]. In our study, the positive results of cannabinoids (p<0.001), and synthetic cannabinoids (p=0.046) were higher in the adult group consistent with their article [24].

Cocaine, which has stimulant properties, is obtained from the Coca plant. Cocaine suppresses the neuronal reuptake of monoamine neurotransmitters serotonin, noradrenaline, and dopamine and increases the concentration of these neurotransmitters in the synaptic gap [25]. The psychic addiction to cocaine is strong [26]. Friedman and Eisenstein indicate cocaine use as an epidemic in their review article [27]. Some studies found higher cocaine usage in women compared to men [28, 29] and some others found just the opposite [30–32]. In our study, positive cocaine results were higher in men and the adult group but statistically, there was no difference among the genders and the age groups. For this reason, our results may not be accepted as in line with the previous studies [28–30].

Heroin, morphine, and some other types of opiates are drugs that are effective in the clinical management of chronic pain [33]. It was stated that men have higher opioid use misuse rates In our study males' positive results of opiates (p=0.026) were statistically significant and consistent with the literature [34]

Strengths and limitations

There are survey studies conducted to investigate drug use in our country. These studies, generally carried out in psychiatric clinics, aim to obtain information about the drugs used and their frequency of use [16]. The fact that drug use is prohibited under Turkish law may have prevented correct answers to the questions. The other strengths of our study are that we evaluated each analyte we analyzed in urine according to gender and age groups and that we included only the first result of each patient. In our hospital, urine drug analysis tests are not performed under strict monitoring, so urine samples are open to dilution and replacement with samples belonging to another person. These are the limitations of our study.

Conclusion

Drug abuse has negative impacts on people's health. Drug analysis methods used in our country vary, but the results are similar. However, compared to other European countries or the United States, the prevalence of substance use in our country is still lower; it is an increasing problem in our country compared to previous years [17]. We think that sharing drug positivity situations in different age groups and genders may help to draw attention to this problem and may be preventive. More studies including more than one year of results may be beneficial.

Ethics Committee Approval: The study was approved by The Ankara Bilkent City Hospital no 1 Clinical Research Ethics Committee (No: E1-23-3776, Date: 12/07/2023).

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Research Article

IL28B rs12979860 gene polymorphism and sofosbuvir-based therapy response in HCV-infected Pakistani patients

Maleha Asim[1], Anoshia Javed[1], Ammarah Saeed[2], Bushra Adeel[3], Shazia Siddique[4], Saddaf Ayub[5]

[1]Department of Biochemistry, Islamic International Dental College, Riphah International University, Islamabad, Pakistan
[2]Department of Medicine, PAF unit 2, Fazaia Medical College, Air University, Islamabad, Pakistan
[3]Department of Pathology, Rashid Latif Medical College, Lahore, Pakistan
[4]Department of Biochemistry, Sahiwal Medical College, Sahiwal, Pakistan
[5]Department of Biochemistry, Islamic International Medical College, Riphah International University, Rawalpindi, Pakistan

Abstract

Objectives: Interleukin-28B (IL-28B) gene polymorphisms play an important role in response prediction of direct-acting antivirals (DAAs) treatment, including Sofosbuvir and Daclatasvir with or without Ribavirin. The purpose of this study was to assess the IL-28B polymorphism SNP (rs12979860) and other clinical factors as response predictors for the sustained virological response (SVR) in chronic HCV-infected patients taking DAA therapy.

Methods: A cross-sectional and observational study was carried out among 104 HCV-infected patients who completed a course of Sofosbuvir and Daclatasvir along with Ribavirin. Patients were classified according to their response to therapy. Genotyping of IL-28B was determined through polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay, and HCV genotyping was identified by PCR method. We analyzed the response prediction of IL-28 gene polymorphism among patients receiving DAA therapy.

Results: Overall, IL-28B CC, CT, and TT genotypes were found in 56 (53.8%), 22 (21.2%), and 26 (25.0%) patients, respectively. Higher early virological response (EVR) and SVR were observed in patients with the rs12979860 CC alleles (82.1% and 75%) as compared to CT/TT alleles (54.2% and 20.8%). IL-28B CC genotype (OR=0.14; 95% CI=0.04-0.44; p=0.001) and EVR (OR=0.20; 95% CI=0.05-0.71; p=0.013) remained significantly associated with SVR in the multivariate regression analysis. However, the FIB-4 score (OR=4.24; 95% CI=1.46-11.75; p=0.008) is a strong predictor of non-SVR.

Conclusion: The antiviral efficacy of triple therapy (sofosbuvir, daclatasvir, and ribavirin) is influenced by the variability of the IL-28B gene, as well as the EVR and FIB-4 score. These variables also play a significant role in predicting the treatment response of patients with chronic HCV infection in Pakistan.

Keywords: Early virological response, HCV genotype, hepatitis C virus, IL-28B gene, sustained virological response

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With an estimated 71 million cases of chronic hepatitis C virus (HCV) infection worldwide, 80% of the burden is centered in lower-middle income nations, making this disease a serious global health concern [1]. Furthermore, chronic HCV is thought to be the primary risk factor for cirrhosis (15–35%), a condition that results in decompensated liver damage and eventual mortality [2]. In Pakistan, where a countrywide survey carried out in 2007–2008 estimated the prevalence of HCV at 4.8%, the virus is extremely prevalent. As part of its efforts to eradicate HCV infection, the World Health Organization (WHO) has set 2030 targets for a 65% decrease in HCV-associated death and an 80% decrease in international incidence [3].

For the past 20 years, combination therapy consisting of ribavirin and pegylated interferon alfa has been considered the gold standard treatment for HCV infection. However, this treat-

Address for correspondence: Anoshia Javed, MD. Department of Biochemistry, Islamic International Dental College, Riphah International University, Islamabad, Pakistan
Phone: +923215300115 **E-mail:** anoshiajavad@gmail.com **ORCID:** 0000-0002-2845-0543
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ment plan was hampered by the frequent incidence of adverse effects that made it difficult to adhere to the prescribed course of action, which led to substantially elevated rates of treatment cessation and failure [4]. Direct-acting antiviral drugs (DAAs), like NS5A complex, NS5B polymerase, and NS3/4A protease inhibitors, have been available for a decade now and have shown promise in lowering viral levels in patients with different genotypes of HCV while remaining safe [5, 6].

The management of chronic hepatitis C is influenced by various parameters, including the level of HCV-RNA, the stage of hepatic fibrosis, and the genetic status of the host, despite the progress made in viral pathogenesis and treatment [7]. The IL-28 gene is also known as interferon-gamma 3 because it codes for the protein known as interferon-gamma 3, which is encoded by the cytokine gene Interleukin-28B (IL-28B), one of the host genetic factors [8, 9].

According to recent research, genetic variations in IL-28B may impact the way that various infectious diseases decrease HCV replication and produce the cytokines that are linked to it, which modify the host immune response [3, 10]. A SNP (rs12979860), which is situated just three kilobases upstream from the IL28B gene, has been identified by genome-wide association studies as a critical factor affecting the HCV clearance from infected patients [11, 12].

The disparities in treatment response rates observed between Caucasians, African-Americans, and Asians are mostly caused by one significant factor, which is the differences in allele frequencies among ethnic groups [13]. In HCV genotype-1, the IL-28B gene polymorphisms were found to be a significant predictor of both a sustained and rapid virological response to treatment. Similarly, compared to patients with non-CC genotypes, those with HCV genotype-3 who followed the treatment plan and had the CC genotype of rs12979860 were more than twice as likely to respond favorably to therapy [14]. It has been reported that the IL-28 CC gene polymorphism is more common in Pakistan than the CT/TT genotype, yet there are minor variances in the incidence of other genotypes in different parts of the country [15]. However, little research has been done on how this genetic difference affects how well DAA medication works in patients with HCV.

In this study, we aimed to evaluate the association between viral influential factors and IL-28B rs12979860 genetic variation in response to the DAAs therapy outcome in chronic HCV-infected patients.

Materials and Methods

This was a cross-sectional and observational study enrolling one hundred and four HCV-diagnosed patients who had received the specific combination therapy of sofosbuvir and daclatasvir from three different tertiary care hospitals in Islamabad, Pakistan, between April 2018 and August 2020. Before beginning this study, we obtained approval, by the Declaration of Helsinki and Nuremberg Code [16], from the Ethical Committee of Army Medical College, Rawalpindi (letter number 02/CREAM-A/Maleha). The 104 patients were divided according to their response to DAA therapy into a responder group (n=52) and a non-responder group (n=52). Informed consent was taken from each patient, and they were informed about the outcome of the study. Inclusion criteria were: 1) Positive with HCV-RNA with a lower limit of detection of 600 IU/mL, 2) Age not less than 18 years, and 3) Patients who have completed 12 weeks of sofosbuvir and daclatasvir combination therapy. Patients positive for HIV or hepatitis (A, B, D, or E) infection and evidence of metabolic, genetic, or autoimmune liver disease were excluded from this study.

All patients underwent a comprehensive medical assessment, which included gathering a detailed medical history, conducting clinical examinations, and performing anthropometric measurements such as body weight and height. Peripheral blood samples were collected and routine investigation was performed, including serum hemoglobin, albumin, platelets count, and liver function tests (serum bilirubin, ALT, AST & ALP) using kit Innoline by Martin Dow Marker Specialties (Pvt.) Ltd, and results were recorded carefully in the liver clinic of Holy Family Hospital, Pakistan.

FIB-4 index was calculated for each participant using: FIB-4=[age (yr) × AST (IU/L)/PLT (10^9/L) × √ALT(IU/L)]. Significant fibrosis was associated with a positive predictive value when the FIB-4 index was greater than 3.25, whereas severe fibrosis was associated with a negative predictive value when the index was less than 1.45 [17, 18].

All RNA preparation and HCV RNA determination steps were carried out under RNase-free conditions. HCV RNA quantification was determined by Smart Cycler II system (Applied Biosystem, Foster City, Calif; detection limit 20 IU/ml). Positive RT-PCR cases underwent HCV genotype detection using a specific HCV genotyping assay as described earlier [19]. An internal control was employed to amplify each sample, and both positive and negative controls were incorporated in each tested batch.

The major endpoint was the assessment of SVR, as responder patients who attained sustained virological response (defined as the absence of HCV RNA from serum by a sensitive PCR assay 12 weeks following discontinuation of therapy). Non-responder patients were with detectable (>2 log10) HCV RNA after 12 weeks of end of therapy. Relapse defined as patients with recurrence of HCV RNA levels during the follow-up evaluation after therapy is discontinued. Another term, EVR (early virological response), is defined as a ≥2 log reduction or complete absence of serum HCV RNA at week 12 of therapy compared with the baseline level [20].

PCR amplification and sequencing of rs12979860 single-nucleotide polymorphism was used for genotyping of IL28B. Blood samples from all patients were collected and subjected

to RNA extraction by RNA Mini prep Super Kit (Bio Basic Inc, Canada). First strand cDNA was synthesized by Revert-Aid Premium First Strand cDNA Synthesis Kit (Thermo Scientific Inc,#K1652, USA), using RNA as a template. The polymerase chain reaction-based restriction fragment length polymorphism assay was used for genotyping IL-28B rs12979860 SNP [21]. The TaqMan custom-designed primers and probes used for the genotyping procedure were; IL-28B forward and reverse primer: 5'-GCTTATCGCATACGGCTAGG-3' and 5'-AGGCTCAGGGTCAATCACAG-3'. The PCR mixture had a total volume of 20 µl, comprising 1 µl of DNA template (ranging from 120 to 480 ng), each primer (1 µl), and commercial Super Mix (10 µl), which included Taq polymerase, dNTPs, magnesium chloride, 10 x PCR buffer, and Syber green fluorescent dye. Before conducting the PCR reactions, genomic DNA extraction from whole blood samples was done by the non-enzymatic salting-out method. The mixture was loaded into 96-well MicroAmp Optical Reaction Plates (Applied Biosystems). All Real-time PCR reactions were carried out on the 7500 Fast Real-Time PCR System. The procedure involved initial denaturation for 5 minutes at 95°C, then 35 cycles at 95°C for 30 seconds, annealing at 62°C for 30 seconds, and extension at 72°C for 30 seconds with a final extension at 72°C for 10 min. On 2% agarose gel electrophoresis, bands of 160 and 82 bp indicated the TT genotype, 135, 82, and 25 bp bands indicated the CC genotype; 160, 135, 82, and 25 bp bands indicated the CT genotype and were visualized by a gel doc unit (Bio-Rad, Hercules, CA, USA).

Statistical analysis

Statistical analyses were performed using SPSS v. 26.0. Qualitative variables were measured by number and percentage of patients, whereas mean and standard deviations (SDs) were used to describe quantitative data. Student's t-test was applied to compare independent samples from two groups, and the χ^2 test was performed to compare categorical data. The association between IL-28B polymorphisms and HCV genotyping with SVR, EVR, and relapse was evaluated using a one-way analysis of variance (ANOVA) test. Multivariate logistic regression analysis was performed to determine the role of genetic and bio-clinical variables as predictors of treatment outcomes. A 95% confidence interval (CI) was included in the calculation and reporting of odds ratios (ORs). A p-value with two tails less than 0.05 was deemed statistically significant.

Results

A total of 104 HCV-infected patients participated in the study with a male predominance of 68.3%. The average age was 42.15 years with a standard deviation of 3.78 years. Fifty-two (50%) patients failed to achieve SVR among the 104 total enrolled and have been labeled as non-responders. Among responders, 35 participants (67.7%) were male and 16 (32.7%) were females, however, among the non-responder group, 36 participants (69.2%) were males and 16 (30.8%) were females. The most prevalent IL-28B genotype and HCV genotype were genotype CC (53.8%), and genotype 3 (76%) in our test population.

When the study groups' laboratory parameters were taken into account, significant variations in serum ALT levels (p=0.046) and FIB-4 score (p<0.001) were found between the responder and non-responder groups. Other pretreatment factors that were found to be associated with achieving a positive virological response were low baseline HCV-RNA level <0.4 log6 IU/ml (p=0.020), HCV genotype (p=0.01), and EVR (p<0.001). There were no significant differences in gender, age, hemoglobin concentration, serum albumin, serum bilirubin, serum AST, BMI, and platelet count between these groups (p>0.05). More detailed information about these 104 patients can be found in Table 1.

Regarding IL-28B genotype frequency, a comparison was made between IL28B CC [56 out of 104 patients (53.8%)] and IL28B CT/TT [48 out of 104 patients (46.2%)]. The percentage of early response rate among patients with IL28B CC genotype was 82.1% as compared to 54.2% of the CT/TT genotypes (p=0.002). Important laboratory parameters including ALT and FIB-4 score had a highly statistically significant relationship between the IL-28B genotypes (p=0.001 and p=0.026 respectively). A statistically significant relation was determined between IL-28B (rs12979860) SNP and HCV genotype with 91.1% of IL-28B CC having HCV genotype 3 (p=0.001). However, the relationship between IL28B (rs12979860) and relapse in patients was insignificant with a 10.4% relapse rate among IL-28CT/TT patients and a 1.8% in IL28B CC (p=0.07). Furthermore, as depicted in Table 2, there was a statistically insignificant relation between the two groups when other biochemical parameters were compared such as BMI (p=0.808), hemoglobin concentration (p=0.057), serum bilirubin (p=0.399), serum albumin (p=0.941), platelet count (p=0.268) and serum AST (p=0.177).

Multivariate logistic regression analysis was conducted, which included IL-28B genotypes, HCV genotype, EVR, and various biochemical variables, such as serum ALT value, FIB-4 score, and HCV-RNA levels (which showed significant associations with SVR in the univariate analyses). FIB-4 score (OR=4.26; 95%CI=1.46–11.75) remained significantly associated with failure to achieve SVR to therapy, whereas EVR (OR=0.20; 95%CI=0.05–0.71) and IL-28B CC genotype (OR=0.14; 95%CI=0.04–0.44) remained significantly associated with response to DAA therapy as shown in Table 3.

Figure 1 depicts the frequency of IL28B genotype distribution according to response rate. It also gives a picture of the ongoing treatment virological response rate and relapse rate by HCV genotype. In SVR, the frequency of the IL-28B rs12979860 CC genotype was 42 out of 52 (80.8%), while in EVR, it was 46 out of 72 (63.9%). The frequency of HCV genotype 3 was considerably greater in the SVR (n=48/52, 92.3%) and EVR (n=61/72, 84.7%) groups.

Discussion

HCV is a significant global public health concern, but it is especially so in Pakistan. Pakistan has the highest death rate among the most severely affected countries. Direct-acting

Table 1. Univariate analysis of non-genetic & genetic characteristics of Pakistani patients with chronic hepatitis C infection

Characteristics	Responders n=52		Non-responders n=52		p
	n	%	n	%	
Gender					0.500
Male	35	67.3	36	69.2	
Female	17	32.7	16	30.8	
Age (years) (mean±SD)	40.04±5.43		42.52±7.745		0.062
min–max	32–62		30–62		
Hemoglobin (g/dl) (mean±SD)	13.9±1.69		13.89±1.88		0.880
min–max	11.0–17.2		10.9–17.9		
Albumin (g/L) (mean±SD)	4.37±0.43		4.29±0.53		0.479
min–max	3.40–4.92		3.20–5.00		
Bilirubin (mg/dl) (mean±SD)	0.71±0.28		0.80±0.29		0.136
min–max	0.36–1.34		0.36–1.56		
ALT (IU/L) (mean±SD)	33.71±11.53		40.40±20.21		0.046
min–max	14–82		16–134		
AST (IU/L) (mean±SD)	37.15±14.41		44.90±16.24		0.822
min–max	12–80		16–104		
BMI (kg/m^2) (mean±SD)	29.11±3.59		30.62±4.07		0.320
min–max	22.6–34.2		23.3–42.4		
Platelet (×10^9/L) (mean±SD)	209.15±71.40		208.25±69.44		0.094
min–max	102–356		128–387		
Pre-treatment viral load (log_{10} IU/ml)	1.35±0.64		1.79±1.1		0.020*
(mean±SD) min–max	0.65–5.24		0.21–8.65		
FIB-4 score (mean±SD)	0.92±0.48		1.59±0.46		<0.001**
min–max	0.33–2.08		0.33–1.97		
EVR					
Yes	46	88.5	26	50.0	<0.001**
No	06	11.5	26	50.8	
IL-28B gene polymorphism					
CC	42	80.8	14	26.9	<0.001
CT/ TT	10	19.2	38	73.1	
HCV genotype					
3	45	57.0	34	43.0	0.010
Non-3	07	28.0	18	72.0	

Data expressed as mean±SD or n (%) as appropriate. *: p<0.05: Statistically significant; **: p≤0.001: Highly significant statistically. SD: Standard deviation; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index; FIB-4: Fibrosis-4; EVR: Early virological response; IL28-B: Interleukin 28-B; HCV: Hepatitis C virus.

antivirals (DAAs), which have recently been developed, have dramatically changed HCV therapy by increasing virus eradication rates to around 90% in a variety of patients globally [22]. The global influence of DAAs on the total burden of disease remains limited, notwithstanding these advancements. A significant portion of people with long-term HCV infection remain at risk for serious liver problems such as hepatocellular carcinoma and liver cirrhosis [23].

The current study thoroughly examined the relationship between host genetic variations and clinical factors and the sustained virological response to DAAs. Differential clinical outcomes in response to DAAs against HCV infection are connected with factors related to viruses (such viral load) and hosts (SNPs). In this study, CC (53.8%) was the most common genotype for IL-28B rs12979860 among patients with chronic hepatitis C, followed by TT (25%) and CT (21.2%). These results corroborate those of Calisti et al. [24], who found that among chronic HCV patients, regardless of treatment-naive or experienced, the CC genotype was most common. More research is necessary to fully understand the connection because the precise mechanism is still unknown.

Table 2. Correlation of IL-28B genotypes with baseline demographics and clinical data of HCV-infected patients

Characteristics	IL-28BCC n=56		IL-28B CT/TT n=48		p
	n	%	n	%	
Gender					
Male	40	71.4	31	64.6	0.296
Female	16	28.6	17	35.4	
Age (years) (mean±SD)	41.27±6.53		41.29±7.11		0.986
min–max	32–62		30–62		
Hemoglobin (g/dl) (mean±SD)	13.6±1.64		14.28±1.89		0.057
min–max	10.9–17.9		10.9–17.9		
Albumin (g/L) (mean±SD)	4.34± 0.500		4.33±0.47		
min–max	3.30–5.00		3.20–5.00		0.941
Bilirubin (mg/dl) (mean±SD)	0.83±0.27		0.88±0.35		
min–max	0.36–1.34		0.36–1.56		0.399
ALT (IU/L) (mean±SD)	32.14±12.98		42.79±19.54		0.001
min–max	14–82		16–134		
AST (IU/L) (mean±SD)	40.54±14.66		44.81±17.40		0.177
min–max	12–80		16–104		
BMI (kg/m^2) (mean±SD)	30.04±3.23		29.85±4.41		0.808
min–max	22.6–36.4		22.8–42.4		
Platelet (×10^9/L) (mean±SD)	215.79±74.95		200.44±63.77		0.268
min–max	102–356		102–356		
Pre-treatment viral load (log$_{10}$ IU/ml)	1.414±1.07		1.75±0.79		0.078
(mean±SD) min–max	0.21–5.65		1.0–8.24		
HCV genotype					
3, no (%)	51	91.1	31	64.6	0.001
Non-3, no (%)	05	8.9	17	35.4	
FIB-4 score (mean±SD)	1.00±2.50		1.22±3.49		0.026
min-max	0.33–1.48		0.33 – 2.41		
EVR					
At week 4	46	82.1	26	54.2	0.002
No	10	17.9	22	45.8	
Response to treatment					
Yes	42	75	10	20.8	<0.001
No	14	25	38	79.2	
Relapse					
Yes	01	1.8	05	10.4	0.071
No	55	98.2	43	89.6	

Data expressed as mean SD or n (%) as appropriate. IL28-B: Interleukin 28-B; SD: Standard deviation; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BMI: Body mass index; HCV: hepatitis C virus; FIB-4: Fibrosis-4; EVR: Early virological response.

According to IL-28B polymorphism-based analyses, the IL-28B (rs12979860) CC variation was found to be significantly correlated with the rate of response to therapy (42/52, 80.8%) and EVR (46/72; 63.9%) when compared to patients infected with IL-28 CT and TT genotypes (p<0.001 and p=0.001, respectively). According to Abd Alla et al. [25], compared to non-CC genotypes, the pre-treatment detection of the IL-28B CC allele could indicate an increased probability of full clearance of the HCV. The TT genotype was significantly more expressed in relapse-prone patients (60%; p=0.116), indicating that the genotype is thought to be a risk factor for relapse following a first response to DAA treatment. According to Zaki SM, the current findings were consistent [1]. They demonstrated that the likelihood of non-response was considerably elevated by the TT and CT genotypes of IL-28B, respectively, by 10.364 and 8.768 folds. On the other hand, a previous finding demonstrated a different expression, with IL-28B CT being the most common genotype in the SVR group and demonstrating a statistically significant cor-

Table 3. Predictors of sustained virological response among HCV-infected patients after sofosbuvir and daclatasvir treatment

Predictors	ß	p	Odd ratio	95% CI for odds	
				Lower	Upper
IL-28B CC genotype	–1.94	0.001	0.14	0.04	0.44
ALT (IU/L)	0.003	0.83	0.99	0.96	1.02
HCV Genotype	–1.29	0.08	0.27	0.06	1.20
EVR	–1.57	0.013	0.20	0.05	0.71
FIB-4 Score	1.45	0.008	4.26	1.46	11.75
Pre-treatment viral load (log_{10} IU/ml)	0.10	0.75	1.11	0.64	1.93
Constant	1.58	0.23	4.88		

The reference category is non-responder. HCV: Hepatitis C virus; CI: Confidence interval; ALT: Alanine aminotransferase; HCV: Hepatitis C virus; EVR: Early virological response; FIB-4: Fibrosis-4.

relation with triple therapy consisting of peg-interferon, sofosbuvir, and ribavirin [5, 26]. The discrepancies in treatment plans, HCV genotypes, and the features of the population under study are probably to blame for the inconsistent outcomes [27].

The FIB-4 is a noninvasive diagnostic tool to evaluate the presence of fibrotic liver. Our data confirmed that the patient's FIB4-scores are recognized as the strongest pre-treatment predictor of sustained viral clearance (p=0.008; OR=4.26). Presence of advanced fibrosis and cirrhosis seems to remain the strongest pretreatment predictor of response. In the study by Watanabe et al. [28], patients with high FIB-4 index developed hepatocellular carcinoma after DAAs therapy. Statistical analysis revealed that early virological response could be a predictive marker for the response rates of ongoing antiviral therapy (p=0.013; OR=0.20). Patients having low baseline viral load showed higher response rates as compared to patients who have higher baseline viral load (p=0.02). This finding points to a strong, independent correlation between SVR and the baseline viral load along with EVR which was also proved by similar other studies [29, 30]. Patients with HCV genotype 3 had a significantly higher frequency of rs12979860 genotype CC compared to those with HCV genotype non-3 (91.1% vs. 8.9%). Recent data, several previous studies reported that the patients with HCV genotype 3 had a higher incidence of IL-28B CC genotype than those with HCV genotype non-3 [31]. The underlying mechanism of this association may be either due to rs12979860 C allele carriers having higher rates of HCV genotype 3 infection, or that carriers of the CC genotype are more susceptible to HCV genotype 3a/b infection [14, 32].

In Pakistan, genotype-3 is the most common, followed by genotype-1. In the current study, HCV genotype-3 was found to be more prevalent among HCV-infected patients, a finding consistent with the study done by Tayyab et al. [33]. The frequency of HCV genotype 3 in patients attaining early virological response (61/72, 84.7%) and sustained virological response (48/52, 92.3%) is higher than other non-3 genotypes (p=0.001 and p=0.028,

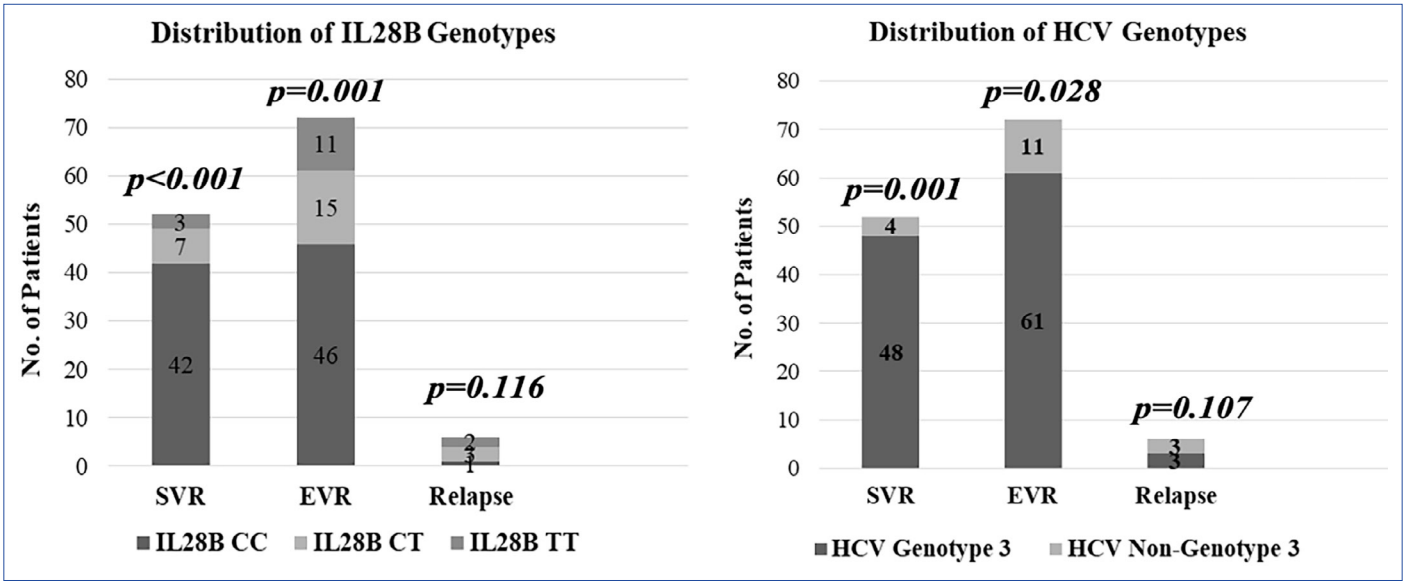

Figure 1. Distribution of IL-28B polymorphism and HCV genotype in patients with SVR, EVR, and Relapse.

IL28-B: Interleukin 28-B; HCV: Hepatitis C virus; SVR: sustained virological response; EVR: Early virological response.

respectively). These findings align with the outcomes reported by Mahmoud et al. [8], Junaid et al. [12], and Attallah et al. [34]. IFNλ3 triggers Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling after interacting to its receptor, triggers the expression of interferon-stimulated genes (ISGs) and ultimately establishes a potent antiviral condition [35].

This study had some limitations. We believed that HCV viremia reflects chronic infection rather than recent infection based on baseline data. A single-center sample collection method and a limited participant count were the study's shortcomings. Another drawback is that we did not assess the impact of IL28B gene polymorphisms on the emergence of various adverse effects of DAAs, particularly hepatocellular carcinoma and liver decompensation, or if IL-12 influenced the frequency of DAA-related adverse effects. The various extrahepatic effects of DAAs and the relationship between IL28B and IL-12 were not assessed. In addition, the effects of IL10 (rs1800896) polymorphisms could have been assessed for recent data in the Pakistani population as IL10 (rs1800896), along with IL28B (rs12979860), is also a good predictor of treatment response in chronic hepatitis C patients [3]. An association between the IL-28 B genotype and poor response to DAAs is required to be studied on a large scale, to formulate national health policies. It is essential to discover cost-effective and specific molecular factors responsible for virological response to antiviral therapy.

Conclusion

Our findings indicate that IL28-B variability influences the antiviral efficacy of DAAs and serves as a significant genetic predictive factor of treatment response in Pakistani HCV patients. The favorable CC genotype of IL-28B rs12979860 was higher and significantly associated with EVR and SVR in HCV-infected patients. Moreover, we found a strong association between high FIB-4 score and non-response to DAA therapy. These findings contribute to anticipating the response to therapy and have implications for reducing the cost of treatment in HCV patients. Altogether, IL-28B genotyping plays an essential role in the therapy choice algorithm.

Ethics Committee Approval: The study was approved by The Army Medical College, Rawalpindi Ethics Committee (No: 02/CREAM-A/Maleha, Date: 17/04/2014).

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Research Article

Investigation of Prothrombin Time, International Normalized Ratio and Activated Partial Thromboplastin Time reference ranges in children

Gozde Ulfer

Department of Biochemistry, İstanbul Medipol University Faculty of Medicine, Istanbul, Türkiye

Abstract

Objectives: This study aimed to ascertain pediatric age-specific reference ranges for prothrombin time (PT), international normalized ratio (INR), and activated partial thromboplastin time (aPTT). Retrospective data were obtained from healthy children who had undergone preoperative tests and compared with those obtained from a group of adult patients.

Methods: Reference individuals were determined by the indirect method. A total of 15,179 patients who presented to our hospital in 2022 and 2023 were retrospectively reviewed. Pediatric patients were divided into three age groups: 1–5 years (n=1949), 6–10 years (n=1563), and 11–17 years (n=508). The adult age group consisted of healthy individuals aged 18–50 years (n=11165). The tests were run in a coagulation autoanalyzer with the mechanical coagulometric measurement method. Reference ranges were analyzed using the non-parametric method statistically.

Results: The mean PT, INR, and aPTT values were found to be 14.34±1.99 s, 1.07±0.16, and 31.43±3.47 s, respectively, in children aged 1–5 years; 14.48±1.40 s, 1.08±0.11, and 31.30±2.66 s, respectively, in those aged 6–10 years; and 14.73±1.12 s, 1.10±0.09, and 31.21±2.91 s, respectively, in those aged 11–17 years. Among the adults aged 18–50 years, the mean PT, INR, and aPTT values were 13.95±1.40 s, 1.04±0.11, and 30.21±2.86 s, respectively. The mean PT, INR, and aPTT values of children aged 1–5 years, 6–10 years, and 11–17 years were statistically significantly higher than those of adults aged 18–50 years (for each group $p<0.01$).

Conclusion: It is important for laboratories to employ age-specific reference ranges for coagulation tests performed on children to ensure accurate diagnosis and avoid unnecessary further investigations. In this study, the reference ranges of the PT, INR, and aPTT parameters were determined for pediatric patients and found to be significantly higher than those of adults, which will be useful for clinical evaluation and diagnosis.

Keywords: Age group, coagulation tests, pediatric, reference ranges

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Prothrombin time (PT) is a measure used to examine the extrinsic pathway of coagulation. This test relies on the measurement of fibrin clot formation time after the addition of whole tissue thromboplastin (from lung, brain, or placenta-phospholipid extract) and calcium to citrate-containing plasma samples using the one-step Quick method [1]. PT is used to determine the risk of bleeding before surgical interventions, evaluate coagulation disorders, and assess liver function. In 1983, a model based on the international normalized ratio (INR) was defined for the standardization of PT. This method reports PT results in the form of INR, ensuring consistency across different laboratories, thromboplastins, and devices [2]. Activated partial thromboplastin time (aPTT) is a coagulation parameter used to evaluate the intrinsic coagulation pathway. This test measures the interaction between clotting factors and the conversion of fibrinogen to fibrin.

Address for correspondence: Gozde Ulfer, MD. Department of Biochemistry, İstanbul Medipol University Faculty of Medicine, Istanbul, Türkiye
Phone: +90 212 460 77 77 **E-mail:** gozde.ulfer@medipol.com.tr **ORCID:** 0000-0003-2350-6381
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aPTT is used to monitor heparin therapy, detect disorders in clotting factors, and diagnose bleeding disorders [3].

Reference ranges are one of the most frequently used decision-making tools to determine whether an individual is healthy. It is recommended that each laboratory determine its own reference ranges, and the reference population that best reflects the society should be carefully selected. Reference individuals are determined by direct or indirect methods. The direct method is a prospective method in which well-defined exclusion criteria are applied before the determination of reference individuals. In the indirect method, retrospective laboratory values from the hospital database are used to determine reference intervals. This method is preferred especially in the pediatric group, where it is very difficult to collect samples from healthy subjects. For an accurate assessment, it is of great importance to establish reference ranges correctly and determine the changes based on factors such as age and gender [4]. The International Society on Thrombosis and Haemostasis (ISTH) has reported that the use of adult reference ranges for the determination of coagulation disorders in pediatric patients may lead to erroneous assessments and recommends that each laboratory establish their own population-specific pediatric reference ranges, considering the combination of the analyzer and reagent used [5, 6].

Since the physiology of hemostasis in pediatric patients is very different from that in adults, reference ranges vary depending on the age of the children. Therefore, the use of adult reference ranges makes it harder to interpret pediatric coagulation tests accurately, and laboratories are required to develop age-specific reference ranges for children to ensure accurate diagnosis and avoid unnecessary further investigations [7, 8].

The objective of this study was to ascertain pediatric age-specific reference ranges for PT, INR, and aPTT. For this purpose, retrospective data were obtained from children aged between 1–17 years who had undergone preoperative tests and compared with reference values obtained from a group of adult patients. It is considered that the establishment of age-specific reference ranges for pediatric patients will be useful for clinical evaluation and diagnosis.

Materials and Methods

Approval was received for this study from Istanbul Medipol University Ethics Committee (No: E-10840098-772.02-7732, Date: 12/12/2023). Reference individuals were determined by the indirect method. The data on coagulation tests (PT, INR, and aPTT) performed at Medipol Mega University Hospital from January 2022 to December 2023 were retrospectively reviewed. The study was carried out based on the data of a total of 15,179 patients of whom 61.1% were female (n=9279) and 38.9% were male (n=5909). Pediatric patients were divided into three age groups: 1–5 years (n= 1949), 6–10 years (n=1563), and 11–17 years (n=508) [5]. The adult age group consisted of healthy individuals aged 18–50 years (n=11165).

The PT, INR, and aPTT results of preoperative tests required for minor surgical procedures (inguinal and umbilical hernia operations, excision of rectal polyps, diagnostic cystoscopy, tonsillectomy, septoplasty, dental treatment, etc.) were evaluated. The exclusion criteria included having a history of bleeding or thrombus, receiving anticoagulant therapy, and having an acute infection, malignancy, or cirrhosis.

The plasma obtained after centrifugation at 3000 rpm for 10 minutes in citrated tubes was analyzed using the mechanical coagulometric measurement method in a Stago Compact Max 3 coagulation autoanalyzer. The PT test relies on the use of calcium thromboplastin to measure the clotting time of the plasma obtained from the patient and its comparison to the normal standard. In principle, the aPTT test involves the re-coagulation of plasma in the presence of a standard amount of cephalin and kaolin.

PT was analyzed as PT (sec), PT activity (%), and INR using a STA NeoPTimal kit. INR was calculated using the ratio of the patient's PT value to the mean value of the normal reference range raised to the power of the reagent international sensitivity index (ISI). The ISI for the PT reagent used was 1.01. aPTT was analyzed as aPTT (sec) using a STA C.K PREST kit.

Calibration and control samples were assayed using the standard methods of the manufacturer. These tests were evaluated with normal and abnormal controls on a daily control basis, as well as by following a monthly ECAT external quality control program.

Statistical Analyses

The Statistical Package for the Social Sciences (SPSS) 2020 program was used for statistical analyses when assessing the findings obtained from the study. Quantitative variables were represented using mean, standard deviation, median, minimum, and maximum values, and qualitative variables were represented using descriptive statistical methods, such as frequencies and percentages. A non-parametric data distribution was observed for the PT, INR, and aPTT values. The Shapiro-Wilk test and box plots were used to evaluate the conformity of the data to the normal distribution. The Mann-Whitney U test was conducted to compare the variables that did not display a normal distribution between paired groups. The chi-square test was used to compare qualitative data. The results of reference ranges were evaluated at a 95% confidence interval and a significance level of $p<0.05$.

Results

The reference range values for PT, PT activity, INR, and aPTT were analyzed for the three pediatric age groups. These values were compared to the reference values obtained from the adult group. The comparison of the data by age groups is shown in Table 1. The age distributions of PT, PT activity, INR, and aPTT are presented in Figures 1, 2, 3, and 4, respectively.

Table 1. Data comparison by age

	Age groups						
	1–5 years (n=1949)	6–10 years (n=1563)	11–17 years (n=508)	18–50 years (n=11165)	p (1–5/ 18–50)	p (6–10/ 18–50)	p (11–17/ 18–50)
Gender, n, (%)							
Female	790 (40.5)	574 (36.7)	204 (40.2)	7711 (69.1)	[a]0.001**	[a]0.001**	[a]0.001**
Male	1162 (59.5)	989 (63.3)	304 (59.8)	3454 (30.9)			
PT(s)							
Mean±SD	14.34±1.99	14.48±1.40	14.73±1.12	13.95±1.40	[b]0.001**	[b]0.001**	[b]0.001**
Median (min-max)	14.2 (11–80)	14.3 (10.6–47.9)	14.6 (11.6–23.3)	13.8 (8–53.5)			
95% CI	14.25–14.42	14.41–14.55	14.63–14.82	13.91–13.97			
PT activity (%)							
Mean±SD	91.47±10.53	89.66±9.81	87.08±9.43	95.39±11.12	[b]0.001**	[b]0.001**	[b]0.001**
Median (min-max)	92 (11–120)	90 (19–120)	88 (46–120)	96 (17–120)			
95% CI	91.00–91.94	89.17–90.15	86.26–87.90	95.21–95.62			
INR							
Mean±SD	1.07±0.16	1.08±0.11	1.10±0.09	1.04±0.11	[b]0.001**	[b]0.001**	[b]0.001**
Median (min-max)	1.06 (0.89–6.47)	1.07 (0.9–3.89)	1.09 (0.9–1.8)	1 (0.9–4.1)			
95% CI	1.07–1.08	1.08–1.09	1.10–1.11	1.039–1.043			
aPTT (s)							
Mean±SD	31.43±3.47	31.30±2.66	31.21±2.91	30.21±2.86	[b]0.001**	[b]0.001**	[b]0.001**
Median (min-max)	31.2 (18.4–74.9)	31.1 (20.4–68.7)	31 (19.6–66.0)	30 (14.9–115.4)			
95% CI	31.28–31.59	31.17–31.43	30.96–31.46	30.16–30.26			

**: p<0.01. [a]: Pearson Chi-Square; [b]: Mann-Whitney-U Test. PT: Prothrombin time; SD: Standard deviation; CI: Confidence interval; INR: International normalized ratio; aPTT: Activated partial thromboplastin time.

The mean PT, INR, and aPTT values of children aged 1–5 years, 6–10 years, and 11–17 years were statistically significantly higher than those of adults aged 18–50 years (p=0.001, p=0.001, p=0.001, and p<0.01, respectively for PT; p=0.001, p=0.001, p=0.001, and p<0.01, respectively for INR; and p=0.001, p=0.001, p=0.001, and p<0.01, respectively, for aPTT). The mean PT activity values of children aged 1–5 years, 6–10 years and 11–17 years were significantly lower than those of adults 18–50 years (p=0.001, p=0.001, p=0.001, p=0.001 and p<0.01 respectively for PT activity).

The PT, PT activity, INR, and aPTT values were also evaluated in each age group by gender (Table 2). The mean PT, PT activity, and INR values did not show any statistically significant differences according to gender in the pediatric group aged 1–5 years (p>0.05), while the mean aPTT value of the male children was statistically significantly higher than that of the female children in this group (p=0.038; p<0.05). In the pediatric group aged 6–10 years, there were no statistically significant differences in PT, PT activity, INR, or aPTT values according to gender (p>0.05). The PT, PT activity, and INR values did not statistically significantly differ between the male and female children in the 11–17-year-old group (p>0.05); however, the mean aPTT value of the male children in this group was sta-

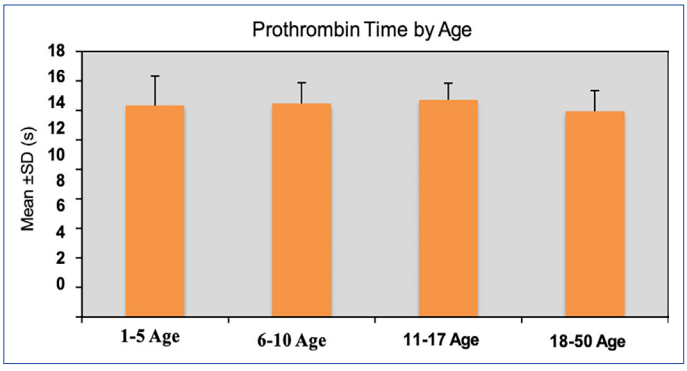

Figure 1. Distribution of prothrombin time by age.
SD: Standard deviation.

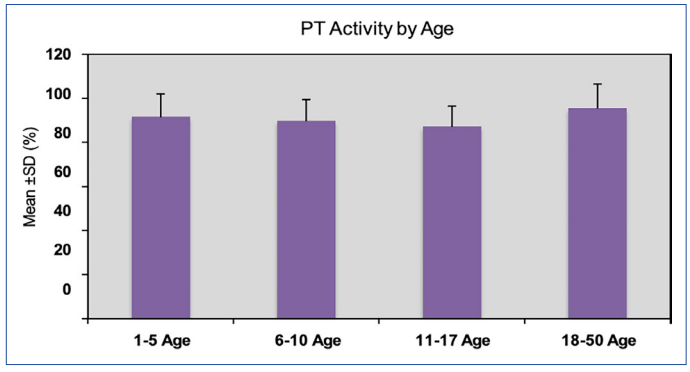

Figure 2. Distribution of PT activity by age.
PT: Prothrombin time.

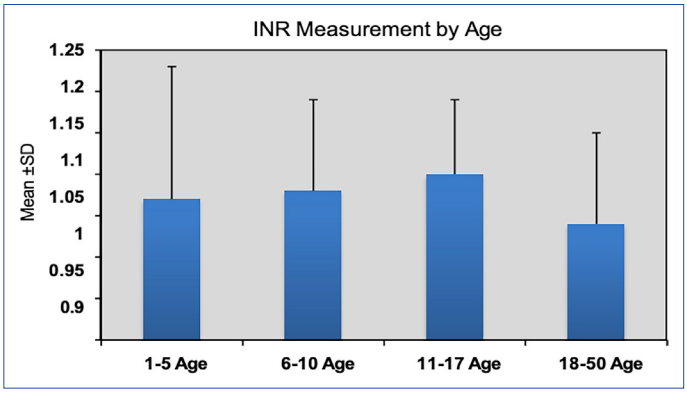

Figure 3. Distribution of INR measurement by age.
INR: International normalized ratio.

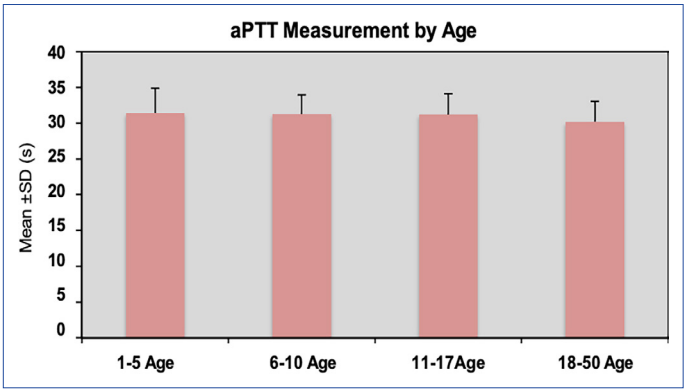

Figure 4. Distribution of aPTT measurement by age.
aPTT: Activated partial thromboplastin time.

tistically significantly lower than that of the female children (p=0.007; p<0.01). Lastly, no gender-related statistically significant differences were observed in the PT, PT activity, INR, or aPTT values of the adult group aged 18–50 years (p>0.05).

Discussion

PT and aPTT are generally considered routine tests to assess preoperative bleeding risk. PT and its mathematical derivative, INR, allow for monitoring vitamin K antagonist therapy. Age-related reference ranges may vary due to differences in physiologic hemostasis in children. Therefore, reference ranges for pediatric age groups should be used [8, 9]. Population, reagent, and analyzer-specific differences in reference ranges have been previously shown [5, 6].

Currently, there are many coagulation analyzers and reagents available for the analysis of PT, INR, and aPTT. Some earlier studies used different analyzers and reagents and established pediatric and adult reference ranges for PT, INR, and aPTT tests.

Liu et al. [10] found age-related differences between children and adults for PT. For aPTT, all pediatric age groups had higher median values than adults. Aral et al. [11] reported that PT levels differed according to age and gender, but there were no age-related differences in aPTT levels. Greenway and Monagle [7] suggested that many children might be misdiagnosed with von Willebrand disease due to prolonged aPTT values compared to adults. Sivrikaya et al. [12] reported that the average PT level of the 0-14-year-old group was 0.4 seconds longer than that of adults. In contrast, Ercan et al. [13] determined that the PT reference ranges were similar between the pediatric and adult groups. On the other hand, the determined reference ranges for aPTT were significantly higher in all pediatric groups compared to adults. In a study by Flanders et al. [14], the PT values were found to be higher in the 7-17-year-old group compared to the adult group, and they showed no difference in aPTT values between the groups.

In our study, the reference ranges of PT, INR, and aPTT were determined for all pediatric age groups, including 1–5 years, 6–10 years, and 11–17 years, and all three parameters were found to be significantly higher when compared to the adult reference ranges (for each group p<0.01).We think that low levels of coagulant proteins (II, V, VII, IX, X, XI, XII) in children caused prolonged PT, INR and low levels of Vitamin K–dependent coagulation factor IX concentrations and von Willebrand Factor caused prolonged aPTT [7, 9]. Our study is one of the rare studies in the literature that is compatible with physiological hemostasis in children.The study was conducted with a large reference population. Age-related reference ranges will be very useful in terms of accurate diagnosis in the clinics.

The discrepancies in age-related reference ranges between studies may be due to the differences in the coagulation analyzer and reagent used, as well as the reference intervals of the population and analyzer. This may also result from the variance of pediatric age groups in each study.

Some studies have evaluated differences in PT, INR, and aPTT by gender in various age groups. Sultana et al. [15] reported that male and female individuals had significantly different PT and INR values. The authors noted that men and women aged 15–50 years were more likely to have increased PT, INR, and aPTT readings than the pediatric group aged 0–14 years.

Based on the results of our study, the pediatric group aged 6–10 years and the adult group (aged 18–50 years) showed no significant differences in the PT, INR, or aPTT values when evaluated according to gender (p>0.05).

The PT and INR values also did not statistically significantly differ by gender in the 1-5-year-old and 7-11-year-old groups (p>0.05); however, the aPTT value of the male children in the 1-5-year-old group was higher than that of the female children (p=0.038; p<0.05), while the aPTT value of the male children in the 11-17-year-old group was significantly lower than that of the female children (p=0.007; p<0.01). The difference in aPTT levels in the 1-5-year-old group may be due to the numerical difference between male and female

Table 2: Data comparison by gender

	Age groups							
	1–5 years		6–10 years		11–17 years		18–50 years	
	Female	Male	Female	Male	Female	Male	Female	Male
PT (s)								
Mean±SD	14.35±1.57	14.33±2.23	14.53±1.83	14.45±1.08	14.77±1.09	14.7±1.15	13.93±1.38	13.98±1.44
Median (min-max)	14.2 (11.8–42)	14.2 (11–80)	14.4 (12.1–47.9)	14.3 (10.6–21.7)	14.7 (12.1–17.9)	14.5 (11.6–23.3)	13.8 (8–53.5)	13.8 (11.3–48.6)
[b]p	**0.761**		**0.603**		**0.255**		**0.093**	
PT activity (%)								
Mean±SD	91.37±10.6	91.54±10.48	89.61±9.99	89.69±9.71	86.71±9.47	87.33±9.41	95.53±11.13	95.07±11.09
Median (min-max)	92 (22–120)	92 (11–120)	90 (19–120)	90 (50–120)	86 (65–115)	88 (46–120)	96 (17–120)	95 (19–120)
[b]p	**0.920**		**0.839**		**0.308**		**0.171**	
INR								
Mean±SD	1.07±0.13	1.07±0.18	1.09±0.15	1.08±0.09	1.11±0.1	1.1±0.09	1.04±0.11	1.04±0.11
Median (min-max)	1.1 (0.9–3.4)	1.1 (0.9–6.5)	1.1 (0.9–3.9)	1.1 (0.9–1.7)	1.1 (0.9–1.8)	1.1 (0.9–1.8)	1 (0.9–4.1)	1 (0.9–3.9)
[b]p	**0.914**		**0.740**		**0.269**		**0.125**	
aPTT (s)								
Mean±SD	31.33±3.83	31.50±3.2	31.31±2.41	31.29±2.79	31.53±2.48	31.00±3.15	30.18±2.88	30.28±2.8
Median (min-max)	31.1 (18.4–74.9)	31.2 (21.3–55.3)	31.1 (23.6–40.2)	31 (20.4–68.7)	31.4 (25.3–44)	30.6 (19.6–66)	30 (14.9–115.4)	30.1 (21.3–108.6)
[b]p	**0.038***		**0.585**		**0.007****		**0.061**	

*: p<0.05; **p<0.01. [b]: Mann-Whitney-U test. PT: Prothrombin time; SD: Standard deviation; INR: International normalized ratio; aPTT: Activated partial thromboplastin time.

children. The difference in aPTT levels at puberty (11-17-year-old group) according to gender may be due to the menstrual cycle. Von Willebrand Factor levels vary according to the days of the menstrual cycle, and estrogen increases the synthesis of coagulation factors such as II, VII, IX, X, which are dependent on vitamin K [16]. Therefore, the menstrual cycle may affect the results.

The varying results reported by studies in terms of gender comparisons may be due to the different groupings of individuals included in each sample.

Laboratory findings should be interpreted in light of the child's age, the analyzer used, and the appropriate reagent reference ranges to provide accurate treatment of hemostatic disorders in the pediatric population. Erroneous test findings are likely to result in the need for additional diagnostic examinations and consultations, inappropriate treatment, cancellation of surgical operations, and an extra financial burden on patients.

Laboratories cannot always rely on adapting literature data on the combination of analyzers and reagents to align with their own needs. It is also often not possible for each laboratory to develop their own reference ranges. It can therefore be concluded that using adult reference ranges in the pediatric population has the potential to result in false-positive assessments in distinguishing if individuals are healthy. It is evident that our study will provide benefits for the clinical evaluation of coagulation tests in pediatric patients. It will also serve as a guide for laboratories using the same kit and analyzer.

Limitations

There are two limitations of the study. First, reference individuals were determined by the hospital database retrospectively. There may be unhealthy individuals in this data. Second, menstrual cycle information for girls aged 11–17 is not available. Therefore, multicenter prospective studies can be conducted in the future.

Conclusion

In conclusion, the hemostatic system in children continues its maturation until adulthood. Pediatric reference ranges of PT, INR, and aPTT are necessary in laboratories and they are very important for clinical diagnosis. Our study revealed age-specific reference ranges to be used in order to avoid erroneously high PT, INR, and aPTT results in children. It is considered that the study will contribute to the literature by presenting data obtained from a large case group.

Ethics Committee Approval: The study was approved by The Istanbul Medipol University Non-Interventional Clinical Research Ethics Committee (No: E-10840098-772.02-7732, Date: 12/12/2023).

Conflict of Interest: The authors declare that there is no conflict of interest.

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Research Article

Assessment of IL-6, TGF-β1 and CTX-II in the diagnosis of early Post-Traumatic Osteoarthritis of knee

Keerthy Rethinam Meenakshisundaram[1], Thiagarajan Keddin Alwar[2], Ramchand Nannapan[3], Vellora Mohanakrishnan Vinodhini[4], Karthikeyan Rajamani[5], Kuppusamy Mahesh Kumar[6], Santhi Silambanan[1]

[1]Department of Biochemistry, Sri Ramachandra Institute of Higher Education and Research, Chennai, India
[2]Department of Sports Medicine, Sri Ramachandra Institute of Higher Education and Research, Chennai, India
[3]Saksin Lifesciences Pvt Ltd, Chennai, India
[4]Department of Biochemistry, SRM Medical College Hospital and Research Centre, Chennai, India
[5]Department of Public Health, Sri Ramachandra Institute of Higher Education and Research, Chennai, India
[6]Department of Physiology and Biochemistry, Government Yoga and Naturopathy Medical College and Hospital, Chennai, India

Abstract

Objectives: Osteoarthritis is an inflammatory and degenerative disorder characterized by the degradation of the extracellular matrix. It commences with injuries to the knee that activate inflammatory pathways. Trauma may predispose to osteoarthritis, called post-traumatic osteoarthritis (PTOA). The disease is diagnosed in the later stages with (KL grade>2) as seen by X-ray; measuring the biomarkers present in the body fluids holds significant promise for the early detection and evaluation of PTOA. The study aimed to establish the role of inflammatory and collagen markers such as serum interleukin 6 (IL-6), transforming growth factor beta 1 (TGF-β1), and urine C-terminal cross-linked telopeptide of type II collagen (CTX-II) in the diagnosis of early post-traumatic osteoarthritis of the knee.

Methods: This case-control study was conducted among 80 participants, of which 40 were apparently healthy individuals, and 40 were cases with a history of trauma to the knee joint in the past ten to 12 weeks. Baseline characteristics, body mass index (BMI), Visual Analog Score (VAS), and Western Ontario McMasters Universities Osteoarthritis Index (WOMAC) were collected from all the participants. X-ray and MRI were done in the cases. Serum IL-6 and TGF-β1, and urine CTX-II were analyzed by ELISA. Statistical analysis was done with SPSS version 16. A P value ≤ 0.05 was considered statistically significant.

Results: The mean serum IL-6, TGF-β1, and urine CTX-II levels were significantly higher in cases than in controls, with P values of 0.025, 0.033, and 0.040 respectively. IL-6 showed correlations with age, WOMAC score, and urine CTX-II values. TGF-β1 showed a positive correlation with VAS.

Conclusion: Individuals with previous knee joint trauma exhibited notably elevated serum IL-6, TGF-β1, and urine CTX-II levels. Among the three biomarkers, IL-6 seemed to be a potential biomarker of early post-traumatic osteoarthritis in patients with knee injuries.

Keywords: Collagen break down products, interleukins, post-traumatic osteoarthritis, pro-inflammatory markers

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Osteoarthritis (OA) is the predominant arthritis type, affecting millions of individuals globally. The Osteoarthritis Research Society International (OARSI) defines osteoarthritis as a disorder affecting movable joints characterized by cell stress and degradation of the extracellular matrix (ECM). The process is triggered by micro- and/or macro-injuries to the knee joint that activate adaptive responses, which include inflammatory pathways of innate immunity [1]. Risk factors of the disease in-

Address for correspondence: Santhi Silambanan, MD. Department of Biochemistry, Sri Ramachandra Institute of Higher Education and Research, Chennai, India
Phone: +9840324406 **E-mail:** santhisilsambanan@gmail.com **ORCID:** 0000-0003-0720-6063
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clude obesity, older age, joint injuries, genetics, and metabolic diseases [2]. Osteoarthritis following joint trauma is called post-traumatic osteoarthritis (PTOA). The prevalence of PTOA accounts for 12% of all symptomatic OA [3]. The injury can be in the form of a fracture, cartilage damage, ligament injury, or instability (or a combination). In many cases, injuries involve more than one structure in the knee. Pain, swelling, and instability are the most common features of knee osteoarthritis. Complications of osteoarthritis include difficult ambulation, decreased range of movement, and joint malalignment [2]. The time span required to clinically measure PTOA is highly variable, ranging from two years to decades depending on the severity of the joint injury [4]. The increasing incidence, age of presentation, rate of progression, and type of injury warrant a broader and in-depth approach to understanding PTOA. To date, X-ray is the investigation of choice, and the disease is classified into four grades by Kellgren Lawrence [5]. However, the radiographic techniques and the physical examination identify only a fraction of the individuals, especially in the later stages of Kellgren Lawrence (KL) grading. The radiographic features are found to have low inter-rater reliability. It has been shown that when the disease becomes radiographically apparent, significant joint damage could have occurred. As the molecular derangements appear much earlier in the course of the disease, analysis of the biomarkers, which indicate the molecular derangements, may have diagnostic and prognostic potentials, as well as serve as therapeutic targets. The biomarkers may be inflammatory, oxidative stress, collagen breakdown, genetic, and epigenetic markers. These markers may be measured in the blood, synovial fluid, urine, and the involved joint tissues. The markers have the potential to identify the different pathological processes involved in the causation of PTOA.

Interleukin 6 (IL-6) is a pro-inflammatory and a soluble mediator having a pleiotropic impact on inflammation, immune response, and hematopoiesis. Studies have shown that circulating levels of IL-6 are increased in osteoarthritis and cartilage loss in the knee joint occurring three to fifteen years after the injury [6]. Transforming growth factor beta 1 (TGF-β1) regulates cell proliferation, differentiation, apoptosis, and synthesis and degradation of the extracellular matrix (ECM). High levels of TGF-β1 alter the cartilage metabolism, as found in cases of PTOA [7]. Elevated levels of C-terminal cross-linked telopeptide of type II collagen (CTX-II) in urine have been observed to correlate with osteoarthritis, and increased concentrations are linked to the advancement of the disease [8]. The hypothesis of the study is that alterations in the levels of inflammatory and collagen biomarkers help in the diagnosis of early post-traumatic osteoarthritis of the knee. The study aimed to establish the role of inflammatory markers IL-6 and TGF-β1 in serum and collagen breakdown product, CTX-II in urine, in the diagnosis of early post-traumatic osteoarthritis (PTOA) of the knee.

Materials and Methods

This case-control study consisted of 80 participants in the age group of 20 to 50 years of both genders. The study was conducted in the Departments of Sports Medicine and Biochemistry at Sri Ramchandra Institute of Higher Education and Research. Ethics approval was obtained from the institutional ethics committee, SRIHER (IEC-N1/21/FEB/77/25). Participants satisfying inclusion criteria were inducted after obtaining written informed consent. The participants were administered VAS and WOMAC questionnaires [9, 10]. Cases included 40 participants with a history of trauma to the knee joint, for which they were treated conservatively or underwent surgery depending on the degree of trauma. They were subjected to X-ray of the knee to obtain the KL grading with a history of trauma to the knee joint in the past ten to 12 weeks. Controls included age- and sex-matched 40 apparently healthy individuals, with no history of trauma to the knee joint in the past two years. Individuals with osteoarthritis, autoimmune disorders, post-menopausal women, osteomyelitis, tumors, and septic arthritis of the knee, metabolic/systemic illnesses, participants on anti-resorptive therapy for bone or joint disorders, anticancer drugs, anti-metabolite drugs, hormone replacement therapy, oral contraceptive pills, calcium, vitamin D, intraarticular steroids, viscosupplementation in the past three months, and a history of previous surgery to the knee joint were excluded from the study.

Laboratory methods

Venous samples were collected for IL-6 and TGF-β1 by trained phlebotomists and were centrifuged for 15 minutes at 2000 X g; the serum was separated and stored at –80°C until analysis. Spot urine was collected for analysis of CTX-II; it was centrifuged at 1500 Xg for 10 minutes, and the supernatant was stored at –80°C until analysis. The levels of IL-6 and TGF-β1 in serum and CTX-II in urine were analyzed by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. As urine markers are usually expressed as a ratio to urinary creatinine concentration, the concentration of CTX-II in the urine was corrected for urine creatinine with the following formula:

$$\text{Corrected CTX II (ng/mmol)} = \frac{\text{CTX-II from ELISA (ng/mL)}}{\text{Con from creatinine (mmol/L)}} \times 1000 \text{ [11]}$$

Statistical analysis

Data analysis was performed using SPSS version 16. The variables were tested for normality of distribution using the Kolmogorov-Smirnov test. Continuous variables were expressed as mean and standard deviation or median and interquartile range. Categorical variables were expressed as frequency and percentage. The unpaired Student's t-test and Mann-Whitney U test were used to compare the variables. Pearson's and Spearman correlation coefficients were used to test the correlation between the variables. Receiver operating characteristic (ROC) curves were plotted to compare the discriminatory strengths of different mediators in the serum and urine

Table 1. Baseline characteristics of cases and controls

Variables	Case (n=40)		Control (n=40)		p
	n	%	n	%	
Age (years)#	31.05 (9.37)		27.58 (5.71)		0.2
Sex					<0.001**
Female	9	22	26	65	
Male	31	78	14	35	
Weight (kg)#	67.48 (12.13)		70.14 (15.88)		0.4
Height (cm)#	162.51 (8.35)		164.38 (8.58)		0.3
BMI (kg/m^2)					0.9
(≤23.4)	12	30	12	30	
(23.5 to 27.4)	16	40	14	35	
(≥27.5)	12	30	14	35	
VAS					<0.001**
No pain	6	15	39	98	
Mild pain	14	35	1	2	
Moderate pain	20	50	0	0	
WOMAC score#	76.55 (16.55)		100.00 (0.00)		<0.001**

#: Data expressed in mean and standard deviation. **: P value highly significant. BMI: Body mass index; VAS: Visual analog score; WOMAC: Western Ontario and McMaster Universities Osteoarthritis Index.

of study participants. Sensitivity, specificity, and area under the curve were calculated. A P value ≤0.05 was considered statistically significant.

Results

The study was conducted with 80 participants (40 cases, 40 controls) at Sri Ramchandra Institute of Higher Education and Research.

Age, gender, BMI, VAS, and WOMAC scores were the baseline characteristics. Sex, VAS, and WOMAC scores showed statistical significance between cases and controls (p<0.001). The mean age of the participants in the cases and controls were 31.05 (9.37) and 27.58 (5.71) years, respectively, with no statistical significance between the two groups (p=0.2). Among the cases, 50% experienced moderate pain, and 14% experienced mild pain, which was statistically different (p=0.001). The WOMAC score was found to be 76.5 in cases and 100 in controls with statistical significance (p<0.001). Isolated anterior cruciate ligament (ACL) injury accounted for 30%, and anterior cruciate ligament tear with medial meniscus tear injury accounted for 20%. Among the cases, 39 cases had grade 0, and one patient had grade 1 changes as seen in X-ray (Table 1).

The level of IL-6 was higher in cases when compared to controls with a P value of 0.025 and was statistically significant. Serum TGF-β1 levels were significantly higher in cases compared to that of the controls (p=0.033). Urine CTX-II was significantly higher in cases compared to controls (p=0.040) (Table 2).

Table 2. Serum and urine levels of biochemical mediators in cases and controls

Variable	Case (n=40)	Control (n=40)	p
Serum			
IL- 6 (pg/mL)#	10.96 (6.16–88.28)	8.36 (5.50–11.40)	0.025*
TGF-β1 (ng/mL)#	2.58 (1.92–3.19)	2.34 (1.59–2.66)	0.033*
Urine			
CTX-II (ng/mmol)#	6.91 (2.03–17.29)	3.15 (0.72–13.87)	0.040*

#: Expressed as median and interquartile range (IQR). *: P value significant. IL-6: Interleukin 6; TGF-β1: Transforming growth factor-β1; CTX-II: C -telopeptide fragments of type II collagen.

The correlation study indicated that VAS positively correlated with BMI (r=0.35, p=0.04) and negatively correlated with WOMAC scores (r=–0.45, p=0.007) with statistical significance. IL-6 showed a positive correlation with age (r=0.46, p=0.001) and a negative correlation with WOMAC scores (r=–0.24, p=0.04). TGF-β1 showed a positive correlation with VAS (r=0.32, p=0.04) and weight (r=0.21, p=0.04). Urine CTX-II showed a negative correlation with IL-6 (r=–0.11, p=0.34); however, it was not statistically significant (Table 3).

Figure 1 shows the ROC curves of IL-6, TGF-β1, and CTX-II. The area under the curve, sensitivity, and specificity for IL-6 were 0.55, 72%, and 52%, respectively. The area under the curve for TGF-β1 was 0.54, with a sensitivity and specificity of 68% and 49%, respectively. The area under the curve for CTX-II was 0.58, with sensitivity and specificity of 54% and 41%, respectively. They did not show statistical significance (Table 4).

Discussion

Trauma causes a cascade of biochemical reactions, leading to the deterioration of the structural stability of the joint, resulting in post-traumatic osteoarthritis (PTOA). This condition causes significant physical disabilities, such as an inability to perform routine physical activities, which further results in mental disturbances such as depression, anxiety, fatigue, and inadequate sleep [12]. Hence, it is imperative to understand the early biochemical changes in the joint, which would help in identifying the biomarkers that have diagnostic, prognostic, and therapeutic potential. The indicators of inflammation are demonstrated by the changes in the levels of markers found in different body fluids like blood, synovial fluid, and urine. The study aimed to determine the significance of inflammatory markers such as IL-6 and TGF-β1 in serum and collagen breakdown product, CTX-II in urine, in diagnosing early post-traumatic osteoarthritis (PTOA) of the knee. In a cohort study conducted by Gelber et al. [13], it is observed that middle-aged individuals who experienced knee injuries faced a significantly heightened risk of developing osteoarthritis in the future. Similarly, in the present study, participants were in the middle-aged group demographic population. Though females are more susceptible to OA development, an associ-

Table 3. Correlation of demographic details and biochemical parameters among study participants

	Expressed as correlation coefficient 'r' value and 'p' value								
	Demographic details				Pain score		Biochemical mediators		
	Age	Weight	Height	BMI	VAS	WOMAC	IL-6	TGF-β1	CTX-II
Demographic details									
Age	1	0.02 (0.89)	–0.2 (0.07)	0.12 (0.27)	0.22 (0.21)	–0.38 (<0.001)**	0.46 (0.001)*	–0.03 (0.79)	–0.01 (0.92)
Weight		1	0.36 (0.001)**	0.87 (0.00)	0.15 (0.39)	0.04 (0.72)	–0.05 (0.68)	0.21 (0.049)*	0.07 (0.51)
Height			1	–0.14 (0.23)	–0.29 (0.049)*	0.03 (0.79)	–0.12 (0.31)	0.15 (0.19)	0.02 (0.86)
BMI				1	0.35 (0.041)*	0.02 (0.85)	0 (0.96)	0.15 (0.17)	0.07 (0.55)
Pain score									
VAS					1	–0.45 (0.007)*	0.08 (0.69)	0.32 (0.048)*	–0.03 (0.87)
WOMAC						1	–0.24 (0.04)*	–0.1 (0.38)	0.07 (0.53)
Biochemical mediators									
IL-6							1	–0.09 (0.42)	–0.11 (0.34)
TGF-β1								1	0.07 (0.54)
CTX-II									1

*: P value significant; **: P value highly significant. BMI: Body mass index; VAS: Visual analog score; WOMAC: Western Ontario and McMaster Universities Osteoarthritis Index; IL-6: Interleukin 6; TGF-β1: Transforming growth factor -beta 1; CTX-II: C -telopeptide fragments of type II collagen.

Table 4. Area under the curve and cut off value of IL-6, TGF- β and Urine CTX- II

Variables	AUC	SE	p	95% CI	Cut off value	Sensitivity (%)	Specificity (%)
IL-6	0.55	0.06	0.37	0.42–0.68	0.62	72	52
TGF-β1	0.54	0.06	0.51	0.41–0.66	2.36	68	49
CTX-II	0.58	0.06	0.21	0.45–0.70	1.44	54	41

IL-6: Interleukin 6; TGF-β1: Transforming growth factor β1; Urine CTX-II: Urine C -telopeptide fragments of type II collagen; AUC: Area under the curve; SE: Standard error; CI: Confidence interval.

ation between gender and disease prevalence in post-traumatic osteoarthritis (PTOA) is yet to be established [3]. In the present study, 22% were females and 78% were males. Among the sports activities, male skiers sustain more knee injuries than females [14]. Female athletes are more prone to anterior cruciate ligament (ACL) injuries than males [15]. The type and number of injuries depend on the type of routine work and also any sports activity involved.

People who suffer from OA undergo varying levels of pain and restricted mobility of the affected joint. Visual Analog Scales (VAS) and the Western Ontario and McMaster University (WOMAC) scale are the most commonly employed self-administered scales to assess the intensity of joint pain in patients with osteoarthritis of the knee or hip. The WOMAC pain subscale uses a five-item questionnaire, each about a different activity type (e.g., walking, standing, etc.), whereas VAS is based on a single-item questionnaire measuring any kind of pain specific to the index joint. The pain expressed by the VAS was categorized as no pain (score: 0), mild (score: 1–3), moderate (score: 4–6), and severe pain (score: 7–10) [9]. In the present study, among cases, 50% of the individuals experienced moderate pain, and 14% experienced mild pain, which was statistically significant ($p<0.001$) (Table 1). The Western Ontario and McMaster Universities Osteoarthritis (WOMAC) questionnaire consists of three subscales: pain, stiffness, and physical function. The extent of pain is noted to range from none to extreme. A score of 100 indicates no symptoms or functional disability, and 0 indicates extreme symptoms and functional disability [10]. As per Roos et al. [16], healthy individuals typically exhibit

a mean score ranging from 88 to 89, while the OA group usually demonstrates a mean score ranging from 52 to 84. Similarly, in the present study, the WOMAC score was notably lower among cases compared to controls with statistical significance ($p<0.001$). (Table 1) It showed a negative correlation with age among the participants ($r=-0.38$, $p<0.001$). (Table 3) This indicated that lower WOMAC scores in the advancing age groups are probably due to increasing pain thresholds with advanced age and also the variations in their daily physical activities.

In the present study, with regard to magnetic resonance imaging (MRI) findings, there were a maximum number of isolated ACL tears (30%). This is similar to a study by Maffulli et al. [17], which suggests that ACL tears are the most common type of knee injuries and can also be associated with other injuries, including meniscal tears, femoral condylar fractures, and cartilaginous loose bodies. X-ray is a commonly used investigation to assess KL grading, but it helps to diagnose the disease in later stages, especially from grade II onwards [18]. Hence, early diagnosis of the disease is possible by analyzing the molecular biomarkers, so that early management strategies can be implemented to slow down the progression of the disease.

Osteoarthritis is driven by inflammatory mediators in its initial stages, producing metalloproteinases that degrade the cartilage matrix. IL-6, a proinflammatory marker, has a crucial role in the pathogenesis of OA. Elevated levels of IL-6 in the serum or synovial fluid of osteoarthritis (OA) patients are linked to both the incidence and severity of the disease. It plays a significant role in the development of cartilage pathology by inducing matrix-degrading enzymes [6]. It increases sensitization to mechanical stimulation by activating the neurons through trans-signalling pathways; both peripheral and central sensitization can cause chronic pain [19]. In the present study, when the levels of IL-6 were compared between cases and controls, the mean levels were significantly higher in cases than in controls ($p=0.025$) (Table 2). IL-6 production is stimulated by IL-1 after trauma and is higher in the synovial fluid of ACL-deficient knees [20]. The injuries lead to high levels of inflammatory cytokines like IL-6, IL-8, interferon-gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α) in synovial fluid, reflecting local inflammation in the joint [21]. As reported by Beekhuizen et al. [22], there is an increased level of IL-6 in OA, implying the involvement of inflammatory processes. The levels in serum reflect the efflux of inflammatory cells into the systemic circulation. A cross-sectional study showed that IL-6 remained high for a maximum of 50 weeks after ACL injury [23].

The present study showed a positive correlation between IL-6 levels and age and a negative correlation with WOMAC scores among the participants (Table 3). With advancing age, the regulation of IL-6 gene expression becomes less effective, contributing to cartilage degradation and the pain associated with PTOA [24]. The cross-sectional study by Orita et al. [25] revealed a negative correlation between IL-6 levels and WOMAC scores. Elevated IL-6 levels in participants with trauma were directly related to pain and malfunction, with lower WOMAC scores indicating more severe symptoms and functional disability, thus establishing an inverse relationship between WOMAC score and IL-6 levels.

TGF-β1 is a growth factor that plays a significant role in the maintenance of homeostasis of articular cartilage. The isoforms and receptors are expressed in bone, cartilage, and synovial tissues, and the signaling by TGF-β1 is cell-dependent [26]. It stimulates chondrocyte proliferation and osteoblast terminal maturation, induces synovial tissue fibrosis, and plays a vital role in tissue formation, repair, and inflammation, essential for cartilage homeostasis [26]. The predominant form of TGF-β1 in articular cartilage is TGF-β1 (60–85% of total). High quantities in articular cartilage and synovial fluid indicate severe OA changes [27, 28]. In a study by Waly et al. [29], the levels of TGF-β1 are elevated in the OA group compared to the healthy individuals. It is suggested to be a helpful marker in assessing the prognosis of osteoarthritis. In post-traumatic osteoarthritis, there is an activation of TGF-β1 in the subchondral bone during osteoclast bone resorption and is found to be elevated in the synovial fluid of the knee joints [30]. In a study by Sarahrudi et al. [31], TGF-β1 levels are measured in patients with trauma in the form of long bone fractures and are higher in the early healing period. It started decreasing by two weeks and then returned to normal by eight weeks.

In the present study, serum TGF-β1 levels were significantly higher in cases compared to controls ($p=0.033$) (Table 2). Molloy et al. [32] describe the healing process of tendons and ligaments in five phases in sequence from the immediate post-injury phase, followed by inflammation, proliferation, reparation, and remodeling phases over 21 days. Levels of TGF-β1 are active in almost all stages of tendon healing. TGF-β1 mRNA levels increase after tendon injury and are found to be high in the third week, and the levels gradually decrease to normal levels by the end of the 14th week. In the present study, correlation studies of TGF-β1 with demographic variables showed a positive correlation with weight among the participants. TGF-β1 levels showed a positive correlation with the VAS score (Table 3). In line with the present study, Lin et al. [33] found a positive correlation between TGF-β1 and obesity in both men and women in the Japanese population. The study by Davidson et al. [34] shows that TGF-β1 levels are linked to pain and cartilage damage. Studies have shown that TGF-β1 decreases IL-6 receptor expression, which in turn decreases IL-6 signaling in chondrocytes and suppresses the action of IL-6 [35]. TGF-β1 levels in plasma have been found to correlate negatively with VAS in osteoarthritic patients [36]. When the cartilage is damaged, it releases TGF-β1, which can induce nerve growth factor (NGF). NGF is a neuron survival factor and a sensitizer of nociceptors that causes pain [37].

Proteases release C-telopeptide fragments of type II collagen from the matrix and cartilage. Type II collagen comprises the majority of the extracellular matrix. The levels of CTX-II can be detected in urine, offering a precise measure of the degradation

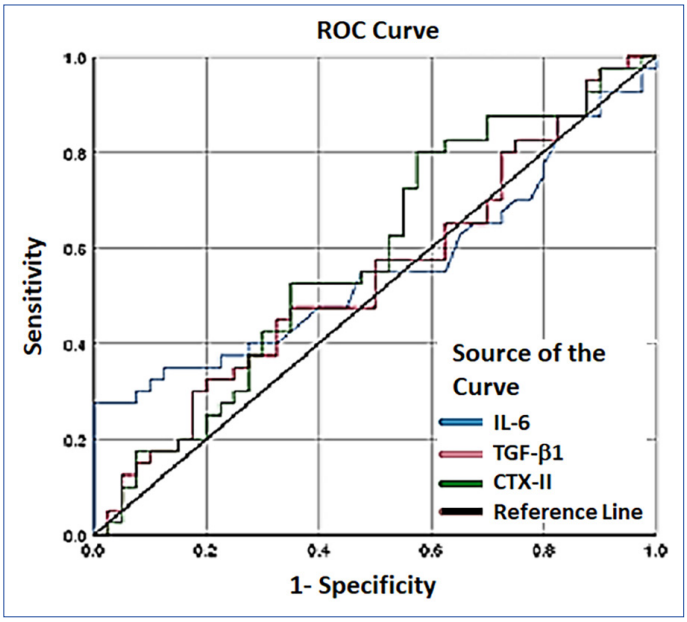

Figure 1. Receiver operating characteristics curve for the biochemical mediators IL-6, TGF-β1 and CTX-II.

ROC: Receiver operating characteristic; IL-6: Interleukin 6; TGF-β1: Transforming growth factor-β1; CTX-II: C -telopeptide fragments of type II collagen.

of type II collagen. Various studies indicate CTX-II levels in urine as a promising marker in the diagnosis of osteoarthritis [38, 39]. In the present study, urine CTX-II was significantly higher in cases compared to controls (p=0.040) (Table 2). In a study conducted by Arunrukthavorn et al. [11], the urine CTX-II levels are significantly elevated in the osteoarthritic (OA) group compared to that of the control group. These levels correlate with the radiological severity of OA. In patients with anterior cruciate ligament reconstruction, the levels of CTX-II are found to be significantly high through 16 weeks after adjusting for BMI [4]. In a study by Xin et al. [40], the level of urine CTX-II in grade-1 OA did not significantly differ from the control group, and there are significantly higher with higher grades of OA. In the present study, urine CTX-II showed a negative correlation with IL-6; however, statistical significance was not obtained, probably due to the small sample size (Table 3). This finding agrees with a study by Poree et al. [41], which concludes that IL-6 and sIL-6 R or a combination of both decrease the Sp1:Sp3 ratio and downregulate the expression of type II collagen at the transcriptional level.

In the present study, the measured biomarkers did not give statistically significant results in the ROC curve analysis. The area under the curve (AUC) for urine CTX-II was higher than other markers, but the sensitivity was 54%, which was the lowest compared to other parameters. IL-6 had better sensitivity than the other two variables, with the area under the curve being 0.55. However, the biomarkers did not show any statistical significance (Fig. 1). In the study by Panina et al. [42], the area under the curve for IL-6 is reported to be 0.753 with a sensitivity of 65.5% and specificity of 84.2%. According to Wang et al. [43], AUC for CTX-II is 0.886, with a sensitivity of 84% and specificity of 86%. All three mediators in the present study did not show statistical significance, probably due to the small sample size. It was a single-center trial with a small sample size. Measurement of the mediators in the synovial fluid could reflect the exact pathogenesis of the disease.

Limitations of the study

The sample size was small. As this study was part of a longitudinal cohort study, compiling the results at the end of the survey could be more informative and may shed more light on the diagnosis and prognosis of the disease. The future scope of the study can include long-term follow-ups with the participants to identify early biomarkers that are predictive of PTOA development and progression.

Conclusion

Post-traumatic osteoarthritis occurs after an injury to the joint. The disease occurs at an early age when compared to primary osteoarthritis. Since it goes undetected until advanced stages, early identification through analysis of biochemical alterations can forecast the onset of the disease. The study indicated that serum levels of interleukin-6 and serum transforming growth factor β1 and urine CTX-II were significantly higher in cases compared to controls. Interleukin-6 showed associations with age, WOMAC scores, and urine CTX-II levels while transforming growth factor β1 levels were associated with BMI and VAS scores. Among the three markers, IL-6 appeared to be a potential biomarker for the diagnosis of early arthritic changes in patients with knee injuries. Thus, these markers will help in initiating targeted therapies to prevent or slow down post-traumatic osteoarthritis and the associated morbidities.

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Research Article

Creatinine normalization approach to diluted urine samples screened by LC-MS/MS method

Cigdem Karakukcu[1,2], Derya Kocer[3], Veysel Uzen[3], Hatice Saracoglu[1,2]

[1]Department of Medical Biochemistry, Erciyes University Faculty of Medicine, Kayseri, Türkiye
[2]Medical Biochemistry Laboratory, Drug Application and Research Center, Erciyes University, Kayseri, Türkiye
[3]Department of Medical Biochemistry, University of Health Sciences, Kayseri, Türkiye

Abstract

Objectives: Urine is the most used matrix in drug analysis; however, it is susceptible to adulteration or tampering. Urine creatinine is the most important urine integrity parameter used as an indicator of dilution. This study aimed to evaluate the prevalence of diluted urine samples and the change in positivity after creatinine normalization.

Methods: Urine samples screened by the LC-MS/MS method over a 3.5-year period (n=21,927) were included in the study. Positivity rates were evaluated in both total and diluted urine samples. Additionally, the impact of creatinine normalization on samples with substance concentrations above the limit of quantitation (LOQ) and below the cut-off was investigated.

Results: A total of 350,832 tests were conducted on 21,927 urine samples, resulting in an overall positivity rate of 21.2% (n=4652). The ratio of diluted urine was 1.6% (n=343), with 61.5% (n=211) testing negative (<LOQ), 23.3% (n=80) testing positive (at least one substance >cut-off), and 15.2% (n=52) testing above LOQ and below cut-off. After creatinine normalization in diluted urines, the sample positivity rate increased from 23.3% (n=80) to 33.8% (n=116) (p<0.001), and the substance positivity rate increased from 2.3% (n=125) to 3.9% (n=212) (p<0.001).

Conclusion: Precautions should be taken in reporting diluted urine samples to avoid reporting false negative results. The creatinine normalization approach shows promise in laboratories using quantitative screening methods such as LC-MS/MS for samples with substance concentrations above the LOQ and below the cut-off. However, more clinical and laboratory collaboration is needed for its routine application.

Keywords: Creatinine normalization, diluted urine, illicit drug, screening test

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The urine is the most preferred biological sample for toxicology screening analysis. It has some advantages such as easy sampling, availability of sufficient samples, a higher concentration of substances or metabolites than blood, a wider detection window (average 2–3 days), and availability of validated analysis methods [1, 2]. However, the most important disadvantage is that urine is open to manipulation. For this reason, urine integrity tests are necessary to evaluate whether a urine sample has been diluted or tampered with by mixing with any external chemicals to produce a negative result [3, 4].

The Substance Abuse and Mental Health Services Administration (SAMHSA) recommends creatinine, specific gravity (if creatinine is below 20 mg/dL), and pH tests for urine integrity assessment. Additionally, nitrite, oxidants, or glutaraldehyde tests are preferable. A sample is considered not to be urine or has been replaced if its creatinine level is below 5 mg/dL and the specific gravity is below 1.001 kg/L. If the creatinine is between 5–20 mg/dL and/or the specific gravity is between 1.001–1.003 kg/L, it is classified as a "diluted urine sample" according to both SAMHSA guideline (revised 2018) and Australian Standard As/NZS4308:2008 [5, 6].

Address for correspondence: Cigdem Karakukcu, MD. Department of Medical Biochemistry, Erciyes University Faculty of Medicine, Kayseri, Türkiye; Medical Biochemistry Laboratory, Drug Application and Research Center, Erciyes University, Kayseri, Türkiye
Phone: +90 352 207 66 66 - 13854 **E-mail:** ckarakukcu@hotmail.com **ORCID:** 0000-0001-9858-3272
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Urine samples can be diluted through external means such as adding water, or internal means such as consuming large volumes of water or ingesting diuretics. Drug test results in diluted urine make it difficult to determine if the participant is using drugs, as they may provide inconclusive results. In diluted urine samples, analyte concentrations can be achieved below the cut-off value. It is known that urine dilution affects the test results of many substances, including marijuana, amphetamines, cocaine, morphine, codeine, and phencyclidine by lowering their concentrations below the cut-off value [7].

There is an ongoing debate regarding whether diluted urine samples should be rejected or tested in the laboratory. However, a more dominant opinion suggests that these samples should be accepted, as some may still yield positive results. In cases of a positive drug result from diluted urine, the laboratory has detected the presence of an illicit substance despite the dilution. Conversely, when a negative result is obtained from a diluted urine sample, it is unclear whether the donor used any drugs or not [1]. Substances that are below the cut-off value are typically reported as negative [7, 8]. For that reason, detecting results for substances above the limit of quantitation (LOQ) and below the cut-off in diluted urine samples can provide valuable data.

Creatinine is a metabolic waste product that is converted from creatine and creatine phosphate in muscle and excreted by the kidneys. Creatinine production depends on muscle mass, age, gender, water consumption, and diet, but the excretion level is maintained within certain limits over 24 hours in healthy subjects [9, 10]. Therefore, urine creatinine concentration is used as an indicator of urine dilution in urine integrity tests [5]. Creatinine normalization is the process of dividing the analyte concentration by the creatinine concentration in the same urine sample and multiplying by the reference creatinine level [11]. The creatinine normalization of urinary drug concentrations is used by athletic organizations and pain management programs to compensate for dehydration, overhydration, and changes in glomerular filtration rate [11–14]. However, similar procedures have not been yet adopted by drug analysis programs.

Immunoassays are frequently used as a substance screening method. However, liquid chromatography-mass spectrometry (LC-MS/MS) is encouraged for screening analysis nowadays, which has many advantages over immunoassay, such as low determination and quantitation limits, analyzing the drug and their metabolites separately, and lack of cross-reactivity, etc. [15].

This study aimed to investigate the frequency of diluted urine samples accepted to the laboratory over 3.5 years, to determine positivity and negativity rates according to cut-off or LOQ values, and the impact of creatinine normalization on the results of these samples.

Materials and Methods

Samples

This retrospective study received approval from the Clinical Research Ethics Committee of Kayseri City Hospital, in compliance with the Declaration of Helsinki, on July 11, 2023 (Decision No: 866).

Data from drug abuse tests conducted on urine samples accepted from psychiatry, the Alcohol and Drug Abuse Treatment Center, and the Probation Clinic between June 2018 and November 2021 were screened from the laboratory information management system (LIMS), in the Medical Biochemistry Laboratory of Kayseri City Hospital, in Türkiye. Diluted urine samples with creatinine levels between 5–20 mg/dL were identified, and the drug screening results, and demographic data of the subjects were collected for these samples.

Chain of custody was applied to all urine samples. Informed consent was obtained from all subjects. Before sample transfer to the laboratory, urine temperature was measured within 4 minutes after sampling and those not between 32–37°C were rejected.

Urine integrity test

Urine integrity tests (creatinine, specific gravity, nitrite, and pH) were performed before drug screening analysis. Urine creatinine levels were measured on Cobas c701 (Roche Diagnostic, Germany) with the compensated Jaffe method. Specific gravity and pH were evaluated with Dirui H10 urinalysis test strips. Nitrite was measured by a colorimetric method with TEST TRUE™ Nitrite Assay kit (Axiom Diagnostics).

The sample was considered a diluted urine sample if the creatinine was 5–20 mg/dL. Acceptable values for specific gravity are 1.003–1.020.

Analysis of drugs

All urine drug screening analyses were performed by LC-MS/MS method using a Restek Allure PFPP 5µm column (length 50 mm, inner diameter 2.1 mm) on AB-SCIEX 4500 Q-TRAP with a validated in-house method (Table 1). The total flow rate was 0.5 mL/min, the oven temperature was 40°C, and the total analysis duration was 18 minutes. Two-level internal control samples were injected in every single run. Samples were prepared by the "dilute and shoot" technique. After dilution with methanol, the internal standard was spiked and injected into the LC-MS/MS system.

The screened substances, their LOQ determined by verification studies, and administratively determined cut-off values were presented in Table 2. The cut-off values were based on SAMHSA LC-MS/MS cut-off concentrations. For opiate group drugs, the optimal cut-off values were determined by our previous published study [16]. Targeted analytes were amphetamine, methamphetamine, 3,4-methylenedioxymethamphetamine

Table 1. Method validation results

Substances	Linear range (ng/mL)	Precision (RSD%)	Accuracy (Bias%)
Amphetamine	5–2000	3.97	15.07
Methamphetamine	5–2000	2.4	15.1
MDMA	5–2000	1.91	–3.07
Benzoylecgonine	1–2000	0.33	9.37
THC-COOH	5–200	1.75	–1.25
Morphine	25–2000	1.31	2.1
Codeine	25–2000	0.79	5.39
6-MAM	5–500	0.87	0.35
Diazepam	12.5–500	2.7	–0.92
Clonazepam	25–500	2.25	–0.29
Lorazepam	12.5–500	2.33	1.16
JWH-18	6.25–100	2.23	–11.31

RSD% and bias% values less than 20% were acceptable. RSD: Relative standard deviation; MDMA: 3,4-methylenedioxymethamphetamine; THC-COOH: 11-Nor-9-carboxy-Δ9-tetrahydrocannabinol; 6-MAM: 6-monoacetylmorphine.

(MDMA) for amphetamines; benzoylecgonine for cocaine; 11-Nor-9-carboxy-Δ9-tetrahydrocannabinol (THC-COOH) for cannabinoids; morphine, codeine, and 6-monoacetylmorphine (6-MAM) for opiates; diazepam, clonazepam, and lorazepam for benzodiazepines; AB PINACA, AB FUBINACA, UR-144, AM-2201, and JWH-18 for synthetics. Carbamazepin-d3 and methadone-d3 were used as internal standards.

Creatinine normalization study

Similar to the study conducted by Cone et al. [11], we established our reference creatinine values to ensure suitable adjustments for our specific population. From the mean urinary creatinine concentration of 200 healthy subjects (100 female and 100 male), we determined 97 mg/dL for females and 118 mg/dL for males. For diluted urine samples with drug screening results above the LOQ and below the cut-off, creatinine normalization was applied for each substance using the following formula. Subsequently, the results were re-evaluated in terms of positivity.

$$\text{Substance concentratio after normalized creatinine} = \text{Substance concentration} \times \frac{\text{Reference Creatinine}}{\text{Sample Creatinine}}$$

Study data were analyzed on Analyse-it for Microsoft Excel (Analyse-it Software, Ltd, The Tannery, 91 Kirkstall Road, Leeds, United Kingdom). In addition, positivity rates before and after creatinine normalization were compared using the Pearson Chi-square Test on SPSS 22.0 package program (IBM Corp., Armonk, NY, USA).

Results

Over 3.5 years, a total of 21,927 urine samples were accepted to the laboratory for drug screening, with 350,832 tests (16 parameters for each urine sample) performed. 91% (n=19,958) were men, and the median age was 31 years (min-max, 17–68). The overall positivity rate was 21.2% (n=4,652).

Table 2. Substances screened in drug screening analysis and their LOQ and cut-off values

Substance	LOQ (ng/mL)	Cut-off (ng/mL)*
Amphetamine	1.5	250
Methamphetamine	1.8	250
MDMA	1.32	250
Benzoylecgonine	2.34	150
THC-COOH	1.65	15
Morphine**	11.02	300
Codeine**	4.69	300
6-MAM	2.24	10
Diazepam	2.89	300
Clonazepam	8.13	300
Lorazepam	6.5	300
AB PINACA	1.31	10
AB FUBINACA	1.27	10
UR-144	1.38	10
AM-2201	1.21	10
JWH-18	1.35	10

*: The used cut-off values were taken from "Standard Drug Testing Cut-Off Levels from SAMHSA Certified Labs"; **: For morphine and codeine, the cut-off values were lowered to 300 ng/mL [16]. LOQ: Limit of Quantitation; MDMA: 3,4-methylenedioxymethamphetamine; THC-COOH: 11-Nor-9-carboxy-Δ9-tetrahydrocannabinol; 6-MAM: 6-monoacetylmorphine.

1.6% (n=343) of urine samples were categorized as "diluted". Of these, 84.5% (n=290) were from men, and the median age was 32 years (min-max, 19–68). When the drug screening results were examined, 61.5% (n=211) tested negative for all substances (<LOQ), while 23.3% (n=80) tested positive for at least one substance (>cut-off), and 15.2% (n=52) had substance concentration(s) above LOQ and below cut-off (Table 3). The most frequently detected substances in diluted urine samples were amphetamine and methamphetamine.

After creatinine normalization, the sample positivity rate was achieved from 23.3% (n=80) to 33.8% (n=116) (p<0.001), and the substance positivity rate was increased from 2.3% (n=125) to 3.9% (n=212) (p<0.001) in diluted urine samples (Table 4, Fig. 1). As a result, 69.2% (n=36) of the samples with substance concentrations above the LOQ and below the cut-off became positive.

Discussion

In most drug analysis laboratories, creatinine measurement is a standard component of urine integrity testing. Urine samples with creatinine levels between 5–20 mg/dL are classified as diluted, and it is advisable to report results from these samples [5]. This approach helps to avoid both

Table 3. Positivity and negativity rates of total and diluted urine samples

	Total urine sample		Diluted urine sample	
	n	%	n	%
Sample	21927	100	343	1.6
Negative samples (<cut-off)	17275	78.8	263	76.7
Negative samples (<LOQ)	15112	68.9	211	61.5
Negative samples (>LOQ and <cut-off)	2163	9.9	52	15.2
Positive samples (>cut-off)	4652	21.2	80	23.3
Substance	350832	100	5488	1.6
Negative substances (<cut-off)	339528	96.8	5363	97.7
Negative substances (<LOQ)	304808	86.9	5235	95.4
Negative substances (>LOQ and <cut-off)	34720	9.9	128	2.3
Positive substances (>cut-off)	11304	3.2	125	2.3

LOQ: Limit of Quantitation.

Table 4. The effect of creatinine normalization on positivity rates of diluted urine samples

	Before creatinine normalization		After creatinine normalization		p
	n	%	n	%	
Negative samples (>LOQ and <cut-off)	52	15.2	16	4.7	<0.001
Positive samples (>cut-off)	80	23.3	116	33.8	
Negative substances (>LOQ and <cut-off)	128	2.3	41	0.7	<0.001
Positive substances (>cut-off)	125	2.3	212	3.9	

The positivity rates before and after creatinine normalization were compared with Pearson Chi-square Test. LOQ: Limit of Quantitation.

wasted time and the possibility of missing a positive result with potential forensic implications.

Consuming a large volume of water and using diuretics can dilute urine, providing a simple way to obtain a test result below the cut-off concentration [3]. Attempts to dilute urine before substance analyses are common, even when chain of custody is applied. In this study, despite the chain of custody, the prevalence of diluted urine was 1.6% (n=343). However, despite the number of attempts, cheating on drug tests is not as straightforward as it may seem; most cheaters are apprehended, and, in fact, the majority of diluted urine samples still tested positive [17, 18]. In this study, 23.3% of diluted urine samples tested positive for at least one substance (>cut-off value). However, the presence of substance concentration(s) above the LOQ and below the cut-off was observed in 15.2%. Such a result could be a "true negative" or a "false negative."

One approach to reducing the false negative rate in urine drug analysis programs has used lower screening and confirmation cut-off concentrations (e.g., LOQ) for diluted urine samples instead of administrative cut-offs. In this context, the Correctional Services Canada (CSC) program accepts cut-off values of amphetamine 100 ng/mL, benzoylecgonine 150 ng/mL, opiates 120 ng/mL, and cannabinoids 20 ng/mL for diluted urine samples [19]. Fraser and Zamecnik reported that 25.9% (n=2054) of 7912 diluted urine samples were positive according to SAMHSA cut-off concentrations. When the same samples were evaluated with lower cut-off concentrations defined by CSC, the positivity rate increased to 39.9% (n=3154). This study by Fraser and Zamecnik showed that the false negative screening rate in diluted urine samples can be effectively reduced by using lower cut-off concentrations [19, 20]. However, there is still no worldwide consensus on cut-off values. Therefore, lowering cut-off values for diluted urine samples is challenging.

There are several studies examining the applicability of creatinine normalization for drug analysis in diluted urine samples [11–14]. Athletic organizations and pain management programs employ creatinine normalization of urinary drug concentrations to account for dehydration, overhydration, and variations in glomerular filtration rate. However, similar procedures have not yet been adopted by other drug analysis programs.

In this study, the creatinine normalization procedure was applied and 32 of 52 urine samples with substance concentrations above the LOQ and below the cut-off became pos-

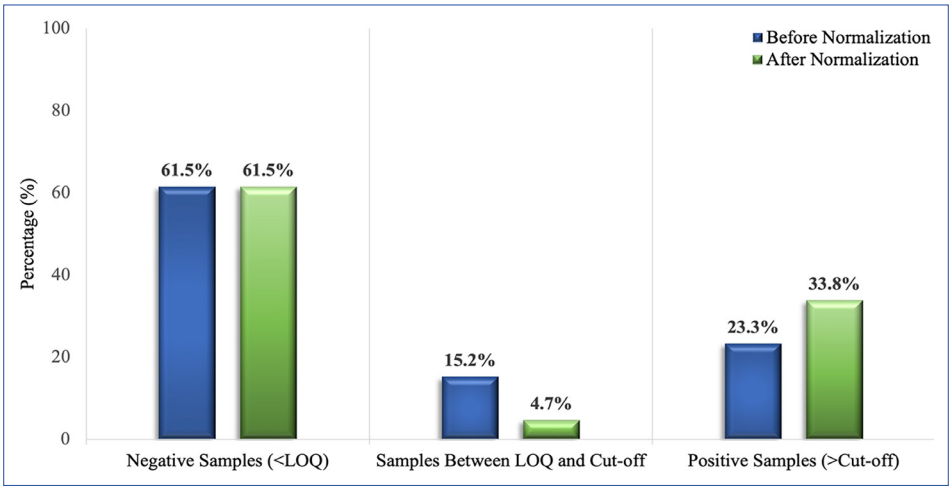

Figure 1. Change in positivity rates of diluted urine samples after creatinine normalization.
LOQ: Limit of Quantitation.

itive. This increased the positivity rate from 23.3% to 33.8%. The creatinine normalization approach seems to be potentially beneficial to decrease the possibility of false-negative results in diluted urine samples. Across diluted positive samples, the highest positivity rate was for methamphetamine and amphetamine. Methamphetamine abuse is growing, and it is the most commonly used substance in the last two years in Middle Anatolia in Türkiye [21].

There are a few points to consider in this application. Urine water content varies according to fluid intake throughout the day. Therefore, the analyte/creatinine ratio is a common approach used to normalize analyte levels in random spot urine samples, as it helps to account for variations in urine concentration (such as urine protein/creatinine, albumin/creatinine, cortisol/creatinine ratios). The use of analyte/creatinine ratios is indeed preferred over absolute analyte concentrations alone [22, 23]. In healthy individuals, the analyte/creatinine ratio is a reasonable way to account for variations in urine concentration. Nevertheless, in the presence of conditions, such as tubular dysfunction, that could potentially alter (decrease or increase) the renal excretion of the substance and/or creatinine, the accuracy of the creatinine normalization approach could be compromised. Therefore, information on "chronic diseases" should be requested for each sample. Furthermore, creatinine normalization is applicable only in laboratories using quantitative screening methods. Semi-quantitative methods, such as immunoassays, do not incorporate this application.

The Jaffe method, widely preferred for creatinine measurement similar to our study, is not specific to creatinine and may be affected by various interferences. Interference from bilirubin, glucose, protein, ketone bodies, and cephalosporins is notable. Bilirubin causes negative interference, while the others cause positive interference, potentially measuring values up to 25% higher than the true value. Although kinetic measurements can largely mitigate this effect, interferences stemming from alpha-ketoacids may persist, particularly affecting low creatinine levels (e.g., diluted urine samples) [24]. Therefore, it will be particularly important to choose more reliable methods for the creatinine normalization approach. Enzymatic-based methods may enhance measurement specificity, while the development of techniques for simultaneous creatinine and substance measurement on LC-MS/MS could offer a promising approach.

We recommend that an approach should be chosen according to the reason for requesting drug screening. A creatinine normalization approach is preferable in cases under follow-up, such as probation. However, even if creatinine normalization is not applied, laboratory specialists should report absolute analyte concentrations together with creatinine values in their reports and interpret the results more carefully. When a negative result is detected in a diluted urine sample, "diluted urine sample" information should be added to the laboratory report, and the interpretation of the analysis result should be left to the authority requesting the test. In addition, these samples should be kept for the legal storage period even if they are negative [21, 25].

The strength of our study is the large number of samples. However, a limitation is that the screening panel had to be restricted to only 16 substances due to the large daily flow of samples, which would be very time-consuming and troublesome if included in a wide variety of drugs/substances.

Conclusion

In conclusion, precautions should be taken to avoid reporting false negative results in diluted urine samples. The creatinine normalization approach, for the samples with substance concentrations between the LOQ and cut-off values, may be applied. However, more laboratory-cooperated studies are needed to enable routine application.

Ethics Committee Approval: The study was approved by The Kayseri City Hospital Clinical Research Ethics Committee (No: 866, Date: 11/07/2023).

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Review

Unveiling the latest insights into Androgen Receptors in Prostate Cancer

Suleiman Zakari[1,2,4], Christogonus Chichebe Ekenwaneze[1,2], Emmanuel Chimuebuka Amadi[1,2], Abrar AbuHamdia[3], Olubanke Olujoke Ogunlana[1,2]

[1]Department of Biochemistry, College of Science and Technology, Covenant University, Ogun State, Nigeria
[2]Covenant Applied Informatics and Communication - Africa Centre of Excellence (CApIC-ACE), Covenant University, Ogun State, Nigeria
[3]Department of Medical Laboratory Science, Faculty of Medicine and Health Sciences, An-Najah National University, Nablus, Palestine
[4]Department of Biochemistry, College of Medicine, Federal University of Health Sciences Otukpo, Benue State, Nigeria

Abstract

Prostate cancer (PCa) is a major cause of cancer-related mortality worldwide, with a rising incidence observed over the years. The androgen receptor (AR) signaling pathway plays a pivotal role in male development and maintaining masculine characteristics. Dysregulation of AR signaling in PCa can lead to disease progression and resistance to standard therapies. Understanding the intricate regulation and function of AR in both healthy and diseased states is crucial for developing effective treatment strategies. This review comprehensively explores the role of androgen receptors in PCa susceptibility, disease progression, and treatment response by analyzing recent literature. An extensive search of peer-reviewed publications in major databases, including PubMed, Scopus, and Web of Science, was conducted using specific keywords related to androgen receptor, prostate cancer, disease progression, and treatment resistance. Relevant conference abstracts and clinical trial reports were also included. The review presents an overview of the role of androgen receptors in PCa initiation, progression, and treatment resistance. It also highlights the role of SPOP as an emerging biomarker associated with AR signaling dysregulation and their potential utility for early detection and personalized treatment approaches. Additionally, recent advances in targeting the AR pathway for novel therapeutic strategies to improve patient outcomes and overcome treatment resistance in advanced PCa are discussed. The findings contribute to a comprehensive understanding of the AR signaling pathway in PCa and offer insights into its multifaceted role in disease development and treatment response. They may pave the way for innovative therapeutic interventions and precision medicine approaches based on specific AR signaling profiles, enhancing patient care and reducing the burden of this lethal disease.

Keywords: Androgen deprivation therapy, androgen receptor, AR-targeted therapies, disease progression, prostate cancer, therapeutic resistance

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Cancer, characterized by uncontrolled cell growth, remains a significant concern worldwide [1]. Prostate cancer (PCa) specifically targets the walnut-shaped prostate gland in the male reproductive system [2]. It stands as one of the most commonly diagnosed cancers among men and a leading cause of global cancer-related deaths [3]. The incidence of PCa has surged in recent years, with an estimated increase of 1 in every 52 males aged 50 to 59 [4, 5]. 1.4 million new cases were documented in 2020 [6]. Incidence rates for PCa vary among different populations and regions. Men from Europe, Latin

Address for correspondence: Olubanke Olujoke Ogunlana, PhD. Department of Biochemistry, College of Science and Technology, Covenant University, Ogun State, Nigeria; Covenant Applied Informatics and Communication - Africa Centre of Excellence (CApIC-ACE), Covenant University, Ogun State, Nigeria
Phone: +2348080454316 **E-mail:** banke.ogunlana@covenantuniversity.edu.ng **ORCID:** 0000-0001-5781-592X
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America, the Caribbean, and Northern America exhibit higher incidence rates but lower mortality compared to those from Africa and Asia, where incidence rates are lower yet PCa mortality rates are higher [6, 7]. Disparities in PCa incidence and mortality could stem from geographic and racial factors, as well as environmental influences, genetic variations, advancements in diagnostic tests, access to healthcare, and disease awareness [8]. Family history, black ethnicity, and aging are the main risk factors for PCa. To gain a more comprehensive understanding of prostate cancer's natural history and true prevalence, more extensive studies are imperative [9].

Over time, the prostate-specific antigen (PSA) test has been used to diagnose PCa. However, PSA is not specific to prostate cancer; elevated PSA levels might indicate non-cancerous conditions, like prostatitis. Yet, there is a need for precise biomarkers for early PCa detection. A digital rectal exam is conducted alongside the PSA test to strengthen the diagnosis. Technological advancements have led to innovative methods for detecting and managing PCa. Sophisticated imaging techniques, including magnetic resonance imaging (MRI), have significantly improved diagnostic precision, enabling earlier detection of the disease [9, 10]. Prostate cancer cases are predicted to rise as a result of technological advancements in diagnostic and screening tests.

The intricate process of cancer development is influenced by various genetic abnormalities, with individuals bearing tumors often exhibiting more genetic mutations [10–12]. Prostate cancer (PCa) is intensively associated with abnormalities in androgen receptor (AR) activity [11]. AR is responsible for the development and maintenance of male genitals. In PCa, the expression of AR is significantly increased, promoting the growth and survival of cancerous cells. Research indicates inhibition of AR signals hinders prostate cancer cell proliferation [12, 13]. Thus, androgen deprivation therapy (ADT) was developed. It is a common treatment for PCa patients [14]. It reduces the levels of AR-ligand interration, thus eliminating/reducing the activity of AR. However, many PCa cells eventually resist ADT and develop what is known as castration-resistant prostate cancer (CRPC). Recent research has focused on targeting AR rather than its ligand. Some approaches involve suppressing AR signaling pathways and developing AR antagonists. Moreover, studies highlight AR's interactions with other pathways, like the PI3K/Akt pathway, suggesting a potential target for PCa treatment. This review aims to outline AR's role in PCa susceptibility, diagnosis, and treatment, highlighting potential therapeutic targets for PCa [15]. Additionally, we provided an overview of androgen and androgen receptor signaling pathways to help readers comprehend the object.

Methodology and search strategy

We extensively searched various reputable databases, such as Google Scholar, PubMed, Scopus, and WOS, for relevant literature to compile this review. We employed specific search terms like "androgen receptors" or "androgen deprivation therapy" alongside "prostate cancer susceptibility," "prostate cancer progression," or "prostate cancer treatment." The search included literature until February 2023. The search terms were tailored to fit each database's unique features. Articles, theses, and dissertations that report quantitative insights into androgen receptor functions in prostate cancer, as well as information about prostate cancer susceptibility, progression, or treatment, were included. Non-English publications and those not adhering to the conclusion criteria were excluded. Two reviewers (S.Z and E.C.A) independently screened the retrieved articles by assessing titles and abstracts, followed by detailed scrutiny of the full-text versions. Discrepancies between reviewers were resolved through a third reviewer (O.O.O).

For efficient comparison and interpretation of findings, we categorized samples into three groups based on information included: (1) PCa susceptibility; (2) PCa progression; and (3) PCa treatment. Moreover, to improve the grasping of various mechanisms of AR in PCa, we visually presented these mechanisms using data visualization tools such as Cloud SmartDraw and BioRender platforms.

Results and Discussion

We conducted a thorough review of the literature for studies, theses, and dissertations that reported information on the roles of AR in PCa. Our review highlights the contribution that AR plays in PCa susceptibility, progression, and treatment.

Brief history of androgen receptor (AR)

Androgen receptors are ubiquitous ligand-dependent transcription factors and are found across various target tissues. AR activity and levels change during certain cellular processes, such as malignant transformation and sexual development [16]. The earliest proof of androgen receptors came from research on the effects of androgens on the reproductive system conducted in the 1930s and 1940s. Researchers began investigating the androgens' mode of action in the 1950s and 1960s. In 1958, a group at the University of Illinois under the direction of Paul Zamboni discovered that androgens encourage the development of the prostate gland in rats, postulating that androgens exert their effects by interacting with certain cell receptors. The AR was independently identified and characterized by three researchers in the late 1960s: Ian Mainwaring, Nicholas Bruchovsky, and Shutsung Liao [17]. After eight years, a team at the University of Chicago under the direction of Elwood Jensen identified androgen receptors in rat prostate tissue. The receptors were discovered to be androgen-specific and to have a strong affinity for testosterone. This finding opened the door for understanding the role of androgens and AR in the male reproductive system [18].

AR family members

The androgen receptor (AR), glucocorticoid receptor (GR), and progesterone receptor (PR) are transcription factors that belong to the nuclear receptor superfamily. They control gene ex-

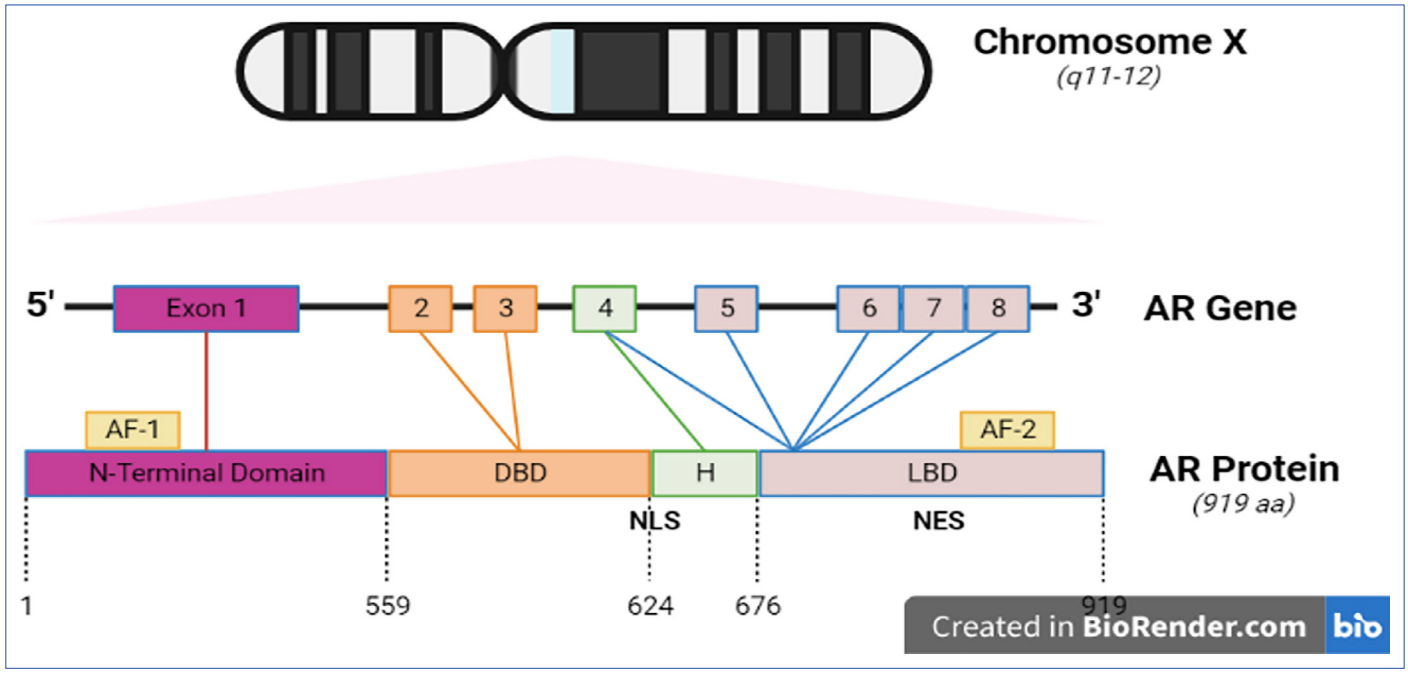

Figure 1. Androgen receptor gene and protein.

AR: Androgen receptor; AF-1: Activation function 1; DBD: DNA-binding domain; LBD: Ligand-binding domain; H: Hinge region; NLS: Nuclear localization signal; NES: Nuclear export signal.

pression in response to a hormone binding to its receptor. Each of these receptors has distinct functions and is activated by different hormones. AR is a protein that functions as an intracellular transcriptional factor. It is activated mainly by testosterone and dihydrotestosterone (DHT). Activated AR initiates sequential conformational changes in its structure, affecting receptor-protein and receptor-DNA interactions [19–21]. GR is activated by the hormone cortisol and mediates the effects of glucocorticoids in cells [22]. PR is activated by the hormone progesterone and functions in the reproductive system of females and mammary glands. PR is necessary for ovulation, implantation of the fertilized egg, and the maintenance of pregnancy [23]. These receptors play a role in many other physiological processes, including bone density, and immunological function. Deregulation of these receptors has been linked to several diseases, including cancer and autoimmune disorders [22].

AR structure

Androgens exert their effects through the binding to Androgen receptor (AR) [24, 25]. AR belongs to the superfamily of nuclear hormone and steroid receptors, including glucocorticoids, mineralocorticoids, progesterone, estrogens, and vitamin D. Steroid receptors, including the AR, have three functional domains: an NH2-terminal domain (NTD) that contains the transcriptional activation function 1 (AF-1), a central DNA-binding domain (DBD) linked to a hinge region, and a COOH-terminal ligand-binding domain (LBD), which is linked to the DBD by a hinge region and contains the transcriptional activation function 2 (AF-2) [26, 27]. The AR gene is situated on Xq11-12 and creates a protein that weighs 110 kDa and has 920 amino acids [28]. The AR gene consists of eight exons, with exon 1 encoding the NTD, exons 2–3 encoding the DBD, exon 4 encoding the HR, and exons 5–8 encoding the LBD as shown in (Fig. 1). This ligand-dependent transcription factor controls the expression of genes that are involved in the growth and differentiation of the prostate gland [29]. Family members differ in the amino-terminal domain and the hinge region that joins the core DBD to the C-terminal ligand-binding domain [30].

The NH2-terminal domain: About half of the receptor's 919 amino acid core sequence is taken up by the NTD, which ranges from amino acids 1 to 559 [31]. The AR-NTD differs most from other members of the steroid receptor family in terms of amino acid variability, sharing less than 15% of its amino acid sequence with those of other steroid receptor-NTDs. It produces the AF-1, which has the Tau1 and Tau5 transcriptional activation units (Tau). When the AR-LBD is removed, the Tau5 area (amino acids 360–528) exhibits constitutive transcription of the AR-NTD without the need for ligands, whereas the Tau1 region (amino acids 141–338) is necessary for ligand-dependent transactivation of the AR [28]. Tau-5 is a signal-dependent transactivation site, in contrast to Tau-1, and is activated by signaling events from the protein kinase C related kinase (PRK-1). Other steroid receptor-NTDs do not have the three distinct homo-polymeric amino acid repeats found in the AR-NTD. There are three types of repeats: poly-glutamine (poly-Q), poly-proline (poly-P), and poly-glycine (poly-G). The poly-P tract is 9 residues long and begins at amino acid 327. The poly-glycine tract is 24 residues long and begins at amino acid 449. The poly-Q tract is found at amino acid 59 and has a usual range of 17–29 residues. Although the particular relevance of these three repetitions is unknown, the poly-Q tract has been the subject of intense study to under-

stand its involvement in AR activity. It has been demonstrated that the length of the poly-Q tract and AR transcriptional activity are inversely correlated. The AR-poly-Q tract's length may also affect how directly AR interacts with its co-regulatory proteins, which control AR-mediated transcription. Shortening the poly-Q tract of AR to 17 amino acids or less, as was described earlier, has been linked to an increased risk of prostate cancer. The AR-NTD is appealing for AR-specific protein interactions due to its distinctive sequences and characteristics, which may be crucial for guiding AR-specific responses. Finding novel protein partners that interact with the AR-NTD may help to clarify the process by which cells are able to respond to androgenic ligands in an AR-specific manner. In the reverse yeast two-hybrid system (RTA), our group has discovered a number of novel AR-NTD interacting proteins by using the N-terminus of AR as bait. An example of these proteins is the TATA binding protein Associated Factor 1 (TAF1).

The central DNA-binding domain and hinge region: The DBD and hinge region of the AR are respectively comprised of amino acids 560–623 and 624–676. These areas perform a variety of tasks, such as dimerization of active AR molecules, nuclear localization of activated receptors, and binding to DNA at consensus sequences in the promoter/enhancer region of AR-regulated genes [32]. Moreover, the DBD of AR interacts with potential transcriptional co-regulators as well as proteins that make up the basic transcriptional apparatus. It is important for the dimerization of AR and the binding of dimerized AR to certain DNA patterns. The cysteine residues in this domain, which promote the development of two zinc finger motifs, contribute to these DBD activities [28]. Two conserved zinc finger motifs in the DBD of the AR and other steroid receptors interact with DNA regulatory regions. These DNA sequences in the promoters of androgen-regulated genes are referred to as androgen response elements (ARE) for AR. Inverted palindromic sequences with two half-sites and a 3-nucleotide spacer (5'-GGA/TACAnnnTGTTCT-3') make up the ARE. Whereas the second zinc finger of the DBD stabilizes receptor-DNA connections, the first NH2-terminal zinc finger of the DBD is in charge of detecting ARE sequences and selectively binding to AREs in the main groove of DNA. The AR-second DBD's zinc finger may have an impact on how well the receptor binds to AR-specific ARE. Nuclear localization sequence (NLS) (amino acids 613–633) found in the hinge region of the AR directs the activated receptor to the nucleus. The bipartite NLS is made up of two basic amino acid clusters spaced apart by ten amino acids. Because of the disruption caused by Lys-to-Ala mutations of these residues, the hinge region's lysine residues (K630, 632, and 633) that are acetylated during receptor activation are thought to be crucial for nuclear translocation [33].

The Ligand Binding Domain: The ligand binding domain (LBD) of AR is a region within the AR protein that is responsible for binding to androgens, which are hormones that play a key role in the development and maintenance of male characteristics [34]. The LBD is located at the C-terminus of the AR protein and

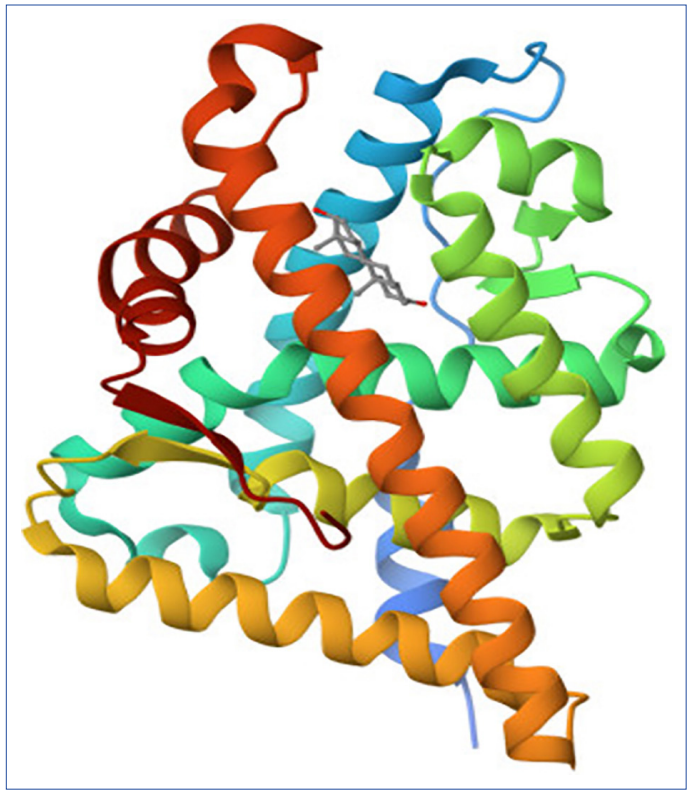

Figure 2. Structure of AR bound to DHT.
AR: Androgen receptor; DHT: Dihydrotestosterone.

is composed of several structural elements, including 12 alpha-helices and several beta-strands. The LBD of AR, which consists of amino acids 616–919, includes a hydrophobic pocket that accepts androgenic ligands such as DHT and testosterone. The LBD is well conserved among different species such as human, rat, and mouse, with degrees of homology ranging from 20–55% with LBDs of other members of the steroid receptor family. When an androgen hormone binds to the LBD of the AR, it causes a conformational change in the receptor, which allows it to translocate to the nucleus of the cell and bind to androgen response elements (AREs) on DNA (Fig. 2).

This binding leads to the activation of target genes involved in the regulation of a wide range of physiological processes, including male sexual development, muscle growth, and bone density. The AR-LBD is particularly critical for prostate cancer because it is the main target of current androgen deprivation therapies. Despite the availability of potent androgen antagonists in clinics, mutations in the AR-LBD can result in the improper activation of AR by non-androgenic substances, leading to ligand-binding promiscuity. Over 30% of prostate cancers possess AR mutations, and several AR variants have been discovered that lack receptor specificity in the absence of traditional ligands. The majority of mutations in the AR affect the ligand binding pocket and are found in three primary regions of the LBD, specifically amino acids 670–678, 710–730, and 874–910 [30]. The most frequently observed variants in tumors are T877A, T877S, and H874Y. The T877A mutation is

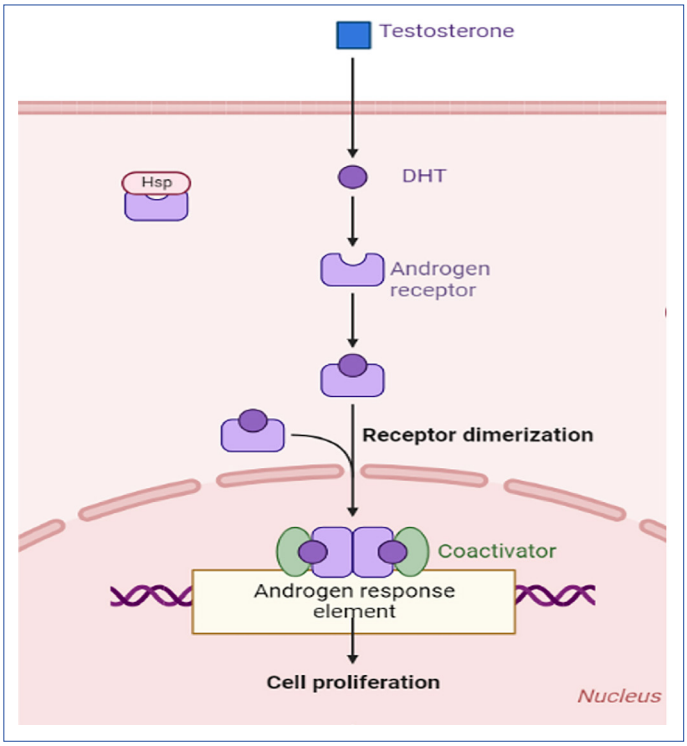

Figure 3. Androgen signaling pathway.
DHT: Dihydrotestosterone.

particularly well-known as it is found in the LNCaP human PCa cell line, as well as cases of advanced prostate cancer. Overall, these mutations make the receptor more sensitive to adrenal androgens or other steroid hormones compared to the wild type AR. This may be due to the recruitment of various co-activators, which enable the AR to bind other steroid ligands, and allow antagonists to act as agonists to activate the AR in an androgen-depleted environment [29].

AR signaling

The androgen receptor (AR) plays a vital role in the development and maintenance of male characteristics, including the development of male reproductive organs, the growth of muscle and bone mass, and the deepening of the voice during puberty. In addition, AR has been shown to play a role in the growth and function of other tissues, such as the skin, hair follicles, immune system, and brain. AR has been implicated in the development of certain diseases, including prostate cancer [22, 35].

The AR signaling pathway (Fig. 3) is a complex process that involves multiple steps and regulatory factors. AR is found in the cytoplasm, bound by several cochaperones, such as HSP90 and HSP70/HSC70, which maintain receptor conformation and prevent its degradation. When androgens bind to the AR, the receptor undergoes a conformational change, dissociates from HSP90, and translocates to the nucleus [36]. The AR subsequently attaches to motor and transport proteins, such as dynein and importin-α/-β, which recognize the nuclear localization signal of the AR and facilitate the AR complex's translocation to the nucleus. Once in the nucleus, the AR dimerizes with another AR molecule and binds to specific regions of DNA known as androgen response elements (AREs), which are located in the promoters of androgen-responsive genes. The binding of the AR to AREs initiates a cascade of events that result in the transcription of androgen-responsive genes. This process is regulated by several co-regulators, including co-activators and co-repressors, which can modulate the activity of the AR by either enhancing or inhibiting its transcriptional activity [24, 37, 38].

AR signaling is also subject to crosstalk with other signaling pathways such as the PI3K/AKT pathway, Wnt/β-catenin pathway, MAPK/ERK pathway, Hedgehog pathway, and Notch pathway [39].

Biosynthesis of androgens

Androgens are a group of steroid hormones crucial for the development, differentiation, and maintenance of the male reproductive system [35]. The androgens include testosterone, androstenedione, dehydroepiandrosterone (DHEA), DHEA sulfate (DHEA-S), and dihydrotestosterone (DHT). Androstenedione serves as a precursor for both testosterone, the male hormone, and estrogen, the female hormone. Testosterone and DHT affect male genital organs via the androgen receptor (AR) [40]. The testis and adrenal glands synthesize all androgens from cholesterol [35].

Concentrating on Leydig cells, they synthesize testosterone under the control of the pituitary gonadotropin LH. Once LH binds to its receptor on Leydig cells, it activates the cAMP/PKA pathway. This pathway leads to the activation of several enzymes involved in testosterone synthesis. Moreover, LH regulates the expression of several genes involved in cholesterol biosynthesis and uptake, such as HMG-CoA reductase, HSL, and ACAT [41].

Leydig cells could either de novo synthesize cholesterol or use stored cholesterol ester to produce testosterone. Cholesterol is first converted to pregnenolone by the enzyme CYP11A1. Pregnenolone is then converted to progesterone by the enzyme 3β-HSD. Progesterone is then converted to androstenedione by the enzyme CYP17A1. Finally, androstenedione is converted to testosterone by the enzyme 17β-HSD (Fig. 4) [41].

Androgen-binding proteins, such as sex hormone-binding globulin (SHBG), are carrier proteins that transport androgens like testosterone and dihydrotestosterone into the bloodstream. They attach to these androgens to move them through the bloodstream, and most circulating androgens (98%) are bound to these transport proteins [31]. This bound fraction of androgens is reversible and can be released from the protein binding sites as needed to exert their biological effects in target tissues. The binding of androgens to transport proteins is an important mechanism for regulating their distribution and availability in the body, and alterations in the levels of these transport proteins can have significant effects on androgen bioactivity.

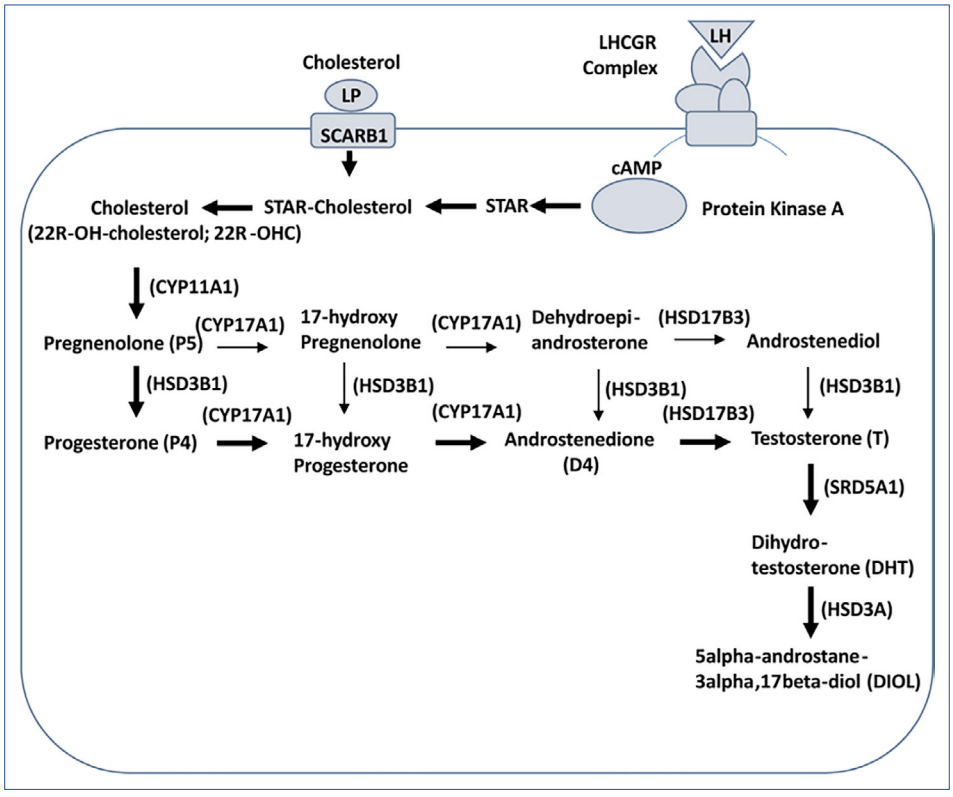

Figure 4. Androgen biosynthesis.

In males, androgen-binding proteins transport androgens in the testis and epididymis, vasa deferentia, seminal vesicles, and ejaculatory ducts. These structures are androgen-sensitive tissues that respond to testosterone during fetal development and at puberty [42, 43]. However, the effect of testosterone and DHT differs in various tissues. For instance, DHT binds to the AR with higher affinity than testosterone in certain tissues, such as the prostate gland, scrotum, urethra, and penis [31].

Association of genetic variation with PCa susceptibility

Genetics, alongside lifestyle and environmental factors, significantly influence prostate cancer susceptibility [44]. Factors such as family history and specific ethnic backgrounds increase the likelihood of developing prostate cancer. It is estimated that hereditary factors contribute to about 5–15% of prostate cancer cases. Alterations in the androgen receptor (AR) play a pivotal role in prostate cancer development. Studies indicate that genetic variations in the AR gene heighten the risk of prostate cancer. These variations can modify AR function, intensify its activity, and subsequently elevate the risk of prostate cancer development. Investigations into polymorphisms in androgen-related genes have revealed that variations in the androgen receptor impact prostate cancer risk [45]. A specific polymorphism sequence involves CAG repeats encoding polyglutamine found in the first exon of AR, responsible for the N-terminal domain crucial for transactivational regulation. The length of CAG repeats varies across racial/ethnic groups, with white individuals typically exhibiting longer repeats compared to African-Americans [46]. Longer CAG repeats are associated with androgen insensitivity syndrome, while shorter repeats correlate with heightened AR transcriptional activity and an increased risk of prostate cancer.

Notably, certain other genetic mutations, including those in mismatch repair genes (MMR, MLH1, MSH2, MSH6, and PMS2) and homologous recombination genes (BRCA1/2, ATM, PALB2, and CHEK2), are frequently associated with prostate cancer susceptibility [47, 48]. Additionally, mutations in BRCA1 and BRCA2 genes are established risk factors not only for prostate cancer but also for breast and ovarian cancer. Although mutations in HOXB13, BRP1, NSB1, RNASEL, and MSR1 genes are linked to prostate cancer development, further research is imperative [49]. Understanding the genetic underpinnings of prostate cancer has facilitated the development of genetic testing to identify high-risk individuals [50]. This testing empowers at-risk individuals to take proactive measures such as routine screening and lifestyle adjustments to mitigate their risk.

Crosstalk between AR and other pathways

The androgen receptor (AR) orchestrates gene expression linked to cell proliferation, differentiation, and survival. Yet, its signaling pathway doesn't operate in isolation; it frequently intersects with other pathways, shaping AR activity and influencing prostate cancer development. Multiple pathways engage in cross-talk with AR signaling, namely

the PI3K/AKT, Wnt/β-catenin, MAPK/ERK, SRC, IL-6/STAT3, Hedgehog, and Notch pathways. Activation of these pathways follows genomic changes in PCa, bolstering tumor growth, genotype-phenotype connections, and responses to the tumor microenvironment. These active signals trigger epithelial-mesenchymal transition (EMT), cancer stem cell (CSC)-like characteristics, and neuroendocrine differentiation (NED), impacting PCa behavior [51].

Phosphatidylinositol 3-kinase (PI3K) is an essential enzyme for cellular processes like cell division,growth, and proliferation. The intricate interplay between AR and hosphatidylinositol 3-kinase (PI3K)signaling revealed synergistic suppression when both pathways were inhibited, leading to reduced prostaticcell proliferation and enhanced apoptosis [52]. Of note, the AR-driven metabolic program hinges on mTORpathway activation [39].The PI3K/AKT/mTOR pathway governs cell-cycle regulation. Impairment of this pathway has contributedto 20–40% of PCa and 50% of metastatic castration-resistant PCa [53]. The PI3K enzyme converts PIP2 (phosphatidylinositol-(4,5)-bisphosphate) into PIP3 (phosphatidylinositol(3,4,5)-triphosphate). PIP3 attractsproteins with pleckstrin homology domains to the cell membrane, including AKT kinase, and activates it.Then, the activated AKT moves to the cell nucleus and triggers downstream pathways, such as mTOR. mTORsignaling is involved in angiogenesis, growth, migration, cell division, and survival. After that, PTEN acts asnegative feedback, removes phosphate from PIP3, and converts it back to PIP2. Dysregulation of PTEN--either by bi-allelic loss or hotspot mutations--or PIK3CA/B mutations, amplifications, and activating fusionsor AKT activating mutations, often triggers hyperactivity in this pathway, promoting prostate cancerdevelopment and progression. An *in vitro* study showed mutual feedback mechanisms betweenPI3K/AKT/mTOR and AR signaling: when deleting PTEN, the PI3K/AKT/mTOR pathway is eitherupregulated leading to downregulation of AR, or the opposite [53].

Interactions between AR and CDK/pRb drive cell cycle progression, presenting a promising therapeutic strategy in prostate cancer (PCa), particularly with combined AR and CDK4/6 inhibition, AR regulates the cell cycle and G1-S phase transition, enhancing CDK activity and inactivating pRb [52]. Additionally, in yeast and mammalian two-hybrid tests, β-catenin directly interacts with AR. The interaction sites were found in the AR's LBD and β-catenin's armadillo repeats. This interaction modifies transcriptional signaling of the p160 coactivator transcriptional mediators/intermediary factor 2 (TIF2) and NTD. In the absence of androgen, β-catenin primarily resides in the cytoplasm, while in the presence of DHT, it co-localizes with AR in the nucleus. This translocation seems unique to AR, as other liganded receptors fail to move β-catenin into the nucleus. Moreover, the presence of agonist-bound AR is necessary for β-catenin translocation, indicated by the inability of AR antagonists like bicalutamide and hydroxyflutamide to facilitate this translocation. Notably, co-translocation of β-catenin and AR occurs independently of several pathways, including GSK3, p42/44 ERK/MAPK, and PI3K. E-cadherin expression in E-cadherin null PCa cells redistributes cytoplasmic β-catenin to the cell membrane and reduces AR signaling. Thus, the absence of E-cadherin increases β-catenin and AR signaling, contributing to PCa development and progression [54].

Studies using gene editing mouse models demonstrate Wnt/β-catenin signaling's oncogenic roles in CRPC proliferation maintenance, EMT and NED encouragement, and transition of stem cell-like properties to PCa cells. β-catenin enhances AR to advance CRPC, acting as a coactivator with mutant AR (W741C and T877A) and recruiting AR to specific promoter regions (Myc, cyclin D1, and PSA). Conversely, increased AR expression amplifies Wnt/β-catenin signaling's transcriptional activity. Activation of SOX9 transcriptional factor facilitates Wnt/β-catenin-AR feedback signaling. Notably, AR possesses the capability to induce β-catenin translocation into the nucleus, whether in AR-expressing LNCaP cells or AR-lacking PC3 cells [51].

SPOP dysregulation as emerging biomarkers associated with AR signaling

Recent findings suggest that mutations in the speckle-type POZ protein (SPOP) gene significantly contribute to the development and progression of prostate cancer [55]. SPOP operates as an E3-ubiquitin ligase. It mediates the proteasomal breakdown of various substrate proteins, including the androgen receptor (AR). It has emerged as a key regulator of AR signaling, directly influencing AR protein stability and transcriptional activity by targeting it for ubiquitination and subsequent degradation. Notably, SPOP mutations occur predominantly in the substrate-binding MATH domain of SPOP [56]. Their impact on AR signaling varies based on context. In certain instances, SPOP mutations heighten AR protein stability, increasing transcriptional activity and causing persistent androgen-dependent growth of PCa even in castration-resistant prostate cancer (CRPC). Conversely, specific SPOP mutations hinder AR binding, reducing AR protein stability and diminishing AR signaling. This intricate interplay between androgen receptor (AR) signaling and SPOP dysregulation is a critical aspect of prostate cancer pathogenesis [57].

Here are several ways in which AR signaling and SPOP dysregulation interact in prostate cancer:

1. **AR protein stability and degradation:** Mutations in SPOP's substrate-binding MATH domain disrupt its interaction with AR by impairing AR ubiquitination and increasing AR protein stability [57, 58]. This results in increased AR resistance to degradation and higher transcriptional activity, fostering abnormal cell growth and survival in prostate cancer.

2. **AR-ARV interaction:** SPOP dysregulation can influence the stability of AR variants (ARVs). ARVs are truncated forms of AR that lack the ligand-binding domain and are risk factors for castration-resistant prostate cancer (CRPC). Specific SPOP mutations target ARVs, enhancing ARV sta-

bility and transcriptional activity [59]. This contributes to resistance to androgen deprivation therapy and the development of CRPC.

3. **Impact on other signaling pathways:** Dysregulated SPOP can affect pathways intersecting with AR signaling, such as the phosphoinositide 3-kinase (PI3K) pathway. The interaction between SPOP and these signaling pathways could modulate AR signaling, impacting disease progression and treatment resistance.

4. **SPOP-AR signaling feedback loop:** Dysregulated AR activity due to SPOP mutations can drive the expression of genes linked to tumorigenesis and treatment resistance [60].

Understanding this intricate crosstalk between AR signaling and SPOP dysregulation is crucial for developing targeted therapies for prostate cancer. Targeting the AR signaling axis or restoring SPOP function holds therapeutic promise for treating SPOP-mutated prostate cancers or those with dysregulated AR signaling.

Targeting AR in prostate cancer

Androgen deprivation therapy (ADT): Androgen Deprivation Therapy (ADT) has been a standard treatment option for PCa for many years [36]. ADT slows the growth of PCa by reducing the levels of androgens, thus reducing AR activity, the main player in PCa development and progression [61]. Often, ADT is used alone or combined with radiation therapy or chemotherapy, depending on the stage and severity of the cancer. Despite its benefits in symptom alleviation and impeding PCa spread, many patients start to develop resistance to the drug, leading to the development of castration-resistant prostate cancer (CRPC) [4, 33, 61, 62].

Over the past two decades, discoveries have revealed that AR signaling is responsible for tumor growth, even post-castration. This insight spawned novel hormonal drugs like Abiraterone, Enzalutamide, Darolutamide, and Apalutamide, designed to enhance anticancer activity [63]. Additionally, LHRH analogs, mostly administered via injection, are part of this therapeutic landscape. However, these treatments often have adverse effects like metabolic syndrome, cardiovascular risk, and cognitive and sexual symptoms, necessitating the quest for alternatives. One such development is Relugolix, an orally available nonpeptide LHRH antagonist. It competitively binds to and blocks the LHRH receptor in the pituitary gland, decreasing LH secretion and subsequently testosterone production in the testes.

The limitations of ADT therapies have moved recent research focuses to targeting AR rather than androgens in PCa, exploring AR antagonists, interference with AR signal transduction, and second-generation anti-androgens and androgen receptor signaling inhibitors (ARSIs). These therapies, showing promise in clinical trials, especially in CRPC, aim to enhance outcomes for advanced PCa patients [36, 64].

Castration-resistant state

Castration-resistant state (CRPC) is developed when cancer cells gain the ability to grow in the absence of androgens. Several mechanisms underlie this resistance, including AR gene mutations, increased levels of co-activators, or enhanced synthesis of potent androgens like dihydrotestosterone [13]. These mechanisms ultimately result in androgen-independent AR activation, conferring resistance to anti-androgen therapy such as flutamide, bicalutamide, and enzalutamide. The hyperactive AR in CRPC increases cell proliferation and survival, stemness, resistance to apoptosis (programmed cell death), and cell migration and invasion, leading to metastasis. Research shows that the AR gene consistently up-regulates with over 80% of CRPCs having high nuclear AR and metastasis of cancer to the bone [65].

The shift from androgen dependence to a castration-resistant state involves molecular mechanisms divided into pathways that either bypass or operate through the AR receptor (Fig. 5). These pathways are not mutually exclusive and often coexist in castration-resistant prostate cancer. These pathways are intricate and still not fully understood. Studies reported that, in the AR-bypass pathway, castration resistance can be reached by impacting apoptotic genes like PTEN and Bcl-2, which are downregulated, boosting cell survival. While in the AR-operating pathway, prostate cancer cells manage to survive via dysregulated cytokines, anomalies in receptor genetics or amplitude, autocrine synthesis of active androgens, altered co-activator expression, and the presence of alternatively spliced AR variants.

Early-stage disease can be managed with radical prostatectomy or radiation ablation of the prostate gland [66]. However, once cancer cells spread beyond the prostate capsule, treatment becomes considerably more challenging. For patients where surgery is no longer an option—representing one-third of PCa patients—androgen withdrawal is used. Androgen ablation therapy eliminates hormones, preventing the growth-promoting effects of androgens, leading to cancer cell apoptosis and tumor regression. However, the average overall survival time is less than 2–3 years. While these therapies have significantly improved outcomes, their limitations necessitate exploring alternative strategies.

Androgen receptor signaling inhibitors (ARSIs): Androgen receptor signaling inhibitors (ARSIs) are a diverse class of medications used to treat prostate cancer (PCa). These drugs interfere with androgen receptor (AR) signaling, impacting tumor growth [27]. ARSIs operate through various mechanisms, not solely by blocking androgen receptors. They include:

1. **Anti-androgens:** These drugs impede androgen binding to receptors, hindering cancer cell growth. Examples include bicalutamide, flutamide, and nilutamide.

2. **GnRH agonists:** These drugs suppress gonadotropin-releasing hormone (GnRH), lowering testicular androgen

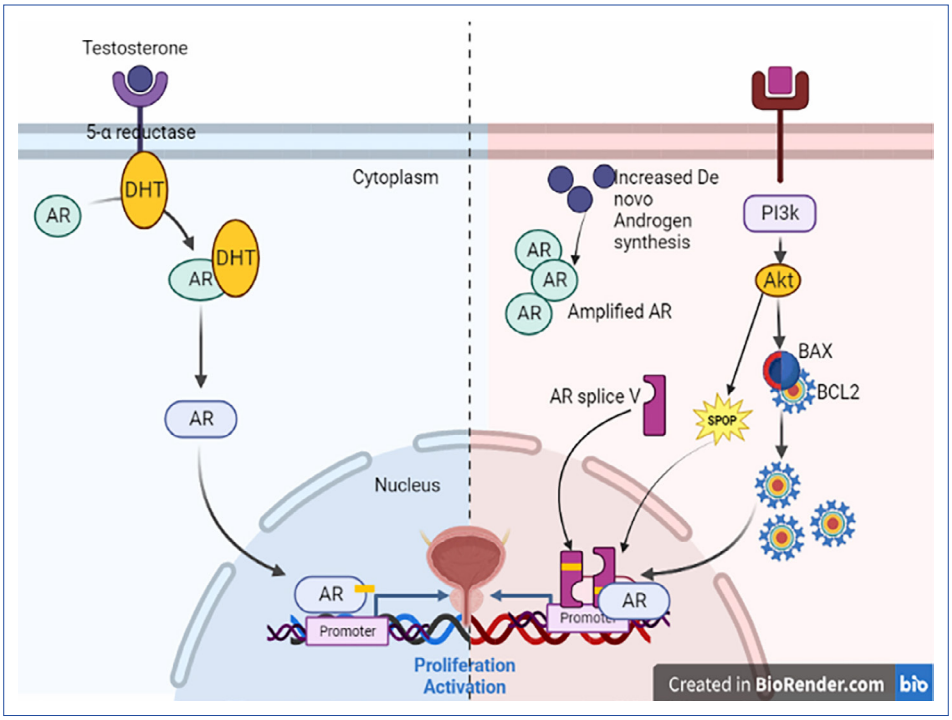

Figure 5. Androgen-dependent vs. castration-resistant PCa progression.
PCa: Prostate cancer.

production and slowing prostate cancer growth. Notable examples are leuprolide and goserelin.

3. **CYP17 inhibitors:** These drugs target the enzyme CYP17, disrupting androgen synthesis in both adrenal glands and prostate cancer cells. An example is abiraterone acetate.

4. **Androgen receptor antagonists:** These drugs directly impede androgen receptor activity, curbing prostate cancer cell growth. Examples include enzalutamide and apalutamide.

These drugs could be used alone or in combination with other treatments, such as chemotherapy or radiation therapy, to treat prostate cancer and manage its symptoms. Abiraterone, enzalutamide, apalutamide, and darolutamide are recommended by the NCCN, aiming to restore balance and impede AR signaling [67].

Abiraterone is an FDA-approved CYP17A1 inhibitor. Although it effectively suppresses androgen synthesis in the testis and adrenal glands, improving prostate cancer outcomes, it is associated with significant adverse reactions. Prednisone is added to abiraterone to mitigate its adverse effects. Studies have reported that the co-administration of these drugs has increased the survival rate of CRPC patients. However, some patients eventually develop resistance to abiraterone. Increased CYP17A1 expression and mutations activate de novo androgen synthesis, promoting pathways like "backdoor" and "alternative" androgen formation. Concurrently, aberrant expression of 3-HSDs and AKR1C3 elevates these pathways while reducing the metabolism of active androgens, contributing to abiraterone resistance. Additionally, exogenous glucocorticoids, used to mitigate adverse effects, might inadvertently activate mutated AR, fostering drug resistance. Various factors like truncated androgen receptor variants (e.g., AR-V7) and the activation of pathways like PI3K/AKT/mTOR and ErbB2 also play roles in abiraterone resistance.

New-generation AR inhibitors like apalutamide and darolutamide demonstrate improved central nervous system (CNS) safety compared to enzalutamide. However, resistance could develop due to AR mutations, splicing variants, and PI3K pathway activation. Darolutamide is an oral non-steroidal AR inhibitor that inhibits AR function and cell growth in PCa without crossing the blood-brain barrier (BBB), resulting in fewer CNS side effects. Studies on darolutamide resistance are limited, but there is evidence of cross-resistance with other AR inhibitors and significant inhibition of AR-mutated variants.

Enzalutamide, a second-generation androgen receptor antagonist, impedes AR translocation and induces apoptosis in CRPC cells. However, resistance may arise due to changes in AR structure or quantity, over-activation of GR, and other signaling pathways like Wnt, and genetic alterations, leading to neuroendocrine trans-differentiation of CRPC cells.

The majority of these AR-targeted therapies target the LBD. The limitations of these LBD-specific therapies are due to genetic variations and the presence of AR variants in many cases. Thus, targeting the DNA-binding domain (DBD) and N-terminal domain (NTD) has emerged as a potential strategy to combat this

Table 1. Combination therapies targeting AR and PI3K/AKT/mTOR pathways

Target	Agent	Phase	Administration	Condition	I.D on Clinicaltrial.gov
AKT	AZD5363	III	Docetaxel	mCRPC	NCT05348577
	MK2206	II	Bicalutamide	High-Risk of Progression	NCT01251861
	Capiversertib	II	Abiraterone acetate	High Risk Localized PCa	NCT05593497
PI3K	AZD8186	I	Docetaxel	mPCa with PTEN Mut	NCT03218826
	GSK2636771	I	Enzalutamide	PTEN(-) mCRPC Mut	NCT02215096
mTOR	Sepanisertib	II	Monotherapy	CRPC	NCT02091531
	Everolimus	I	+ standard radiation therapy	PCa with rising PSA	NCT01548807

AR: Androgen receptor; PI3K: Phosphatidylinositol 3-kinase; AKT: Protein kinase B also known as PKB; mTOR: Mammalian traget of rapamycin.

resistance and enhance the efficacy of existing therapies [68]. The amino-terminal domain (NTD) of the androgen receptor contains AF-1, a crucial element for AR transcriptional activity. EPI-001, pioneered by Marianne Sadar and Raymond Andersen, represents the first inhibitor targeting this domain [29]. EPI-001 operates as an antagonist, disrupting vital protein-protein interactions necessary for AR transcriptional activity by covalently binding to the AR's NTD [69]. It showed potential in managing advanced prostate cancer cases resistant to traditional anti-androgens like enzalutamide, holding immense promise in the clinical development of treatments for castration-resistant prostate cancer (CRPC). By inhibiting NTD, it may be possible to enhance men's longevity and living conditions, and acquired resistance to current therapies will all be improved.

Emerging therapies targeting AR: Precision medicine is emerging as a critical avenue, aiming to tailor treatments to individual needs across various prostate cancer types. Emerging therapies for PCa encompass diverse approaches, such as immunotherapy, targeted therapies, radiopharmaceuticals, gene therapy, and nanoparticle-based therapies [70].

- **Immunotherapy:** A form of therapy that aids the immune system's recognition and destruction of cancerous cells [70]. Several immunotherapy drugs, such as checkpoint inhibitors, CAR-T cells, and cancer vaccines, are being developed and tested in clinical trials for prostate cancer.
- **Targeted therapies:** Drugs that specifically target cancer cells based on their genetic mutations or other specific characteristics. For prostate cancer, several targeted therapies are being developed, including drugs that target the androgen receptor pathway and drugs that target specific enzymes and proteins involved in prostate cancer growth.
- **Radiopharmaceuticals:** Drugs that include radioactive materials and can be used to target and eliminate cancer cells. Several radiopharmaceuticals, such as radium-223 and lutetium-177, are being developed and tested for prostate cancer.
- **Gene therapy:** A type of treatment that involves inserting or altering genes in a person's cells to treat or prevent disease. For prostate cancer, several gene therapies are being developed, including therapies that target the androgen receptor pathway and therapies that use viruses to deliver therapeutic genes to cancer cells.
- **Nanoparticle-based therapies:** Nanoparticles are tiny particles that can be used to deliver drugs directly to cancer cells. Several nanoparticle-based therapies, such as liposomes and polymer nanoparticles, are being developed and tested for prostate cancer.

It is crucial to mention that many of these treatments are still in the developmental or research phase and might take time before becoming widely accessible [70].

Combinational therapies: There's a well-established correlation between PI3K/AKT/mTOR and AR signaling in PCa [61]. This understanding has fueled interest in combination therapies targeting these pathways, showing promise in both preclinical and clinical studies. One potential strategy involves combining AR inhibitors like enzalutamide or abiraterone acetate with PI3K inhibitors such as buparlisib or idelalisib. These combinations have demonstrated a synergistic impact, reducing cancer cell proliferation and boosting apoptosis. Table 1 outlines several of these dual-targeting combination therapies.

In a preclinical study, it was observed that merging a PI3K/AKT inhibitor with an anti-androgen prolonged disease stabilization in a CRPC model [71]. An example of such an inhibitor is AZD5363. It exhibited anti-cancer activity in both androgen-sensitive and castration-resistant phases of the LNCaP mouse xenograft, reducing cell propagation and inducing apoptosis in AR-expressing PCa cell lines [72]. However, resistance to AZD5363 emerged after around 30 days of treatment, marked by rising PSA levels. Investigations revealed that AZD5363 boosted AR transcriptional activity, AR binding to androgen response elements, and AR-dependent gene expression, including PSA and NKX3.1 [71]. Combining AZD5363 with the antiandrogen bicalutamide effectively countered these effects, prolonging tumor growth inhibition and stabilizing PSA levels in CRPC *in vivo*.

It's worth noting that combination therapy targeting both AR and PI3K/AKT pathways might entail increased toxicity compared to using a single agent. Hence, careful selection and monitoring are crucial to ensuring the safety and efficacy of these treatments. Overall, combinational therapies in prostate cancer present a hopeful avenue for enhancing patient outcomes. Nonetheless, further research is necessary to understand the action of these combinations and their long-term side effects better.

Conclusion

Androgen receptors (AR) play a pivotal role in prostate cancer (PCa) susceptibility, progression, and treatment. This pivotal role warrants continued exploration and strategic intervention. Integrating advanced diagnostic strategies and technologies represents a promising avenue for improving PCa detection. Further investigation into the intricate mechanisms underlying AR's influence on PCa initiation and progression is imperative. Overcoming resistance to androgen deprivation therapy necessitates novel strategies targeting AR, informed by identifying genetic variations impacting AR activity and innovating AR-targeted therapies. Future research should intensify efforts to elucidate AR's molecular mechanisms, circumvent resistance, and develop predictive biomarkers, thereby optimizing treatment strategies tailored to individual patient profiles. Such initiatives hold profound potential to revolutionize PCa diagnosis and treatment paradigms by deepening our comprehension of AR's multifaceted involvement.

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Review

Fibroblast growth factors: properties, biosynthesis, biological functions, therapeutic applications and engineering

Nitin Tiwari[1], Ashok Tiwari[2], Lalita Mehra[2], Arjun Ganguly[3], Kamlesh Darji[2], Muzafar Pandit[4], Rachana Rachana[5]

[1]Department of Microbiology, Atal Bihari Vajpayee Institute of Medical Sciences And Dr. RML Hospital, New Delhi, India
[2]Department of Pathology, All India Institute of Medical Science, New Delhi, India
[3]Department of Burns and Trauma, All India Institute of Medical Science, New Delhi, India
[4]Department of Biosciences, Jamia Millia Islamia, New Delhi, India
[5]Department of Biotechnology, Jaypee Institute of Information Technology, Uttar Pradesh, India

Abstract

Fibroblast Growth Factors (FGFs) function as signaling molecules within various signaling pathways, regulating the proliferation, migration, and differentiation of soft connective tissues, nerves, epithelial tissue, and bone. The FGF family comprises 22 members, with acidic Fibroblast Growth Factor (aFGF/FGF-1) and basic Fibroblast Growth Factor (bFGF/FGF-2) being of primary significance. This article explores the biochemical and biological properties of different FGFs, elucidating their roles in various biological processes. Additionally, it delves into the interactions between FGFs and Receptor tyrosine kinases (RTKs), which activate several cell signaling cascades, such as the RAS/MAPK (Mitogen-activated Protein Kinase) pathway, PI3K (phosphoinositide 3-kinase)/AKT (v-akt murine thymoma viral oncogene homolog) pathway, PLC-γ (Phospholipase C-γ) pathway, and Signal Transducer and Activator of Transcription (STAT) pathway, to facilitate diverse cellular functions. The article also examines methods for engineering FGFs, including N-terminal truncation, point mutations, or combinations thereof, for therapeutic applications in tissue regeneration, angiogenesis, and repairing damaged tissues such as cartilage, bone, ligaments, and skin. Finally, it concludes with a discussion of the delivery systems for FGFs, encompassing scaffolds, hydrogels, as well as nano- and micro-particulate methods.

Keywords: Angiogenesis, engineered FGFs, fibroblast growth factor, RAS/MAP kinase pathway, tissue regeneration

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Growth factors are among the most studied and explored proteins of the human body. They have been in the limelight since the 1980s and have shown tremendous scope in therapeutics over the last few decades. They regulate cellular functions such as stem cell differentiation [1], cell proliferation [2], growth of cells, migration of cells, angiogenesis [3], adhesion in the epithelium, cartilage, bone, some soft connective tissues, nerve cells, and maintaining the stemness of stem cells [4]. They can also be modified genetically or structurally following their use in therapeutics and their commercial production. Growth factors are named based on their tissue of origin, such as the Cartilage-Derived Growth Factor (CDGF), Retina-Derived Growth Factor (RDGF), Astroglial Growth Factors (AGF), and Eye-Derived Growth Factor (EDGF); while some are named according to the tissue they stimulate, like endothelial cell growth factor (ECGF) [5], fibroblast growth factors (FGFs), and vascular endothelial growth factors (VEGFs). Among these, this review paper will emphasize Fibroblast growth factor and their therapeutic applications, structural modifications, and binding aspects of FGFs with FGFRs.

The first-ever discovered Fibroblast growth factor was bFGF. It was initially referred to as a polypeptide that acted

Address for correspondence: Rachana Rachana, MD. Department of Biotechnology, Jaypee Institute of Information Technology, Uttar Pradesh, India
Phone: +919315434437 **E-mail:** rachana.dr@iitbombay.org **ORCID:** 0000-0002-8686-0372

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like a cation and had a pI of 9.6. The bFGF was found in the pituitary gland and brain and could stimulate cell division in NIH/3T3 fibroblast cells [6]. Fibroblast growth factors have been observed in both vertebrates and invertebrates. Some vertebrates like zebrafish, Xenopus, chicken, mice, and humans have FGF genes in their genomes [7], while invertebrates like Drosophila and Caenorhabditis elegans also have FGF genes in their genomes [8]. Since this article is centered around the human Fibroblast Growth Factor, we won't go beyond these limits.

Biologically, FGFs are as important as other growth factors; they are key for cellular functions such as cell growth, repair, differentiation, proliferation, migration, and adhesion. The FGFs trigger receptor tyrosine kinases (RTKs), resulting in the initiation of various cellular signaling pathways, which leads to the transcription of genes into mRNAs, ultimately sequencing various cellular processes mentioned above [9]. FGFs have been implicated in imparting a series of biological functions, including the development, branching, and morphogenesis of limbs; patterning of the brain; helping the metabolism of Vitamin D and bile acid; and some cytoprotective functions [10]. Some of these functions are highlighted in this article.

The FGF family

The members of the FGF family are single polypeptide chain proteins that share some structural characteristics in common, most of them showing high avidity with heparin. Many of them are secreted into extracellular matrices where they bind to heparan sulfate (HS) or heparan-like glycosaminoglycans (HLGAGs). All the members of this growth factor family share a common 140-amino-acid-containing homologous core, which forms twelve folds of antiparallel β-sheets resulting in a barrel-shaped cylinder with variable amino acids and carboxy-terminal stretches covering it around. All the members of the FGF family are grouped because they are structurally similar. The initial FGF doesn't imply that they all stimulate fibroblast cells; for example, FGF7 doesn't stimulate fibroblast cells [11].

As shown in Figure 1, the human FGF family has 22 members so far, i.e., FGF1 (aFGF), FGF2 (bFGF), FGF3 (int-2), FGF4 (hst-1/kFGF), FGF5, FGF6 (hst-2), FGF7 (KGF), FGF8 (AIGF), FGF9 (GAF), FGF10, FGF11, FGF12, FGF13, FGF14, FGF16, FGF17, FGF18, FGF15/FGF19, FGF20 (XFGF-20), FGF21, FGF22, and FGF23, which are further subcategorized into 7 subfamilies, i.e., FGF1, FGF4, FGF7, FGF9, FGF8, FGF19, and FGF11 families, based on phylogenetic relations [12]. The FGFs contained in the FGF1, FGF4, FGF7, FGF8, and FGF9 subfamily act in a paracrine fashion and are referred to as canonical FGFs as they bind to the FGFRs with the help of Heparin/HS, which acts as a cofactor [13]. The FGF15/19 (human FGF19 is an ortholog of rodent Fgf15) subfamily members show low affinity to the FGFRs as well as the Heparan Sulfate and depend on the Klotho proteins to bring about their metabolic effects in the target tissues, while the FGF11 subfamily includes intracrine FGFs, which themselves act as a cofactor for voltage-gated sodium channels and other molecules [14].

Structure of FGF proteins

The FGF family members are closely related to each other, both structurally and to some extent functionally. Their molecular weight ranges from 17 to 34 kDa, and the amino acid sequence length ranges between 126 and 268 aa, with a core region of 120 to 140 amino acids. Additionally, a sequence of 28 amino acids from this core region is conserved in all members [15]. Out of all these FGFs, aFGF and bFGF are the most extensively discussed and studied. The secondary and tertiary structures of human FGF1, which has a molecular weight of around 15,900 Da, have eight tyrosine residues exposed to solvent and only a single tryptophan, while the secondary structure of native human aFGF comprises 52% β-sheet, 28% turns, and 11% α-helices. The remaining 9% of the structure is disordered [16, 17]. On the other hand, bFGF is entirely composed of β-sheets, in which each antiparallel β-strand is bound to the adjacent β-strand in its primary sequence through hydrogen bonding. The continuous turns or β-meanders give it the shape of a barrel, which is closed by the amino and carboxyl-terminal strands. The core of bFGF consists of hydrophobic and aromatic amino acid side chains, while the charged amino acids, particularly arginine and lysine, make up the surface of the molecule [18]. The tertiary structure of bFGF comprises three copies of β-meander motifs, where the first copy consists of residues 18–59, the second one involves residues 60–100, and the third one consists of residues 101–143. Each one has four β-strands, while the first seventeen amino-terminal residues, which are rich in serine, glycine, and proline residues, appear to be disordered. There are four cysteine residues in bFGF, i.e., Cys25, Cys69, Cys87, and Cys92, out of which Cys25 and Cys92 are conserved in the FGF family. Out of those four cysteine residues, Cys69 and Cys87 are supposed to be involved in the dimerization of bFGF molecules. Residues 106–115 provide a binding site for FGFRs where Tyr-114 and Trp115 determine the binding strength between bFGF and FGFRs [19].

Biosynthesis of FGFs

The FGFs are primarily expressed in embryonic or adult tissue. Although they are expressed in various cells like neurons, smooth muscle cells, fibroblast cells, cartilage, and bone cells, the reason behind their expression is unclear. Some features of FGF biosynthesis have been understood to some extent; for instance, signal sequences of some FGFs help in their secretion, the mRNA sequences from 5′ to the starting AUG codon may play a significant role in FGF biosynthesis, and upstream and in-frame CUG triplets can behave like an alternative translation initiation site in bFGF and INT-2 mRNAs. However, it is well established that stress related to the liver and heart prompts the production of FGF21 [20].

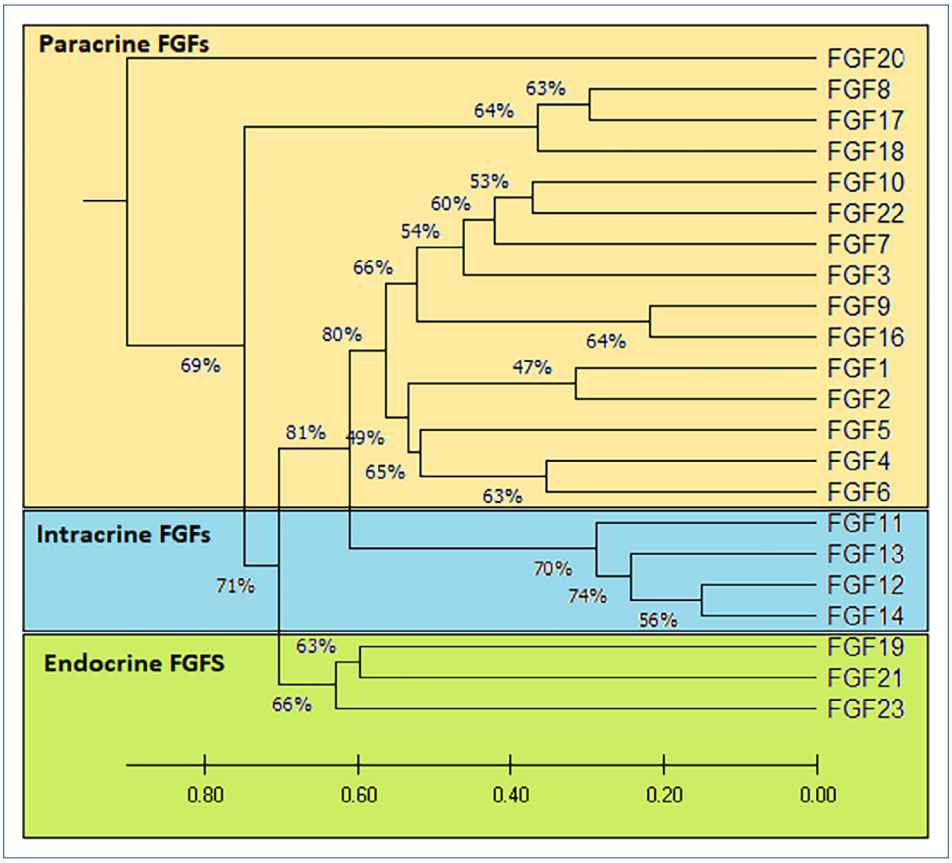

Figure 1. The phylogenetic tree of the FGF family based on the evolutionary relationships of FGFs [Phylogenetic and molecular evolutionary analyses were conducted using MEGA version X [83].

FGFs: Fibroblast growth factors.

Molecular interactions of FGF

Heparan Sulfate

Heparan Sulfate acts as a necessary cofactor for all paracrine FGFs, all of which show moderate to high affinity toward Heparan Sulfate. The binding of HS to FGF1 protects it from thermal denaturation, and the binding to FGF2 protects it from both thermal denaturation and proteolysis. The binding of FGFs to Heparan sulfate proteoglycans limits their diffusion and secretion into the interstitial space, therefore aiding them in exerting their effect at the site of their production. Cells that are unable to synthesize HS on their surfaces require heparin for effective and strong binding between FGFs and FGFRs. Studies have also shown that heparin/HS increases the half-life of the FGF/FGFR complex [21].

HS consists of heterogeneously sulfated linear polymers of repeating disaccharide subunits of hexuronic acid (iduronic or glucuronic acid) and amino acetylglucosamine, linked through α-1,4-glycosidic bonds. HS is sulfated at the 2-O position of glucuronic acid and amino acids and at the 6-O position of N-acetylglucosamine. HS shows covalent relationships with selective serine residues of proteoglycans, such as membrane-bound syndecans and glypicans and extracellular matrix (ECM) perlecan, which means they are present within the extracellular matrix of tissues and are also located on the surface of the cells [22].

HS binds to the β1-β2 loop and β10-β12 region of FGFs, which are composed of solvent-exposed basic amino acids and backbone atoms of FGFs. The HS-FGF binding affinity depends on the primary variable sequence of the HS binding region of different FGF molecules. Consequently, to some extent, this differential affinity between HS and different FGFs results in distinct biological functions of FGFs [23].

FGF-receptors

Many cell types that express FGFs also bear FGF receptors (FGFRs) on their surfaces. These FGFRs are tyrosine kinase receptor proteins with a molecular weight of 125–160 kDa, which transduce the FGF signals via phosphorylation of tyrosine residues of FGFR polypeptides, elicited by the binding of FGFs to FGFRs [24]. The first FGF receptor, FGF receptor 1 (FGFR1), was isolated from membrane fractions of chicken embryos by tagging it with crosslinked FGF2 and I^{125}. It showed a resemblance with a partial human cDNA clone called FLG (Fms-like gene). Subsequently, three more FGFRs were discovered, along with their isoforms. These FGFRs are characterized as transmembrane

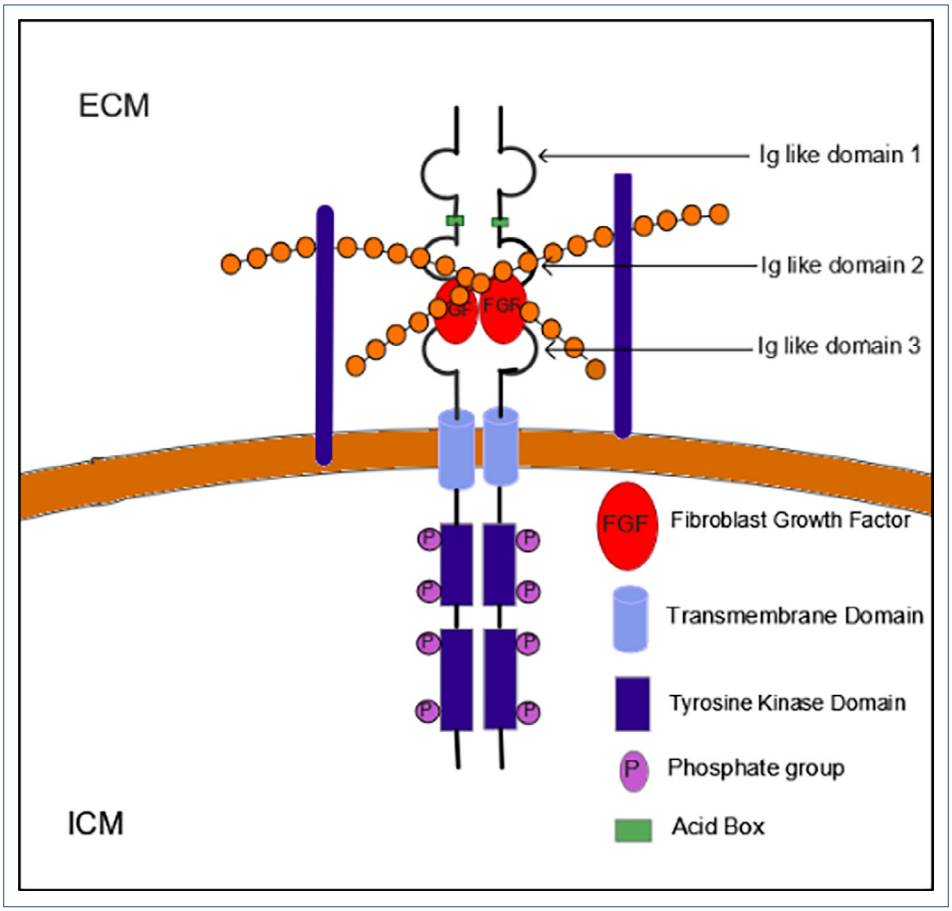

Figure 2. The schematic shows an Auto phosphorylated transmembrane FGFR dimer bound to the FGF molecules. Each FGFR molecule has one extracellular domain with three Ig-like subdomains, one transmembrane domain and one split type Tyrosine kinase domain. The alternative splicing of exon 8 and exon 9 of gene encoding Ig like domain III of FGFR 1, FGFR2, and FGFR3 results in Ig-IIIb (green) and Ig-IIIc (orange) isoforms of these FGFRs [25].

ECM: Extracellular matrix; ICM: Inner cell mass; FGF: Fibroblast growth factor; FGFR: Fibroblast growth factor receptor.

proteins consisting of three domains: an N-terminal extracellular domain with three immunoglobulin-like subdomains (IgI, IgII, and IgIII), a transmembrane domain that is a single α-helix, and an intracellular single split-type tyrosine kinase domain (Fig. 2) [25]. These FGFRs can be placed under the Ig superfamily of receptors, along with other tyrosine kinase receptors like the platelet-derived growth factor-α receptor (PDGFαR), PDGFβR, and interleukin receptor-1 (IL-1R). These FGFRs form dimers after binding to the FGFs. The FGFs bind to the Ig-like domain II in the presence of HSPG, and these altogether form a dimer with a similar complex [26].

FGF-activated cell signaling pathways

As all Tyrosine kinase receptors transduce extracellular signals to cytoplasmic transduction signal pathways by phosphorylating tyrosine residues, similarly, FGF receptors also transduce the extracellular signals to cytoplasmic transduction signal pathways by auto-transphosphorylation of tyrosine residues. The dimerization of FGFRs is necessary for their activation and signal transduction. The lateral dimerization of FGFRs in the plasma membrane brings the two tyrosine domains nearby, resulting in the trans-autophosphorylation of the tyrosine residues present in their activation loops, which results in the activation of kinase domains. These activated kinase domains interact with adapter proteins and other cytoplasmic substrates, finally triggering the cascade process of signaling pathways that control cellular functions like differentiation, proliferation, regeneration, repair, growth, etc. [27].

When FGFs act as signaling molecules for the activation of four major cellular pathways, they bind to the FGF tyrosine kinase receptors to induce dimerization and auto-trans-autophosphorylation in their kinase domains. The phosphorylation of the tyrosine kinase domain of FGFR1 is completed in three phases: in phase one, Y653 residue is phosphorylated, resulting in a 50- to 100-times increased activity of the kinase domain; the second phase phospho-

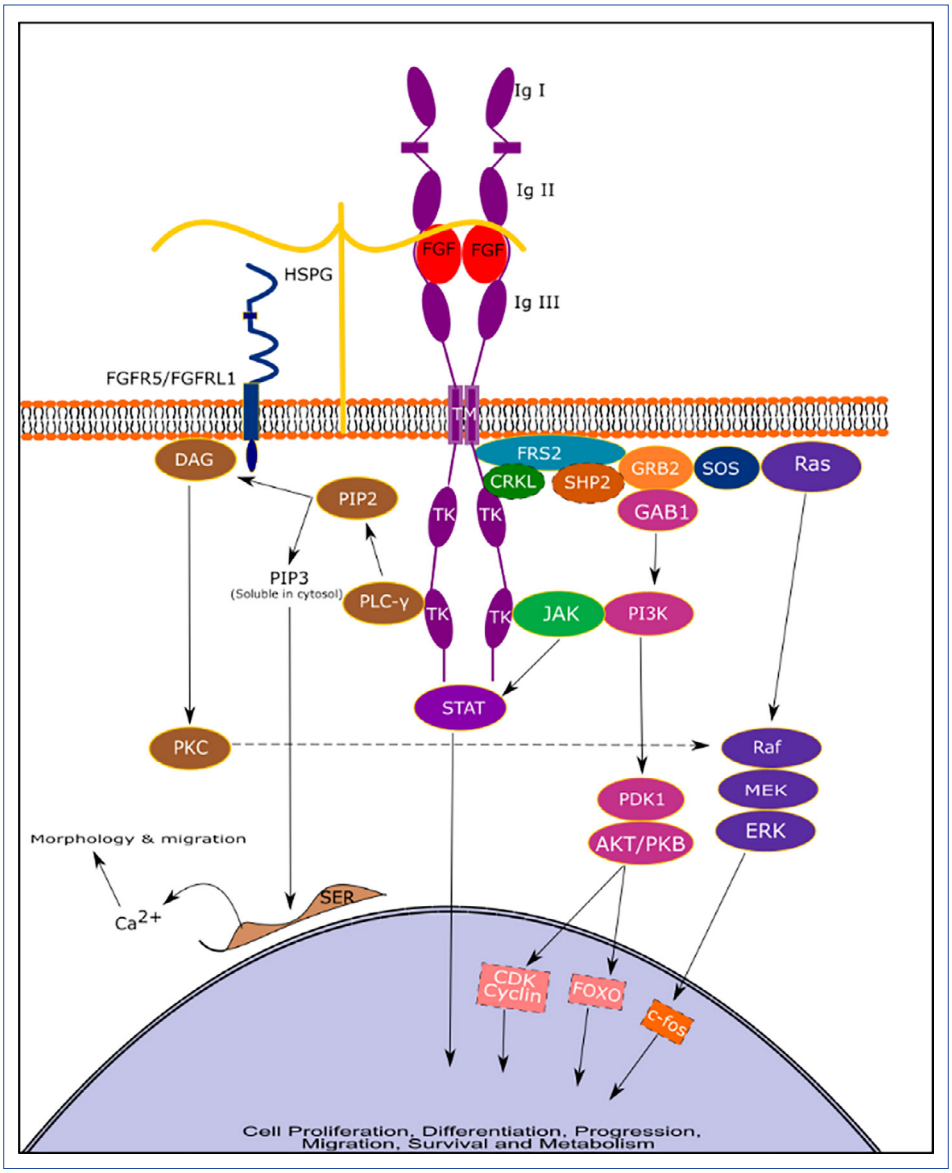

Figure 3. The FGF binding to the FGFRs triggers the dimerization of FGFRs which in turn leads to the trans-autophosphorylation of the Tyrosine kinase domains of FGFR dimer which ultimately leads to the initiation of cellular signaling cascades like RAS-MAPK, PI3K-AKT, PLCγ, and (STAT). Now, these cascades induce the activation of several genes in the nucleus leading to the transcription of respective mRNAs which are transferred out to the cytosol for the respective protein synthesis [84].

FGF: Fibroblast growth factor; FGFR: Fibroblast growth factor receptor; Ig I, II, III: Immunoglobulin-like domain I, II, III respectively; HSPG: Heparan sulfate proteoglycan; FGFRL1: Fibroblast Growth Factor Like 1; SER: Smooth endoplasmic reticulum; JAK: Janus kinase; CRK: CT10 regulator of a tyrosine kinase; CRKL: Crk-like protein; CDK: Cyclin-dependent kinase; FOXO - forkhead box transcription factor.

rylation of Y583, Y463, Y766, and Y585 does not result in any increment in kinase activity; whereas, during the third and final stage, Y654 phosphorylation leads to a further 10-times increase in the activity of the kinase domain [28]. This phosphorylation of six tyrosine residues of the tyrosine domain of FGFR1 completely activates it and increases the overall activity of the tyrosine kinase by 500–1000 folds. The activated FGFRs trigger the chain reactions of various cellular cascades including Ras/Raf/MAPK, PI3K/AKT, PLCγ, and STAT (Fig. 3). The binding of STAT3 and PLCγ requires the phosphorylation of two additional tyrosine residues, i.e., Y677 and Y766 [29].

RAS/RAF/MAP kinase pathway

The RAS/MAP Kinase Pathway is involved in important cellular functions like cell growth, proliferation, and migration. The phosphorylation of FRS2α by the tyrosine kinase domain of FGFR activates the RAS/MAP Kinase Pathway. The phos-

phorylation of FRS2α is dependent on the phosphorylation of Y463 residue of the intracellular domain of FGFR and the presence of CRKL at the docking site of the FGFR tyrosine kinase domain. A complex comprising GRB2 and the tyrosine phosphatase SHP2 is recruited by this activated FRS2α. Further, GRB2 recruits the son of sevenless (SOS), which activates the Ras molecule by exchanging one molecule of GTP for GDP, which leads to the activation of the Ras/MAP Kinase Pathway. Activation of this pathway by FGF1 relates it with the protection of the heart, development of nerve tissue, tumor invasion, biosynthesis of cholesterol, differentiation of adipocytes, and metastatic cancer [30].

According to studies, the negative feedback loop mediated by MAP-Kinase is induced by FGF signaling which phosphorylates the threonine of FRS2α, resulting in decreased engagement of GRB2 to FRS2α due to hampered phosphorylation of tyrosine residues of FRS2α. This ultimately results in the attenuation of the Ras/MAP Kinase Pathway [31].

PI3K/AKT pathway

Indeed, in addition to the Ras/Raf/MAP Kinase Pathway, the PI3 Kinase/AKT Pathway is also activated by FRS2α. Following the formation of the FRS2α, GRB2, and SHP2 complex, GRB2 recruits GAB1, subsequently triggering PDK1 and AKT to initiate the PI3K pathway. The PI3K/AKT pathway is implicated in cell survival, determination of cell fate, cell growth, cell proliferation, and cell migration. Regarding the FGF1-related PI3 Kinase/AKT Pathway, it plays a role in various physiological processes, including angiogenesis, lung development, maintenance of neuronal phenotype, preservation of neuronal structure and/or function, and ApoE-HDL (Apolipoprotein E-high-density lipoprotein) release [32].

PLCγ pathway

In this pathway, the activated FGFR tyrosine kinase domain phosphorylates PLCγ, which is activated by the formation of IP3 and DAG from the breakdown of PIP2 (Fig. 3). This membrane-bound DAG activates Protein kinase C, and the soluble IP3 stimulates the smooth endoplasmic reticulum (SER) to release high amounts of calcium ions, affecting cellular morphology and migration. Activated PKC likely expresses the gene for adhesion purposes [33]. Here, GRB14 acts as an inhibitor of PLCγ by inhibiting the tyrosine phosphorylation by binding to activated FGFR1 at pY766 [34].

Biological functions of FGFs

The FGFs exert their effect on cells by binding to the tyrosine kinase receptors and phosphorylating them. As a result of this phosphorylation, many cellular signaling cascades are activated, which result in cellular functions through gene transcription and then protein synthesis. Thus, some of the cellular functions are regulated directly or indirectly by FGFs, which are summarized in Table 1.

Cell proliferation

Cell proliferation implies the division of cells at a war scale, meaning repetitive division of cells. This is mostly observed at the developmental stage and to some extent in adults during tissue regeneration or repair, and in cancerous conditions. The essentiality of FGFs in cell proliferation begins with the proliferation of the inner cell mass (ICM) in mice [35]. Whereas FGF4 is expressed in epiblast cells [36] and FGF1 is involved in the proliferation of human preadipocytes, promoting adipogenesis in humans [37]. Similarly, FGF10 also facilitates the proliferation of epithelial cells in prostate cancer [38]. It has been established that FGF2 promotes cell proliferation by activating the Ras and RAF cascade process. FGF2 binds to the FGFRs which leads to the dimerization of FGFR, and this leads to the autophosphorylation of the tyrosine domain of the receptor. This phosphorylated domain acts as the binding site for some of the intracellular signal transducers like Grb2, which makes a complex with Ras guanine nucleotide-releasing factor Sos. This Grb2-SOS complex recruits the Ras oncogene in the plasma membrane. This recruited Ras is further activated by SOS by exchanging GDP for GTP. Further, this fully activated Ras activates the Raf-mediated MAP kinase signaling cascade, which finally results in cell proliferation [39]. This clearly shows the involvement of FGFs in cell proliferation.

Cell differentiation

Developmental biology defines cell differentiation as the process by which more specialized cells develop from less specialized ones, as observed in the development of the human zygote. The zygote develops from a single cell into an embryo and then into an adult human being. The process of differentiation can be observed in early life during development and in adults during normal cell turnover and tissue repair. Various FGFs play significant roles in the process of cellular differentiation during both the developmental and adult stages [40].

Many FGFs have been identified as crucial for cellular differentiation. FGF2, for example, is used in the divergence of neural stem cells into fully-fledged neurons and glial cells. Similarly, the morphogenesis and differentiation of suprabasal keratinocytes without FGF7 are unimaginable. Additionally, the differentiation of monkey stem cells into dopamine-synthesizing neurons would not be possible without in vitro treatment with exogenous FGF20 [41].

Cell migration

During intrauterine development, wound healing, tissue repair, and immunological responses, chemotactic movement of cells or cell migration are key processes involving many FGFs at some stages. FGF7 stimulates the migration of human keratinocytes and also regulates plasminogen activity in these cells. Similarly, FGF2 and FGF8 act as chemoattractants in the migration of mesencephalic neural crest cells [42].

Table 1. Biological functions of FGFs and their target cells/Tissues

Function	FGF involved in the function	Target cell/Tissue/ Organ	Reference
Cell proliferation	FGF1, FGF2	Preadipocyte, Endothelial cell, epithelial cell, fibroblast cell, neural stem cell	[8]
	FGF2	hematopoietic stem cells, embryonic stem cells, dental pulp stem cells and periodontal ligament stem cells	[62]
	FGF4	Trophoblast stem cell	[8]
	FGF7, FGF10	Epithelial cell	[8]
	FGF18	Osteoblast, chondrocytes, osteoclast	[63]
Cell migration	FGF2	Vascular endothelium	[64]
	FGF4	mouse Embryonic Skeletal Muscle cells	[65]
	FGF7	Human Pancreatic Duct Epithelial cells	[66]
	FGF8	Neural crest cell	[67]
Cell differentiation	FGF1, FGF2	Neuroepithelial	[68]
	FGF4	Embryonic stem cell	[27]
	FGF2	mesenchymal stem cells	[62]
		rabbit bone marrow stromal cells	[69]
	FGF7	mouse Progenitor cells	[70]
	FGF20	Monkey embryonic stem cell	[55]
Angiogenesis	FGF1, FGF2	Endothelial cell	[8]
	FGF9	Mouse Bone tissue	[71]
Metabolism	FGF21	Adipocytes and β-cells of the pancreas	[44]
	FGF23	Parathyroid gland and Kidney (Phosphate and vitamin D metabolism)	[72]
	FGF15/19	Hepatocytes (glucose metabolism)	

FGFs: Fibroblast growth factors.

Angiogenesis

During intrauterine life development, wound healing, tumor development, and tissue repair, angiogenesis is a central process. It refers to the development of new vessels from pre-existing vessels. FGFs are established to play a crucial role in inducing angiogenesis. FGF1, FGF2, and FGF4 have prominently defined angiogenic properties. These FGFs have been reported to upregulate urokinase-type plasminogen activator (uPA) and metalloproteinases (MMPs) in endothelial cells, consequently resulting in the proliferation of endothelial cells and the organization of endothelial cells into tube-like structures [43].

Metabolism

Many FGFs also play important roles in metabolism. They bind to their receptors on cells and induce effects related to metabolism. For example, FGF21 is primarily expressed by the liver to reduce hepatic glucose output. It also increases the uptake of glucose by adipocytes and improves or preserves the functions of β-cells in the pancreas [44]. The FGF19 subfamily regulates many mainstream metabolic pathways, including those involving carbohydrates, lipids, and bile acids, as well as vitamin D and phosphate homeostasis [45].

Engineered fibroblast growth factor: The new hope

The natural FGFs are strongly mitogenic and also have a very short half-life, making it difficult to control their mitogenicity and maintain their viability at the site of administration. Thus, the use of structurally modified/engineered FGFs for therapeutics or tissue engineering is becoming increasingly popular among biotechnologists.

As discussed earlier, FGFs have been very effective in the repair and regeneration of tissue. They stimulate FGFRs by acting as signaling molecules, which results in the initiation of a cascade of several cellular signaling pathways, such as RAS/MAP kinase, PLCγ, SNT-1/FRS2, Crk-mediated signaling, and PI3 kinase/AKT pathway. These pathways ultimately result in cell differentiation, proliferation, migration, and angiogenesis. Due to this broad range of biological activities, FGFs have attracted significant interest for their application in therapeutics and tissue regeneration and repair. However, due to their mitogenic nature and the challenges in delivering them to the target tissue, their use has been limited. To combat these issues, researchers have engineered FGFs both structurally and genetically and have developed several delivery systems for administering FGFs to the target tissue.

Table 2. Different types of FGF medication and their resulting recombinant FGF examples.

Type of Modification	Mutation/ modification	Example	References
Point mutation	Point Mutation (such as amino acid substitutions, deletions, additions, or combinations) of at least one amino acid residue	Q40P rFGF1, S47I rFGF1 and H93G rFGF1 respectively	[50]
Chain truncation	Deletion of continuous amino acids from one end or both	FGF1ΔNT; K25 to D155 and FGF1ΔNT2; L29 to D155	[73]
Combination of point mutation and chain truncation	FGFs modified both with point mutations and chain truncation altogether	FGF1 ΔNT1 (1-140 αα) M 1	[73]
Polypeptide chain extension	The addition of a few more amino acids to the natural polypeptide chain of FGFs	H6-FGF2 (rFGF2)	[62]
Chimeric proteins	Combination of specific sequences of one FGF to another mutated FGF.	FGF19/21-1	[68]
Extra group addition	Addition extra functional group or chemical compound	PEGylated FGF2	[74]
FGF-mAb fusion	The fusion of monoclonal human Ig fragments with FGFs	Fc-FGF21	[75]
rFGF-VLP conjugate	A recombinant FGF is conjugated with a virus-like particle (HBsAg in this case)	(Trx-FGF2)-HBcAg	[76]
SUMO ubiquitination	Small ubiquitin-related modifier (SUMO) chaperone protein attached to recombinant human FGF21	srhFGF-21	[77]

Table 3. Use of recombinant FGFs in Tissue regeneration

FGF	rFGF variant	Target tissue	Treatment	Reference
FGF1	TTHX1114	Corneal epithelial cells	Short-term Corneal Nitrogen Mustard Injury (In rabbit)	[78]
FGF2	-	Alveolar epithelium	COPD and Emphysema	[79]
	rhFGF-2	Osteocytes	Tibial Shaft Fractures	[80]
FGF18	rhFGF18	Cartilage	Repair of Articular Cartilage	[81]
FGF19	M70	Biliary and hepatic cells	Liver injury (in mice)	[82]

COPD: Chronic obstructive pulmonary disease; rFGF: Recombinant Fibroblast Growth Factor.

Engineered/recombinant FGFs represent a new hope for use in therapeutics. Recent clinical trials and concept studies are exploring the use of recombinant FGFs for tissue engineering and other applications. There are various methods for modifying FGFs, including point mutations targeting specific amino acids and the formation of chimeric proteins by combining sequences of two different FGFs [46, 47].

FGFs can be modified both at the genetic and protein levels, and various recombinants of FGFs have been developed so far. Mutated FGF proteins retain their ability to bind with FGFRs with the same specificity but without altering their properties that trigger cell growth, proliferation, survival, etc. [48]. Based on modifications done at the genetic level of FGFs or in the protein structure of FGFs, modifications can be classified into various categories (Table 2). Table 2 illustrates almost all modifications/mutations performed to date on the native forms of FGFs (with examples) to improve their activity—such as reducing their mitogenic properties while maintaining other desired properties intact and also obtaining desired characteristics like structural stability and longer half-life.

Engineered FGFs in tissue regeneration

The regeneration of damaged and injured tissues remains a significant challenge for humanity. It has been a major focus for researchers and biotechnologists for decades. The tissue regeneration capability in mammals, including humans, is very limited. In the case of tissue injury, cells from adjacent tissue and progenitor cells recruited from the bone marrow migrate and proliferate at the site of injury. Both the repair and regeneration of tissue are controlled by various cytokines and growth factors, with FGFs being among them. For example, FGF9 promotes long bone repair, and a combination of FGF7 and FGF10 promotes wound re-epithelialization in mice [49]. However, the challenge with FGFs lies in their almost uncontrollable mitogenesis and their controlled administration to specific sites.

Low stability and susceptibility to degradation by proteolytic enzymes also hinder the therapeutic use of FGFs. To overcome these problems, researchers have introduced mutations and alterations in the native sequences of FGFs, finding it feasible to some extent. For instance, the Q40P/S47I/H93G variants of

recombinant/mutated FGF1 have shown 10-fold higher activity in DNA synthesis [50]. In recent years, the use of mutated FGFs in tissue regeneration has become increasingly popular. Table 3 shows the mutated FGFs used in tissue regeneration or repair over the past few years.

Engineered FGFs in angiogenesis

The process of forming new blood vessels or capillaries from pre-existing ones during embryonic wound healing, embryonic development, or the menstrual cycle in females is known as angiogenesis. FGFs regulate the stimulation of endothelial cells to secrete proteases and plasminogen activator, which in turn degrade the basement membrane. This degradation allows new cells to migrate to the area, where they proliferate and differentiate into new vessels. bFGF was the first-ever FGF identified as an angiogenesis-stimulating growth factor [51]. Several trials, such as FIRST (FGF Initiating Revascularization Trial), AGENT (Angiogenic GENe Therapy), and TRAFFIC (Therapeutic Angiogenesis with recombinant Fibroblast Growth Factor for Intermittent Claudication), have been conducted to treat ischemic diseases with recombinant/engineered FGFs. However, none of these trials have yielded significant results [52], although the clinical trial TALISMAN (Therapeutic Angiogenesis with Intramuscular NV1FGF) did reduce the mortality rate in patients with Critical Limb Ischemia, though it was not found to be significant [53]. The hopes do not end here, as there are expectations that recombinant and improved FGFs will soon be used for revascularization/angiogenesis processes.

Methods of FGF administration

As mentioned above, the low stability and susceptibility to degradation by proteolytic enzymes also hinder the therapeutic use of FGFs. Various strategies have been employed to increase the stability, reachability, and efficacy of FGFs. FGFs have been directly used for healing wounds and administered in vivo to induce the regeneration of various tissues such as nerve, bone, cartilage, skin, endothelial tissue, and dental tissue. However, studies have shown that FGFs either become functionally degraded by enzymes or suffer diffusional loss [54]. To combat this situation, FGFs are either engineered or delivered to the site using some delivery systems listed below. The following are examples of different drug delivery systems used to administer FGF to the site of action [55, 56].

Scaffolds

FGFs or other growth factors are immobilized on matrices made of materials like collagen, beta-tricalcium phosphate (β-TCP), polyhydroxy ethyl methacrylate, polyurethane, etc. These are then molded into scaffolds using several techniques such as particulate leaching, solvent casting, freeze-drying, phase separation, melt molding, in situ polymerization, gas foaming, and phase emulsion. An excellent example of this is the delivery of FGF2 immobilized into a polyhydroxy ethyl methacrylate scaffold for bone regeneration [57]. Due to the specific biochemical interactions between natural polymers of scaffolds and FGFs, these structures are better suited for long-term delivery and stability of FGFs [58]. Scaffolds need to be implanted at the site of tissue defects.

Hydrogels

Similar to scaffolds, hydrogels also impregnate growth factors onto gels such as a carboxymethylcellulose-based topical gel, hydroxypropyl cellulose (HPC) gel, and fibrin gel. These impregnated growth factors in hydrogels are then injected into the tissue defect site. A notable example of FGF delivery using this technique is the delivery of FGF2 via a 3% hydroxypropyl cellulose gel for the regeneration of periodontal tissue [59].

Nano- and micro-particulates

The delivery of FGFs using nano- and micro-particulate techniques is even easier than the scaffold and hydrogel methods. In this growth factor delivery system, many natural and synthetic polymers are designed to be spheres a few micrometers to a few nanometers in size, and these spheres incorporate the growth factors. These FGF-containing polymers are administered either via the bloodstream or orally. The particulate method of growth factor delivery is easily manipulated to safely deliver FGFs to the defect site, and this method also ensures the full utilization of the roles and functions of FGFs. An example of this particulate method of delivery is the physical and chemical conjugation of FGF to magnetic iron oxide nanoparticles for targeting the nasal olfactory mucosa [60, 61].

Conclusion

FGFs are crucial growth factors for various physiological processes, including cell proliferation, growth, metabolism, angiogenesis, cell survival, and migration. They play an active role in embryonic growth and other physiological processes like tissue regeneration. FGFs, along with their receptors, have been associated with various pathophysiological conditions, including cancers. Their mitogenic activity has been linked to several types of cancer, including lung, breast, and prostate cancer. Due to their broad range of activities, they are a focal point for therapeutic use. Several recombinant and engineered FGFs have been shown to be effective in conditions such as bone injuries, COPD, and emphysema. Research on metabolic disorders like diabetes is ongoing to determine the therapeutic use of FGFs in these conditions. Since the half-life of FGFs is very short, various methods of site-specific delivery of FGFs have also been developed, including hydrogel, scaffold, and nano- and micro-particulate methods. These methods enhance the application of FGFs and improve their therapeutic use. In conclusion, FGFs could potentially offer solutions for various human health issues, including cancers, some metabolic and cardiac disorders,

and bone injuries. Several studies have been conducted, and others are ongoing, to determine the therapeutic effects of FGFs on various disorders; however, the number of studies, especially in the context of cancer, is still limited. Although many studies have established the role of FGFs in tumorigenesis and cancer progression, only a few have explored their therapeutic roles in cancer biology.

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Letter to the Editor

The changes of oxidative stress markers and Vitamin E in patients with diabetes using SGLT2 inhibitors

Pradnya Padalkar

Department of Biochemistry, Bharati Vidyapeeth Deeemed To Be University Medical College, Pune, India

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Dear Editor,

I read, reviewed carefully, and meticulously the article published by Banu Buyukaydin et al., in which the authors aimed to show changes in oxidative stress and vitamin E in diabetes using an SGLT2 inhibitor. Int J Med Biochem 2023;6(3):185-190. They concluded their evaluation of the antioxidant properties of SGLT2 inhibitors along with oxidative stress markers. I want to congratulate the authors for this research publication and want to contribute by emphasizing a few important points of this study.

First, this original study includes the measurement of oxidative stress markers and antioxidants like vitamin E (non-enzymatic) and catalase (enzymatic). As catalase was measured by a colorimetric method, the author can add the word "antioxidants" to the title instead of only "vitamin E," which is a non-enzymatic marker. According to the study, both antioxidants do not show a significant relationship with or without an SGLT2 inhibitor, yet catalase has proven to be a better enzymatic marker to study antioxidant status. Otherwise, if the patient is treated with vitamin E supplements, then only vitamin E measurement is sufficient to study changes in diabetes, and catalase measurement can be avoided.

Second, if total oxidant status (TOS) and total antioxidant status (TAS) were measured along with oxidative stress index (OSI), myeloperoxidase, vitamin E, and catalase measurement was just an addition of tests and even the study shows all these do not show a significant relationship in the study population.

Third, HbA1c values mentioned in "Table 1" of the article show a mean value of 7.1% and in "Table 2," without an SGLT2 inhibitor, it was 7.0%, and with an SGLT2 inhibitor, it was 7.2%. "Table 1" does not mention the mean value of glucose. Also, blood glucose and HbA1c mean values show a positive correlation but +1% is not such a big significant difference. The study explains the reason for the reduction in HbA1c concentration is increased glucose excretion, but that could be a sign of diabetic nephropathy, which should also be taken into consideration by the author. WHO criteria for diabetes for HbA1c need to be considered if a baseline study was not done [1].

Fourth, the study needs to emphasize other factors like the duration of the disease and comorbidities if any, because, as mentioned, the mean age of the study population was 55.7 years, the duration of the disease and comorbidities could affect this age group and may result in a non-significant relationship with study objectives [2]. Also, the adverse effect of an SGLT2 inhibitor in a patient on diuretics may lead to osmotic diuresis, though it is rare yet to be considered [3].

Fifth, a patient on only antidiabetics drug like metformin may not be considered; ADA criteria equally have importance for diabetic patients because many young women on metformin for the treatment of Polycystic Ovarian Disease (PCOD) [2].

Address for correspondence: Pradnya Padalkar, MSc. PhD. Department of Biochemistry, Bharati Vidyapeeth Deeemed To Be University Medical College, Pune, India
Phone: +7758066651 **E-mail:** pradnya.padalkar79@gmail.com **ORCID:** 0000-0002-9217-7633

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