**Review and Research on Cancer Treatment**
Volume 5, Issue 1 (2019)

Review and Research on Cancer Treatment
Volume 5, Issue 1 (2019)





ISSN 2544-2147

Publisher:
Fundacja na rzecz promocji nauki i rozwoju TYGIEL
ul. Głowackiego 35/348, 20-060 Lublin
www.fundacja-tygiel.pl

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Volume 5, Issue 1 (2019) ISSN 2544-2147

# Application of the tape stripping method in the research on the skin condition and its diseases

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**Abstract:** Tape Stripping (TS) is a minimally invasive but yet a very useful procedure which enables the partial removal of the *stratum corneum* (SC) almost layer by layer. Being the outer layer of the epidermis, SC is responsible for the mechanical protection of the skin and deeper lying organs, as well as the protection against water loss from the body. In addition, the occurrence of the lipid coat on the surface of the SC regulates the process of absorption and penetration of various substances into the skin. The corneocyte samples obtained by the use of adhesive tapes may provide a lot of useful information concerning the skin condition and also its proper functioning as a barrier. This data could be helpful in the investigation and diagnosis of several skin diseases.
In this review, we present the history of the tape stripping method, some critical parameters of the procedure as well as the possible applications of the combined non-invasive tape stripping method with the other more advanced methods to analyze corneocytes taken from volunteers or patients.

## 1. Introduction

The minimally invasive procedure which allows the collection of the cells from the *stratum corneum* by a single or multiple use of the appropriate adhesive tape is the tape stripping method. It was invented in the 1940s and can be used in many studies carried out on humans as well as on animals such as pigs, rats, mice and guinea pigs (Arct, Koazyra et al. 2013). This method is based on the interaction of the adhesive forces of the tape with the cohesion forces occurring between the cells.

The general procedure (Fig. 1) of the tape stripping method is based on placing an adhesive tape on the surface of the skin (previously selected area – head, arm, leg), a gentle pressure on the tape ensuring a good contact of the tape with the cells and next by the removal of the tape with the cells through tearing the tape from the surface of the epidermis. The sample should be secured on a glass slide or in a tube. If more samples are needed or the study requires an analysis of certain part of the stratum corneum, the procedure should be repeated. Tests should be performed within 24-48 h for freshly collected cells or they should be frozen and stored at the temperature of -80°C.

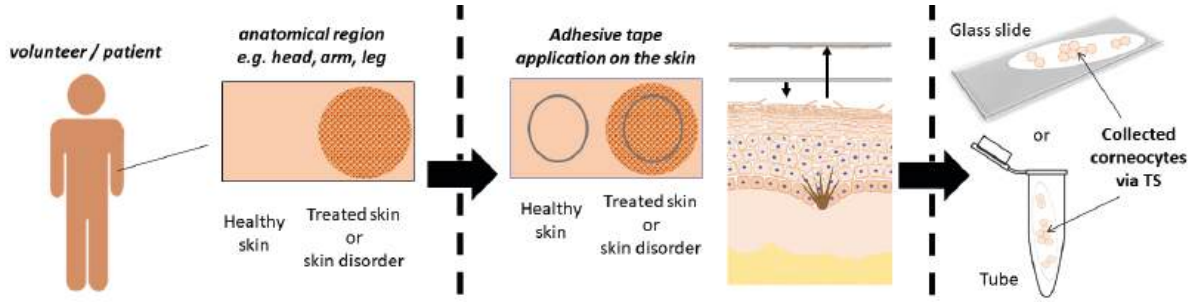


Figure 1. The basic procedure of the tape stripping method.

The procedure is painless and practically non-invasive, that is why it can be used routinely for testing skin conditions and in case of skin diseases in diagnostics. To receive high quality results, one must remember of some crucial parameters not only during the sample collection but also when measuring these kinds of samples.

## 2. Search strategy and selection criteria

Terms used for the preparation of this article are a combination of the words "tape stripping – skin/ corneocytes – selected methods – skin disorders – skin diseases".

As the selection criteria we have chosen articles in which the tape stripping method was used for some skin analysis mainly on human. For a better understanding of some issues some additional articles were also investigated.

## 3. State of the Art

The largest organ with a complex layered structure is the skin. Its surface in the adult human is around 2 $m^2$ and it weighs approximately 4 kg. The thickness of the skin depends on the place on the body that it covers e.g. on the eyelids it is the thinnest (0.5 mm), while on the palms and feet it is the thickest (4 mm). The skin consists of the epidermis, the dermis and the subcutaneous tissue. The surface of the skin is covered with exfoliated keratin and lipid coat (oil-water slurry), which protects the skin mainly against harmful chemical agents and microorganisms.

In addition, applying cream to the skin protects it to some degree against mechanical damage (Noszczyk, 2018).

The epidermis is the outermost layer of the skin. It is made of a constantly renewing multilayer epithelium (basic, spinous, granular and translucent) and an outer horny layer. The epidermis is basically built of keratinocytes (about 90%) and other cells, i.e. the melanocytes and the Langerhans cells (cells of the immune system) as well as the Merkel cells (cells of the nervous system). Depending on the type of cells and layer, epidermal cells differ in shape, size, structure and production of various chemical substances like the pigment melanin, hormones α-MSH (α-melanocyte-stimulating hormone) and ACTH (adrenocorticotrophic hormone), some cytokines from the interleukins families, the tumor necrosis factor (TNF) and the transforming growth factor (TGF) superfamily (Gasque, Jaffar-Bandejee, 2015; Hirobe, 2014). The process of keratinization, that is the passing of keratinocyte from the basal layer to the cornified layer and then subject to exfoliation, takes about 26-28 days. The daily amount of exfoliated epidermis is 6-14 g and it is caused by the continuous separation of corneous plaques from the cornified layer of the epidermis (Jabłońska, Majewski, 2010).

The most outer layer of the epidermis is the *stratum corneum*. It is composed of flattened corneocytes surrounded by the intercellular lipid cement. The dry mass of the corneocytes from the horny layer is about 50-80% lower than the cells from the granular layer. This is mainly due to two major changes that occur during the keratinization process while keratinocytes transform into corneocytes, i.e. they lose the cell nuclei and almost all of the cytoplasm (except for the intermediate filaments mostly keratin). The lipid matrix of the SC consists of ceramides, cholesterol and free fatty acids, that are responsible for the layer function as a barrier (Alikhan, Maibach, 2010). Moreover, the occurrence of the supramolecular structure, the glycosaminoglycan polymer hyaluronan (HA, hyaluronic acid) scaffold for the sulfated proteoglycans and the matrix proteins, enables water and ions trapping and prevents skin dehydration and turgor loss (Guzmán-Alonso, Cortazár, 2016).

As a consequence, corneocytes create a strong layer quite resistant to mechanical damage of the whole skin and to water loss from the body. In addition, the appearance of the lipid coat on the surface of the SC regulates the process of absorption and penetration of the substance into the skin (Jabłońska, Majewski, 2010). An important role in the SC formation, cohesion and desquamation is played by the expression of sugar moieties (glycans) on the surface of viable keratinocytes. Here, the formation of the permeability barrier in SC is possible due to the deglycosylation of glycosyl ceramides (Abdayem, Formanek et al. 2016).

Skin, equally like other organs, is affected by aging due to the intrinsic and extrinsic aging factors like thermal and radiation skin damage. This complex and multifactorial phenomenon progressively leads to a loss of structural integrity and physiological function of the skin (Farage, Miller et al. 2007). One can mention a few dermatological diseases like: the bacterial infection (e.g. impetigo); the fungal infection (e.g. nail fungus); the viral infection (e.g. herpes); the inflammatory and the allergic diseases (e.g. atopic dermatitis, psoriasis); the autoimmune diseases (e.g. systemic lupus erythematosus); moles, marks and benign tumors (e.g. fibroma); and last but not least the most dangerous skin malignancies like the basal cell carcinoma (BCC), the squamous cell carcinoma (SCC) and melanoma skin cancer (Burns, Breathnach et al. 2010). Some diseases may be easily diagnosed without sample collection just by observation. However, in some cases blood sample, skin swabs or scraps and even biopsy must be taken directly from the patient

for investigation. In these cases, the selection of a proper diagnostic biomarker covering the wide range of phenotypes and disease is obligate, but at the same time the analysis (e.g. mRNA) may be costly and quite laborious (Berekméri, Latzko et al. 2018). Other diagnostic possibilities randomly

used are allergy tests or skin imaging. Nowadays, a label-free imaging by the confocal Raman microscopy or the attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) are often used for skin investigations performed both *ex vivo* (skin biopsy) as well as non-invasively *in vivo* (Ashtikar, Matthäus et al. 2013; Tosato, Orallo et al. 2015; Bindera, Kulovitsa, 2018).

In this way the profiles of human *SC* were obtained and different components of the tissue were detected. Nevertheless, one must be aware that developing non – or less – invasive (than biopsy) methods are more comfortable for the patient under the condition that they are still reliable. Especially, in case of the requirement of repetitive skin sampling before and after treatment or to prevent possible infections after biopsy (Clausen, Slotved et al. 2016). The introduction of the tape stripping method gave a significant expansion to experimental tools in the subject of skin research, both its condition and diseases. This is mainly due to the specificity of the method which enables one to obtain only the *stratum corneum* layers and in this way, it minimalizes the main side effects of other methods like biopsy – pain, itching or possible infections in case of improper wound dressing.

The first description of the method to remove cells from the epidermis with a cellophane tape was described by Wolf in 1939 (Wolf, 1939). With this method it was then possible to study the morphology of the cells. In 1951 with the use of the TS method Pinkus demonstrated that the removal of almost all of the horny layer stimulates the epidermal proliferation (Pinkus, 1951). Next, in 1953 Sper and Natzel used the TS to reduce the cornified layer before applying the patch test (a method to identify substances causing allergic reaction in patients). The reduction of the corneocyte layer increases the bioavailability of the substance to the deeper epidermal layers (Spier, Natzel, 1953; Dickel, Goulioumis et al. 2010). However, the basic tape stripping procedure could be further modified for wider examinations like: to study epithelial regeneration, keratinocytes' kinetics, skin microcirculation, cornified layer impermeability, the distribution of SC lipids (Berrutti, Singer et al. 2000), to confirm dermatological disorders, or to increase epidermal DNA/lipid synthesis and lamellar body production/secretion. Unfortunately, in some cases TS application may also suppress the mitotic activity of cells, increase the epidermal cytokine production and dermal inflammation (the occurrence of TNF and IL-1α in the skin) (Alikhan, Maibach, 2010).

## 4. RESULTS New insight

The application of the tape stripping method in the skin research should be followed by the selection of some crucial parameters like the place of sampling on the skin, the type of the adhesive tape and precise method of use. In some investigations the amount of the collected cells is a very important factor, that is why some methods enabling a direct cell counting or others based on the comparison of samples have been developed. Moreover, TS is used in the field of epidermal biology and dermatology.

## 5. The crucial parameters of the tape stripping procedure

In theory, the use of an adhesive tape should allow the removal of one full layer of corneocytes (about 0.5-1 μm). According to some sources, the complete removal of all the SC layers can occur after the removal of dozens of layers (about 60-100 strips) (Jacobi, Weigmann et al. 2005). However, some scientists claim that a complete removal of the horny layer with this method is impossible because still about one third of the layer remains (Schaefer, Schalla et al. 1982).

As research shows, in case of patients who had 30 layers removed the complete recovery of the SC was observed after two weeks (Berrutti, Singer et al. 2000). Nevertheless, the amount of the corneocytes removed by the TS in the same place on the skin decreases with each collected strip from different depths of the *stratum corneum* (Lademann, Jacobi et al. 2009). In the lower layers (*stratum compactum*), the corneocytes more closely adhere to each other using modified desmosomes, while in the upper layers (*stratum dysjunctum*) they are loosely laid on the surface, which allows them to be easily peeled off (Jabłońska, Majewski, 2010). If a long-term tape strip application is planned, the skin dressing becomes a very important issue. As in many cases, occasionally and in specific conditions at the site of the tape injury several pathological microorganisms may be colonized. For example, there is a described case of a 77-year-old patient, where tape stripping was applied and gave an inexpedient result when handling a postoperative wound. The patient sustained a TS type injury due to bandaging his periorbital after a cataract surgery and not having received any immunosuppressive medication. At the same time, he regularly used recreational hot tubs, where the injury was infected with achlorophyllic algae, one of the *Prototheca* species. Fortunately, the infection was completely cured after oral treatment with fluconazole, an antifungal medicine (Humphrey, Martinka et al. 2009).

The amount of collected corneocytes *via* TS method can be affected by numerous factors like skin moisturizing, cohesive forces occurring between the cells, the part of the body from which the corneocytes' samples were obtained, the individual features of the volunteer or patient (Arct, Koazyra et al. 2013). Before the main experiment the compatibility of the selected tape with the patients' skin should be checked.

Some important factors are also directly connected with the procedure of tape stripping: the way the tape is applied on the skin (creating folds), the pressure adjusted during tape application, the speed of the tape peeling off as well as the direct properties of the adhesive tape (Arct, Koazyra et al. 2013). The place for corneocytes sampling should be smooth and without scars. In case of furrows, their occurrence in the skin may cause difficulties, while performing the depth-penetration studies of SC (van der Molen, Spies et al. 1997). The skin should be previously prepared for the cells' collecting by hair removal with scissors and not with a razor that can also damage the skin layer. To create the optimal adhesive bond of the tape with the SC cells, a uniform pressure is crucial. It is also necessary to obtain comparable amounts of the removed SC layer. The high speed of the SC peeling may result in low amounts of corneocytes present on the tape, whereas low speed could cause an increase in the amount of corneocytes. What is more, to identically press the tape each time onto the skin surface as has been done up till now – spatulas, rollers as well as constant weights have been applied. Some tests might need a precise tape size. This may be provided by the usage of adhesive discs (e.g. the comparison of corneocytes dry mass), whereas for others it is possible to use tape from the spool. The tape transparency (e.g. for microscopic analysis) or its flexibility (e.g. for RNA or protein isolation) may be important features likewise (Lademann, Jacobi et al. 2009; Wong, Tran et al. 2006).

## 6. The determination of the amount of the collected corneocytes

For some tests the quantification of the collected corneocytes is essential for comparing samples, like those taken from the skin before and after the application of cosmetic/medical products as well as skin with or without dermatological changes. What is more, with this knowledge an interlaboratory comparison of the results would be possible. However, up till now a satisfactory method of determining the amount of collected cells has not been developed yet. The most common methods used to determine the amount of SC are:

a) the differential weighing – in this method, each tape must be weighed before and after stripping. This allows to determine the amount of tissue removed (m) on the basis of the difference in tape weights. For these experiments adhesive tapes with a defined area must be used (A) and the tissue density (ρ) can be known from the literature data. With these described factors, it is possible

to determine the depth or distance (x) of the interference in the tissue based

on the formula $x=m/(A*\rho)$ (Herkenne, Alberti et al. 2008). In general, the method is quite accurate and reproducible. However, it is time-consuming and, in some cases, due to the moisture content (e.g. topical application of the product) it might be deceiving. Only a few micrograms of the SC usually adhere to a single tape strip (Dreher, Modjtahedi et al. 2005).

b) the optical density measurements – in the optical spectroscopy, corneocytes exhibit pseudo--absorption during measurements using a UV-VIS spectrophotometer at the wavelength of 430 nm (Jacobi, Kaiser et al. 2005), but at the same time their aggregates reduce the transmission of radiation by the reflection, diffraction or dispersion of the light rays, which results in a linear increase in absorbance with a decrease in the wavelength (Arzt, Kozyra et al. 2013). The calculation includes the thickness of the completely removed SC (100%) by adding up values of the absorbance for every single tape strip taken. This allows one to obtain a non-linear correlation between the relative amounts of stratum corneum (y) removed with multiple tape stripping (n) as in the equation $y=a--be^{(-n/c)}$, where "a", "b" and "c" are variables (Jacobi, Weigmann et al. 2005). Also, for this kind of measurements the near-infrared (NIR) densitometry can be used. The beam of light at the 850 nm goes directly through the tape strips with cells and the decrease in light intensity is measured (Jacques-Jamin, Jeanjean-Miquel et al. 2017).

c) various microscopic techniques are used to determine the density of the cells on a specific area using a selected microscope. The cells can be viewed on a light microscope (like optical or fluorescent) after staining the cells with a selected dye. For the optical microscope, corneocytes may be stained with the solution of gentian violet or brilliant green (Lademann, Jacobi et al. 2009).

The intercorneocyte cohesion can be observed after the cells' incubation with the solution of toluidine blue and basic fuchsine in 30% ethanol (Gao, Wang et al. 2013). The fluorescent staining of corneocytes is possible with a fluorescein green dye (the general cell staining) or specific fluorescent antibodies (for example, corneodesmosomes staining) (Guz, Gaikwad et al. 2009; Igawa, Kishibe et al. 2013). In some cases, it is better to use the confocal laser scanning microscopy (CLSM) that

allows to obtain images of the cells/specimens' layer by layer. The sample must also be stained by the specific fluorophore, but the obtained images are sharper due to the removal of reflections not originating directly from the focal point (Lindemann, Wilken et al. 2003, Hanrahan, Harris et al. 2011). Results obtained with these methods (like images or calculations of corneocyte geometry) may also be influenced by artifacts occurring on the tape or skin exposed to stripping and the staining process. The manual data processing in such a situation might be time-consuming. That is why, a good automatic image analyzing software may be useful. Another problem is the occurrence of corneocyte clusters on the tape after the stripping procedure.

For the calculations of a single corneocyte area or geometry, clusters are a serious problem. To tackle this problem, it is possible to place a new adhesive tape on the first one (with the already collected cells) and next to peel it off creating another sample with a smaller number of cells (Li, Guz et al. 2011).

More advanced microscopic methods may be used like the scanning electron microscopy (SEM) and the atomic force microscopy (AFM) (Ezerskaia, Pereira et al. 2018; Li, Guz et al. 2011; Milani, Chlasta et al. 2018). These methods enable the visualization of the cells through imaging the cells' surface (topography). However, obtaining a proper number of images is time-consuming and requires proper operator training to achive this task.

d) the spectrophotometric determination of protein content in the collected corneocytes – it is possible to measure the maximum absorbance of keratin, which mainly remains in corneocytes, at the wavelength of 278 nm or 850 nm (Lademann, Jacobi et al. 2009; Mohammed, Hirata et al. 2014).

The absorbance can be meas red directly on a tape strip when taken with a proper transparent adhesive tape and by using a special UV-VIS spectrophotometer. Moreover, to increase the absorption of proteins and shift it to the visible range, corneocytes could be stained with dyes like Brilliant Blue R 250 (595 nm), crystal violet (540 nm) or trypan blue (652 nm). In practice, the more selective reagent applied to cells, the better (Lademann, Jacobi et al. 2009). Another accurate and reproducible method was developed for the soluble proteins' extraction from the SC by 1 M sodium hydroxide solution followed by the sonication process and next by the sample neutralization with 1 M hydrochloric acid. Finally, the amount of proteins is determined by a similar method to the Lowry assay one. The protein reacts with an alkaline copper tartrate solution as well as with the Folin phenol reagent. The appearing products are measured at 750 nm (Dreher, Arens et al. 1998). Furthermore, the method can be accustomed to 96-well plates, which shortens the time of analysis (Dreher, Modjtahedi et al. 2005). However, the modified Lowry assay may give a positive result in case of some adhesive tapes, especially those made of polyester and polyacrylate. That is why, a modified Bradford assay is also possible to apply with the absorbance read at 595 nm (Chao, Nylander-French, 2004).

On the contrary, the protein content in tape stripped samples may be investigated due to the activity of desq amatory proteases (for example, kallikrein 5 and kallikrein 7). The extraction with a Tris-HCl buffer enables the quantification of aminomethyl coumarin released from peptide substrates by the high-performance liquid chromatography (HPLC) (Mohammed, Hirata et al. 2014).

e) the transepidermal water loss (TEWL) measurements – though the SC is not homogenous, the corneocytes and the intercellular lipids should prevent the water loss from the skin and the loss of the skin barrier integrity. Water loss is carried out on the principles of Fick's laws of passive diffusion (Alikhan, Maibach, 2010). This parameter may be measured with an evaporimeter (tewameter®) directly on the tape stripped sample and with each tape strip number the TEWL value increases. Still, many factors may influence the result. TEWL measurements may be performed in open or closed chambers with similar results in values expressed in $g/m^2h$ (Yosipovitch, Duque et al. 2007; Kikuchi, Asano et al. 2017).

## 7. Review and discussion

Due to the simplicity of the TS method and its possible usage with the more advanced methods, tape stripping can be used in various fields of epidermal biology. As the main advantage of TS, the ability to estimate the general condition of the *stratum corneum* and its re-analysis is made possible after the application of the skin care products (Escobar-Chávez, Merino-Sanjuán et al. 2008). The bioavailability, the bioequivalence and the penetration profiles are important assessments in the subject of the active substances. These substances being the potential ingredients for cosmetics or dermocosmetics are possible to detect with TS (Jacobi, Weigmann et al. 2005). Moreover, it is used in research of the disturbance of the skin barrier functioning usually together with the measurements of water loss (Kikuchi, Asano et al. 2017) or in the skin microbiome analysis (Ogai, Nagase et al. 2018). Valuable analysis can also be made on the tape stripped samples taken from patients

with various diseases like atopic dermatitis or psoriasis (Dyjack, Goleva et al. 2018; Berekméri, Latzko et al. 2018). TS may likewise be used prior to the direct *in vivo* measurements of the skin components, lipids and water with the short-wave infrared (SWIR) Raman spectroscopy (Ezerskaia, Pereira et al. 2018), ATR-FTIR spectroscopy (Bommannan, Potts et al. 1990) or the reflectance confocal microscopy (RCM) (Peppelman, van den Eijnde et al. 2015). On the other hand, skin after tape stripping may likewise be used as a model of the injury in the wound healing research (the study of epidermal growth kinetics) (Surber, Schwarb et al. 2001).

**a) The investigation of the potential ingredients for cosmetic industry**

The main ingredients in cosmetic formulations are water, emulsifiers, emollients, polymers, preservatives, fragrance compositions, natural and synthetic dyes, alcohols, protein hydrolysates, peptides, free amino acids, vitamins and plant extracts. Some commonly used moisturizing ingredients are glycerin and urea.

The *in vivo* 9-day treatment of the skin with glycerin resulted in the increase of the collected corneocytes geometrical characteristics like cell surface. Conversely, sensitivity to glycerin depends on the skin type (dry or oily skin) (Li, Guz et al. 2011). Moreover, low concentrations of the glycerin solution cause the mechanical deformation of corneocytes (the decrease in Young's modulus value measured by the AFM), whereas higher concentrations increase the adhesion forces between the cells (Yanagiya, Takahashi et al. 2015). On the other hand, the therapeutic effect of urea depends on its concentration which can be transferred from the formula to SC. The conventional radiocarbon (14C) or radioactive isotopes (RIs) labeling methods to determine urea concentration in the formula are complicated and rather carry a high risk for the analyst. That is why, TS method combined with colorimetry can easily be applied to measure the urea nitrogen, however, the accurate urea measurement requires the application of at least 1400 mg of a 20% urea solution on a 50 $cm^2$ skin area – the detection threshold (Goto, Morita et al. 2016).

Another widely used ingredient group are natural and synthetic colorants.

To obtain a golden color, curcumin alone or in the extract as well as fluorescein sodium (D&C Yellow NO. 8®) could be used (Modasiya, Patel, 2012; Winter, 2005). Their usage in microemulsions and hydrogels causes the need for the substance's penetration depth evaluation into the SC. The curcumin concentration measurement procedure is as follows: the application of the formula with curcumin on the skin, corneocytes collection via tape stripping, substance dissolvement in the pure ethanol during the sonication process and finally the UV/VIS spectroscopy analysis at 425 nm of the obtained curcumin solution. Similarly, in the fluorescein sodium quantification the salt from inside of the corneocytes is dissolved in a phosphate buffer during the substance release by sonication and the obtained solution is analyzed by the fluorescence spectroscopy at the excitation wavelength of 485 nm and the emission wavelength of 535 nm (Klang, Schwarz et al. 2012).

The ultraviolet (UV) radiation is a major risk factor of skin aging and many diseases including cancer, that is why protection of the skin barrier against the damage on the level of epidermis is very important. This may be provided by the usage of active compounds of dietary phytochemicals (Sobiepanek, Milner-Krawczyk et al. 2016) or the UV filters present in the day care creams and sun creams formula. The chemical filters are organic compounds with an aromatic structure that absorb radiation, whereas physical filters are inorganic compounds that reflect or scatter radiation (Tampucci, Burgalassi et al. 2018). The application of the potential UVA filters (like butyl methoxydibenzoylmethane) in the O/W emulsions has been determined in many investigations (Jacobi, Weigmann et al. 2005; Lindemann, Wilken et al. 2003).

The corneocytes may be collected with TS and the compound extraction can be made with ethanol during the sonication process. Next, the measurements are performed with the UV/VIS spectrophotometer or HPLC (Tampucci, Burgalassi et al. 2018).

The compound concentration in the collected tape strips can be determined due to the prepared calibration curve (Jacobi, Weigmann et al. 2005). Another possibility is to define the penetration profiles of the applied formulations with UV filters due to corneocytes image analysis performed with the laser scanning microscopy and light microscopy. The density of the stripped cells can be determined microscopically with the corneocytes pseudoabsorption (Lindemann, Wilken et al. 2003).

Significant cosmetic formula compounds are the organic solvents, which enable the dissolvement of various active compounds, as well as the anionic and non-ionic surfactants for their washing properties. The lower potential of irritancy has been assigned to nonionic surfactants, while anionic surfactants can damage the skin barrier (Hoppel, Holper et al. 2015). However, due to strong properties for both groups the penetration profile into the SC must be checked. For this purpose, the combination of the TS method with ATR-FTIR spectroscopy can be applied. The decrease in the relative surfactant or solvent concentration is visible with the following tape strip number (Bindera, Kulovitsa, 2018).

**b) The investigation of the proper skin barrier function**

The skin acts as a protective barrier for the whole body, but in case of the incorrect barrier function as the main result – the increase in the transepidermal water loss is mainly pointed out (Barba, Alonso et al. 2016). As it is known, cross-linked proteins ceramides, including transmembrane glycoproteins and proteoglycans, are covalently bound to the monolayer of the cell surface.

These structures participate in the maintenance of the SC barrier function (the hydration and the potential protective role against premature proteolysis) and in the regulation of SC desquamation. However, the presence and the distribution

of glycans on the corneocytes is still investigated. Samples collected via TS and labeled with antibodies or lectins may be later observed with SEM. As it was found, the peripheral distribution of α-D-mannosyl and N-acetyl-D-glucosamine-labelling patterns is higher than of α-linked fucose and α-(2,3) linked sialic acid and it is mainly concentrated in corneodesmosome (Abdayem, Formanek et al. 2016).

Gaining every knowledge about the SC barrier structure, the intercellular lipids properties and the level of hydration may help with the optimization of the damaged skin therapy and improve the design of percutaneous penetration enhancement strategies (Bommannan, Potts et al. 1990). For some cosmetics, to facilitate the pass through the barrier, skin penetration enhancers (SPEs) are used. Some examples are isopropyl myristate, propylene glycol or propylene glycol laurate. However, their exact effect on the skin is not yet fully understood. One of the possible investigation pathways in this research area is the SPE influence on the activity of the desquamatory proteases like kallikrein 5 and kallikrein 7 in healthy skin.

The amount of aminomethyl coumarin released during the extraction of TS samples with Tris HCl buffer was analyzed with HPLC. The result shows that propylene glycol may significantly elevate the kallikrein 7 activity to alter the skin barrier at the macroscopic level. At the same time TEWL was compared before and after SPE applications and the results suggest a significant extent of the TEWL value, which is consistent with the previous results (Mohammed, Hirata et al. 2014). Nowadays Nanoparticles (NPs) like magnetic nanoparticles (MNPs), magnetic beads (MB) and quantum dots (QDs) gain a lot of interest, especially in medicine.

This is due to their unique properties, for example, their significant affinity for cancer cells (Sobiepanek, Kobiela, 2018). Some investigations have been performed to study the penetration profile of the QDs from a proper formula applied topically.

Due to their fluorescent properties, the accumulation of QDs could be detected in the collected samples of corneocytes via TS method on the confocal microscope. The results show that the concentration of QDs progressively decreased with the investigated depth (15 tape stripped samples). Thus, QDs were able to penetrate through the SC, but not to the living epidermis which was revealed through the punch biopsy and the energy diverse X-ray (EDX) spectra to determine the elemental composition of particles assumed to be QDs (Jeong, Kim et al. 2010).

The barrier impairment associated with the SC lipid composition and structure changes, may also cause skin disorders. To confirm this statement, differences in intrinsic and extrinsic aging (UV light) were investigated on the corneocytes by comparing their shape and size with SEM, thickness with AFM and chemical changes with the time-of-flight secondary ion mass spectrometry (ToF-SIMS). Significant changes were observed in the sterol cholesterol sulfate, lignoceric acid and hexacosanoic acid (Starr, Johnson et al. 2016). On the other hand, for the inflammatory diseases glucocorticoids (GCs) like clobetasol proprionate or betamethasone diproprionate could be used, however, they might induce skin atrophy including excessive keratinocyte proliferation, synthesis of extracellular matrix proteins or the reduction of the skin barrier integrity. For a detailed lipidomic analysis the highly sensitive liquid chromatography–mass spectrometry (LC/MS) as well as the ultra-high performance liquid chromatography coupled to the time-of-flight mass spectrometry (UHPLC-ToF--MS) were used. Samples were collected via the TS method and lipids were extracted by homogenization in the chloroform/methanol mixture. The application of GC on the skin caused a significant reduction in lipids, whereas the most effected were ceramide classes consisting of the ester-linked fatty acids (Røpke, Alonso et al. 2017).

**c) Diagnostics of skin diseases by means of the TS method**

In inflammatory skin diseases, like psoriasis and atopic dermatitis (AD), several defects in the proper functioning of the skin barrier can be observed (Barba, Alonso et al. 2016). However, a proper diagnosis when the inflammation is minimal or located in certain anatomical regions may cause diagnostic difficulties. The current gold standard in their diagnosis is the histopathological examination of the tissue obtained through biopsy, but it is invasive and often unavailable in the primary care setting (Berekméri, Latzko et al. 2018).

As the main causes of AD filaggrin mutations, changes in the lipid composition and the altered antimicrobial response are pointed out. However,

due to the fact that the pathogenesis of AD is still unclear, research focused on SC barrier is required for a better understanding of these diseases (Clausen, Slotved et al. 2016).

The whole-transcriptome analysis of corneocytes collected via TS allowed the identification of the gene expression dysregulation in the AD molecular pathology by type 2 inflammation (Dyjack, Goleva et al. 2018). On the next posttranslational level, a significant decrease in the soluble and insoluble protein amount between AD lesional, AD non-lesional and healthy skin collected via TS was noted (Clausen, Slotved et al. 2016). The distribution patterns of corneodesmosomal components like desmoglein 1, corneodesm sin and desmocollin 1 can be followed by the immunofluorescent staining. In healthy skin they are detected at cell periphery. However, in case of AD skin peripheral, sparse diffuse, dense diffuse and partial diffuse pattern were observed (Igawa, Kishibe et al. 2013). Some reliable biomarkers for psoriatic and AD inflammation are the neutrophil-recruiting chemokines like (C-X-C motif) ligand 1 (CXCL1), (C-C motif) ligand 20 (CCL20) and interleukin IL-8, which can be detected on tape strip with the enzyme-linked immunosorbent assay (ELISA). What is more, with this approach it is possible to distinguish healthy skin from AD, and AD from psoriasis due to the increasing amounts of IL-36γ. In case of the first pair the difference was only 1.5 times higher, but in case of the second pair it was 10 times higher (Berekméri, Latzko et al. 2018). On the other hand, while comparing mRNA, profiled with the semiquantitative reverse transcriptase-PCR, the overexpression of TNFα, IFNγ, Krt-16, CD2, IL-23A, IL-12B, and VEGF in the psoriatic lesion can be traced. Additionally, samples collected via the tape stripping method revealed mRNA markers that were not detected in biopsy samples, that is why the application of both methods (non-invasive and invasive) to obtain more information is recommended (Benson, Papenfuss et al. 2006).

## 8. Short conclusion

The tape stripping method is a very simple and minimally invasive tool to obtain the maximum knowledge about the *stratum corneum* properties as well as to diagnose some skin disorders. In combination with other simple or more advanced methods it may help in the basic research as well as in the detailed one. It can be applied with a very low risk of side effects during a standard medical consultation. However, one must be aware that the selection of the crucial parameters of the tape stripping procedure (tape type and size, force and pressure during the skin stripping as well as the amount of the collected cells) are important factors for obtaining reliable results in any analytical method later used. That is why, it is recommended to optimize each parameter of the collecting procedure for one's own use. Collected samples may be analyzed directly via the microscope or they might be used for selected molecule isolation and analysis via spectrometry or chromatography.

**Acknowledgements:**

This work was supported by the Warsaw University of Technology and the Medical University of Warsaw.

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**Review and Research on Cancer Treatment**
Volume 5, Issue 1 (2019) ISSN 2544-2147

# Comorbid targeting of TPPII and proteasome in leukemic cells

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**Abstract**: Tripeptidyl peptidase II is the largest known protease complex in eukaryotic cells acting primarily downstream of the ubiquitin-proteasome system (UPS), and digest peptides generated by the proteasome. However, TPPII can replace the proteasome function by working parallel to proteasome, and is recruited into aggresomes upon proteasome inhibition. Proteasome inhibitors are used as drugs in hematological malignances. Also TPPII inhibitor shows anti-leukemic activity in vitro.
Thus we decided to test whether comorbid inhibition of TPPII and proteasome inhibitors can increase their cytotoxic effect against U937 leukemia cells. We used AAF-cmk as TPPII inhibitor and PSI as proteasome inhibitor.
We show that AAF-cmk effectively inhibit TPP II in U397 but do not affect main proteasome activities. AAF-cmk did not induce accumulation of proteasome substrates in cells. Both TPPII inhibitor AAF-cmk and PSI reduced U937 cell viability, but without synergistic effect. AAF-cmk augments cytostatic effect of TRAIL, but do not enhance comorbid action with PSI against U937 cells.
Thus, in this study we have confirmed anti-leukemic action of TPPII inhibitor AAF-cmk against U937 cells *in vitro* but we did not observed augmented effect of comorbid TPPII and proteasome inhibitors on U937 cells viability. Therefore, our results suggest that in U937 cell TPPII more likely works downstream to proteasome in the same protein degradation pathway in U937. However to answer the question if TPPII works more subsequent or more parallel to proteasome a complex biochemical research is needed.

## 1. Introduction

Leukemias are neoplastic diseases of the white blood cell that manifest themselves by presence of leukemic clones in the bone marrow, in the blood, and in the internal organs – that originate in the early stages of hematopoesis (Kierszenbaum, Tres 2015) The pathogenesis of leukemia is still not fully understood, and its basic elements can be considered together with other cancerous diseases at the current state of knowledge. Leukemic transformation can be the result of both maturation errors or deregulation of cell division as well as the effect of impaired apoptosis (Kierszenbaum, Tres 2015). Increased expression of genes that inhibit apoptosis, such as, for example, proteins from the c-myc or bcl-2 family, or a weakening of the system of regulators and apoptosis-inducing factors such as the Fas-FasL system has been observed in many leukemic cells and other cancers (Cui, Wang et al. 2009; Kanno, Maeda et al. 2012; Karawajew, Wuchter et al. 1997; Kierszenbaum, Tres 2015; Peterson, Mitrikeska et al. 2011).

In 2019, 22,840 people are expected to die from leukemia (13,150 males and 9,690 females) (Al-Asadi, Ibrahim 2018; Howlader N). From 2011 to 2015, leukemia was the sixth most common cause of cancer deaths in both men and women in the US (Al-Asadi, Ibrahim 2018; Howlader N). The incidence of deaths for various leukemia is about 8.2/100,000 (Howlader N). Some authors point that leukemia consist 3.5% of all new cancer cases in the USA and 3.8% of cancer death (Howlader N 2019).

Leukemia treatment is long-term and burdening the patient's body and the prognosis, especially in old age and with adverse cytogenetic changes, is uncertain. Especially acute myeloid leukemia in adults, according to recent data, has the lowest survival (Howlader N). Especially in this type of leukemia, there is a great demand for new treatment strategies supporting existing therapy regimens (Norgaard, Friis et al. 2019; Redaelli, Lee et al. 2003).

Knowing cell physiology and signal transduction pathways, therapies are sought that are effective, non-toxic, and by acting on several elements of this pathway simultaneously, reduce the resistance of leukemic cells against chemotherapy (Norgaard, Friis et al. 2019; Ohno, Asou et al. 2003).

Proteins, in addition to lipids and carbohydrates, are the main building blocks of living organisms and their proper metabolism, i.e. synthesis from amino acids and proteolysis to peptides and amino acids, condition functioning of any cell. In all cell compartments, proteolysis occurs constantly, serving many tasks – from removing some amino acid sequences from proteins to complete hydrolysis of polypeptide chains. The degradation of protein molecules determines such important cell

functions as modulating the activity and amount of key enzymes and regulatory proteins, removing abnormal molecules that arise as a result of biosynthesis errors or post-synthetic damage, as well as the construction of multi-component complexes, e.g. ribosomes, mitochondria or multi-subunit proteins (Alberts 2015).

Cytoplasmic protein degradation depend on proteasome-ubiquitin pathway (UPS) (Alberts 2015). UPS is composed of ubiquitin conjugation machinery and the proteasome that is a proteolytic complex degrading proteins into oligopeptides (Hershko, Ciechanover 1998).

Proteasome inhibitors were shown to be effective against various cancer cell types in preclinical studies (Nowis, McConnell et al. 2007; Szokalska, Makowski et al. 2009) and they are used in the treatment of multiple myeloma and mantle cell lymphoma (Teicher, Tomaszewski 2015). Proteasome inhibitors induce accumulation of undigested proteins in aggregates termed aggresomes (Johnston, Ward et al. 1998; Wojcik 1997a, 1997b; Wojcik, Schroeter et al. 1996). Formation of aggresome is postulated as one possible mechanism of cancer cell death induction by proteasome inhibitors and recently, is was shown that TPPII is also recruited into aggresomes upon proteasome inhibition (Bialy, Kuckelkorn et al. 2019).

Tripeptidyl peptidase II (TPPII) is the largest known protease complex in eukaryotic cells (6MDa) (Rockel, Peters et al. 2005; Tomkinson 2019). TPPII acts primarily downstream of the ubiquitin-proteasome system (UPS), and digest peptides generated by the proteasome (Tomkinson 1999, 2019) TPPII is involved in several cellular processes, however, its exactly function in cells is enigmatic (Mlynarczuk-Bialy 2008). Moreover, TPPII is postulated to substitute for some functions of the proteasome (Geier, Pfeifer et al. 1999). TPPII was shown to assist the UPS in degradation of proteins especially of polypeptides, different studies show that when the UPS is impaired TPPII overtakes its function (Bialy, Kuckelkorn et al. 2019; Bury, Mlynarczuk et al. 2001; Guil, Rodriguez-Castro et al. 2006; Mlynarczuk-Bialy 2008; Zhang, Wong et al. 2011). Moreover, TPPII inhibitor AAF-cmk was shown to display anti-leukemic activity against U937 cells in vitro (Bialy, Fayet et al. 2018; Mlnarczuk, Mroz et al. 2004; Pleban, Bury et al. 2001).

Taking into account that TPP II can work not only not only downstream to proeasome but also replace the proteasome function by working parallel to proteasome, and is recruited into aggresomes upon proteasome inhibition, we decided to test whether comorbid inhibition of these both intracellular proteoltic systems can incerase their cytotoxic effect against U937 leukemia cells.

## 2. Materials and methods

### 2.1. Chemicals

PSI (Calbiochem-Novabiochem, Nottingham, England) was dissolved in DMSO and stored as a 5 mM solution. As a control factor for PSI, a calpain inhibitor: ZLL (Z-leu-leucinal) (Calbiochem-Novabiochem, Nottingham, England) was used which was also dissolved in DMSO as a 5 mM solution. AAF--cmk and AAF-amc (Sigma, St. Louis, MO, USA) were dissolved in DMSO and stored as a 10 mM solution. All substances were stored at -20$^0$C prior to use.

Recombinant human LZ-TRAIL, later referred to as TRAIL, was provided by Immunex Corporation, Seatle, WA, USA. The stock solution was prepared according to the manufacturer's instructions at a concentration of 100 μg/ml and stored at -70$^0$C. Recombinant human rhTNF, later called TNF, was provided by Dr. Wojciech Stec (Department of Bioorganic Chemistry, Center for Molecular and Macromolecular Research of the Polish Academy of Sciences, Łódź, Poland). TNF was stored at -20$^0$C at a concentration of 200 μg/ml. TNF activity was $4.3 \pm 1.1 \times 10^7$.

### 2.2. Cell culture

U937 cell line (human promonocytic leukemia) ATCC was used in the experiments (Mlnarczuk, Mroz et al. 2004; Pleban, Bury et al. 2001).

Cells were cultured in a Heraeus incubator at 37°C, 100% humidity and 4.5% $CO_2$ saturation, in RPMI 1640 medium (Gibco BRL, UK) with the addition of 2-mercaptoethanol (50 μM), L-glutamine (2 mM), and 10% calf serum a standard mixture of antibiotics (amphotericin with penicillin) (all substances from Gibco BRL) in plastic bottles with a bottom area of 25 cm2 (Nunc, Denmark). Cells were passaged every 3-4 days in a 1:5 ratio.

### 2.3. Viability assay

Cells in the exponential growth phase were taken from culture bottles, washed in RPMI-1640 and resuspended in culture medium at $5 \times 10^4$ cells/ml. Subsequently, $5 \times 10^3$ cell aliquots suspended in 100 μL of culture medium were added to the wells of a 96-well plate (Corning, NY, USA)

followed by addition of inhibitors. The final volume in each well was 200 µl.

After 48-hour incubation for PSI and/or AAF--cmk (37$^0$C, 5% $CO_2$) an MTT test was performed (Bialy, Fayet et al. 2018). Each group was in quadruplicates.

To perform the MTT test, 25 µL (concentration 2.5 mg/mL) of 3 – (45-dimethylthiazol-2-yl) – difeyl-tetrazole bromide (MTT) (Sigma-Aldrich) was added to each well. After a 4-hour incubation with MTT, the cell plates were centrifuged for 300 minutes for 300 g. Then 200 µl of supernatant was removed from each well and 200 µl of isopropyl alcohol solution with 0.1 N HCl was added (mixed in a 25:1 volume ratio). After 15 minutes, the well contents were thoroughly mixed to completely dissolve the formazan crystals. A colorimetric absorption reading was then performed (using a 540 nM filter) using a Spectra II reader (SLT-Labinstrument GmbH, Salzburg, Austria).

The cytostatic/cytotoxic effect of the inhibitors tested was presented as relative viability (% control) and was calculated according to the formula:

Relative viability = (Well read – Background read) ÷ (Control read – Background read) x 100%

## 2.4. Western blott

Cells were harvested, centrifuged, washed with PBS, and then the dry pellet was lysed in lysis buffer containing 50 mM Tris-HCl, 5 mM MgCl2, 1 mM EDTA, 0.5% Triton X-100 and protease inhibitors (PMSF 1 mM, Aprotinin 6 µg / ml, Pepstatin 1 µg / ml, Leupeptin 5 µg / ml) by freezing three times in liquid nitrogen and thawing and mixing the sample. After cell dissolution and centrifugation of the insoluble fraction, protein concentration was determined by conjugation with bicinchononic acid (BCA Protein Assay) (Pierce Inc., Ilfold, IL, USA), according to manufacturer instructions. The absorbance value was read in a Spectra II plate reader (SLT GmbH, Salzburg, Austria) at 550 nm. The protein concentration in the solutions was calculated by comparing with the curve obtained after measuring the absorbance of ten known BSA dilutions. Appropriate amount of protein (2.5 mg/ml) was mixed with sample buffer containing: 40% glycerol, 10% SDS, 0.25M Tris-HCl pH 6.8; 0.2% bromophenol blue solution and 20% β-mercaptoethanol. After 5 min incubation of each sample in 96°C, samples were placed on precasted polyacrylamide gel.

Electrophoresis was performed using a Mini Protean 3 (Bio-Rad, Hercules, CA) vertical apparatus at an initial voltage of 60 V for 15 minutes, then 100 V was applied for 1.5 hours from the Power 200 apparatus (Bio-Rad). The gel was thereafter subjected to a semi-dry transfer. The MilliBlot graphite electroblotter (Millipore, Berofd, MA) was used for this purpose. On the anode, filter paper soaked in anode buffer I (0.3 M Tris, 20% methanol v / v, pH 10.4) was applied, a layer of filter paper soaked in anode buffer II (25mM Tris, 20% methanol, pH 10.4), gel, Immobilin-P transfer membrane (Millipore, Beford, MA) soaked in methanol and paper soaked in cathode buffer (40 mM 6-aminocaproic acid, 0.01% SDS and 20% v / v methanol, pH 9.4). After installation, semi dry transfer was performed at 15 V and 0.45 A. After the transfer, the membrane was blocked overnight in a solution of 10% FCS, 3% BSA and 0 , 2% Tween-20 in TBS, at 40C. The next day, the membrane was washed 4x for 10 minutes in TBST (TBS with 0.2% Tween-20) and then incubated for 2 hours in a solution of the appropriate antibody (1: 500-1: 1000) in TBST with 1% BSA and 0.01% thiomerosal. Thereafter the membrane was washed 4 times for 10 minutes in TBST. The reaction was then developed using a ready alkaline phosphatase (Sigma-Aldrich) development kit. The color reaction was stopped by rinsing in distilled water, after which the membrane was dried and scanned.

## 2.5. Measurement of proteasome and TPP II activity by hydrolysis of fluorogenic substrates:

After incubation with appropriate inhibitors the activity of the proteasome and TPP II in cell lysates as well as using the purified proteasome was tested. Substrates of enzymatic activities specific for the given peptidases were used. They give fluorogenic products which, when excited with the right length, emit fluorescent light whose intensity is directly proportional to the initial enzymatic activity.

Substrates for activity assay:

- Suc-LeuLeuValThyr-amc – chymotrypsin-like activity (ChTL) of proteasome
- Z-GlyGlyLeu-amc – chymotrypsin-like activity (ChTL) of proteasome
- Val-Gly-Arg-amc – trypsin-like activity (TL) of proteasome
- z-LeuLeuGlu-βNA – PGPH activity of proteasome
- AAF-amc – TPP II activity

The substrates for enzymatic activities listed above were dissolved in dimethylformamide (Sigma-Aldrich) at a concentration of 20 mM. The test was performed on a black 96-well plate (Greiner, Schwarz, Germany). To each well, 100 ng of purified proteasome (Institut for Biochemistry,

Charite Medical School, Berlin, Germany) or cell lysate was added in a volume of 10 µl and made up to 100 µl with a solution of the substrate of the enzymatic activity tested. The concentration of fluorogenic substrate for the purified proteasome preparation was 10 µM, for the cell lysate it was higher due to the lower content of the proteasome and was 20-50 µM. After 30 minutes of incubation at $37^0$C, the value of excited fluorescence, which was generated as a result of the enzymatic reaction, was measured using a Fluostar Reader fluorometer (SLT, Germany) at an excitation wavelength of 390 nM (AMC) or 355 nM (βNA) and an emission wave of 460 nM for AMC or 405 nM for βNA. The results were analyzed using the Easy software program (SLT, Germany).

## 3. Results and disscusion

### 3.1. AAF-cmk effectively inhibit TPP II in U397

U937 cells were incubated with AAF-cmk at 10 and 20 µM for 24 hours and TPP II activity was determined by fluorogenic substrate methods as AAF-amc hydrolyzing activity in cell lysates.

As shown in Fig.1 incubation with AAF-cmk caused a significant reduction of AAF-amc hydrolyzing activity in both used concentrations (40% for 20µM and 18% for 40µM remaining TPPII activity).

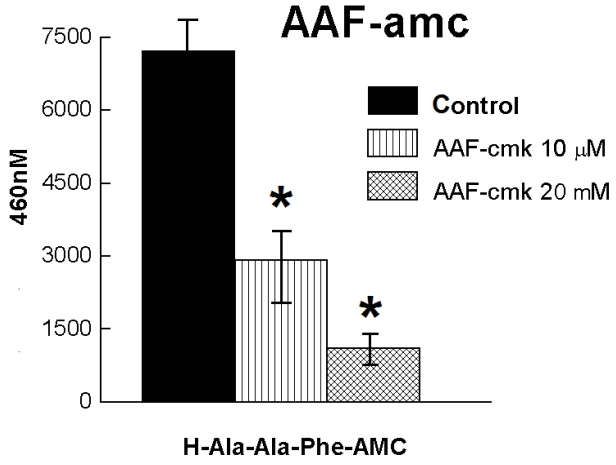


Figure 1. TPP II activity in U937 cells

*Cells were incubated with AAF-cmk in given concentrations for 24h, subsequently lysed and TPP II activity was measured using AAF-amc fluorogenic substrate in cell lysates. Bars represent mean values ± standard deviation, asterisks indicate groups statistically significantly different from the control $p < 0.05$ (student's t test)*

### 3.2. AAF-cmk do not affect main proteasome activities

Du to the fact that AAF-cmk has also been shown to slightly chymotrypsin-like activity of the proteasome without affecting its other activities [Bury Folia] we tested weather AAF-cmk affects three main activities of purified protasome. Thus to exclude unspecific proteasome inhibition by AAF-cmk the purified 20S proteasome was incubated with AAF-cmk and proteasome activity was measured using flourescent substrates.

As shown in Fig. 2 there was no significant inhibition of two main proteasome activities: chymotrypsin-like (Fig. 2A and B) or trypsin-like proteasome activity (Fig. 2C) using appropriate two various substrates. However, AAF-cmk inhibition resulted in a dose-dependent decrease in PGPH hydrolyzing activity starting form 10µM for 1h incubation and 1µM for 24h (Fig. 2D). Nevertheless, this activity is not essential for cell survival and inhibition of only one activity is not sufficient to prevent protein degradation (Kisselev, Callard et al. 2006).

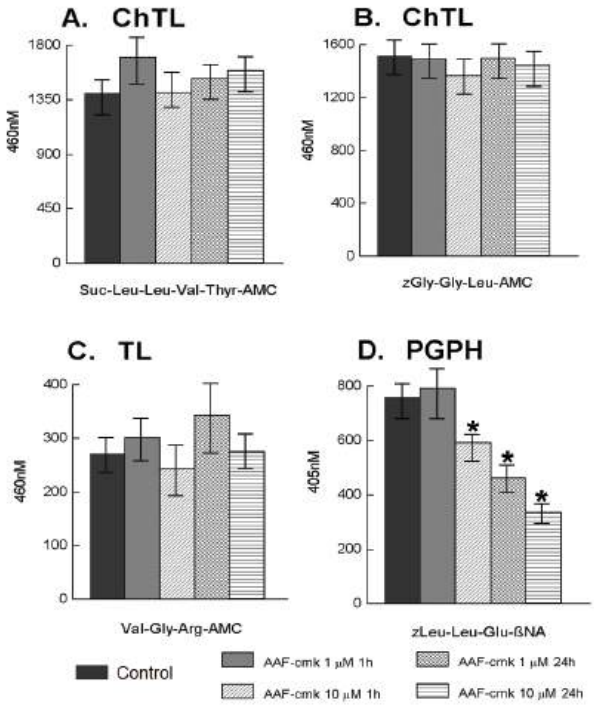


Figure 2. The effect of AAF-cmk on purified proteasome main activities

A and B: chymotrypsin-like activity (ChTL); C: Trypsin-like (TL); D: Postglutamyl-hydrolyzing (PGPH) proteasome activities.

Purified 20S proteasome was incubated with AAF-cmk in given concentrations and times and subsequently the activities were measured using Suc-Leu-Leu-Val-Tyr-AMC (A) and zGly-Gly-Leu-AMC (B) for ChTL; Val-Gly-Arg-AMC (C) for TL; zLeu-Leu-Glu-βNA (D) for PGPH

Bars represent mean values ± standard deviation, asterisks indicate groups statistically significantly different from the control $p < 0.05$ (student's t test)

### 3.3. AAF-cmk do not induce accumulation of proteasome substrates in cells

To analyze the effect of AAF-cmk on proteasome function in vivo we have done Western-blott analysis for ubiquitinated proteins as proteasome substrates in lysates made from U937 cells incubated previously with AAF-cmk. As displayed in Fig 3. there was not any accumulation of poly-ubiquitinated proteins in U937 cells upon AAF-cmk in given concentrations.

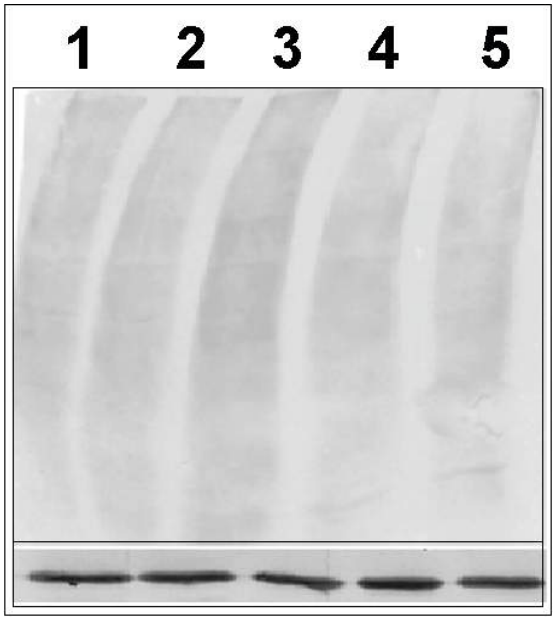


Fig. 3. Western-blott analysis of polyubiquitinated proteins in U937 cells
Top pannel:polyubiquitinated proteins.
The lower pannel: control of protein loading (tubulin)
Numbers: 1 – control group; 2 – AAF-cmk 20 μM, 24h; 3 – AAF-cmk 40 μM, 24h;
3 – AAF-cmk 20 μM 48h; 5 – AAF-cmk 40 μM 28h.

### 3.4. Comorbid anti-tumor activity of TPPII inhibitor AAF-cmk and a proteasome inhibitor PSI

In order test whether inhibition of TPPII and proteasome can increase their cytotoxic effect against U937 leukemia cells we incubated the U937 cells with AAF-cmk and PSI alone and in their combination. Moreover we added both inhibits alone and in combination to TRAIL, which is a potent anti-cancerous cytokine

### 3.5. Both TPPII inhibitor AAF-cmk and a proteasome inhibitor PSI reduce U937 cell viability, but without synergistic effect

To analyze the effect of AAF-cmk and PSI the MTT assay was performed. As shown in Fig 4. AAF-cmk (10μM) reduced cell U937 viability to 90% after 24h and to 30% after 72h of incubation. Whereas 50 nM PSI alone to 80 and 30% respectively. The combination of AAF and PSI did not produce any additional significant effect on cell viability compared to the group incubated with PSI alone (Fig 4.).

### 3.6. AAF-cmk augments cytostatic effect of TRAIL, but do not enhance comorbid action with PSI against U937 cells

Since formerly it was published that both proteasome inhibitor PSI and TPP II inhibitor AAF-cmk increased anticancerous action of TNF and TRAIL (Mlnarczuk, Mroz et al. 2004; Mlynarczuk, Hoser et al. 2001) in this study we tested whether the combination of both substances would further enhance TRAIL action. In this aim U937 leukemia cells were incubated with AAF-cmk or with AAF-cmk and PSI followed by TRAIL and the effects of therapy were assessed by MTT test.

As displayed in Fig.5 Compared to the group in which AAF-cmk was combined with TRAIL, no statistically significant differences were observed in the group that received the triple therapy (Figure 5).

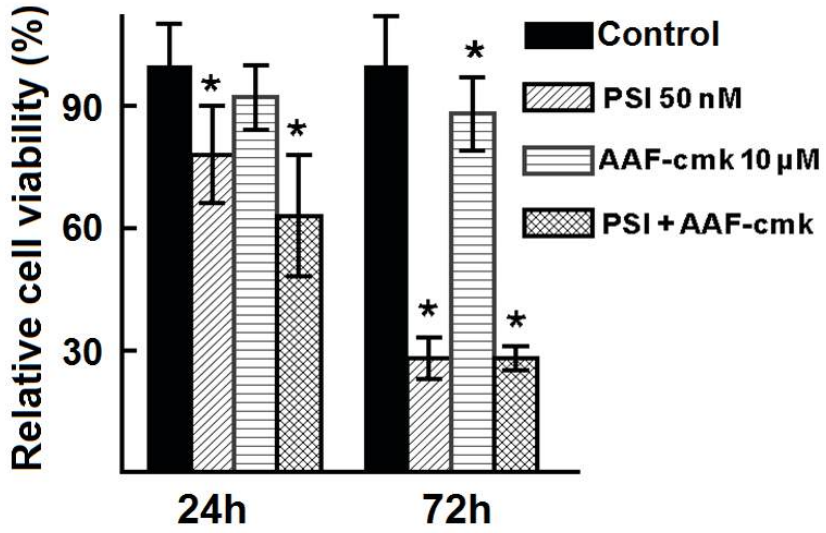


Figure 4. The effects of the combination of PSI and AAF-cmk on U937 cell viability in MTT test.

Bars represent mean values ± standard deviation, asterisks indicate groups statistically significantly different from the control $p < 0.05$ (student's t test).

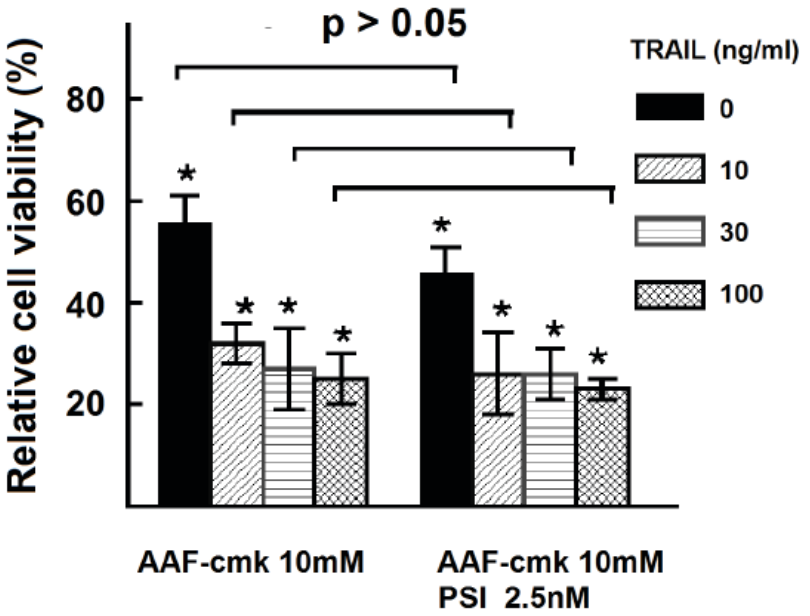


Figure 5. Effect of the combination of PSI, AAF-cmk on U937 cell viability.

U937 leukemia cells were incubated with AAF-cmk or with AAF-cmk and PSI in given concentrations followed by TRAIL. The viability was analyzed by standard MTT test. Bars represent mean values ± standard deviation, asterisks indicate groups statistically significantly different from the control $p < 0.05$ (student's t test).

Individual groups marked with a clamp do not differ statistically significantly (t-student test $p > 0.05$).

As displayed in Fig 5. both AAF-cmk reduced U937 viability to 56% (black columns, left panel) and if combined with PSI to 51% and there were no statistical significance between these two groups. Compared to the group in which AAF-cmk was combined with TRAIL (Fig.5; left panel), no statistically significant differences were observed in the group that received the triple treatment with PSI in all investigated concentrations ($p > 0.05$) (Fig. 5; right panel)

## 4. Concluding remarks

In this study we have confirmed anti-leukemic action of TPPII inhibitor AAF-cmk against U937 cells *in vitro*. We observed no signs of polyubiquitinated proteins accumulation upon AAF--cmk and no inhibition of either chymotrypsin--like or tyrpsin-like activity of proteasome. Only PGPH activity was reduced by AAF-cmk. Thus, the observed cytostatic effects of AAF-cmk do not depend on proteasome inhibition by AAF-cmk. However, we cannot exclude effect of AAF-cmk on other proteases in U937 cells.

No enhancement of cytostatic/cytotoxic effects was achieved by combining AAF-cmk and PSI compared to substance separately (Figure 5). Thus, it gives the argument for rather sequential protein degradation than parallel action of TPPII and proteasome in U937 cells.

It is worth to mention and discuss that some research suggested that TPP II could replace the proteasome in its function, however, it turned out that cells adapted to high concentrations of proteasome inhibitors require some low proteasome activity for life (Princiotta, Schubert et al. 2001). However, TPP II may assume certain proteasome functions, e.g., the generation of certain peptides presented by MHC molecules (Seifert, Maranon et al. 2003). Therefore, it is believed that the proteasome and TPP II interact with each other in intracellular proteolysis processes and complement each other's functions (Wang, Miura et al. 1996).

Under basic conditions, the proteasome generates peptides with a chain length of up to several amino acids, which are most likely degraded to tripeptides by TPP II (Yao, Cohen 1999). In this way, the pool of substrates for downstream peptidases is increased and the pool of substrates for the synthesis of new proteins is returned faster. Cancer cells that have an intense metabolism and synthesize large amounts of proteins may be dependent on the rapid supply of free amino acids resulting from proteolysis. Then full TPP II activity may also play an important role. Burkitt's lymphoma cells overexpressing c-myc oncogene have been shown to have reduced proteasome and elevated TPP II activity and are sensitive to blocking of TPP II by AAF-cmk (Orlowski, Eswara et al. 1998). Another studies showed that overexpansion the *c-myc* oncogene in Burkitt's lymphoma cells results in increased sensitivity to TPPII inhibition and reduced chymotrypsin-like activity of the proteasome, increased activity of deubiquitinizing enzymes (Duensing, Darr et al. 2010; Gavioli, Frisan et al. 2001). These cells under the influence of incubation with proteasome inhibitors do not accumulate polyubiquitinylated proteins and no DNA fragmentation was found in them. Moreover, recently it was shown that TPPII is involved in formation of aggresome in murine C26 adenocarcinoma cells under proteasome inhibition (Bialy, Kuckelkorn et al. 2019) demonstrating possible simultaneous role of TPPII and proteasome in protein homeostasis. Thus all citied research above supports the idea that TPPII can work parallel to protesomes and can be responsible for degradation of protasome substrates.

In this paper we did not observed augmented effect of comorbid TPPII and proteasome inhibitors on U937 cells viability. Moreover, there was no potentiating of their cytotoxicity when both inhibitors together were used with preinduction of apoptosis with TRAIL. Therefore, our results suggest that in U937 cell TPPII works downstream to proteasome in the same protein degradation pathway. However to answer the question if TPPII works more subsequent or more parallel to proteasome a complex biochemical research is needed.

**Acknowledgment:**

The publications costs were provided by Praktyka Lekarska dr n. med. Łukasz Biały NIP 9591228106 REGON 143234827

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**Review and Research on Cancer Treatment**
Volume 5, Issue 1 (2019) ISSN 2544-2147

# Correlation between endothelial growth factors family and development of cancer in metabolic syndrome

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**Abstract:** Vascular endothelial growth factors (VEGF) family is a key to regulation of angiogenesis, lymphangiogenesis or endothelium physiology. These proteins play a crucial role in pathogenesis of cancer, cardiovascular or ocular disorders. Patients suffering from metabolic syndrome (MetS), which is, in brief, compose of obesity, insulin resistant, dyslipidemia, and hypertension, also develop colon, hepatic, cervical and pancreas cancer more often. The aim of this paper is to summarize the knowledge, which connects these two very frequently pathologic statements – cancer and metabolic syndrome in a few aspects. The emphasis was placed on factors, which signaling pathways are downstream via type 2 of a receptor to VEGF. VEGF-A and VEGF-B are the best knows proteins, which are qualified in this group. Few of isoforms of these molecules regulates endothelial functions, like proliferation, migration and forming new vessels from preexisted ones. They are also involved in other processes like regulation of transport of fatty acids. Chronic inflammation and impaired function of vascular endothelium is characteristic of metabolic syndrome and may probably influence on endothelium functioning also in tumors. VEGF pathways are important in the treatment of cancer because many therapies are focused on VEGFs or their receptors. Dysregulation of this signaling pathway via antibodies or kinases inhibitors results in the reduction of pathological angiogenesis in the tumor. A potentially pathological condition of the endothelium can modulate response for these therapies. MetS and tumors are a hot topic in research, due to widespread in the developed countries, but data about the correlation of VEGFs family in cancer in a group of patients with MetS are limited. Further studies in this field can be useful in answer to a question about the role of VEGF in cancer in the MetS.

## 1. Introduction

The vascular endothelial growth factor was first thoroughly characterized in 1986. Initially, it was called the vascular permeability factor (VPF)(Senger, Perruzzi et al., 1986). The VEGF (Vascular Endothelial Growth Factor) proteins family and their associated receptors are key factors regulating angiogenesis or lymphangiogenesis. They are crucial in physiological and pathological conditions, and in embryonic development. Cardiovascular diseases, cancers, or some ophthalmologic pathologies are directly dependent onthe VEGF/VEGFR pathway. The most important elements included in this family are VEGF-A, VEGF-B, VEGF-C, VEGF-D, PLGF (Placenta growth factor), VEGF-E (present in viruses) and VEGF-F (present in snakes). Furthermore, the appropriate function of this pathway is also dependent on VEGFR-1, VEGFR-2 and VEGFR-3 receptors and other co-receptors like neuropilins (Karaman, Leppanen et al., 2018, Peach, Mignone et al., 2018). Genetic and environmental factors causing insulin resistance, abdominal type obesity, lipid metabolism disorders leading to atherosclerotic plaque development, elevated blood pressure, prothrombotic status, and endothelial dysfunction make up the clinical symptoms of metabolic syndrome(MetS) (Kaur, 2014). The association of more frequent occurrences of tumors, mainly of the large intestine, liver in men and the large intestine, endometrium and pancreas in women, suffering from metabolic syndrome is well-proven(Esposito, Chiodini et al., 2012). Moreover, oncological treatment can be an important factor to initiate the development of MetS (Westerink, Nuver et al., 2016).

Due to the important role of the family of vascular endothelial growth factors in the tumor development, which is, for example in regulating the formation of blood vessels in a tumor mass, it was decided to summarize the knowledge on this subject. The aim of the paper was focused the most on VEGFs, the functioning of which is mainly dependent on VEGFR-2. VEGF pathways are important in the treatment of cancer because many therapies are focused on VEGFs or their receptors. Dysregulation of this signaling pathway via antibodies or kinases inhibitors results in reduction of pathological angiogenesis in tumor. Potentially pathological condition of endothelium can modulate response for these therapies. MetS and tumors are hot topic in research, due to widespread in the developed countries, but data describing the cor-

relation of VEGFs family in cancer in a group of patients with MetS is limited. Additionally, an attempt was made to answer the question about the correlation between the development of tumors, VEGF signaling impairment and the metabolic syndrome. The paper was based on data obtained from the PubMed database using key words such as: "VEGF","VEGF-A", "VEGF-B", "angiogenesis", "tumor", "cancer" and "metabolic syndrome". Particular attention has been paid to literature reports from the last five years.

## 2. Vascular endothelial growth factor-A

VEGF-A is produced by many different cells. Its presence was detected in endothelial cells, fibroblasts, smooth myocytes, platelets and peripheral blood mononuclear cells such as neutrophils and macrophages (Uchida, Uchida et al., 1994, Namiki, Brogi et al., 1995). In addition, about 60% of tumors are capable of secretion of vascular endothelial growth factor (Peach, Mignone et al., 2018). VEGF-A secretion is also stimulated by hypoxia and proinflammatory cytokines (Imaizumi, Itaya et al., 2000).

In angiogenesis, the most important role is played by VEGF-A. It is a large protein with a non-globular structure, which in the secondary structure consists of a tightly twisted opposite structure β, which creates two distorted hairpin loops, cysteine knot on one side and a single loop on the other. This protein occurs in the form of homodimers, which are maintained by hydrophobic interactions. Each monomer consists of two α-helixes and seven β-chains. Three of them are slightly in contact with central parts. They are form very irregular structure. This region is a soluble part of β-structure. Moreover, this area features a very twisted structure, with the greatest intensity in the position β4. In this region of the protein is the main concentration of hydrophobic bonds. The dimeric structure is stabilized by the cysteine knot and the middle, hydrophobic protein motif. The cysteine knot is formed by the first two disulfide bonds and the polypeptide backbone joining with the disulfide bonding. The system also includes two other disulfide bonds that bind the monomers together. Structure of the protein is crucial in thermostability and protection against proteolytic enzymes (Muller, Heiring et al., 2002, Iyer and Acharya, 2011).

The alternative splicing of the Vegfa gene, which causes the formation of few functionally different proteins, is significant in the functioning of vascular endothelial growth factor A (Woolard, Bevan et al., 2009). This gene in a human is located in the chromosome 6p21.1. It consists of eight exons and seven introns (Venables, 2006). Due to the alternative splicing, it is possible to modify the pre-mRNA by creating many different proteins from the same gene (Tischer, Mitchell et al., 1991). So far, sixteen VEGF-A isoforms have been described. The most important, however, are six - VEGF-$A_{111}$, VEGF-$A_{121}$, VEGF-$A_{145}$, VEGF-$A_{165}$, VEGF-$A_{189}$, VEGF-$A_{206}$ (Woolard, Bevan et al., 2009, Gu, Li et al., 2013). In addition, differently functioning molecules with the same amount of amino acids have been characterized. The first described VEGF-$A_{165}$a, act in the opposite manner to VEGF-$A_{165}$b (Eswarappa, Potdar et al., 2014). Alternative splicing is regulated by a complex of proteins - spliceosom. It is also regulated by other proteins e.g. SRSF1 (serine / arginine-rich splicing factor 1), which is phosporylated by its kinase - SRPK1 (Guyot, 2015). Dysregulation of SRPK1kinase was observed in tumors for example in colon carcinoma cell line, prostate cancer and melanoma. This was reflected in the attempts to apply a therapy modulating the alternative assembly of VEGF (Oltean, Gammons et al., 2012).

Differences in the presence of specific exons in the protein determine the different properties of VEGF-A isoforms. Furthermore, in tumors are present in high level specific to cancer type isoforms of VEGF-A (Vempati, Popel et al., 2014). The first five exons are of constitutive nature and they are present in each of the isoforms of type A vascular endothelial growth. In this part of the protein there is a signal sequence between the first and second exons , a glycosylation site - Asp74 and a potential site of action for plasmin - Arg110 and Ala111 (Keyt, Berleau et al., 1996). The remaining part of the protein, i.e. exons 3 and 4, is a binding site for VEGFR-1 and VEGFR-2 (Holmes and Zachary, 2008, Woolard, Bevan et al., 2009, Vempati, Popel et al., 2014). The most significant differences in the functioning of the different VEGF isoforms are exons 6a and 7. They are responsible for the interaction between electronegative heparan sulfate in the extracellular matrix (ECM), which determines the bioavailability of certain isoforms of VEGF type A (Krilleke, DeErkenez et al., 2007, Lee, Folkman et al., 2010). The key binding site for neuropilin-1 (NRP-1 - Neuropilin-1) is the product of exon 7 (Soker, Takashima et al., 1998). This domain has ability to create a complex NRP-1/VEGF/ VEGFR2 (Kawamura, Li et al., 2008). Shorter isoforms such as VEGF-$A_{111}$, VEGF-$A_{121}$ are devoid of these exons and they do not bind to the ECM, which makes them diffuse freely (Houck, Leung et al., 1992). Longer isoforms - VEGF-$A_{145}$, VEGF-$A_{189}$, VEGF-$A_{206}$ contain exons 6a and 7 in their structure, thus they can be bound to glycoproteins containing heparan

sulphate (Houck, Ferrara et al., 1991). VEGF-$A_{165}$ is an intermediate form between a completely soluble and bound form (Houck, Leung et al., 1992).

Another domain where there are differences between the isoforms is C - the end of the protein. $VEGF_{xxx}a$ isoforms contain exon 8a, and $VEGF_{xxx}b$ exon 8b (Bates, Cui et al., 2002). As a result of this modification, the last six amino acids are changed at the C-terminal of the protein. This difference between the CDKPRR sequence in the VEGFxxxa isoforms, and SLTRKD in the VEGFxxxb isoforms results in significant functional implications (Ladomery, Harper et al., 2007). VEGFxxxa isoforms have proangiogenic activity, increase the permeability of blood vessels, increase proliferation, survival, and cell migration (Olsson, Dimberg et al., 2006). In contrast, VEGFxxxb isoforms have a counter-action. By what they become regulators and inhibitors of angiogenesis (Woolard, Wang et al., 2004, Catena, Larzabal et al., 2010). In conditions in which it is not necessary to create new vessels, predominantly there are isoforms having anti-angiogenic properties, whereas in tumor growth a reduction of these inhibitory isoforms is observed (Bates, Cui et al., 2002). The presence of exon 8a is important site of binding to neuropilin-1 (Kawamura, Li et al., 2008). The formation of these two different forms is also regulated by other molecules. Insulin-like growth factor-1 (IGF1) and tumor necrosis factor-α (TNFα) affect the increased synthesis of exon 8a, whereas tumor growth factor-β (TGF-β1) of those with exon 8b (Nowak, Woolard et al., 2008). For regulation of this mechanism are mainly responsible, the SRSF1 protein with which are creating the isoforms $VEGF_{xxx}a$ and SRSF6, which contributes to the formation of $VEGF_{xxx}b$ isoforms. $VEGF_{xxx}b$ isoforms probably do not occur under physiological conditions in human and murine cells (Harris, Craze et al., 2012, Bridgett, Dellett et al., 2017).

Another possibility to increase the diversity of VEGF-A is its proteolytic processing through plasmin (Keyt, Berleau et al., 1996) or metalloproteinases (MMPs), e.g. 1,3,9,7,12,16,19 (Lee, Jilani et al., 2005, Lundkvist, Lee et al., 2007). The proteolysis site is located in exon 5. This process leads to the formation of respectively 13kDa and 16kDa fragments of protein (Lee, Jilani et al., 2005). In this process, domains capable of binding to the receptor are not removed from the protein. Furthermore, the biological activity of VEGF is reduced, because the modified proteins have no possibility of binding to NRP-1 and heparan sulphates (Keyt, Berleau et al., 1996). About 40-80% VEGF undergoes proteolysis under pathological conditions (Lee, Jilani et al., 2005, Lundkvist, Lee et al., 2007, Gutierrez, Konecny et al., 2008). MMPs can also produce a proangiogenic forms of VEGF-A. For example, MMP-9 is responsible for modifications of VEGF, that results in increased angiogenesis in breast, cervical and colon cancer (Vempati, Popel et al., 2014).

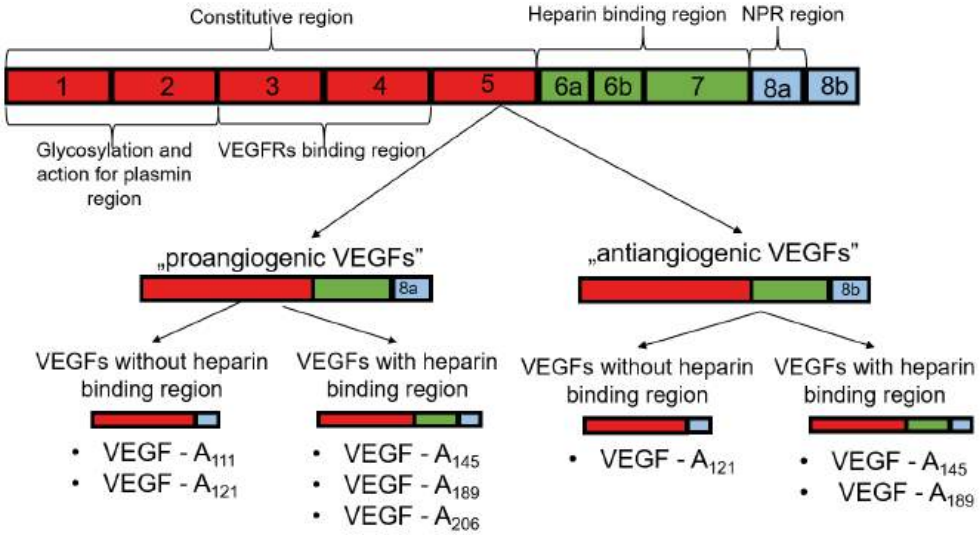


Figure 1. Scheme of VEGF-A isoforms. Formation of each of VEGF-A isoforms is precisely described above. Figure is based on (Ladomery, Harper et al., 2007, Nowak, Woolard et al., 2008, Sargent, Clopton et al., 2016).

## 3. Vascular endothelial growth factor-B

VEGF-B is a growth factor that is encoded by the Vegfb gene, located in the 11q13 chromosome (Olofsson, Pajusola et al., 1996). Two isoforms VEGF-B - VEGF-$B_{167}$ and VEGF-$B_{186}$ are formed as a result of the alternative splicing. They differ in the presence of a domain that has the ability to bind to heparan sulfate. Due to the lack of this fragment of the protein in VEGF-$B_{186}$ it is characterized by a greater ability to diffuse in the intercellular matrix (Grimmond, Lagercrantz et al., 1996, Olofsson, Pajusola et al., 1996). Both isoforms have affinity for the VEGFR-1 receptor and its soluble form, as well as NRP-1. VEGF-$B_{186}$ requires proteolytic treatment to be able to interact with neuropilin-1 (Olofsson, Pajusola et al., 1996, Makinen, Olofsson et al., 1999). In vitro, this protein is able to form heterodimers with VEGF-A, but they have not been observed in vivo (Olofsson, Pajusola et al., 1996).

The expression of VEGF-B is observed in the early stages of embryonic development, in the heart, central nervous system and in brown adipose tissue. Under physiological conditions its production is elevated in cardiomyocytes, skeletal muscle myocytes and in neuronal tissue. The main isoform that occurs in mice is VEGF-$B_{167}$, which is about 80% of the total secreted VEGF-B.(Li, Kumar et al., 2012)

It is believed that the role of this growth factor is to augment the function of VEGF-A (Robciuc, Kivela et al., 2016). VEGF-B is not able to initiate the process of formation of blood vessels - the density or the permeability of blood vessels in the tissue does not change under its influence (Aase, von Euler et al., 2001, Malik, Baldwin et al., 2006, Zhang, Tang et al., 2009). The essential action of this growth factor is also to inhibit apoptosis of endothelial cells and smooth myocytes. In addition, in an environment rich in other factors that stimulate angiogenesis, VEGF-B has antagonistic activity to these molecules (Li, Zhang et al., 2008, Li, Lee et al., 2009). VEGF-B is also associated with the transport of fatty acids to endothelial cells. At its low levels, the decrease of Fatp3 and Fatp4 proteins is also observed, which are involved in this process. The type B endothelial cell growth factor also contributes to the development of insulin resistance (Hagberg, Mehlem et al., 2012).

In cardiac muscle after an ischemic event, VEGF-B expression is reduced (Mehrotra, Wu et al., 2014). In cardiac muscle, VEGF-B activates the Akt/mTORC1 and Erk1/2 MAPK pathways. Activation of the Erk1/2 MAPK pathway results in the phosphorylation of VEGFR-2, thereby increases the effect of VEGF-A (Kivela, Bry et al., 2014).

## 4. Lymphangiogenesis associated endothelial growth factors – VEGF-C and VEGF-D

Part of the VEGF family that is particularly related to lymph vessels is VEGF-C and VEGF-D. Both of these factors play an important role not only in lymphangiogenesis but also in angiogenesis. The “lymphatic” endothelial growth factors undergo proteolysis, which determines the diversity of proteins. This regulation of forming a different isoforms of VEGF-C and VEGF-D is characteristic to “lymphatic” part of family. (Rauniyar, Jha et al., 2018, Stacker and Achen, 2018)

VEGF-C is the main stimulant for the formation of lymphatic vessels. It was characterized in 1996 (Joukov, Pajusola et al., 1996). Like every member of the VEGF family, it contains a domain that is homologous with the rest of the family - VHD (VEGF homology domain. differently to described above VEGF-A and-B, VEGF-C is capable of binding only with NP - 2 (Karpanen, Wirzenius et al., 2006, Xu, Yuan et al., 2010). VEGF-C is produced as a propeptide that needs proteolytic activation. It is this process molecules with different peptide chain lengths are formed, thus diversity of VEGF-C and-D is most related to alternative splicing as “angiogenic” VEGF (Joukov, Sorsa et al., 1997, Stacker, Stenvers et al., 1999). Proteases taking part in this are different in the case of VEGF - C and VEGF - D (Bui, Enis et al., 2016).

VEGF-C has binding properties to VEGFR-2 and VEGFR-3. The strength of this binding depends on the protein proteolytic process. The propeptide has no activity to stimulate the receptor - first it must be processed by furin, ADAMTS3 (a disintegrin and metalloproteinase with thrombospondin motifs 3) or plasmin (McColl, Baldwin et al., 2003, Siegfried, Basak et al., 2003, Jeltsch, Jha et al., 2014). As a result of proteases, two forms of protein are formed that differ in length by in nine amino acids. The longer protein is formed with the participation of ADAMTS3, and shorter by the action of plasmin (McColl, Baldwin et al., 2003, Jeltsch, Jha et al., 2014). The differences between the effects of both forms have not been described so far (Rauniyar, Jha et al., 2018). Nevertheless, pro-VEGF-C only with the participation of NRP-2 can also binds to its receptors. Interaction of immature VEGF-C with NRP-2 and VEGFR-2 or VEGFR-3 results in reduction of activity of the mature proteins (Jeltsch, Jha et al., 2014). Under conditions of inflammation, the level of VEGF-C increases. Its production is mainly stimulated by cytokines secreted from macrophages (Ristimaki, Narko et al., 1998, Krebs,

Tikkanen et al., 2012). Activation of the receptor via VEGF-C leads to stimulation of the MAPK / ERK and AKT pathways, which is associated with the growth, survival and migration of lymphatic endothelial cells (Makinen, Veikkola et al., 2001, Deng, Zhang et al., 2015).

VEGF-D was first described as FIGF (c-fos-induced growth factor) (Orlandini, Marconcini et al., 1996). Like VEGF-C, it has VEGFR-2 and VEGFR-3 receptor-stimulating properties (Ogawa, Oku et al., 1998). The c-FOS transcription factor and factors affecting its activity together with the cadherin - 11 suppress the secretion of VEGF-D (Orlandini, Marconcini et al., 1996, Orlandini and Oliviero, 2001). In addition, β-cadherin increases mRNA stability for VEGF-D (Orlandini, Semboloni et al., 2003). VEGF-D may also exist in the form of a monomer, which is quite unique for VEGF protein family (Stacker, Stenvers et al., 1999).

VEGF-D is produced in the form of a propeptide that undergoes proteolytic treatment, which determines the formation of various forms of the protein. This process occurs in both mice and humans (Stacker, Stenvers et al., 1999, Stacker, Williams et al., 2014).Significantly different proteins with a different polypeptide chain length affect the bioactivity of this growth factor. Pro-VEGF-D also binds to two receptors, thus competing with mature forms to form a negative feedback loop (McColl, Paavonen et al., 2007). Due to the possibility of binding to VEGFR-2 and VEGFR-3, the D-type endothelial cell growth factor may stimulate both angiogenesis and lymphangiogenesis (Achen, Jeltsch et al., 1998, Stacker, Williams et al., 2014). In embryo development, the formation of blood vessels depends on the presence of the transcription factor SOX18, which expression is precisely regulated through VEGF - D (Duong, Koltowska et al., 2014). This growth factor also limits inflammation, which may be an alternative in the treatment of diseases with this background (Huggenberger, Ullmann et al., 2010, Huggenberger, Siddiqui et al., 2011).

## 5. Another proteins from VEGF family - PLGF, VEGF-E and VEGF-F

Another molecule belonging to the VEGF family is PLGF (placenta growth factor). characterized in the early 1990s. This protein forms homodimers. As a result of the alternative splicing, four isoforms are formed - PLGF-1-4, but in the largest number there are only the first and the second isoforms (Maglione, Guerriero et al., 1991, De Falco, 2012). Only the PLGF-2 isoform is found in mice (DiPalma, Tucci et al., 1996). PLGF also has a C-terminal binding domain with heparin. The cooperation between PLGF and heparin is necessary to interaction with NRP-1 and NRP-2. Heparin binding site can be suppressed by proteolysis of the c-terminal fragment of the protein by plasmin. The PLGF-$2_{123 - 144}$ domain also allows interaction with ECM (Migdal, Huppertz et al., 1998, Persico, Vincenti et al., 1999, Hoffmann, Willenborg et al., 2013, Martino, Briquez et al., 2014). This growth factor only binds to the VEGFR-1 receptor and its soluble form – sVEGFR-1, but not to VEGFR-2 (Kendall and Thomas, 1993, Park, Chen et al., 1994). PLGF-2 homodimers via phosphorylation of VEGFR-1 and PLGF-2/VEGF heterodimers via forming a VEGFR-1/VEGFR-2 dimer, are able to stimulate the formation of new blood vessels (Ziche, Maglione et al., 1997, Autiero, Waltenberger et al., 2003). In few cancers is observed overexpression of PLGF-1. This isoform can forms PLGF-1/VEGF heterodimers. This interaction, results in limited activation of VEGFRs. It is a way to regulation of VEGF-A activity (Eriksson, Cao et al., 2002). The placenta growth factor also causes an increase in the synthesis of proangiogenic factors such as VEGF, FGF2 or MMP. By activating VEGFR-1, it influences the interaction of VEGFR-1 with VEGFR-2, which results in increased activation of VEGFR-2 by VEGF-A (Autiero, Waltenberger et al., 2003, Roy, Bhardwaj et al., 2005, Marcellini, De Luca et al., 2006).

In physiological conditions, in most cells high levels of PLGF are not observed, but in pathological conditions there is a significant increase in the level of this protein like in cancers. This growth factor causes growth, survival of vascular endothelial cells. It also stimulates the growth of blood vessels and indirectly through interactions with macrophages contributes to the activation of angiogenesis.(Fischer, Mazzone et al., 2008, Hedlund, Yang et al., 2013)

A molecule that has the ability to bind only to VEGFR-2 and an angiogenic effect similar to VEGF-$A_{165}$ has been characterized in 1998 and named VEGF-E. Initially, it was described in the Orf NZ-7 virus. It also has no ability to bind to heparin due to the lack of a suitable domain. It creates homodimers as most of the VEGF family members(Ogawa, Oku et al., 1998). It is also possible to isolate vascular endothelial growth factors - VEGF-F from the snake venom (Yamazaki, Matsunaga et al., 2009).

## 6. Links between vascular endothelial growth factors and cancer in metabolic syndrome

One of the most significant features of cancer development is a creation of metastasis (Fidler, 2003). As was well described before, VEGF proteins family plays, a crucial role in formation of new vessels. A lot of cancer, such as breast, ovarian, endometrial non-small cell lung, colorectal, head and neck and another cancers (Costache, Ioana et al., 2015). Overexpression of VEGFs in neoplasms result in worsen survivals of patients. This is a reasons of many clinical trials which are focused of inhibition of VEGF signaling, for example with bevacizumab or aflibercept (Ramjiawan, Griffioen et al., 2017). The association of the metabolic syndrome with elevated VEGF-A, -B and -C concentration in the patients' serum is well proven (Zafar, Mills et al., 2018). Despite this, are there any data, which are only focused on difference of expression of VEGF family proteins in the group of metabolic syndrome patients versus without this condition ?

In a small group of patients with colorectal cancer, there were no significant differences between the VEGF concentration in patients with metabolic syndrome as compared to patients without such condition, however above study was performed with limited number of patients, which significantly limits the reliability of results (Liu, Druta et al., 2014). No worse survival was observed in patients with metabolic syndrome and hepatocellular carcinoma treated with sorafenib. Therapy with its results in reduction of pathological angiogenesis, because this medicament is an inhibitor of various tyrosine kinases including VEGFRs (Labenz, Prenosil et al., 2018). Based on this two small studies, probably VEGF expression is not significant upregulated in this cancers in metabolic syndrome patients, but more studies in this field are needed to confirm this observations. Despite this, in MetS condition, there a some genetic background of neoplasm development. Patients with metabolic syndrome and carrying polymorphism (1451C> T or 1725G> A) in the VEGF gene are characterized by a particular predisposition to colorectal cancer (Jeon, Kim et al., 2014).

The data, focused on another then VEGF--A expression in the cancer of MetS patients are indirect. High expression of VEGF-B is observed in metabolically active tissues such as cardiomyocytes and skeletal muscle myocytes. Many mitochondria are present in above tissues and fatty acid transport is intensified, which underlines the important role of this growth factor in regulating the level of fatty acids in cells. Expression of VEGF-B is also noticeable in tumor cells such as adenomas, breast cancer, ovarian cancer, lymphomas, melanomas and sarcomas, which may indicate its significant role in angiogenesis in these tumors (Kivela, Bry et al., 2014, Zafar, Zheng et al., 2017). Metformin, which is a drug used in the treatment of metabolic syndrome, can potentially inhibit VEGF-B signaling, which results in reduced growth of tumors, such as pancreatic cancer (Zhu, Zhang et al., 2016). Moreover, this drug probably reduce the angiogenesis in hepatocellular carcinoma, but this hypothesis must be confirmed in bigger group of patients (Cauchy, Mebarki et al., 2015). Nonphysiological secretion of "lymphatic" endothelial cell growth factors is noticeable in some tumors like acute myeloid leukemia or non-small cell lung cancer. In the solid tumors they are responsible for promotion of metastasis (Chen, Chang et al., 2012). Blocking a VEGF-C and VEGF-D dependent pathways in metabolic syndrome in a one of new strategies in treatment of this MetS (Karaman, Hollmen et al., 2015). So far, limited investigations are described association of inhibition of this pathway in cancer in the group of patients with MetS. Currently, there is little evidence suggesting the importance of these and other members of the VEGF family in the pathogenesis of cancer in patients with metabolic syndrome.

On increased secretion of VEGF in MetS condition impacts upregulated secretion of adipokines in visceral adipose tissue (Lopez-Jaramillo, Gomez-Arbelaez et al., 2014). In the well reported, that leptin, which is highly overexpressed in MetS, stimulated angiogenesis via VEGF signaling especially in mammary cancer (Gonzalez, Cherfils et al., 2006). Furthermore, in some cases, like in chondrosarcoma adiponectin can induce secretion of VEGF-A and VEGF-C (Lee, Lin et al., 2015, Huang, Chang et al., 2016), but another especially hepatocellular carcinoma this adipokine works in opposite way (Man, Ng et al., 2010). Dysregulation of leptin and adiponectin secretion in triple negative breast cancer (TNBC) was propose by Davis and Kaklamani. In their hypothesis angiogenesis in MetS patients with TNBC is regulated by two crucial adipokines. Upregulation of leptin secretion and impaired adiponection expression can results in increased secretion of VEGF, which results in promotion of cancer angiogenesis (Davis and Kaklamani, 2012), but this mechanism is not confirmed in the studies.

Furthermore, to MetS condition characteristic is a chronic low-grade inflammation. Pathologic function of upregulated secretion of cytokines like TNF-α, IL-1, IL-6 and infiltration of immune cells in the tissues are involved in pathogenesis of

endothelial damage, which is observed in MetS (Grandl and Wolfrum, 2018). Moreover, overexpression of this cytokines in the group of MetS patient is significant associated with worsen survivals of patients with prostate cancer (Conteduca, Caffo et al., 2018). Inflammatory cytokines, which are produced by that cells are also induce angiogenesis and VEGF activity (Carmeliet and Jain, 2000). This mechanism can be one of reasons of inappropriate new vessels formation in the MetS related cancers, but there are needed more studies which show it directly.

Interesting that, reduction of body weight by bariatric operation (Ashrafian, Ahmed et al., 2011) or diet (Di Daniele, Noce et al., 2017) limit the many cancers development. Furthermore, it was estimated that in the cohort of women with endometrial cancer bariatric operation improve survivals of patients, which undergoes surgical procedure compare to control group (Neff, Havrilesky et al., 2015). In was reported that after operation levels of VEGF family proteins are downregulated (Farey, Fisher et al., 2017). Despite this, the question of potential benefits of inhibition of VEGF signaling in cancers in the group of MetS patients is unsolved.

## 7. Summary

The family of vascular endothelial growth factor proteins play a key role in many physiological processes and maintaining homeostasis. Not without significance is their participation in the progression of solid tumors, which due to the VEGF activity gain the ability to increase size and appropriate conditions for metastasis. The interactions between endothelial cell growth factor and its receptors are very important in the process of tumor angiogenesis, which justifies the use of VEGF blocking antibodies or inhibitors of tyrosine kinases in targeted therapy, which inhibit the signal transmission from these receptors. Due to characteristic features of the metabolic syndrome, in particular chronic inflammation and vascular endothelial cell dysfunction, it can be assumed that the nature of VEGF activity changes. However, the data presented above indicate that in patients with metabolic syndrome, despite the higher levels of serum endothelial cell growth factor, there are no significant differences VEGF activity. There are many spots in VEGF signaling regulation, that can be targeted in therapy of cancer essentially in the group of MetS. Particular attention should be paid to the limited literature data on this issue, which, firstly, limits the information about the differences in the functioning of these proteins in patients with metabolic syndrome, and secondly encourages further research into this issue, especially in this group of patients. This is potentially important due to the possibility of optimizing targeted cancer therapy aimed at the formation of new blood vessels in solid tumors. Vascular endothelial growth factors family plays an important role in pathogenesis of cancer and metabolic syndrome, so further investigations in this area can show unexpected associations of these widespread pathologic conditions.

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**Review and Research on Cancer Treatment**
Volume 5, Issue 1 (2019) ISSN 2544-2147

# Small cell carcinoma of the urinary bladder – literature review

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**Abstract:** Neuroendocrine neoplasms (NENs) are epithelial neoplasm with prominent neuroendocrine differentiation. NENs are frequent in the respiratory and gastrointestinal tract, but they can arise in any organ. Extra-pulmonary localization of NENs are the larynx, salivary glands, the uterus, the cervix uteri, the vagina, the prostate and the urinary bladder. Small cell carcinoma of the urinary bladder (SCCB) is a type of neuroendocrine neoplasm. In order to be included in the review, articles from PubMed (NCBI), Google Scholar, Web of Science and Scopus archive had to fit the following criteria:
- They had to be original articles, case studies and reviews connected with the following key words: neuroendocrine neoplasms, small cell carcinoma, urinary bladder, epidemiology, pathogenesis, diagnosis, histopathology, treatment and prognosis.
- They had to be written in English.
- They had to be published between 1981 and 2018, as the first article about small cell carcinoma of the urinary bladder was written by Cramer et al. in 1981.
SCCB is an extremely rare tumor, however it is highly malignant and is characterized by very aggressive behavior. The origin of the disease is unknown; the majority of patients are male in their sixties and seventies with a history of smoking tobacco products. Clinical symptoms of SCCB are not characteristic and include severe pain and hematuria. During cystoscopy macroscopically it is visible as a polypoid tumor. The image in a microscopic sample is dominated by small, round tumor cells infiltrating the urinary bladder wall. Immunoreactivity of these cells is very low for conventional neuroendocrine markers such as synaptophysin, chromogranin A and CD56. Since SCCB is so rare, no universal standard treatment has yet to be developed. Treatment includes chemotherapy, radiotherapy as well as surgical procedures. The prognosis of SCCB is very poor and the tumor tends to spread to the bones, the brain and the liver. Average survival rates of patients diagnosed with SCCB range from 12 to 24 months.

## 1. Introduction

Neuroendocrine neoplasms (NENs) are epithelial tumors with prominent neuroendocrine differentiation. NENs can arise from any type of epithelium, but frequently develop from epithelia rich in enterochromafin cells. These cells are numerous in the gastrointestinal tract, and also appear in the respiratory tract, which develops from the primary gastrointestinal bud. Small amounts of enterochromafin cells are also found in the genitourinary system as well, particularly in the urinary bladder and prostate (Ghervan, Zaharie et al. 2017). Neuroendocrine neoplasms belong to a very rare neoplasm in the human body. They occur at a rate of 2.5 to 5 incidences per 100,000 people per year (Oberg, Castellano 2011). Current research shows that there are year on year increases in the incidence of NENs, both in the United States and in other countries (Dasari, Shen et al. 2017, Hauso, Gustafson et al 2008, Hallet, Law et al. 2015).

NENs most often develop in the respiratory and gastrointestinal tracts but can arise in any organ. Extra-pulmonary localization of NENs are the larynx, salivary glands, the uterus, the cervix uteri, the vagina, the prostate and the urinary bladder (Zhao, Flynn 2012). Neuroendocrine neoplasms are a heterogenous group of tumors very different in clinical behavior depending on where they appear. In 2017, experts of the International Agency for Research on Cancer (IARC) and the World Health Organization (WHO) finally classified them as neuroendocrine neoplasms and decided that "the key feature of the new classification is a distinction between differentiated neuroendocrine tumors (NETs) also designated carcinoid tumors in some systems, and poorly differentiated neuroendocrine carcinoma (NECs), as they both share common expression of neuroendocrine markers". At the time of discussion of the new classifica-

tion, experts took into account six major points: anatomy, tumor category definition, tumor family definition, tumor-type definition, tumor sub-type definition, and tumor grading procedures. According to the above classification regarding the genitourinary system, experts distinguished between well-differentiated NENs - neuroendocrine tumors (NETs) G1, G2, G3, as well as poorly differentiated NENs – neuroendocrine carcinoma (NECs) G3 as small cell type, and large cell type (Rindi, Klimstra et al. 2018).

Small cell carcinoma (SCC) belongs to a very rarely occurring type of neoplasm and develops in various systems and organs, including the gastrointestinal tract, pancreatobiliary system, larynx, salivary glands, uterus, cervix uteri, vagina, prostate and urinary bladder (Davis, Ludwig et al. 1983, Kim, Lin et al. 1984, Dores, Qubaiah et al. 2015).

The most frequently occurring location of SCC is in the lungs, which account for 15% of all bronchogenic carcinoma cases (Zheng, Ettinger et al. 2013, Zheng, Liu et al. 2015). Extrapulmonary small cell carcinomas make up only about 4% of all small cell carcinomas (Jemal, Bray et al. 2008). Despite its appearance in different organs, SCC is characterized by a similar histological structure, whereas its clinical behavior is very different depending on its location.

Small cell carcinoma of the urinary bladder (SCCB) is a type of small cell carcinoma. The goal of this work is to review the literature concerning the extremely rare occurrence of small cell carcinoma of the urinary bladder.

## 2. Search strategy and selection criteria

The goal of this work is to collate up-to-date knowledge on the subject of the etiology, pathogenesis, clinical symptoms, diagnosis, treatment and prognosis of the course of small cell carcinoma of the urinary bladder. To carry out the study, the following databases were used: PubMed (NCBI), Google Scholar, Web of Science and Scopus, using the following key words: neuroendocrine neoplasms, small cell carcinoma, urinary bladder, epidemiology, pathogenesis, diagnosis, histopathology, treatment and prognosis. We searched through original articles, case studies, and reviews published between 1981 to 2018. The results of the study includes alternative keywords submitted to put together a complete picture of this cancerous condition.

## 3. Epidemiology

Small cell carcinoma of the bladder is a very rare disease, occurring in 0.3 to 0.7% of all primary tumors in this organ, and is characterized by very aggressive and quick growth, as well as negative prognosis. (Blomjous, Vos et al. 1989, Holmang, Borghede et al 1995, Mackey, Au et al. 1998). The first case of SCCB was described in 1981 by Cramer, Aikawa et al. Patients with SCCB were mostly men, with an overall ratio of men to women being 5:1, with a range from 1:1 to 16:1 (Quek, Nichols et al. 2005, Bex, Nieuwenhuijzen et al. 2005, Mukesh, Cook et al. 2009, Mangar, Logue et al. 2004). Most SCCB patients are over 60 years old (Dores, Qubaiah et al. 2015). The average age of patients diagnosed for the first time with SCCB is 66.9 years old with a range from 32 to 91 years of age (Choong, Queveda et al. 2005, Cheng, Pan et al. 2004, Iczkowski, Shanks et al. 1999, Siefker-Radtke, Dinney et al. 2004). Similar to patients suffering from transitional cell carcinoma, SCCB patients are smokers (from 65% to 79%) (Chen, Liu et al. 2017, Lohrish, Murray et al. 1999, Ismaili, Elkarak et al. 2008). It is therefore assumed that smoking may be one of SCCB's etiological factors. Smoking may cause similar genetic lesions in the urinary bladder (Abbosh, Wang et al. 2008). Other environmental and individual risk factors are not mentioned in the etiology of this neoplasm, but can include chronic cystitis, bladder stones, bladder manipulation or cytoplasty (Anthony, Douglas 2013, Church, Bahl 2006)). The majority of SCCB patients are white men, making up 74-97% of all cases (Choong, Queveda et al. 2005, Abrahams, Moran et al. 2005, Siefker-Radtke, Dinney et al. 2004).

## 4. Pathogenesis

The pathogenesis of SCCB still remains unknown. It is presumed that small cell carcinoma of the urinary bladder can be associated with chromosomal aberrations, such as hypermethylation of tumor-suppressor gene CpG islands (Abbosh, Wang et al. 2008). Similarly the origins of neuroendocrine cell carcinoma have not yet been clarified. Several theories exist that attempt to explain the origin of neuroendocrine cell carcinoma. The first theory states that malignant transformation of neuroendocrine cells leads to the development of small cell carcinoma of the urinary bladder. (Ali, Reuter et al. 1997, Trias, Algaba et al. 2001). The second theory posits that metaplastic changes of transitional epithelium lead to the progression of SCCB (Iczkowski, Shanks et al. 1999, Oesterling, Brendler et al. 1990). The third theory proposes that multipotential common stem cells affect

a specific transformation in a progression-related gene that can differentiate itself in various types of cells, including small cell carcinoma, but also in transitional cell carcinoma. (Terracciano, Richter et al. 1999, Christopher, Sefteil et al. 1991, van Hoeven, Artymyshyn 1996). The last of these theories attempts to clarify the relatively frequent coexistence of small cell carcinoma and transitional cell carcinoma of the bladder as well as the diversity of the immunohistochemical reaction encompassing both neuroendocrine markers as well as cytokeratin (Terracciano, Richter et al. 1999, Christopher, Sefteil et al. 1991, Abenoza, Manivel et al.1986, Hailemariam, Gaspert et al. 1998).

## 5. Clinical features

The clinical symptoms during the course of SCCB are not typical and are often displayed at an advanced stage of the neoplasm's progression. The primary symptom of SCCB is hematuria, which occurs at a frequency of 63% to 88%. (Cheng, Pan et al. 2004, Chen, Liu et al. 2017). In addition, patients complain of dysuria. (Blomjous, Vos et al. 1989, Abrahams, Moran et al. 2005). Less frequent symptoms are urinary obstruction, abdominal pain, urinary tract infection, and weight loss (Lohrish, Murray et al. 1999, Choong, Queveda et al. 2005, Abrahams, Moran et al. 2005). Occasionally, in the course of small cell carcinoma of the urinary bladder, there occur paraneoplastic syndromes characterized by hypercalcemia, hyperphosphatemia and ACTH secretion (Choong, Queveda et al. 2005, Reyes, Soneru 1985). SCCB develops insidiously and is typically discovered at an advanced stage. It also causes early metastasis in the liver, brain and bones (Saeed, Cramer et al. 2018). The average life expectancy for patients with SCCB is 12 to 24 months. (Chen, Liu et al. 2017).

## 6. Histopathology

Small cell carcinoma of the bladder usually forms a large polypoid or nodular tumor with extensive bladder infiltration (Zhang, Niu et al. 2017). The tumor is most often characterized by diffuse growth and sometimes develops by forming nests and trabeculae (Zhao, Flynn 2012). In the majority of cases, there is visible necrosis on the surface of the neoplasm. Macroscopically, small cell carcinoma of the bladder is indistinguishable from urothelial carcinoma of the bladder. (Cheng, Jones et al. 2005, Mukesh, Cook et al 2009). Microscopically, the structure of a small cell carcinoma of the bladder is identical to small carcinomas of other organs, i.e. the lungs or the prostate (Zhao, Flynn 2012). Tumor cells are small round or oval cells, sometimes spindled (Moretto, Wood et al. 2013, Manunta, Vicendeau et al. 2005, Soriano, Navarro et al. 2004, Yoshida, Ishida et al. 2014, Podesta, True 1989). These cells are loosely attached to one another, sometimes forming sheets or nests. The cancer cells have sparse cytoplasm with few organelles and pyknotic round or oval nuclei crowding and molding (Ismaili 2011, Zhao, Flynn 2012). The nuclei contain a hyperchromatic coarsely granular chromatin. Sometimes nuclei of these cells have chromatin that look like "salt and pepper," with a dusty appearance, inconspicuous nucleoli, and a high nuclear-to-cytoplasm ratio. In the tumor cells high mitotic activity, apoptosis, necrosis, and crush artifact (sharing of cells) is frequently observed. (Ismaili 2011). An electron microscope can show neurosecretory granules inside these cells (Kim, Lin et al. 1984).

Compared to small cell carcinoma of the lungs, approximately half of small cell carcinomas of the urinary bladder have a mixed component. The mixed epithelial component, in the majority of cases, is established as a type of urothelial carcinoma. (Ismaili, Heudel et al. 2009, Siddqui, Shabbir et al. 2006, Grignon, Ro et al. 1992).

Cancer cells exhibit expressed neuroendocrine markers such as synaptophysin, chromogranin A, and CD56, but the sensitivity of these markers is relatively low in SCCB. The least sensitive, but the most specific, of SCCB's neuroendocrine markers is chromogranin A, also referred to as *parathyroid secretory protein 1*. CD56 in turn, is referred to as the *neural cell adhesion molecule*, and is the least specific, but the most sensitive. Synaptophysin, the *major synaptic vesicle protein p38*, is of average specificity and sensitivity in relation to SCCB in comparison to chromogranin A and CD56 (Vakar-Lopez, True 2007, Yoshida, Ishida et al. 2014, Zhang, Niu et al. 2017, Moretto, Wood et al. 2013). In the diagnosis of the immunohistochemical practice, there is also a thyroid transcription factor 1 (TTF-1), the expression of which shows that this marker can also be expressed in SCCs arising in places other than the lungs.

Differential diagnosis of urinary bladder small cell carcinoma must also take into consideration large cell carcinoma and carcinoid tumors of the bladder. Neuroendocrine large cell carcinoma is characterized by large cells of a polygonal shape, a low nuclear/cytoplasmic ratio, coarse chromatin, frequent nucleoli and high mitotic activity. Neuroendocrine markers for large cell carcinoma are similar to those applied in the diagnosis of small cell carcinoma (Ghervan, Zaharie et al. 2016, Serrano, Sanchez-Mora et al. 2007, Coelho, Pe-

reira et al. 2014). Carcinoid tumors present with columnar or cuboidal cells with granular eosinophilic cytoplasm, with round or oval nuclei containing finely stippled chromatin and inconspicuous nucleoli without necrosis (Klimstra, Modlin et al. 2010). Differential diagnosis must also take into consideration small cell carcinoma of the prostate, which may have infiltrated the bladder, urothelial carcinoma, metastases from neuroendocrine tumors from the lung, as well as lymphoma, and lymphoepithelial-like carcinoma from the lung (Klimstra, Modlin et al. 2010). Recommended diagnostic procedures include an abdomen and pelvis contrast-enhanced computer tomography, an MRI to ascertain whether there has been infiltration to adjacent organs, 99mTC-MDP bone scan to make sure the cancer did not spread to the bones as well as gadolinium-enhanced MRI to check for possible spreading into the brain (Zhang, Niu et al. 2017).

## 7. Treatment

Small cell carcinoma of the urinary bladder belongs to a rarely occurring type of neoplasm and for this reason, there has yet to be a uniform standard of established treatment for this type of condition (Ismaili 2011). Standard practice for the treatment of this tumor includes chemotherapy, radiotherapy and invasive surgery. (Vakar-Lopez, True 2007). Chemotherapy is the standard treatment for small cell carcinoma of the lung, where in it is often applied single-handedly. The application of neoadjuvant chemotherapy is also sometimes combined with cystectomy, where cystectomy follows the course of chemotherapy, or the opposite happens, where cystectomy is the first procedure, followed by neoadjuvant chemotherapy. Cystectomy is also sometimes applied alone without chemotherapy. In certain patients, only a transurethral resection of the bladder (TURBT) is performed. There is also a method of treatment restricted solely to radiotherapy. In addition to a treatment where both chemotherapy and radiotherapy are simultaneously applied, a procedure is followed by an additional course of chemotherapy, followed again by radiotherapy.

## 8. Conclusion

Aggressive, quickly advancing SCCB displays distinctive clinical symptoms that give rise to neoplasms. Diagnosis of the illness usually occurs at an advanced stage, which significantly worsens the prognosis and makes effective treatment difficult. Difficulties also arise in histopathological diagnostics, since markers suitable in SCCB may have variable expression. At the same time, the very rare occurrence of these neoplasms has not yet led to the development of a uniform standard in the treatment of SCCB and therefore chemotherapy, cystectomy and radiotherapy are used in various combinations, or alone.

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**Review and Research on Cancer Treatment**
Volume 5, Issue 1 (2019) ISSN 2544-2147

# The application of fecal microbiota transplantation in patients with oncologic diseases

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Praca została wykonana w ramach projektów WNM SUM KNW-1-061/K/8/O oraz KNW – 2- O43/D/8/N.

**Abstract:** Oncologic patients often undergo a long-term hospitalization and exhaustive treatment. For this reason they often suffer from intestinal microbiota violations and dysbiosis, which results in *C. difficle* colonization and infection. Disorders in the composition of microbiota also can predispose to the development of some cancers. There are more than 2,000 bacterial species in the human intestine, with the composition of microbiota changing during a life. These microorganisms, often called "colonization resistance factor" are an important barrier for infections, caused by intestinal pathogens. Fecal microbiota transplantation (FMT, also referred as stool transplantation, fecal transplantation, fecal flora reconstitution, or fecal bacteriotherapy) is a procedure involving the transfer of a specially prepared stool sample from a healthy donor to the recipient, suffering from intestinal dysbacteriosis. The purpose of FMT is to rebuild and restore the normal bacterial microflora, especially the population of anaerobic bacteria. Currently, FMT is used for treatment of the recurrent *C. difficile* infection (rCDI), as well as other diseases related to the gastrointestinal tract, such as Inflammatory Bowel Disease, neurological, hematological and functional disorders. The stool donor may be related or not with the recipient, but most importantly, each donor should undergo many examinations similar to those for organ donors. Besides these, the presence of multi-resistant bacteria in the feces as well as a number of etiological agents of intestinal infections (bacteria, viruses and parasites) should be excluded from donatation. On the other hand, the FMT procedure as an application of biologically-alien material seems to be quite risky, especially in patients who are undergoing anticancer and immmunosuppressive therapy. However, in most cases, when FMT has been used, positive therapeutic effects for eradication of CDI have been described. Moreover a protective action against acute graft-versus-host disease has been observed.
In this review, we will focus on the problem of FMT usage in oncologic patients, in the light of recent publications.

## 1. Introduction

FMT (fecal microbiota transplantation, but also referred to as stool transplantation, fecal transplantation, fecal microbiota reconstitution, or fecal bacteriotherapy); is a procedure involving the transfer of a stool sample from a healthy person, to the recipient who has disturbed composition of the intestinal microbiota. These disorders also are called bacterial or intestinal dysbiosis which are abnormalities in quantitative and qualitative composition of commensal microbiota (Hill, Hoffman et al. 2010). Dysbiosis may lead to the development of inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), diabetes, obesity, cancer, cardiovascular and central nervous system disorders (Belizário, Faintuch 2018). The purpose of FMT is to rebuild and restore normal bacterial population of the recipient's intestinal microbiota. Currently, FMT is used for the treatment of recurrent *Clostridioides difficile* infection (rCDI). Besides rCDI treatment, FMT is also used in other gastrointestinal (GI) inflammatory diseases, such as Inflammatory Bowel Disease and non-gastrointestinal diseases (n-GI). There are attempts made to use FMT in the treatment of: autism, chronic fatigue syndrome, fibromyalgia, idiopathic thrombocytopenic purpura, multiple sclerosis, myoclonus dystonia, and Parkinson disease. However lack of randomized trials makes it impossible to develop strict guidelines for the treatment of these diseases (Vindigni, Surawicz 2017).

The number of microorganisms inhabiting the human intestine is up to $10^{14}$/ml of feces. Based on the bacterial analysis of 16S rRNA, 2172 bacterial species were identified, of which 386 were classified as obligate anaerobes. Anaerobes are classified in 12 different phyla, of which 93.5% belonged to *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroidetes* (Thursby, Juge 2017, Hill, Hoffman et al. 2010). The composition of the physiological mirobiota inhabiting the intestine changes over the course of life. The intestine of newborns after delivery is quickly settled with bacteria. Type of bacteria depends on the type of delivery and the method of feeding. The intestine of newborns with natural delivery is quickly settled mainly with *Lactobacillus* spp., while those after Caesarean section is mainly colonized by *Bacteroides* spp. and *Clostridium* spp. During the first year of life the diversity of intestinal microbial species increases, so that after the child re-

aches 2.5 years, it has a microbiota characteristic for an adult. The composition of this microbiota may also vary depending on diet, accompanying diseases or medication. Subsequent changes occur after the age of 65, when again the number of *Clostridium* and *Bacteroides* genus increases. With the aging of the body, rearrangement in the intestinal microbiota, leads to an increase in butyric acids production and a decrease in short-chain fatty acids production as well as the decrease efficiency of the process amylolysis (Thursby, Juge 2017, Quigley 2017, Arumugam, Raes et al. 2011, Bhatt, Redinbo et al. 2017). Intestinal microbiota is responsible for a large variety of important functions and processes in human life, such as:

– induction of synthesis of antimicrobial particles increasing of mucin gene expression, increasing of IgA secretion

– stimulation of intestinal macrophages to produce IL1β. IL1β is a highly pro-inflammatory cytokine, responsible during infection for neutrophil recruitment to the site of inflammation and others

– differentiation and activation of Th17 lymphocytes (killing of extracellular pathogens)

– production of bacteriocines and short-chain fatty acid (SCFA) inhibitions the growth of *C. difficile*

– the restriction of substances facilitating the germination of spores and the development of vegetative cells of *C. difficile* (taurocholate, mannitol, fructose, sorbitol)

– limitation of carbohydrate and sialic acid, which facilitate the growth of *C. difficile* (El Feghaly, Bangar et al. 2015).

## 2. Search strategy and selection criteria

The aim this study was to find an answer to the question whether the FMT procedure applies in the course of treatment of oncological patients? In order to achieve this goal, the literature was reviewed in the PubMed, Google Scholar, and Medline databases by entering: Fecal microbiota transfer, Fecal microbiota transplantation and cancer, Fecal microbiota transplantation and oncology, *Clostridium difficile* infection and cancer, *Clostridium difficile* infection and oncology. Particular attention was paid to English-language articles from recent years. The search revealed a large number of publications on the FMT procedure and its use in the treatment of intestinal diseases, while information on the use of FMT in patients undergoing oncological treatment is scarce.

## 3. State of the Art

### FMT History

An intestinal microbiota transplantation is not an invention of our time. In ancient China, in the fourth century, attempts were made to treat food poisoning and severe diarrhea by using stool administered to the patient's mouth. The Chinese manual "Handy Therapy for emergencies" said that this therapy is wonderful and brings patient back from the edge of death. In the 16th century, various fecal preparations (fermented fecal solution, fresh fecal suspension, dried feces, feces from infants) were named by the doctors of alternative medicine "yellow soup" or "golden syrup". Li Shizhen described their use in the treatment of severe diarrhea, fever, pain, vomiting and constipation. In the seventeenth century, the Italian doctor Acquapendente tried to apply sick animals an intestinal microbiota transplant from healthy animals basing on the observed coprophagia in animals. In the nineteenth century there was a breakthrough when Antonie van Leeuwenhoek discovered bacteria in the stool. Another scientist was Russian zoologist Ilja Miecznikow, who observed among poor Bulgarian farmers maintaining good health, thanks to drinking fermented milk, and then linked the occurrence of *Lactobacillus bulgaricus* in milk, as a protective and health-improving bacterium. German physician and bacteriologist Alfred Nissle isolated *Escherichia coli* and noted that *Escherichia coli* competes with *Shigella* spp. for growth factors. During World War II, German soldiers in Africa used a Bedouin way against dysentery - eating fresh, warm camels stool. Nazi scientists isolated from this stool *Bacillus subtilis*, which was later used as a treatment against dysentery (Sbahi, Di Palma 2016, de Groot, Frissen 2017).

### 3.2. FMT in XXI century

FMT standards are regulated by EUTCD guidelines (The European Union Tissue and Cells Directives). The consensus was elaborated in 2016 at the European conference, and in 2017 a guide with a set of recommendations was published. FMT guide developed by the European Working Group, set standards for the selection of stool donors, the method of material preparation and the route of FMT administration (Cammarota, Ianiro et al. 2017). A group of researchers from Denmark very precisely developed the FMT transplant procedure. It is a good idea to look for

fecal donors among blood donors who already have some of the diagnostic tests done; such as complete blood counts or serological blood tests for HIV, cytomegalovirus (CMV), hepatitis B and C (HBV, HCV), Epstein-Barr (EBV) and syphilis. Donors are recruited among people aged 25-50, in general healthy, without obesity but also without malnutrition (BMI Body Max Index 18-28 kg/m$^2$). The excluding factor is permanent drug usage, the use of antibiotics in the last 6 months, risky behaviors (sexual relations with unknown partners, tattoos, piercing body parts, trips to countries with low hygiene standards), intestinal diseases and other chronic diseases, including depression. In additional, blood tests are performed to evaluate the functions of specific organs: pancreas (amylase), kidneys (creatinine), liver (ALT, AST, bilirubin, INR, albumin) and the level of electrolytes, CRP, immunoglobulins and glycated hemoglobin. Besides the DNA of the parasites *Strongyloides stercoralis* and *Entamoeba histolytica*, *Cryptosporidium* spp. is also sought. Fecal sample are also tested for presence of enteropathogenic bacteria such as *C. difficile* (toxinogenic and ribotype 027), EPEC, *Salmonella* spp., *Shigella* spp., *C. jejuni*, *Y. enterocolitica*, multi-drug resistant bacteria and several viruses (adenovirus, enterovirus, parechovirus) (Jørgensen, Hansen et al. 2017).

Currently, FMT is mainly used to treat rCDI and IBD. The FMT may be administrated by nasogastric tube, nasoduodenal tube, esophagoduodenoscopy (EGD), colonoscopy, or enema (Khanna, Pardi 2012, Rohlke, Stollman 2012, Landy, Al-Hassi et al. 2011).The volume of material for FMT may also vary, depending on the route of administration. When using a nasogastric tube, 25-50 ml is given, and 200 to 500 ml when during colonoscopy. There are also differences regarding the administration of a fresh or frozen sample, the time that elapses from donating feces to the preparation of FMT (6-24h). Researchers also suggest bowel lavage before FMT and loperamide after colonoscopy to stop diarrhea or probiotics use (Kim, Gadani et al. 2018, Rohlke, Stollman 2012). In a study of 35 patients infected with *C. difficle* and subjected to the FMT procedure, it was confirmed that 30 patients (85,7%), achieved improvement and there was no recurrence of CDI. Patients had previously undergone conventional treatment with metronidazole, vancomycin or fidaxomycin, but this did not work. Among group of 5 patients infected with hypervirulent strain 027, 60% efficacy was achieved, suggesting that the *C. difficile* strain 027 is not easily treated by the FMT (Kim, Gadani et al. 2018). This procedure, however, seems to be very evaluative and gives hope for the treatment of other diseases, e.g. it can be used against multi-resistant microorganisms, such as rod-shaped Gram-negative bacteria possesing New Dehli metalobetalactamase (NDM), extended-spectrum beta-lactamases (ESBL), *Klebsiella pneumoniae* carbapenemase (KPC), OXA 48. Biliński et al. described the case of treating oncology patient colonized by multi-resistant bacteria, including *K. pnemoniae* NDM (+) and *E. coli* ESBL (+) with FMT. The patient with hematologic disease was undergoing neoplastic therapy in strong neutropenia. There was a high risk of systemic multi-drug resistant (MDR) infection. Antibiotic therapy did not work, but after the use of a FMT, MDR strains were not found in the control cultures (Biliński, Grzesiowski et al. 2016).

### 3.3. FMT in oncology

There are indications that bacterial dysbiosis, and in particular the presence of specific groups of bacteria may lead to carcinogenesis. The processes that promote tumor formation are chronic inflammation and damage to the host's DNA. Dysbiosis leads to a decrease in the production of short-chain fatty acids and activation of inflammation through TLRs. Some bacteria produce proteins that promote the separation of β-catenin from E-cadherin, activating β-catenin signal pathway involved in carcinogenesis (Chen, Wu et al. 2018). β-catenin regulates cell proliferation and differentiation by regulating transcription factors, controls the adhesion and migration of cells, while in the complex with E-cadherin, it builds intercellular connections (Tian, Liu et al. 2011). Intercellular junctions provide the integrity of tissues, their development and proper maturation. They enable interaction and transmission of signals between neighboring cells and between them and the extracellular matrix. Weakening of cell adhesion may lead to disturbance of cell cycle control (Kwiatkowski, Godlewski et al. 2009). Changes in the composition of the intestinal microbiota may lead to the development of cancer: colorectal cancer (CRC), hepatocellular carcinoma (HCC), pancreatic cancer, breast cancer, and melanoma. Although the impact of long-term antibiotic therapy on cancerogenesis is debatable. *Helicobacter pylori*, *Bacteroides fragilis*, *Streptococcus gallolyticus*, pathogenic *Escherichia coli*, *Fusobacterium nucleatum*, are species that have been proven in cancerogenesis. *H. pylori* is classified by the World Health Organization (WHO) as a class I carcinogen and results in gastric adenocarcinoma and mucos-associated lymphoid tissue (MALT lymphoma). *H. pylori* virulence factors stimulate the signal path leading to tumor development (Wang, Meng et al. 2014). Chronic inflammation and oxidative stress, which leads to

damage of the host DNA are main causes of carcinogenesis. Very important is *H. pylori* cytotoxin (CagA) and its other virulence factors such as VacA, urease and NapA2 (Ajagopala, Vashee et al. 2017). The bacterium uses a type IV secretion system to translocate CagA to gastric epithelial cells, activating β-catenin signaling pathway (Müller 2012). *B. fragilis* toxin causes inflammation and DNA damage in host cells, leading to the development of colorectal cancer (CRC). In the CRC tissue, *S. gallolyticus* is also present, additionally activating β-catenin signaling pathways in mice. The pathogenic strains of *E. coli* can produce calmodulin, leading to the development of a tumor. *F. nucleatum* promotes proliferation and the ability to adhere tumor cells to tissue (Chen, Wu et al. 2018, Cammarota, Ianiro et al. 2017).

In CRC patients, changes in the composition of the intestinal microbiota were noted - a decrease in the number of *Lactobacillus* and *Bifidobacterium* genus, and an increased number of the *Staphylococcus*, *Fusobacterium* genus, and the *Peptostreptococcus anaerobius* species. On the basis of experiments carried out on mice, it was found that buttyric acid producers such as *Clostridium butyricum* and *Bacillus subtilis* may promote the development of CRC tumor, while the probiotic bacteria *Lactobacillus casei* strain BL23 counteracts. It was also noticed that in germ-free mice after transplantation of the intestinal microbiota from patients with CRC, the tumor develops, whereas after transplantation of feces from wild mice this development is stopped (Chen, Wu et al. 2018).

Oncological patients are exposed to long-term hospitalization, the use of anti-cancer drugs, immunosuppressants and antibiotic therapy. These factors also may predispose to the development of the cancer. The type and malignancy of the tumor may affect predisposition to CDI, but there are no clearly defined correlations between the type of cancer and the risk of development of CDI (Garzotto, García et al. 2015, Abughanimeh, Qasrawi et al. 2018). Researchers estimated the prevalence of CDI in the general hospitalized population at 1-2%, while the incidence was 7-14% in adult oncological patients (Scappaticci, Perissinotti et al. 2017). In patients during chemotherapy, CDI is estimated at 7% (Chung, Kim et al. 2016). The prevalence of CDI in patients undergoing bone marrow transplantation (HSCT) is 9 times higher than in general population and 1,4-fold higher than that of other oncology patients (Chopra, Chandrasekar et al. 2011). CDI also occurs 15 times more often in children with cancer compared to the pediatric population without cancer (Tai, Richardson et al. 2011).

At the present time, FMT seems to be a safe procedure. However, considering that it consists of the administration of biological material from the donor, the question arises whether the method of treatment of recurrent CDI is also safe in oncological patients, especially those with immunosuppression and neutropenia. Hefazi et al. Examined 23 patients with various tumors (hematologic and visceral) using the FMT procedure for the treatment of recurrent CDI. The majority of patients improved, CDI recurrence occurred only in 2 patients, but it was cured with standard therapy (metronidazole, vancomycin and fidaxomycin) without any problems. It turned out that this method is also safe for non-immunocompetent patients with multiple comorbitidies (Hefazi, Patnaik et al. 2017).

CDI is one of the main complications in patients undergoing hematopoietic stem cell transplant. Long-term hospitalization, antibiotic therapy, chemotherapy and radiotherapy damaging the intestinal mucosa and microbiota promote the development of CDI. A higher percentage of CDI is observed among allogeneic compared with autogenous transplant recipients. This can be explained by a higher frequency of hospitalization, a greater impairment of the immune system and longer antibiotic therapy. The development of CDI also predisposes to the development of acute graft-versus-host disease (GI GVHD) (Alonso, Treadway et al. 2012).

The FMT procedure for HSCT recipients in the treatment of CDI, including recurrent cases, is possible. This method is effective regardless of the patient's age and route of administration (gastro-intestinal tube, capsuled feces). There is also evidence that FMT prevents and treats patients with acute graft-versus-host disease GI GVHD, because after administration of FMT in patients' stool the number of bacteria from the genus of *Bacteroides*, *Lactobacillus*, *Bifidobacterium*, and *Faecalibacterium* increases and intestinal microenvironment is restored. Increasing the diversity of microorganisms in the feces of HSCT patients leads to a decrease in side effects after transplantation including CDI development (De Filipp, Hohmann et al. 2019).

## 4. Discussion

FMT is a procedure involving the administration of a specially prepared stool samples from healthy donor to a recipient who has qualitative and quantitative disturbances in the normal intestinal microbiota (Limketkai, Hendler et al. 2019). Bacteria produce different substances (of which the most important are short-chain fatty acids – SCFAs and secondary bile acids, polyamines and vitamins), which may, affect the development of cancer and the effectiveness of anti-cancer therapies (Zitvogel, Daillère et al. 2017).

The FMT procedure was first used in 1958 in 4 patients suffering from pseudomembranous colitis, when Eisman et al. used the enemas from

donor feces (Eisman, Silen et al. 1958). Intestinal microbial disorder can lead to dysbiosis. It was noted that critical patients hospitalized in ICU have a disturbed intestinal microbial composition (Lankelma, van Vught et al. 2017). Dysbiosis is also a predisposing factor for the development of many serious diseases, including cancer and CDI. (Chen D. Wu J. et al. 2018, Cammarota, Ianiro 2017). There is a connection between the composition of the intestinal microbial composition and the development of cancer. Microorganisms as *H. pylori, F. nucleatum,* Epstein-Barr Virus (EBV) and Human Papilloma Virus (HPV) are well known carcinogenesis factors, which may have direct oncogenic effects, by producing toxins, damaging the epithelial barrier, impairing the anticancer immune surveillance, stimulating the production of trophic factors such as growth factors or other proinflammatory cytokines (Zitvogel, Daillère et al. 2017).

In the literature there is a broadly described, close connection between intestinal dysbiosis and the development of colorectal cancer. The composition of the intestinal microbiota of CRC patients differs from the microflora of the control group patients (Helmink, Khan et al. 2019, Sears, Garret 2014, Gao, Guo et al. 2015). In CRC tumor tissues, the presence of *F. nucleatum* was found, moreover this bacterium may also contribute to resistance to anti-cancer chemotherapy (Yu, Guo et al. 2017). Experiments in mice have shown that the use of the FMT procedure may reduce colorectal carcinogenesis (Bel, Elkis et al. 2014).

Recently, an increase in *C. difficile* incidence has been observed. The main factors predisposing to CDI are older age, long-term hospitalization and the use of antibiotics. Important factors contributing to the development of CDI are immunosuppression and achlorhydria (Barlett 2017).

Oncological patients are hospitalized for a long time, undergo immunosuppressive therapy (hematooncological patients undergoing bone marrow transplantation) and preventive antibiotic therapy. All of these increases risk of CDI (by 10%) (Alonso, Treadway et al. 2012). Hematological malignant tumors are an independent risk factor that causing the development of CDI (Dubberke, Reske et al. 2007). The use of fecal transplantation in patients with multiple comorbidities seems to be very risky. Stool is a material containing a large number of microorganisms, including pathogenic ones. Therefore, it is very important to keep all safety procedures during the selection of the donor and during the FMT procedure itself. In oncological patients, the FMT procedure also has positive effects in the treatment of CDI, without causing excessive side effects (Abu- Sbeih, Ali et al. 2019). With all precautions taken, the FMT procedure can be used in cancer patients both for CDI treatment and for the eradication of multiresistant bacteria (Biliński, Grzesiowski. et al. 2016).

## 5. Short conclusion

For most people FMT seems to be a highly controversial procedure, having features of alternative medicine. Since the first application of FMT in 1958, there has been a significant development of medicine. Research methods have developed (including molecular ones, allowing to evaluate the bacterial microbiom), the approach to the concept of bacterial microbiota inhabiting the human body has also changed. While the XX century enraptured with antibiotics, the XXI century, aware of the growing antibiotic resistance among bacteria and the severity of CDI, reduce this admiration. FMT is a procedure that belongs to non-standard therapies. Based on the observations described above, it can be concluded that it is relatively safe, and above all, effective in the treatment of an increasing number of diseases, including comorbidities of oncological patients.

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**Review and Research on Cancer Treatment**
Volume 5, Issue 1 (2019) ISSN 2544-2147

# The effects of miRNA-21 on the epithelial to mesenchymal transition in cancer

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**Abstract:** MicroRNAs (miRNAs) are short, non-coding RNAs which regulate the post-transcriptional gene expression. They have been implicated in many cellular processes in physiological and pathological conditions, including cancer development and progression. miRNAs are important players in epithelial to mesenchymal transition (EMT), which is a phenomenon observed during tumour invasion and cancer metastasis. The most typical changes in gene expression profile occurring in EMT is down-regulation of epithelial genes, such as for E-cadherin, and up-regulation of mesenchymal genes, such as for N-cadherin and vimentin. miRNAs appear to be potentially useful in the clinical diagnostics and as a therapeutic targets. miRNA-21 regulates the cancer development in many ways, including control of genes expression associated with the EMT process.

## 1. Introduction

miRNAs regulate numerous, crucial biological processes. One of them is the epithelial to mesenchymal transition (EMT). Furthermore, miRNAs have been linked to many diseases, including cancer, and potentially can be useful for the clinical diagnosis [1]. For instance, in gastric cancer miR--18a, miR-106b, miR-21, miR-203, miR-146b, miR-192, and miR-200c are released to the plasma, therefore they can be a potential biomarkers of this cancer in specific ethnic group [2]. It is believed that exploring the functional importance of miRNAs in cancer development and progression may open a new ways in tumor treatment. The relationship between miRNA and cancerogenesis was well established for cluster of miR-17-92 in lymphomas and leukaemias, for miR-34 in neuroblastoma [1] as well as for miR-21 in breast cancer [3]. miRNAs which are associated with the development of cancer have been divided into two groups. In first of them, in which miRNAs are overexpressed, they are called oncogenic miRNAs (oncomiRNAs). In the second group they are underexpressed and named suppressive miRNAs (suppressor miRNAs). When the expression of the oncomiRNAs and suppressor miRNAs is upregulated or reduced, respectively, cancer cells proliferation and metastasis are induced [4].

There are many studies on the role of miRNAs in the cancer cells development and tumor progression. Since the great majority of death caused by cancer is due to metastasis formation in the vital organs, also the role of miRNAs in this phenomenon is intensively studied. In many types of cancer the process of EMT has an important role in the regulation of the metastasis by facilitating tumour cell invasion and dissemination to the distant organs. Currently, many clinical trials which modulate EMT process are ongoing. The knowledge on factors which regulate this process may lead to the development of a new strategy for treatment of cancer [5].

The initiation of EMT depends both, on the activation of activators of this process, and on the inactivation of inhibiting proteins. miRNAs, as a regulators of gene expression, may interfere with mRNA for inhibitory proteins and thus, contribute to the activation of the cascade leading to EMT. Many signaling pathways leading to the EMT are known, some of them are regulated by miR-21.

This paper is based on the review of the literature included in the PubMed database, using "EMT", "epithelial to mesenchymal transition", "cancer", "miRNA", "miR-21", as a key words.

## 2. miRNAs biogenesis and regulation of the gene expression

miRNAs are a group of small single-stranded non-coding RNAs that regulate the post-transcriptional gene expression. The first miRNA was discovered over 30 years ago during the study of a nematode, nowadays thousands of miRNAs are reported in many organisms [1]. In human genome, around 2600 miRNA genes are annotated [6]. They consist of 1-5% of all predicted human genes [7]. There is not a simple correlation between miRNA and mRNA expression, which means that multiple miRNAs can target the same mRNA and one miRNA can target many mRNA [8]. miRNAs

repress expression of the genes mainly by binding to complementary sequences in the 3' untranslated region (3'UTR) of mRNA. miRNA-mRNA interaction results latter degradation or translation inhibition [5]. miRNAs can be categorized into different groups, which are called miRNA families. The membership to specific miRNA family is determined by mature miRNA (described also as miR) and by the structure of precursor miRNA (pre-miRNA) [9]. All miRNAs undergo a series of biogenesis steps that convert the primary miRNA (pri-miRNA) transcript into pre-miRNA and finally into active, 20-25 nucleotide (nt) mature miRNA.

About half of all currently identified miRNAs are intragenic and processed mostly from the introns and relatively few exons of the protein coding genes, while the remaining are intergenic, transcribed independently of a host gene and regulated by their own promoters[10]. The biogenesis of miRNA takes place in a multi-step process. The first step occurs in the nuclear, and the second in the cytoplasmic compartment of cells. The most of miRNA genes are transcribed by RNA polymerase II (or less frequently by polymerase III), as a long pri-miRNAs of approximately 80 nt., with a cap and a poly-A tail. These pri-miRNAs having the shape of hairpin consist of a double-stranded (ds) stem of about 30 base pairs, a terminal loop and two flanking single-stranded tails [11]. pri-miRNA is processed in the cell nucleus by a heterotrimeric complex, named Microprocessor into short, approximately 70-nt. stem-loop structure known as pre-miRNA. The Microprocessor consists of the RNAse III enzyme, Drosha, and two molecules of its essential cofactor DiGeorge syndrome critical region 8 (DGCR8) [9]. Drosha contains a double stranded RNA-binding domain and two RNAse III domains. Each of RNAse III domains cleaves the 3' or 5' strand of a pri-miRNA hairpin, and as results pre-miRNA is formed [8].

In the next step pre-miRNA is transported from nucleus to the cytoplasm in a Ran-GTPase dependent manner by an export receptor, exportin 5 (EXP5), where it is further processed with the endoribonuclease III, named Dicer [1, 8]. The main functional domains of Dicer are: ATPase RNA helicase domain, a PAZ domain, two catalytic RNAse III domains, DUF283 domain, and C-terminal a double stranded RNA-binding domains [12]. Binding of Dicer by RNAse domains to the end of the pre-miRNA cuts off the dsRNA stem close to the terminal loop and produces 20-25 nt. long mature duplex of miRNA. In humans, DICER function together with the trans-activation responsive RNA-binding protein (TRBP), which enhances the precision of DICER-mediated cleavage of pre-miRNAs. Upon cleavage by Dicer, the short ds miRNA product is transferred onto an Argonaute family (AGO1-4) proteins in a process termed RNA inducing silencing complex (RISC) loading [13]. AGO consists of N-terminal domain, a PAZ domain, middle domain and PIWI domain, having activity of the endoribonuclease. Next, one strand of the ds pre-miRNA is cleaved by AGO protein and a single-stranded, matured mi-RNA is formed. The strand loaded into AGO is considered the guide strand, the unloaded strand is termed the passenger strand [10].

The important miRNA region of the guide strand interacting with mRNA is termed "seed region". It consists of two to seven nucleotides on the 5' end of miRNA. The further fate of mRNA depends on the complementarity between miRNA and mRNA. If a full complementarity exists, the target mRNA can by degradated via the AGO2, which possesses an endonucleolytic activity. This type of mRNA processing is seldom in animal cells. Most of the target mRNAs do not have fully matched sequence with miRNA, and therefore, cannot be directly cleaved by the AGO2. In such situation, 9-11 nucleotides of miRNA, together with a members of the TNRC6C/GW182 family of proteins, interact with the 3' UTR region of the target mRNA. These proteins are involved in inhibition of the translation of mRNA. This interaction occurs in the processing bodies (P-bodies), which are the cytoplasmic foci of the transcripts storage and degradation [1, 8].

## 3. miRNAs nomenclature

miRNA is called using the prefix "miR" and a numeric suffix, e.g. miR-21, miR-34. The three preceding letters denote the species. For humans (*Homo sapiens*) those letters are "hsa" (e.g. hsa-mir-21), for a mouse (*Mus musculus*) they are "mmu" (e.g. mmu-mir-21).

Evolutionary related miRNAs possess a letter after the number in the suffix, by this, multiple members of the same family can be differentiate (e.g. hsa-mir-34a and hsa-mir-34b). Identical mature products produced by two diverse loci are labeled by additional number after the full name. For instance, hsa-mir-1-1 and hsa-mir-1-2 produce the same final microRNA product: hsa-miR-1. A tag -3' or -5' added to the name indicate from which double-stranded RNA the mature sequence comes from (e.g. [R*attus norvegicus*] rno-miR-21-5p from the 5' arm of the precursor and rno-miR-21-3p from the 3' arm of the precursor). For the first miRNAs discovered, "let" and "lin" prefixes are used, instead of "miR" (e.g. let-7, lin-4) [14, 15].

## 4. Epithelial to mesenchymal transition is regulated by miRNAs in carcinogenesis

Epithelial to mesenchymal transition is a process in which epithelial cells lose cell-cell adhesion and polarity and gain a motile mesenchymal phenotype. This phenomenon can be consider as a continuum, whereby cells exhibit epithelial, transitional and finally mesenchymal phenotypes [16]. The major changes in gene expression profile occurring in EMT are associated with decreased expression of epithelial genes such as E-cadherin, mucin-1, cytokeratins, occludin as well as desmoplakin, and increased expression of mesenchymal genes such as, N-cadherin, vimentin, smooth muscle alpha actin (αSMA), fibronectin, and vitronectin. The EMT has been classified into three categories: type I, associated with embryogenesis; type II, connected with wound healing, what means associated with tissue regeneration and organ fibrosis; and type III, implicated in carcinogenesis and tumor progression [17].

The EMT confers the metastatic properties upon cancer cells by increasing mobility, invasion and resistance to the apoptotic stimuli [18]. Moreover, EMT closely associate with acquisition of stemness and therapy resistance [19, 20]. Regulation of the gene expression through miRs may contribute to both, the initiation of EMT process and its inhibition [21].

The EMT can be induced by many factors, such as Transforming Growth Factor beta (TGFβ), Fibroblast Growth Factor (FGF), Platelet-derived Growth Factor (PDGF), Epidermal Growth Factor (EGF), as well as Wnt and Notch signaling pathways [22]. Many transcription factors, including SNAIL1 (Zinc finger protein SNAIL1), SLUG (SNAIL2), Twist1/2, (ZEB)1/2 (Zinc finger E-Box-binding homeobox), HIF-1α (Hypoxia-Inducible Factor-1-alpha), E12/E47, Dlx-2 (Distal-less homeobox 2), and several regulatory molecules contribute to EMT [23].

One of the transcription factors important for EMT which may be regulated by miRNAs is Snail1. SNAIL1 is able to bind the E-box on E-cadherin gene promoter and to reduce this gene expression. Moreover, SNAIL1 is co-expressed with WNT3a (Wnt family member 3) protein, which is a master regulator of SNAIL1. Wnt3a inhibits SNAIL1 phosphorylation and increases SNAIL1 protein levels. One of miRNAs which can regulate the function of SNAIL1 is cluster miRNA-34 a/b/c located on chromosome 1. These miRNAs are considered as a tumour suppressors and are overexpressed in an epithelial state in EMT. SNAIL1 induces also the zinc-finger transcription factor 281 (ZNF281), involved in initiation of the mesenchymal phenotype in EMT. The zinc-finger transcription factor 281 suppresses the miRNA-34 at transcriptional level. This transcription factor-miRNA interaction provides a feedback loop [24, 25].

Another member of the zinc-finger transcription factors, engaged in EMT, and regulated by miRNAs is SNAIL2 (also known as a SLUG). SNAIL2 is one of the major EMT inducer, but it is a less potential suppressor of a E-cadherin than SNAIL1. It was shown that SNAIL2 expression correlate with a distant metastasis [26]. miR-203, coded by gene located on chromosome 14 interacts with SNAIL2. This miR is considered to be an anti-proliferative agent. The promoter region of miRNA-203 possesses a three different putative SNAIL2 binding sites. This interaction causes the suppression of miRNA-203 function. Transforming growth factor beta, induces EMT by interaction with SNAIL2 and also represses miRNA-203 function. There is a specific feedback loop in which miRNA-203 induces SNAIL2 repression, leading to the overexpression of E-cadherin and to the maintenance of epithelial phenotype [27, 28].

ZEB1 is the zinc-finger transcription factor, which suppresses of E-cadherin expression during carcinogenesis, and interacts with SMAD complex (SMAD1/2/3). Association between ZEB1 and SMAD may cause different activity of this transcription factor; from repression to co-activation of the transcription. Moreover, ZEB1 is a mediator of TGF-β signaling pathway, which is the major inducer of EMT. ZEB1 can be regulated by a potent EMT inhibitors such as a members of miRNA-200 family (miRNA-200a/b/c, miRNA-141, miRNA429) [29]. TGF- β is also target for miRNA-200. The controls of cell plasticity, between the epithelial and the mesenchymal states depends on autocrine TGF-β/ZEB1/miR-200 signaling network. The ZEB1 factor binds to the E-boxes in promoter region of E-cadherin gene and causes its transcriptional repression. It has be demonstrated also that ZEB1 binds to the E-boxes in miR-200 gene promoter, and thereby suppresses its expression. Therefore, while miR-200 causes post-transcriptional repression of ZEB, the latter regulates transcriptional repression of miRNA-200. These feedback mechanisms lead to the increased expression of TGF-β1 and TGF-β2, which is correlated with low miR-200 and high ZEB expression [30-32].

TWIST1 belongs to the class of alpha basic helix-loop-helix transcription factors [33], which also may be regulated by miRNAs. TWIST1 expression down-regulates the epithelial genes (e.g., for E-cadherin and claudin-7) and up-regulates genes for the mesenchymal proteins. TWIST1 is one of the downstream targets of let-7 miRNAs family, which includes let-7e-; and let-7b miRNAs as well as miRNA-98. In many cancers, let-7 miRNA family members, which possess anti-metastatic function, are significantly reduced. The biogenesis of let-7 miRNAs is regulated by RNA-

-binding proteins, LIN28A and LIN28B, which block DICER cleavage activity, and therefore inhibit the formation of these miRNAs. It was also shown that in many human cancers LIN28A/B are over-expressed. This leads to reduction of let-7 miRNA, and in consequence to TWIST up-regulation [34].

## 5. miR-21 (oncomiR) controls EMT in various types of cancer

miR-21 is important in regulation of such oncogenic processes as high cells proliferation and invasion, metastatic potential, and low apoptosis, therefore it is an example of oncomir [35]. miR-21 gene is located in the intronic region which overlaps with the 3' UTR end of the transmembrane protein 49 (TMEM 49) gene in q23.2 on chromosome 17 [36]. TMEM49 protein mediates autophagy and regulates cancer-relevant processes, such as an inhibition of proliferation and metastasis [37]. The overexpression of miR-21 is noted in glioblastoma and in many other types of tumors, including B-cell lymphoma, hepatocellular carcinoma and cancers of head and neck, breast, ovary, cervix as well as lung. Numerous targets for miR-21 have been described, e.g., PTEN, PDCD4, BTG2, HIF1α, TIMP3, TM1 [35, 38], LIF, STAT3 [39], NR2F2 and Smad7 [40]. It was demonstrated that TGF-β increases miR-21 expression in cancer cells, and causes induction of cancer stem cells-like phenotype, and also increases hypoxia-inducible factor 1 alpha (HIF1α) levels. This factor is known to be induced not only by the hypoxia, but also by the growth factors and oncogenes. In addition, there is relationship between miRNA expression and increased HIF1α expression, since miR21 targets PTEN, what leads to enhancement of the HIF1α level [41]. The main target for the tumor supressor PTEN (phosphatase and tensin homolog) is phosphatidylinositol 3,4,5 trisphosphate (PIP3). PIP3 recruits Ser/Thr kinase AKT to the plasma membrane, what leads to the phosphorylation and activation of AKT by phosphoinositide-dependent kinase-1 (PDK1). The direct phosphorylation target of AKT is the family of forkhead transcription factors (FOXO) [42]. One of them, namely, FOXO3a upregulates HIF-1α. miR21 via suppressing PTEN increases the level of PIP3 and HIF-1α as a result of upregulation of AKT-dependent activation of FOXO3a [43].

It is known that HIF-1α directs the expression of many EMT regulators and induces the loss of E-cadherin by transcriptional activation of genes encoding repressors of E-cadherin expression, such as ZFHX1B (Smad interacting-protein 1), ZFHX1A (Zinc Finger Homeobox Protein 1A) and TCF3 (Transcription factor 3) [44].

HiF-1α, by binding via hypoxia response element (HRE) sites in the Zeb1 or Twist1 proximal promotor, can regulate the expression these transcription factors [45-47].

In the breast cancer cells, overexpression of miR-21 promoted EMT by regulation of PTEN and AKT pathway [48].

Moreover, miR-21 has a crucial role in colorectal cancer. It was shown, that in colorectal cancer, miR-21 is upregulated in preneoplastic and neoplastic parenchymal and stromal cells as well as in the serum. It was also demonstrated that the level of miR-21 can be a predictor of tumour relapse and poor survival. In colorectal cancer cell line overexpression of miR-21 contributes to the lose of epithelial marker, such as E-cadherin, and to the acquisition of mesenchymal marker, N-cadherin. miR-21 promotes EMT probably by the regulation of NR2F2/TGF-β/SMAD7 pathway [49] and ITGβ4/PDCD4 network [50]. NR2F2 (Nuclear receptor subfamily 2 group F member 2) is commonly upregulated in cancer cells and is considered to be a key transcription factor in the development of breast, lung, prostate and colorectal cancers. Smad7 protein is a negative regulator of TGF-beta pathway. NR2F2 activates miR-21 expression by binding to its gene promoter. In turn, miR21 inhibits SMAD7 and therefore TGF-beta signaling cascade is activated. In addition, NR2F2 inhibits Smad7 expression and promotes TGF-β-dependent EMT [40].

ITGβ4 (Integrin-β4) plays a role in the regulation of EMT and is expressed in epithelial cells. Expression of the gene for this integrin is another target for miR-21. Modulation of ITGβ4 protein levels, by miR-21, is executed via repression of mRNA translation acting on 3' UTR regions, and also through mRNA degradation. Inhibition of miR-21 results in increased ITGβ4 mRNA expression and the protein level. It has been postulated that high miR-21 level combined with low ITGβ4 and PDCD4 (Programmed Cell Death 4) expression is able to predict the presence of colorectal cancer metastasis [50].

PDCD4 is a tumour suppressor and its upregulation is closely linked to apoptosis. Decreased PDCD4 expression enhances malignant transformation, by intensifying the expression of apoptosis inhibitors, and causes also chemoresistance by induction of multidrug resistance protein, MDR1/P-gp [51]. In addition, downregulation of PDCD4 leads to the low expression of epithelial-specific proteins (α-catenin and γ-catenin), and high expression of mesenchymal-specific proteins (N-cadherin and fibronectin), in HT29 colon cell line. The presumed mechanisms of these phenom-

ena are through activation of β-catenin dependent transcription. A decrease in E-cadherin expression, which is the binding partner of β-catenin, results in an increase of free β-catenins in the cytoplasm. Free β-catenins are phosphorylated by glycogen synthase kinase 3β (GSK3β) in the adenomatous polyposis coli (APC)-axin-GSK3β-casein kinase I complex, and next degradated. Mutations of the APC or block of GSK3β activity, cause translocation of β-catenin into nucleus and initiation of the β-catenin-dependent transcription. A downstream target of β-catenin-dependent transcription is c-Myc, transcription factor commonly up-regulated in tumor cells [52].

It is assumed, that PDCD4 is directly engaged in EMT regulation by inhibition of Snail translation, which is a master transcription factor for this transition. Therefore, downregulation of PDCD4 by miR-21 can lead to Snail overexpression and EMT initiation [52].

miRNA-21 expression may be associated with IL-6 (interleukin 6) upregulation. This interleukin is involved in a variety of phenomena, including the inflammatory response, oncogenesis, regulation of cell growth, survival, differentiation and many other processes. IL6 binding to its receptor (IL6R) leads to the activation of receptor-associated Janus kinases (JAKs), and following phosphorylation and dimerization of STAT3 (Signal transducer and activator of transcription 3), its translocation to the nucleus, and miRNA-21 gene expression [53].

miR-21, as many others molecules, can be loaded into vesicles (exosomes), which after exocytosis are able to integrate into surrounding cells, where they release functional miRNAs. Some data show, that cancer and immune cells cultured *in vitro* interact and cross-talk via IL6 and miRNAs. The tumor-associated immune cells produce IL-6, which binds to IL6R on the cancer cells surface. As a result, a number of oncogenes and miRNAs, including miR-21 is expressed. After secretion into environment, miR-21 is taken up by the tumor-associated immune cells endosomes and binds to TLR8 (Toll like-receptor-8). This interaction induces the NF-kB pathway and further IL-6 secretion [53].

Considering the uncontested functions of miR21 in tumorogenesis, it may be important to inhibit this molecule. A number of studies indicate that miR-21 gene editing and silencing may inhibit the phenomenon of EMT and cancer progression. An example can be application in ovarian cancer cell lines the Lentiviral CRISPR/Cas9 vectors, which mediate mutation in sequences of the miR-21 precursor. This results in upregulation of E-cadherin expression and downregulation of Snail2 and vimentin, and in consequence the reduction of cell proliferation, migration and invasion [43].

Application of miR-21 inhibitor in NSCLC (non-small cell lung cancer) cell line suppresses the phosphorylation of Akt and promotes apoptosis through inhibition of PI3K/Akt/NF-κB signaling pathway. Furthermore, in NSCLC cell down-regulation of miR-21suppresses cell migration and invasion, as well as EMT signaling pathways [44].

It has been documented also, that transfection of the breast cancer stem cell-like cells with human Hsa-miR-21 antagomir reverses EMT phenotype and HIF-1α expression, both of which are consistent with the tumor cells invasion and migration [36].

miR-21 is implicated in the drug resistance to neoadjuvant treatment in trastuzumab and chemotherapy, in HER2-positive breast cancer patients. The DNA damage induced by this therapy upregulates the expression of miR-21, by activating NF-κB, what consequently sustains EMT in breast cancer. Probably, the mechanism of resistance to trastuzumab-chemotherapy in patients with HER2-positive tumors depends on miR-21-mediated silencing of PTEN and PDCD4 proteins. The use of anti-miR-21 inhibitor in human breast cancer cell line leads to increase of susceptibility to trastuzumab, and to the reduction of viability of these cells. It has been postulated, that the increase in miR-21 expression in neoadjuvant trastuzumab-chemotherapy can be a predictive biomarker of resistance to this treatment [3].

## 6. Summary

miRNAs can regulate numerous biological processes, including EMT phenomenon during cancer development. This process contributes to the regulation of cells motion and in consequence to the cancer cells metastasize and tumor progression. In this review we illustrate mainly the role of miR-21, and some other selected microRNAs in the control of EMT process in cancerogenesis. The regulation of miRNAs is complicated and its specificity depends on cancer type. Understanding the molecular mechanisms, which are controlled by miRNAs may contribute to the generation of new strategies in therapy, and therefore to the life extension of patients with cancer.

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