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Original article

Assessment of the performance of Romanian Public Health Units in identifying and conducting antimicrobial susceptibility testing for healthcare-associated infection-causing pathogens

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Abstract

Detecting healthcare-associated infections (HAIs) is crucial as HAIs can pose a significant threat to patient safety, particularly among vulnerable populations such as the elderly, immunocompromised individuals, and those undergoing invasive medical procedures. Also, it is important to monitor the effectiveness of antibiotics and identify the resistance patterns of microorganisms to ensure appropriate treatment options. Bacteriology external quality control programs provide a means for laboratories to assess their performance and identify areas that require improvement. By participating in such programs, laboratories can evaluate their procedures, equipment, and personnel to ensure that they are producing reliable and accurate results because the External Quality Control (EQC) programs are carried out by medical laboratories using identical procedures as those used for handling patient samples. Our investigation centered on the performance of Romanian Public Health Units in identifying bacteria and conducting antimicrobial susceptibility testing in Bacteriology EQC programs associated with some of the most prevalent HAIs, such as blood infections, respiratory infections, and postoperative infections.

Keywords External Quality Control, multidrug-resistant bacteria

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Introduction

The occurrence of HCAIs is a matter of public health concern due to their potential to escalate treatment expenses, reduce quality of life, and raise the risk of morbidity and mortality (E. N. TCHOUAKET & al., 2022 [4]). Antibiotic consumption is the primary factor responsible for the emergence of new mechanisms of microbial resistance, such as multidrug resistance which is a contributing factor to the onset of HAIs. The development of bacterial resistance limits the treatment options for infections, particularly when it comes to healthcare-associated infections. This makes treatment more challenging and increases the risk of unfavorable outcomes (G. A. POPESCU & al., 2020 [1]).

In Romania, the data collected during the years 2018-2019 showed an increase in the consumption of antibiotics compared to 2016 and 2017. Subsequently, the situation in 2020 was significantly influenced by the evolution of the COVID-19 pandemic (G. A. POPESCU & al., 2020 [1]).

Based on statistical reports from most hospitals in Romania, HAIs continue to be a significantly underappreciated condition, with an incidence rate of only 1.04% among all discharged patients in 2020. The incidence of reported HAIs has been continuously rising between 2012 and 2020, with an acceleration observed after 2016. In 2020, the reported cases reached 23.208, which is 2.8 times higher than the minimum recorded in 2012 (G. A. POPESCU & al., 2020 [1]).

Respiratory infections were the most frequently reported in 2020, accounting for 37% of all reported cases, which is twice the number reported in 2019 (G. A. POPESCU & al., 2020 [1]).

Bacterial resistance to antibiotics has increased for most pathogens circulating in hospitals, while remaining relatively stable for germs that are prevalent in the community. This trend can be attributed to the reduction in oral antibiotic consumption, which decreased by 12-13% during 2016-2017 compared to 2015. The resistance level of *Escherichia coli* decreased considerably, and the percentage of *Methicillin-resistant Staphylococcus aureus* (MRSA) stabilized. Although there is an increasing trend in the reporting of infections associated with antimicrobial-resistant microorganisms at the national level, progress remains limited (G. A. POPESCU & al., 2020 [1]). MRSA remains among the top five causes of nosocomial infections as the global prevalence of antibiotic-resistant pathogenic forms of *S. aureus*, including MRSA, is a significant issue in clinical medicine. *S. aureus* is frequently responsible for wound infections following surgery. It also has the potential to cause a large spectrum of illnesses, ranging from minor skin infections to severe conditions like pneumonia, meningitis, and sepsis (F. TUTULESCU & al., 2018 [9]).

Also, the utilization of carbapenems for treating infections caused by Gram-negative bacteria that are resistant to multiple antibiotics, particularly those producing extended-spectrum beta-lactamase (ESBL) or non-fermentative bacilli such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (except for ertapenem), has resulted in the emergence of *Enterobacteriaceae* that are resistant to them. The main concern related to the emergence of these bacterial strains is the increase in the difficulty to treat severe infections caused by them (including those that carry a risk of mortality) as only a limited number of antibiotics are effective against them (G.A. POPESCU & al., 2016 [2]). *Pseudomonas aeruginosa* is a dominant pathogen in people suffering from cystic fibrosis, contributing significantly to morbidity and mortality. Its remarkable adaptability greatly enhances its potential to cause chronic infections in such individuals (I. JURADO-MARTÍN & al., 2021 [3]).

The exponential rise in infections caused by *Enterobacterales, particularly Klebsiella pneumoniae* and *Escherichia coli*, is one of the primary concerns associated with antimicrobial resistance (E. MACHADO & al., 2022 [6]). *Klebsiella pneumoniae* is a type of Gram-negative bacterium that is significant in human pathology as it is responsible for causing a broad spectrum of infections such as urinary and respiratory infections, as well as systemic, digestive, and meningeal infection (L. GIUBELAN & al., 2021 [5]).

Serratia marcescens can lead to various types of infections in both adults and children (such as pneumonia, sepsis, wound infection, meningitis, endocarditis, ocular infections, etc.), including opportunistic and nosocomial infections. The entry point for infections with *S. marcescens* are the urinary catheter, intubation, and the central venous catheter. Catheter colonization increases bacterial survival, particularly in biofilm communities. Therefore, biofilms increase resistance to antibiotics (R. ZIVKOVIC ZARIC & al., 2022 [7]).

Vancomycin-resistant Enterococcus spp. (VRE) has emerged as a significant multidrug-resistant nosocomial pathogen and a primary cause of HAIs, over the past 25 years. Patients who are severely ill and immunocompromised are particularly vulnerable to the threat posed by VRE. Its natural characteristics, including innate and acquired resistance to various antimicrobial drug classes, result in limited therapeutic options and this contributes to the threat it poses (A. JANJUSEVIC & al., 2022 [8]).

The purpose of this study is to form a viewpoint on the proficiency of Romanian Public Health Units in identifying bacteria and conducting antimicrobial susceptibility testing

for certain bacteria that cause healthcare - associated infections: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Escherichia coli*, *Acinetobacter baumannii*, *Klebsiella pneumoniae* and *Serratia marcescens.*

Materials and methods

Our research was focused on examining seven of the primary bacteria that are responsible for the prevalence of HAIs worldwide. We utilized the outcomes from an EQC provider. The study was centered on the results registered from Public Health Units in Romania participating to five EQC bacteriology schemes: i) screening for the detection of multidrug-resistant bacteria, ii) bacteriological examination of blood culture, iii) bacteriological examination of sputum, iv) bacteriological examination of nasal secretion and v) bacteriological examination of purulent secretion. For the screening for the detection of multidrug-resistant bacteria EQC program we used the outcomes registered in 2020 – 2022, whereas for the bacteriological examination of blood culture, sputum, nasal secretion, and purulent secretion we utilized the outcomes registered in 2021 and 2022.

Besides the screening for the detection of multidrug-resistant bacteria program, each EQC scheme includes two components for evaluation: identification and susceptibility testing. The screening for the detection of multidrug-resistant bacteria EQC program is designed to only receive the name of the multidrug – resistant bacteria (MDR bacteria) from the participating laboratories.

Results

During the initial phase of the study, our focus was on bacterial species identification from bacteriological examination of blood culture, bacteriological examination of sputum, bacteriological examination of nasal secretion and bacteriological examination of purulent secretion. We centralized the number of results and the percentages of satis-

Table 1 - Bacteriological examination of blood culture

EQC Round	Strain	Number of results obtained by Public Health Units	Percentage of satisfactory results registered (%)
March 2021	*Methicillin-resistant Staphylococcus aureus*	12	100.00%
May 2021	*Pseudomonas aeruginosa*	21	100.00%
September 2021	*Escherichia coli*	25	100.00%
October 2021	*Serratia marcescens*	16	100.00%
November 2021	*Staphylococcus aureus*	26	100.00%
March 2022	*Serratia marcescens*	16	100.00%
April 2022	*Escherichia coli*	5	100.00%
May 2022	*Staphylococcus aureus*	22	90.91%
September 2022	*Klebsiella pneumoniae*	31	100.00%
November 2022	*Escherichia coli*	29	100.00%

Table 2 - Bacteriological examination of sputum

EQC Round	Strain	Number of results obtained by Public Health Units	Percentage of satisfactory results registered (%)
March 2021	*Klebsiella pneumoniae*	15	100.00%
April 2021	*Staphylococcus aureus*	5	100.00%
October 2021	*Pseudomonas aeruginosa*	16	100.00%
November 2021	*Klebsiella pneumoniae*	42	100.00%
March 2022	*Pseudomonas aeruginosa*	21	100.00%
May 2022	*Staphylococcus aureus*	42	100.00%
September 2022	*Methicillin-resistant Staphylococcus aureus*	60	100.00%
October 2022	*Klebsiella pneumoniae*	25	100.00%
November 2022	*Klebsiella pneumoniae*	52	100.00%

Table 3 - Bacteriological examination of nasal secretion

EQC Round	Strain	Number of results obtained by Public Health Units	Percentage of satisfactory results registered (%)
April 2021	*Staphylococcus aureus*	17	100.00%
September 2021	*Staphylococcus aureus*	153	100.00%
October 2021	*Methicillin-resistant Staphylococcus aureus*	78	100.00%
May 2022	*Staphylococcus aureus*	155	100.00%
October 2022	*Staphylococcus aureus*	58	100.00%
November 2022	*Staphylococcus aureus*	176	100.00%

Table 4 - Bacteriological examination of purulent secretion

EQC Round	Strain	Number of results obtained by Public Health Units	Percentage of satisfactory results registered (%)
September 2021	*Escherichia coli*	150	100.00%
October 2021	*Acinetobacter baumannii*	62	93.55%
March 2022	*Pseudomonas aeruginosa*	89	100.00%
April 2022	*Acinetobacter baumannii*	67	98.51%
November 2022	*Escherichia coli*	158	100.00%

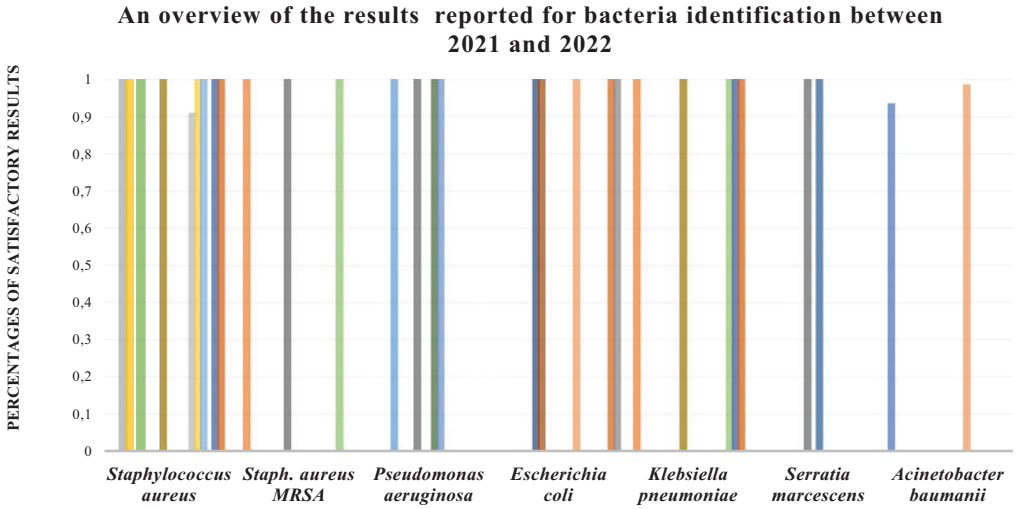

Fig. 1 – An overview of the results obtained by Public Health Units participating on EQC programs – bacterial identification

factory results obtained by the Public Health Units laboratories.

Centralizing the results from the bacteriological examination of blood culture program we observed that nine out of ten EQC rounds analyzed recorded 100% satisfactory results. Only one round registered 90.91% for *S. aureus* (Table 1).

Upon analyzing the outcomes recorded for the bacteriological examination of sputum scheme, we found that in each EQC round, the percentage of satisfactory results was 100% (Table 2).

Upon analyzing the outcomes obtained from the six EQC rounds of bacteriological examination of nasal secretion, we found a similar situation where the percentage of satisfactory results was 100% (Table 3).

Regarding the bacteriological examination of purulent secretion program, our study uncovered that three EQC rounds recorded 100% satisfactory results, while the remaining two registered 93.55% and 98.51%, both for *A. baumannii* (Table 4).

By centralizing the percentages of satisfactory results, we were able to determine that out of the thirty EQC rounds conducted for the four bacteriology programs, the lowest percentage of satisfactory results was 90.91%. This result was recorded for a *S. aureus* strain provided in May 2022 in the Bacteriological examination of blood culture program.

Furthermore, out of the total EQC rounds conducted, only three recorded a percentage of satisfactory results lower than 100%. One of these was for the *S. aureus* strain provided in May 2022, while the other two were recorded in the Bacteriological examination of purulent secretion program for *Acinetobacter baumannii* in October 2021 (93.55%) and April 2022 (98.51%) (Figure 1).

For the next stage of our study, we directed our attention to the techniques utilized by the Public Health Units laboratories to identify bacteria in the thirty EQC rounds analyzed in the first phase of our research.

A total of 1644 results were reported in 2021 – 2022 for the bacteriological examination of blood culture, sputum, nasal secretion and purulent secretion scheme. The number of results obtained utilizing manual methods was significantly higher (1086) than the number of results obtained using automated systems (558) (Table 5).

The subsequent phase of the study was focused on antimicrobial susceptibility testing. Our initial step was to centralize the outcomes and percentages of satisfactory results recorded for the same four EQC bacteriology programs, i.e. bacteriological examination of blood culture, sputum, nasal secretion, and purulent secretion. During this phase, we conducted the analysis on each bacteria strain provided by the EQC organizer to the four EQC programs.

After centralizing the percentages of satisfactory results, we found that out of the forty *S. aureus* EQC rounds performed for the four bacteriology programs, the lowest percentage of satisfactory results was 90.00%, which was recorded in May 2022 for penicillin in the bacteriological examination of blood culture program. Furthermore, only sixteen cases recorded percentages of satisfactory results lower than 100%, ranging from 90.00% to 99.42%. The remaining 24 cases resulted in a 100% satisfactory outcome.

Table 5 – A viewpoint of the techniques utilized by the Public Health Units laboratories for bacterial species identification

EQC Program	Strain	Number of results obtained using manual method	Number of results obtained using automated systems
Blood culture March 2021	*Methicillin-resistant Staphylococcus aureus*	4	8
Blood culture May 2021	*Pseudomonas aeruginosa*	8	13
Blood culture September 2021	*Escherichia coli*	9	16
Blood culture October 2021	*Serratia marcescens*	5	11
Blood culture November 2021	*Staphylococcus aureus*	9	17
Blood culture March 2022	*Serratia marcescens*	5	11
Blood culture April 2022	*Escherichia coli*	1	4
Blood culture May 2022	*Staphylococcus aureus*	6	16
Blood culture September 2022	*Klebsiella pneumoniae*	11	20
Blood culture November 2022	*Escherichia coli*	10	19
Sputum March 2021	*Klebsiella pneumoniae*	8	7
Sputum April 2021	*Staphylococcus aureus*	4	1
Sputum October 2021	*Pseudomonas aeruginosa*	8	8
Sputum November 2021	*Klebsiella pneumoniae*	18	24
Sputum March 2022	*Pseudomonas aeruginosa*	12	9
Sputum May 2022	*Staphylococcus aureus*	24	18
Sputum September 2022	*Methicillin-resistant Staphylococcus aureus*	30	30
Sputum October 2022	*Klebsiella pneumoniae*	13	12
Sputum November 2022	*Klebsiella pneumoniae*	27	25
Nasal secretion April 2021	*Staphylococcus aureus*	11	6
Nasal secretion September 2021	*Staphylococcus aureus*	117	36
Nasal secretion October 2021	*Methicillin-resistant Staphylococcus aureus*	65	13
Nasal secretion May 2022	*Staphylococcus aureus*	123	32
Nasal secretion October 2022	*Staphylococcus aureus*	40	18
Nasal secretion November 2022	*Staphylococcus aureus*	135	41
Purulent secretion September 2021	*Escherichia coli*	110	40
Purulent secretion October 2021	*Acinetobacter baumannii*	44	18
Purulent secretion March 2022	*Pseudomonas aeruginosa*	70	19
Purulent secretion April 2022	*Acinetobacter baumannii*	47	20
Purulent secretion November 2022	*Escherichia coli*	112	46
Total number of results for each technique		Total number of results = 1086	Total number of results = 558

Legend: S = Susceptible

Table 6 -The performance of Public Health Units regarding antimicrobial susceptibility testing of *Staphylococcus aureus* strains

Antibiotics	EQC Program	Expected result	Percentage of satisfactory results (%)
Gentamicin	Blood culture - November 2021	S	100.00%
	Blood culture - May 2022	S	95.45%
	Sputum - May 2022	S	100.00%
	Nasal secretion - April 2021	S	100.00%
	Nasal secretion - September 2021	S	99.30%
	Nasal secretion - May 2022	S	100.00%
	Nasal secretion - October 2022	S	98.21%
	Nasal secretion - November 2022	S	99.41%
Cefoxitin	Blood culture - November 2021	S	100.00%
	Blood culture - May 2022	S	90.91%
	Sputum - April 2021	S	100.00%
	Sputum - May 2022	S	100.00%
	Nasal secretion - April 2021	S	100.00%
	Nasal secretion - September 2021	S	99.27%
	Nasal secretion - May 2022	S	100.00%
	Nasal secretion - October 2022	S	100.00%
	Nasal secretion - November 2022	S	100.00%
Penicillin	Blood culture - November 2021	S	100.00%
	Blood culture - May 2022	S	90.00%
	Sputum - April 2021	S	100.00%
	Sputum - May 2022	S	92.31%
	Nasal secretion - April 2021	S	100.00%
	Nasal secretion - September 2021	S	95.68%
	Nasal secretion - November 2022	S	96.79%
Antibiotics	**EQC Program**	**Expected result**	**Percentage of satisfactory results (%)**

	Blood culture - November 2021	S	100.00%
	Blood culture - May 2022	S	95.00%
	Sputum - April 2021	S	100.00%
Erythromycin	Sputum - May 2022	S	100.00%
	Nasal secretion - April 2021	S	100.00%
	Nasal secretion - September 2021	S	97.24%
	Nasal secretion - November 2022	S	98.82%
	Blood culture - November 2021	S	100.00%
	Blood culture - May 2022	S	90.91%
	Sputum - April 2021	S	100.00%
Clindamycin	Sputum - May 2022	S	100.00%
	Nasal secretion - April 2021	S	100.00%
	Nasal secretion - September 2021	S	99.31%
	Nasal secretion - November 2022	S	99.42%
	Blood culture - November 2021	S	100.00%
Linezolid	Blood culture - May 2022	S	100.00%
	Sputum - April 2021	S	100.00%
	Nasal secretion - September 2021	S	100.00%
Trimethoprim -	Sputum - May 2022	S	100.00%
Sulfamethoxazole	Nasal secretion - October 2022	S	100.00%

Table 7 - The performance of Public Health Units regarding antimicrobial susceptibility testing of methicillin-resistant *Staphylococcus aureus* strains

Antibiotics	EQC Program	Expected result	Percentage of satisfactory results (%)
	Blood culture - March 2021	R	100%
Gentamicin	Sputum - September 2022	R	98.31%
	Nasal secretion - October 2021	R	92.96%
	Blood culture - March 2021	R	100%
Cefoxitin	Sputum - September 2022	R	100%
	Nasal secretion - October 2021	R	91.89%
			100%
Penicillin	Blood culture - March 2021	R	100%
	Sputum - September 2022	R	
	Blood culture - March 2021	R	100%
Erythromycin	Sputum - September 2022	R	100%
	Nasal secretion - October 2021	R	95.95%
	Blood culture - March 2021	R	100%
Clindamycin	Sputum - September 2022	R	100%
	Nasal secretion - October 2021	R	97.30%
Linezolid	Blood culture - March 2021	S	100%
	Nasal secretion - October 2021	S	100%
Trimethoprim - Sulfamethoxazole	Sputum - September 2022	S	100%
Vancomycin	Sputum - September 2022	S	100%

Legend: S = Susceptible; R = Resistant

Linezolid and trimethoprim – sulfamethoxazole were the only antibiotics with 100% satisfactory results in every EQC round (Table 6).

For the eighteen *methicillin-resistant Staphylococcus aureus* EQC rounds performed the study revealed that the lowest percentage of satisfactory results was 91.89% recorded for cefoxitin in October 2021 in the bacteriological examination of nasal secretion EQC program. Thirteen out of the eighteen EQC rounds conducted recorded 100% satisfactory results. On the other hand, five EQC rounds registered percentages of satisfactory results ranging from 91.89% to 98.31%. Penicillin, linezolid, trimethoprim– sulfamethoxazole and vancomycin were the antibiotics with 100% satisfactory results in every EQC round (Table 7).

After centralizing the percentages of satisfactory results, we observed that for the *P. aeruginosa* EQC rounds performed for three of the bacteriology programs, the lowest percentage of satisfactory results was 60.00%, which was recorded in March 2022 for ceftriaxone in the Bacteriological examination of sputum program. Five of the nine EQC rounds conducted recorded 100% satisfactory results. Four EQC rounds registered percentages of satisfactory results ranging lower than 100%, from 60.00% to 98.75%. Gentamicin, tobramycin, and amikacin registered 100% satisfactory results in each of the EQC rounds conducted (Table 8).

Table 8 - The performance of Public Health Units regarding antimicrobial susceptibility testing of *Pseudomonas aeruginosa* strains

Antibiotics	EQC Program	Expected result	Percentage of satisfactory results (%)
Gentamicin	Blood culture - May 2021	S	100.00%
	Sputum - March 2022	S	100.00%
Tobramycin	Blood culture - May 2021	S	100.00%
Ceftriaxone	Sputum - October 2021	R	80.00%
	Sputum - March 2022	R	60.00%
	Purulent secretion - March 2022	R	63.41%
Amikacin	Sputum - October 2021	S	100.00%
Meropenem	Sputum - March 2022	S	100.00%
	Purulent secretion - March 2022	S	98.75%

Legend: S = Susceptible; R = Resistant

Table 9 - The performance of Public Health Units regarding antimicrobial susceptibility testing of *Escherichia coli* strains

Antibiotics	EQC Program	Expected result	Percentage of satisfactory results (%)
Ampicillin	Blood culture - September 2021	R	100.00%
	Blood culture - April 2022	R	100.00%
	Purulent secretion - September 2021	R	95.49%
	Purulent secretion - November 2022	S	97.58%
Ceftazidime	Blood culture - September 2021	S	100.00%
	Purulent secretion - September 2021	S	100.00%
Gentamicin	Blood culture - September 2021	S	100.00%
	Blood culture - April 2022	S	100.00%
	Purulent secretion - September 2021	S	100.00%
	Purulent secretion - November 2022	S	100.00%
Trimethoprim - Sulfamethoxazole	Blood culture - September 2021	S	100.00%
	Purulent secretion - September 2021	S	99.30%
Amoxicillin - clavulanate	Blood culture - September 2021	S	100.00%
	Blood culture - April 2022	S	100.00%
	Purulent secretion - September 2021	S	88.98%
	Purulent secretion - November 2022	S	98.56%
Ertapenem	Blood culture - September 2021	S	100.00%
	Blood culture - April 2022	S	100.00%
	Purulent secretion - September 2021	S	100.00%
Ceftriaxone	Blood culture - September 2021	S	100.00%
	Blood culture - April 2022	S	100.00%
	Purulent secretion - September 2021	S	100.00%
	Purulent secretion - November 2022	S	100.00%
Levofloxacin	Blood culture - April 2022	S	100.00%
Cefuroxime	Blood culture - April 2022	S	100.00%
Ciprofloxacin	Purulent secretion - November 2022	S	100.00%

Legend: S = Susceptible; R = Resistant

Table 10 - The performance of Public Health Units regarding antimicrobial susceptibility testing of *Serratia marcescens* strains

Antibiotics	EQC Program	Expected result	Percentage of satisfactory results (%)
Piperacillin – Tazobactam	Blood culture - October 2021	S	100.00%
Gentamicin	Blood culture - October 2021	S	100.00%
Levofloxacin	Blood culture - October 2021	S	100.00%
Meropenem	Blood culture - October 2021	S	100.00%
Amoxicillin – Clavulanic acid	Blood culture - October 2021	R	100.00%
Ceftriaxone	Blood culture - March 2022	S	100.00%
Ampicillin	Blood culture - March 2022	R	100.00%
Cefepime	Blood culture - March 2022	S	100.00%
Ciprofloxacin	Blood culture - March 2022	S	100.00%
Amikacin	Blood culture - March 2022	S	100.00%
Ertapenem	Blood culture - March 2022	S	100.00%
Cefuroxime	Blood culture - March 2022	R	100.00%

Legend: S = Susceptible; R = Resistant

For *E. coli* EQC rounds the study showed that the lowest percentage of satisfactory results was 88.98% recorded for amoxicillin - clavulanate in September 2021 for the bacteriological examination of purulent secretion scheme. Twenty-six EQC rounds were conducted for *E. coli* and twenty of them recorded 100% satisfactory results. Ceftazidime, gentamicin, ertapenem, ceftriaxone, levofloxacin, cefuroxime and ciprofloxacin registered 100% satisfactory results in each of the EQC rounds conducted (Table 9).

After centralizing the percentages of satisfactory results for *S. marcescens* EQC rounds conducted in October 2021 and March 2022 in the bacterial examination of blood culture program, we found that each antibiotic recorded 100% satisfactory results (Table 10).

For the twenty-six *K. pneumoniae* EQC rounds performed for Bacteriological examination of sputum the study revealed that the lowest percentage of satisfactory results was 76.92% recorded for amoxicillin - clavulanate in March 2021. Twenty - two EQC rounds recorded 100% satisfactory results. On the other hand, four rounds registered lower percentages of satisfactory results ranging from 76.92% to 96.00%. ESBL, ceftazidime, meropenem, ertapenem, trimethoprim –sulfamethoxazole, ceftriaxone, levofloxacin, and amikacin were the cases with 100% satisfactory results in every EQC round (Table 11)

After centralizing the results obtained from *A. baumannii* susceptibility testing we observed that there is no EQC round with 100% satisfactory results recorded. The lowest

Table 11 - The performance of Public Health Units regarding antimicrobial susceptibility testing of *Klebsiella pneumoniae* strains

Antibiotics	EQC Program	Expected result	Percentage of satisfactory results (%)
Ampicillin	Sputum - March 2021	R	93.33%
	Sputum - November 2021	R	100.00%
	Sputum - October 2022	R	100.00%
	Sputum - November 2022	R	100.00%
Amoxicillin - clavulanate	Sputum - March 2021	S	76.92%
	Sputum - November 2021	S	100.00%
	Sputum - October 2022	S	96.00%
ESBL	Sputum - March 2021	Positive	100.00%
	Sputum - October 2022	Negative	100.00%
	Sputum - November 2022	Negative	100.00%
Ceftazidime	Sputum - March 2021	R	100.00%
	Sputum - November 2021	S	100.00%
Ciprofloxacin	Sputum - March 2021	S	80.00%
	Sputum - October 2022	S	100.00%
	Sputum - November 2022	S	100.00%
Meropenem	Sputum - March 2021	S	100.00%
Ertapenem	Sputum - November 2021	S	100.00%
	Sputum - October 2022	S	100.00%
	Sputum - November 2022	S	100.00%
Trimethoprim - Sulfamethoxazole	Sputum - November 2021	S	100.00%
	Sputum - November 2022	S	100.00%
Ceftriaxone	Sputum - November 2021	S	100.00%
	Sputum - October 2022	S	100.00%
Levofloxacin	Sputum - November 2021	S	100.00%
Amikacin	Sputum - October 2022	S	100.00%
	Sputum - November 2022	S	100.00%

Legend: S = Susceptible; R = Resistant

Table 12 - The performance of Public Health Units regarding antimicrobial susceptibility testing of *Acinetobacter baumannii* strains

Antibiotics	EQC Program	Expected result	Percentage of satisfactory results (%)
Levofloxacin	Purulent secretion - October 2021	S	91.38%
	Purulent secretion - April 2022	S	94.92%
Ceftriaxone	Purulent secretion - October 2021	R	68.75%
	Purulent secretion - April 2022	R	71.43%
Amikacin	Purulent secretion - October 2021	S	95.35%
Meropenem	Purulent secretion - October 2021	S	94.64%
Amoxicillin - clavulanate	Purulent secretion - October 2021	R	84.62%
Ampicillin	Purulent secretion - April 2022	R	97.44%
Imipenem	Purulent secretion - April 2022	S	92.98%

Legend: S = Susceptible; R = Resistant

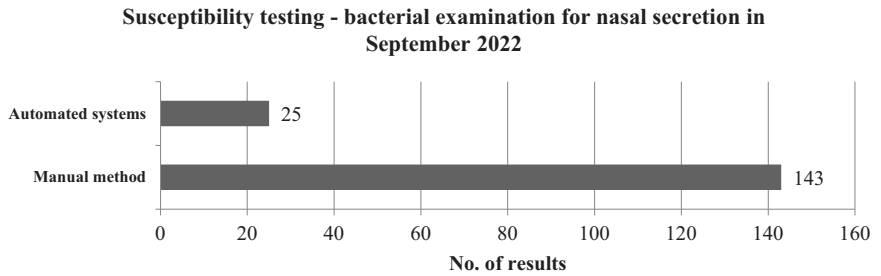

Figure 2 – An overview of the techniques used by Public Health Units laboratories for antimicrobial susceptibility testing on Bacterial examination for nasal secretion EQC program in Septembrer 2022

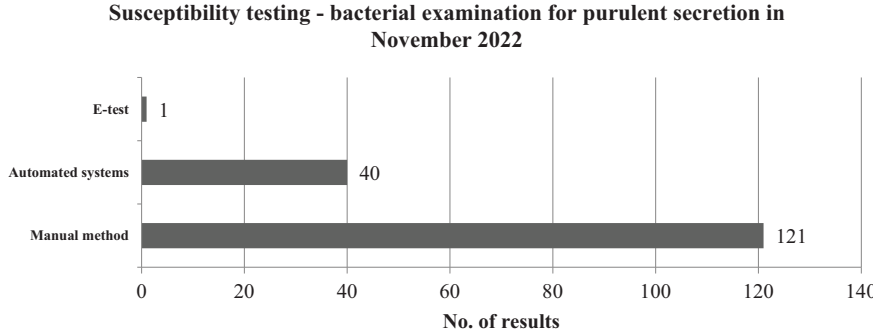

Figure 3 – An overview of the techniques used by Public Health Units laboratories for antimicrobial susceptibility testing on Bacterial examination for purulent secretion EQC program in November 2022

percentage was 68.75% registered for ceftriaxone in October 2021. The highest percentage of satisfactory results was 97.44% registered for ampicillin in April 2022 (Table 12).

During this phase of the study, we noted that the only case where percentages of satisfactory results were lower than 100% for all tested antibiotics was in the *A. baumannii* EQC rounds. On the other hand, *Serratia marcescens* EQC program was the only one with 100% percentages of satisfactory results in all cases.

Ceftriaxone, amoxicillin-clavulanate, and ciprofloxacin susceptibility testing recorded the lowest percentages of satisfactory results, which were below 90.00%.

Among the antibiotics tested in the presented EQC rounds, there are some antibiotics to which the strains provided to the laboratories show intrinsic resistance, thus, we have opted to carry out a more detailed analysis on this topic.

We started by identifying the microorganisms that are intrinsically resistant to certain antibiotics and we identified: *P. aeruginosa* intrinsically resistant to ceftriaxone (with 80.00%, 60.00% and 63.41% satisfactory results registered); *S. marcescens* intrinsically resistant to amoxicillin – clavulanate, ampicillin and cefuroxime (all three antibiotics with 100.00% satisfactory results registered); *K. pneumoniae* intrinsically resistant to ampicillin (with 93.33% and 100.00% satisfactory results registered) and *A. baumannii* intrinsically resistant to amoxicillin – clavulanate (with 84.62% satisfactory results registered) and ampicillin (with 97.44% satisfactory results registered).

One contributing factor to the low percentage of satisfactory results for *A. baumannii* is the incorrect identification of the microorganism (Table 4), leading to inaccurate results for susceptibility testing. On the other hand, for all the other strains provided, in each case 100% satisfactory results were recorded (Tables no. 1, 2 and 4).

According to the procedure of a microbiology laboratory, the intrinsic resistance is reported directly, without the need to test the susceptibility to the respective antibiotic. Since our research also revealed percentages of less than 100% satisfactory results in the case of intrinsic resistance, it turns out that the personnel of some of the laboratories participating in the EQC rounds do not have knowledge about the intrinsic resistance of microorganisms.

We conducted an analysis about the techniques used by Public Health Units laboratories for antimicrobial susceptibility testing (Figure 2 and 3) using the outcomes from Bacterial examination for nasal secretion program in September 2022 and bacterial examination for purulent secretion in November 2022. We only selected the data from September

Table 13 - Bacteriology - screening for the detection of multidrug-resistant bacteria – satisfactory results recorded between 2020 and 2022

EQC Round	Strain	Total number of results registered from Public Health Units	Percentage of satisfactory results registered from Public Health Units
March 2020	*MRSA (Methicilin-resistant Staphylococcus aureus)*	15	100.00%
June 2020	*Klebsiella sp/pneumoniae ESBL positive*	18	83.33%
September 2020	*VRE (Vancomycin-resistant Enterococcus)*	19	94.73%
November 2020	*MRSA (Methicilin-resistant Staphylococcus aureus)*	17	94.12%
March 2021	*VRE (Vancomycin-resistant Enterococcus)*	18	83.33%
May 2021	*Klebsiella sp/pneumoniae ESBL positive*	24	91.67%
September 2021	*MRSA (Methicilin-resistant Staphylococcus aureus)*	29	100.00%
November 2021	*Klebsiella sp/pneumoniae ESBL positive*	22	100.00%
March 2022	*MRSA (Methicilin-resistant Staphylococcus aureus)*	15	100.00%
May 2022	Absence of MDR bacteria	25	88.00%
September 2022	*VRE (Vancomycin-resistant Enterococcus)*	38	86.84%
November 2022	Absence of MDR bacteria	19	100.00%

and November 2022 since these EQC rounds had the highest number of participating laboratories during the 2021-2022 period.

According to the analysis, the number of laboratories that used automated systems for antimicrobial susceptibility testing was considerably low compared to the number of results obtained using manual methods, in both cases. However, the number of results obtained utilizing automated systems experienced a slight increase in November 2022.

The next stage of the study was conducted analyzing the outcomes recorded for Bacteriology - screening for the detection of multidrug-resistant bacteria EQC program (Table 13).

The research showed that the highest percentages of satisfactory results (100.00%) were recorded in the EQC programs with *MRSA, Klebsiella sp/pneumoniae ESBL positive, "Absence of MDR bacteria".* On the other hand, the lowest percentages were recorded for *VRE and for ESBL-positive Klebsiella sp./pneumoniae,* 83.33% (Figure 4).

In general, the most favorable outcomes were observed for MRSA testing, while VRE testing produced the least satisfactory results (with no cases of 100.00% satisfactory results recorded).

Similar to earlier stages, we aimed to examine the methods utilized by laboratories for this EQC program from a particular perspective.

TFor the bacteriology - screening for the detection of multidrug-resistant bacteria EQC program in 2020 – 2022 a total of 259 results were reported, out of which 241 were satisfactory, while only 18 were unsatisfactory. 110 satisfactory results were obtained using automated systems and 131 were obtained using manual methods. Regarding the unsatisfactory results, 5 of them were obtained using auto-

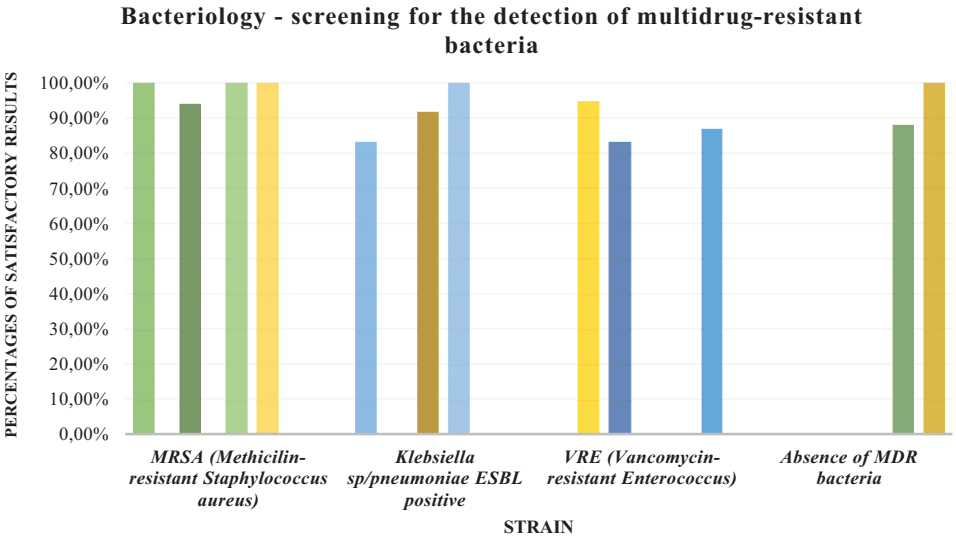

Figure 4 - Satisfactory results recorded between 2020 and 2022 on the bacteriology - screening for the detection of multidrug-resistant bacteria EQC program

Table 14 – An overview of the techniques used by the Romanian Public Health Units participating in Bacteriology - screening for the detection of multidrug-resistant bacteria EQC program in 2020 - 2022

EQC Round	Number of satisfactory results	The method used by Public Health Units with satisfactory results	Number of unsatisfactory results	The method used by Public Health Units with unsatisfactory results
March 2020	15	Automated systems = 7 results Manual method = 8 results	-	-
June 2020	15	Automated systems = 8 results Manual method = 7 results	3	Manual method = 3 results
September 2020	18	Automated systems = 9 results Manual method = 9 results	1	Manual method = 1 result
November 2020	16	Automated systems = 6 results Manual method = 10 results	1	Automated systems = 1 result
March 2021	15	Automated systems = 6 results Manual method = 9 results	3	Automated systems = 2 result Manual method = 1 results
May 2021	22	Automated systems = 11 results Manual method = 11 results	2	Automated systems = 1 result Manual method = 1 results
September 2021	29	Automated systems = 8 results Manual method = 21 results	-	-
November 2021	22	Automated systems = 12 results Manual method = 10 results	-	-
March 2022	15	Automated systems = 5 results Manual method = 10 results	-	-
May 2022	22	Automated systems = 12 results Manual method = 10 results	3	Automated systems = 1 result Manual method = 2 results
September 2022	33	Automated systems = 19 results Manual method = 14 results	5	Manual method = 5 results
November 2022	19	Automated systems = 7 results Manual method = 12 results	-	-

mated systems and 13 were obtained using manual methods (Table 14).

When comparing the number of results obtained through manual methods and automated equipment, there is not a significant difference between the two, regardless of whether the results are satisfactory or unsatisfactory. Most of the unsatisfactory results were obtained by laboratories that used manual methods.

Conclusions

According to our study, a large proportion of the bacterial species identification results in the bacteriology EQC rounds were evaluated as satisfactory, with a percentage of 100% satisfactory results.

Only three EQC rounds had percentages of satisfactory results higher than 90.00%: *S. aureus* in the bacteriological examination of blood culture (90.91%) and *A. baumannii* in the bacteriological examination of purulent secretion (93.55% and 98.51%).

Regarding the techniques utilized by the Romanian Public Health Units for the bacteriology EQC programs, the manual methods are still widely used in many laboratories, both for the bacterial species identification and for antimicrobial susceptibility testing.

For *A. baumannii* susceptibility testing there was no EQC round with 100.00% satisfactory results recorded. On the contrary, *S. marcescens* susceptibility testing concluded with only 100.00% satisfactory results each time.

P. aeruginosa susceptibility testing showed the lowest percentages of satisfactory results (60.00% and 63.41%) for ceftriaxone. One possible reason for this may be the insufficient knowledge regarding intrinsic resistance of this microorganism, given the fact that *P. aeruginosa* is naturally resistant to ceftriaxone.

The findings of the intrinsic resistance part of the research indicated that the intrinsic resistance may not be as familiar as it should be for the laboratory personnel, and they do not consult their susceptibility testing results with specialized standards. As a result, there is a risk of misinterpreting susceptibility results, leading to inappropriate antimicrobial therapy.

Analyzing the bacteriology - screening for the detection of multidrug-resistant bacteria EQC program we observed that medical laboratories managed to provide accurate results.

However, the EQC rounds with *vancomycin – resistant Enterococcus faecalis* posed challenges for the participating laboratories, as these are the only rounds with no cases of 100.00% satisfactory results recorded.

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This document complies with the General Data Protection Regulation and the requirements of the international

standard applicable to the accreditation of Proficiency Testing Schemes providers regarding the confidentiality of data provided by medical laboratories participating in External Quality Control programs.

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Review

Emerging antimicrobial susceptibility methods in monitoring colistin-resistant *Enterobacteriaceae*

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Abstract

One of the most important essential pillars in the fight against antibiotic resistance is to optimize antibiotic treatment by developing and optimizing appropriate methods to establish the antibiotic susceptibility profiles of a specific microbial strain. Moreover, this will contribute to the surveillance and limitation of antimicrobial resistance transmission and spread. Therefore, it is also imperative to harmonize different approaches and techniques and to perform suitable antimicrobial susceptibility tests in microbiology laboratories to achieve precise, reproducible, and comparable results. However, the conventional methods for antimicrobial susceptibility testing are usually based on bacterial culture methods, which are time-consuming, complicated, and labor-intensive. Therefore, other approaches are needed to address these issues. In this mini-review, we will present the common and future perspectives in antimicrobial susceptibility testing. Microfluidic technology and electrochemical devices have recently gained significant attention in infection management. These advantages include rapid detection, high sensitivity and specificity, highly automated assay, simplicity, low cost, and potential for point-of-care testing in low-resource areas.

Keywords Antimicrobial resistance, susceptibility tests, microfluidics, single-cell

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Introduction

The alarming increase in antimicrobial resistance leads to the urgent need to harmonize different approaches and techniques and to perform suitable antimicrobial susceptibility tests (ASTs) in microbiology laboratories (PEELING & al [1]).

Quantitatively, bacterial drug resistance is established by measuring a parameter called minimum inhibitory concentration (MIC), the lowest concentration of a drug that prevents the growth of bacteria. MIC measurements are the basis for establishing resistance breakpoints by agencies such as CLSI or EUCAST. A breakpoint is a drug concentration against which a patient sample is tested – if there is growth, the bacterial strain is resistant; if there is no growth, the strain is susceptible. Setting breakpoint values by medical agencies is based on MIC and pharmacokinetics and pharmacodynamics (PK/PD) of an antibiotic (POSTEK & al [2]). Clinicians use breakpoint values based on the MIC but not the MIC itself. Breakpoint values, although highly useful, do not convey the information that a MIC screen personalized to a given patient would: *e.g.*, a breakpoint does not necessarily take into account a wild-type resistance distribution, which can lead to both false positives and false negatives, or there is a possibility that a tested bacterium does have a resistance mechanism but is still below the breakpoint (CAMA & al [3]).

Since 1976, The Clinical and Laboratory Standards Institute (CLSI) has sought to find the most appropriate values for polymyxin in the clinic by introducing polymyxin disk diffusion breakpoints (NCCLS, 1976). Today, the threat of MDR *Acinetobacter spp*. and *Pseudomonas aeruginosa* persists, and carbapenem-resistant Enterobacterales (CRE) have become significant global health challenges. CRE are endemic in the U.S., Latin America, Asia, Greece, Italy, and Israel, with high rates in some countries (LOGAN & al [4]; TAMMA & al [5]). In 2013, considering this explosion of resistance phenomenon, CLSI and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) reviewed colistin breakpoints for Enterobacterales, *Acinetobacter* spp., and *P. aeruginosa*.

Common AST methods

The use of an accurate method for testing antimicrobial susceptibility (AST) for colistin is critical and urgent, given the continuing increase in the number of multi-resistant strains. EUCAST and CLSI have recommended broth microdilution (BMD) as the reference for identifying MIC (minimum inhibitory concentration) breakpoints in clinical settings. However, the BMD of colistin has some limitations in methodology, which is why it is rarely used in clinical laboratories. First, colistin can bind to polystyrene trays, solving this problem by adding a surfactant (KAUR & al [6]). This situation was analyzed by a joint working group, which recommended that the testing be done using sulfate salt of colistin and standard polystyrene trays, given that the surfactant does not improve the performance of the method. Also, BMD requires antibiotic solutions prepared extemporaneous or frozen solutions; it is a laborious method that consumes time and requires experience to interpret the results correctly (HU & al [7]; RANJAN & al [8]).

Consequently, most laboratories have focused on using automated susceptibility testing systems and disc-diffusion methods. However, international committees have expressed concern about the variable results obtained from these rapid tests. Due to colistin molecules' large size and cationic nature, disc-diffusion and gradient diffusion methods have proved unfeasible. Kulengowski et al. compared the results obtained after performing the BMD and E-test methods in 70 CRE strains. The authors found a considerable discordance between the E-test and BMD (a significant error of 88%).

Most importantly, E-test poorly predicted the polymyxin B MIC for isolates exhibiting elevated polymyxin B MICs by BMD (KULENGOWSKI & al [9]). In another study aiming to analyze these rapid methods, the gradient tests generally underestimated colistin MICs, resulting in many false susceptible results, a significant mistake in the clinic (MATUSCHEK & al [10]). Kananizadeh et al. obtained significantly higher MICs for colistin using the BMD method associated with brain-heart infusion (BHI) medium, Luria-Bertani (LB) broth, tryptic soy broth (TSB), or cation-adjusted Mueller-Hinton broth CA-MHB supplemented with casein, tryptone or peptone. These results suggest that the BMD method using BHI is beneficial when performed with the BMD method using CA-MHB to detect *mcr-9*-positive isolates (KANANIZADEH & al [11]).

Although the BMD has some limitations, EUCAST and CLSI recommend this method as the gold standard for colistin's antimicrobial susceptibility testing. However, considering that many laboratories rarely use this method in clinical routine, alternative AST methodologies are highly desirable.

Emerging AST methods

Microfluidic-based diagnostic is one of the most promising technologies for AST. Microfluidics is an expanding field based on using fluids in micro-volume to obtain a controllable environment in an *in vitro* system characterized by portability, cost-effectiveness, and reproducibility (POSTEK & al [2]; LI & al [12]). Integrated microfluidic devices are based on micro-total analysis systems and are used success-

fully in molecular biology (QIN & al [13]). Given that the amount of biological samples has been a problem over time, using a minimum amount of samples in microfluidic technology makes this system a perfect candidate for solving this problem. Currently, microfluidic systems can analyze a single cell and the interaction of the cell in the signaling network that exists within the cells in culture. However, as mentioned earlier, the techniques commonly used to achieve AST are laborious, time-consuming, high risk of cross-contamination, and require resources that limit their use in certain developed regions (GAJIC & al [14]). Microfluidics systems can be a solution for addressing these shortcomings (KLEIN & DIETZEL [15]).

Another possible strategy for improving AST is to couple microfluidic devices with an optical sensor to detect MIC values within a few hours. Recent studies on single-cell analysis have shown that microfluidic optical sensor-based can detect MIC breakpoints in 30 minutes (QIU & NAGL [16]; HUANG & al [17]).

ATP bioluminescence assay is a luciferase-mediated enzymatic reaction that converts the luciferin substrate to oxyluciferin in the presence of ATP, leading to the emission of a quantum of light (WANG & al [18]). Dong and Zhao analyzed the susceptibility of 13 strains associated with urinary tract infections using this phenomenon. The analysis was performed against eight antibiotics on a microfluidic plate. The resistance is transposed into a bioluminescence phenomenon when the bacteria grow in an antibiotic's presence while the sensitive strains remain neutral. This method provides MIC breakpoints that could be detected in 6-8 hours (DONG & ZHAO [19]).

Another research direction in improving AST is the use of electrochemical devices. One of the most significant studies utilized AC electrokinetic fluid motion and Joule heating-induced temperature elevation for the electrochemical sensing of bacterial 16S rRNA, providing essential information on the analysis of susceptible bacteria (LIU & al [20]). The latest electrochemical biosensor can achieve AST in about 90 minutes and isolate bacteria from blood samples (SAFAVIEH & [21]; ZHANG & al [22]).

Diep et al. combined inexpensive portable components for microbial cytometry to establish the feasibility of rapidly monitoring bacterial motility in the presence of antibiotics. They investigated whether the 3D-printed OpenFlexure microscope using a low-cost Raspberry Pi v2 camera has sufficient magnification and resolution to monitor bacterial motility in microdevices. Adequate magnification and contrast were achieved to view motile bacteria and allowed differences in behavior to be observed in the presence of antibiotics above the organisms' minimum inhibitory concentration (MIC) for that antibiotic. The authors demonstrated that the OpenFlexure microscope combined with microfluidic systems allows rapid antibiotic resistance detection. (DIEP & al [23]).

Lin et al. present a microfluidic device that generates a concentration gradient for antibiotics produced by diffusion in the laminar flow regime along a series of lateral microwells to encapsulate bacteria for antibiotic treatment. All the AST preparation steps were performed in a single chip. After the antibiotic treatment, the viable bacterial cells in each microwell are then quantified by their surface-enhanced Raman scattering (SERS) signals acquired after placing a uniform SERS-active substrate in contact with all the microwells. The authors demonstrated the AST performance of this system on ampicillin (AMP)-susceptible and -resistant *E. coli* strains (LIN & al [24]).

Yamagishi et al. used the drug susceptibility testing microfluidic device (DSTM) to achieve the rapid screening of extended-spectrum β-lactamases (ESBLs) and metallo-β-lactamases (MBLs). β-lactams and β-lactamase inhibitors were pre-fixed in the DSTM for use, and a bacterial suspension in Mueller-Hinton broth was introduced into the device. The effects of β-lactamase inhibitor on morphological changes caused by β-lactam were evaluated after three hours of incubation. The authors conclude that the DSTM method allows rapid detection of β-lactamases and may be a valuable replacement for the disc diffusion method (YAMAGISHI & al [25]).

Future perspectives

The current AST design challenges are the inoculum size and the need to select only a few isolated colonies. The first step in performing AST is to culture bacteria from the original sample on primary inoculum plates. Subsequently, only a few isolated colonies are selected to prepare an inoculum, followed by incubation for 16-18 hours. Performing AST starting from the original sample's inoculation on antibiotic screening flat agar, antibiotic resistance is detected only for certain bacteria in the inoculum. Therefore, resistant bacteria will be at a low frequency, making it impossible to detect them by conventional AST, which is a significant error in clinical settings. This situation is caused by the current standardization of the amount of inoculum and the selection of a small number of individual colonies, reducing bacterial diversity (BRUKNER & OUGHTON [26]). An alternative to the problem of selecting individual colonies is to perform population-based AST via qPCR in the context of the original clinical sample. These amplification tests can detect species-specific growth rates of bacteria in the original samples (MAXSON & al [27]; BRUKNER & OUGHTON [26]).

Microfluidics-based studies of single-cell growth in static chambers are relatively rare, although any static chamber device for population-level studies could be repurposed for single cells (KLEIN & al [28]). However, single-cell approaches could be placed on a distinct niche (research only) and not clinically applicable now due to the need for finding resistant individual cells in the highly-dense bacterial population from clinical samples (e.g., sensitive *P. aeruginosa* and meropenem-resistant *Escherichia coli*). Additionally, single-cell approaches do not capture fine inter- and intra-species communications, allowing certain bacteria to co-exist under selective antibiotic pressure in a complex clinical sample. Thus, this communication between resistant bacteria is missed at the single-cell level, leading to a loss of clinically valuable information. Therefore, clinical microbiology has to implement these bacterial interactions into the predictive models and overcome individual cell approaches.

Author contributions

S.I.T., I.G.B. and I.C.B. conceived and corrected the manuscript. R.E.C., M.C., S.I.T. and I.C. contributed to the literature survey and revised the manuscript. C.O.V. drafted the manuscript. All authors have read and agreed to the published version of the manuscript. The authors have contributed equally to this work and share first authorship.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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Original article

Immunohistochemical evaluation of cellular activities in canine osteoblastic osteosarcoma

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Abstract

Osteosarcoma is the most frequently occurring malignant bone tumor in animals and humans. A better understanding of the etiopathogenetic mechanisms of osteosarcoma, especially biomolecular ones, is fundamental to improving diagnosis and prognosis of the disease. Autophagy is a self-degrative process that removes dysfunctional cellular components, eliminates intracellular pathogens, and promotes cellular senescence. In neoplastic cells, autophagy suppresses tumorigenesis by inhibiting cancer cell survival mechanisms and promoting cell death. The aim of this study was to use immunohistochemistry to identify alterations in the expression of several apoptotic and proliferative biomarkers in multiple cases of canine osteosarcoma. Bcl-2, an intracellular membrane protein, inhibits cell death by blocking the p53-mediated pathway of apoptosis. While Bcl-2 overexpression has been described in many different premalignant and malignant lesions, it has yet to be analyzed in canine osteosarcoma. Our group investigated 10 primary canine osteoblastic osteosarcoma cases from the University of Perugia Department of Veterinary Medicine Teaching Hospital. Immunohistochemical analyses of proteins Bcl-2, Ki-67, and p53 have revealed interesting results as described in this paper. Expression of Bcl-2 was increased in all cases investigated while expression of p53 and Ki-67 was variable and no statistical association was observed between the expression patterns of p53, Bcl-2 and Ki-67.

Keywords osteosarcoma, dog, autophagy, Bcl-2, Ki-67, p53

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Introduction

Osteosarcoma is the most common primary malignant bone tumor in dogs characterized by the formation of osteoid by neoplastic mesenchymal cells [1]. Osteosarcoma represents 80-85% of the primary bone tumors affecting dogs and is defined as a highly aggressive and invasive tumor with a tendency to metastasize [1, 2]. Osteosarcoma usually affects middle-aged to older medium and giant-breed dogs, with a median age range of 7-8 years deferring from humans where it usually involves younger patients [3, 5]. In all species, osteosarcoma predominantly arises within the metaphyseal region of long bones with forelimbs being affected twice as often as hindlimbs. The two most frequently affected regions are the distal radius (35% of cases) and the proximal humerus (18% of cases) [4, 5]. Known etiologic causes of osteosarcoma include irradiation, viral infections, immunodeficiency disorders, environmental and specific genetic mutations such as alterations of pRb genes [5, 6]. The development of osteosarcoma also involves alterations of cell cycle regulators which allows for uncontrolled growth [5]. Several studies on tumorigenesis have hypothesized that molecular mechanisms such as apoptosis and autophagy may be involved in the development of malignancy [7]. Autophagy is a stress-responsive process that regulates the degradation and recycling of cellular components. Autophagy also plays a housekeeping role by removing misfolded or aggregated proteins, clearing damaged organelles, and eliminating intracellular pathogens. In response to certain stimuli, autophagy can induce programmed cell death [8].

Bcl-2 family proteins control cell death through either inhibiting or inducing apoptosis. Bcl-2, the founding member of the Bcl-2 family proteins, regulates cell death through blocking or delaying the mitochondrial apoptosis pathway. Bcl-2 obtained its name from its discovery in B cell leukemia/lymphoma 2. Initially, Bcl-2 was classified as a factor involved in apoptosis and it was only after the discovery of its involvement in autophagy that the scientific community began studying the protein's involvement in numerous biomolecular processes [9]. Bcl-2 is overexpressed in multiple tumors which supports its role as a potent oncogene. In addition, recent studies have demonstrated an upregulated expression of Bcl-2 in multiple cases of human lung cancer supporting its potential involvement in the pathogenesis of these tumors [10].

The p53 protein known as the "guardian of the genome" [11] acts to transmit a variety of stress- responsive signals to multiple anti-proliferative cellular responses. Stimuli such as DNA damage, oncogene activation, and hypoxia, trigger the expression of p53. When activated, p53 initiates several biological processes including apoptosis, cell-cycle arrest, senescence, or autophagy [12-14]. Interactions between Bcl-2 and p53 have been described in several tumors [15]. Multiple studies have demonstrated that apoptosis is tightly regulated and influenced by a series of quantitative and qualitative events that alter p53 activation [16]. P53 can activate the Damage-Regulated Autophagy Modulator (DRAM) gene leading to the production lysosomal proteins and the induction of autophagy [17]. P53 is a potential inducer of autophagy (inhibitor of Bcl-2) its altered activity seems to be responsible for the increase of autophagic activity in many tissues and pathological processes [18].

Ki-67 is a nuclear protein associated with cellular proliferation. Detailed cell cycle analyses revealed that Ki-67 is expressed in the nuclei of cells in S, G1, G2, and M phases of cell division. Quiescent cells in G0 phase lack expression of this protein [19]. Multiple studies have demonstrated that the expression of Ki-67 is strictly associated with cellular proliferation. During mitosis, this nuclear protein relocates to the surface of chromosomes [20]. Given that Ki-67 is expressed by all proliferating cells, evaluating the intracellular presence of Ki-67 may be an excellent indicator of cell growth across several tissues.

Our studies focused on the expression patterns of Bcl-2, p53 and Ki-67 in 10 cases of spontaneous canine osteosarcoma. The goal of the study was to evaluate the potential involvement of these proteins in the development of malignant spontaneous canine osteosarcoma.

Materials and Methods

10 cases of spontaneous canine osteoblastic primary and non-metastatic osteosarcoma were selected to investigate the immunohistochemical expression of Bcl-2, p53, and Ki-67. Tumor samples were fixed in 10% neutral buffered formalin, processed, and embedded in paraffin using routine methods. Hematoxylin and eosin (H&E) staining was performed on 3-5µm sections. Immunohistochemistry (IHC) was performed using the Avidin Biotin Complex (ABCL-2) method. Paraffin was removed with xylene and slides were dehydrated in sequentially diluted ethanol then rinsed in distilled water. To inhibit endogenous peroxidase activity, the tissue sections were treated with 3% hydrogen peroxide in tris phosphate-buffered saline (PBS). Samples were rinsed in normal goat serum for 30 minutes to block non-specific reactions. Immunohistochemistry was performed on serial 3-5µm sections using mouse monoclonal anti-p53 antibodies (clone DO-7, dilution 1:50, Dako), anti-Bcl-2 antibodies (clone 124, dilution 1:100, Dako), and anti-Ki67 antibodies (clone MIB-1, dilution 1:150, Dako). Negative controls for immunohistochemistry were processed identically to test

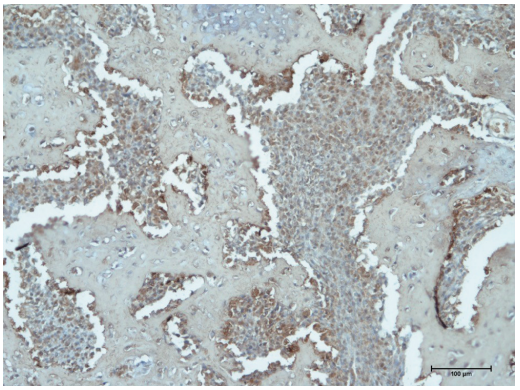

Fig. 1. Osteoblastic osteosarcoma. Immunohistochemical cytoplasmatic reactivity for Bcl-2 in osteoblastic malignant cells (20×).

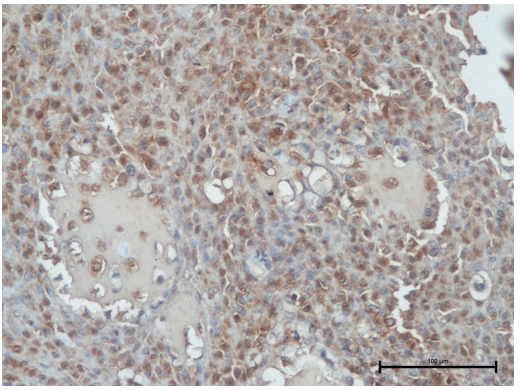

Fig. 2. Osteoblastic osteosarcoma. Immunohistochemical cytoplasmatic reactivity for Bcl-2 in osteoblastic malignant cells (40×).

slides, but the primary antibodies were omitted. Samples of canine fibrosarcoma were used as a positive control for antibodies against p53 and Ki-67 and unaffected canine tonsils were used as a positive control for antibodies against Bcl-2.

Results

Histologically, all tumors examined were highly cellular with polyhedral cells, sometimes round to spindle, hyperchromatic large nuclei, and prominent nucleoli. Tumors also exhibited frequent and numerous mitoses with varying amounts of osteoid production. Immunohistochemistry revealed an increased expression of Bcl-2 in all cases when compared to normal bone samples. The expression of Bcl-2 was rarely correlated with the expression of p53, and Ki-67 proteins and no statistical association was observed between the expression of p53, Bcl-2, and Ki-67 proteins. Bcl-2 is an anti-apoptotic protein that protects cells from a variety of apoptotic stimuli, including cytotoxic drugs, irradiation, heat, and/or growth factor withdrawal.

The overexpression of Bcl-2 has been identified in numerous types of human cancers, including breast, colon, ovarian, and prostate cancer, however, it has yet to be described in osteoblastic osteosarcoma. Although Bcl-2 confers resistance to malignant cells, expression is not always correlated with a poor prognosis [21]. Our results revealed a diffuse overexpression of Bcl-2 in all osteoblastic tumor cells in investigated canine osteosarcoma cases (Figure 1 and 2). The increased expression of Bcl-2 in cases of osteosarcoma suggests that tumor cells upregulate Bcl-2 as a mechanism of inhibiting programmed cell death allowing for the survival and proliferation of neoplastic cells.

The protein p53 is a cell cycle regulator that is often mutated in neoplastic cells. Expression of p53 was mainly detected within the nucleus of tumor osteoblasts, however, in most samples (80% of cases), p53 was also detected within the cytoplasm. The percentage of positive cells in each tumor examined ranged from a few (10-20%) scattered cells with positive nuclei to approximately 90% of the tumor population indicating that the expression of p53 is highly variable. Overall, samples of spontaneous canine osteosarcoma demonstrated significant cytoplasmic expression of p53 in osteoblastic cells (Figure 3). Studies conducted on the role of p53 in autophagy in different human tumors have shown that the cytoplasmic localization of this marker is associated with the inhibition of autophagy. Our results suggest that there is a correlation between the cytoplasmic localization of p53 and the overexpression of Bcl-2 [23].

Ki-67, a nuclear protein, and a marker of cellular proliferation has been upregulated in several forms of human cancer [20]. All osteoblastic canine osteosarcoma samples evaluated in our preliminary study demonstrated strong uniform nuclear expression of Ki-67 suggesting that osteoblastic tumor cells were highly proliferative (Figure 4).

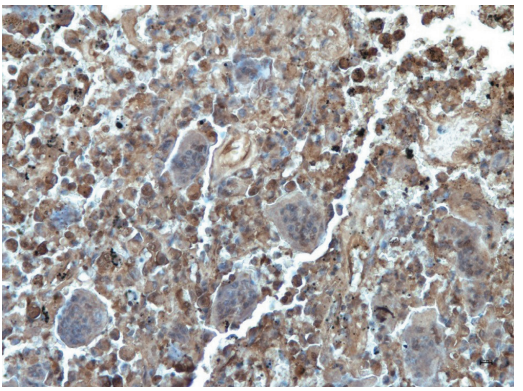

Fig. 3. Osteoblastic osteosarcoma. Immunohistochemical reactivity for p53 in osteoblastic tumor cells (40×).

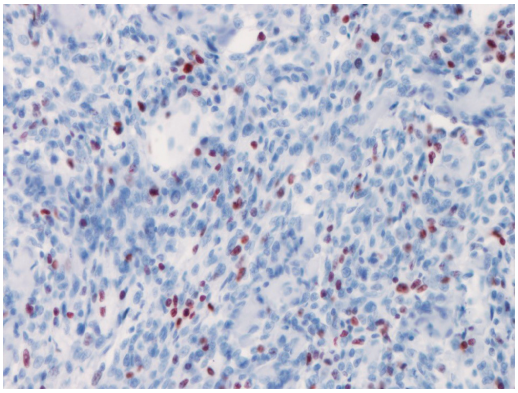

Fig. 4. Osteoblastic osteosarcoma. Immunohistochemical reactivity for Ki-67. Nuclear staining with hematoxylin (20×).

Discussion

The focus of this study was to analyze the mechanisms of cellular proliferation, apoptosis, and autophagy in cases of spontaneous canine osteoblastic osteosarcoma through the evaluation of immunohistochemical expressions of Bcl-2, p53, and Ki-67 proteins. Increased expression of p53 and Ki-67 in all cases of canine osteoblastic osteosarcoma indisputably indicates modifications of the cell cycle in neoplastic cells. We suggest that the increased cytoplasmic expression of p53 plays a role in the inhibition of autophagy and ultimately in the development of malignancy of osteosarcoma [24]. The upregulated nuclear expression of Ki-67 in all neoplastic cells supports aberrant proliferation of neoplastic cells. Bcl-2 is a potent inhibitor of apoptosis and autophagy; however, little is known about the role of Bcl-2 in the development of osteosarcoma. All 10 cases of canine osteoblastic osteosarcoma demonstrated an increased expression of Bcl-2 when compared to normal bone samples. Our results suggest that Bcl-2 acts to inhibit apoptosis in neoplastic osteosarcoma cells which may play a role in proliferation and malignancy of the tumor. Increased expression of Bcl-2 may support the diagnosis of osteosarcoma; however, further studies are required to establish the role of Bcl-2 as a prognostic indicator.

In conclusion, our studies have allowed us to establish alterations in cell cycle regulators, particularly Bcl-2, p53, and Ki-67, in canine osteoblastic osteosarcoma. Increased expression of Bcl-2, p53, and Ki-67 in all cases supports their involvement in the development of osteosarcoma. Our results revealed no statistical significance between the expression of all three proteins. Further investigations are necessary to evaluate the correlation and biomolecular activity between Bcl-2, p53, and Ki-67 proteins in canine osteoblastic osteosarcoma.

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Original article

MicroRNA 138 upregulation is associated with decreasing levels of CCND1 gene expression and promoting cell death in human prostate cancer cell lines

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Abstract

This research intended to discover the significance of miR-138 on the expression profile, proliferation, and the associated regulatory mechanisms in prostate cancer (PCa). RT-qPCR was applied to compare the expression of miR-138 in the PCa cells with a non-cancer cell line, as well as PCa tissue samples with benign prostatic hyperplasia (BPH) samples. The expression of miR-138 notably diminished in PCa tissues and cell lines. Afterward, formerly documented genes, along with bioinformatics analysis, suggested seven possible target genes of miR-138. Among them, *CCND1* seemed to have higher expression in the PCa cell lines and tissues. Also, the negative correlation of miR-138 and *CCND1* in PCa cell line and tissues was validated using Pearson correlation. CCDN1 was revealed to be the target gene of miR138 in the PC3 cell line based on the results of the luciferase reporter gene assay. Over-expression of miR138-5p suppressed the expression of CCDN1 in PCa cell lines as exhibited by RT-qPCR. Finally, the results of the MTT assay exhibited the inhibitory impact of miR-138 on the proliferative capacities in PCa cell lines. Our research introduces miR-138 as a negative regulator of *CCDN1* in the progression of PCa with an inhibitory impact on the proliferation rate of prostate cancer (PCa) cell lines. This regulatory mechanism could be utilized for the design and target selection of remedial miRNA-based approaches.

Keywords miRNA, miR-138, *CCND1*, Prostate cancer, oncogene, tumor suppressor

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Introduction

The second most predominant type of malignant cancer is Prostate cancer (PCa). It is also the fifth primary reason for cancer-related death in men in the world (1). Serum prostate-specific antigen (PSA) measure and digital rectal examination are the most extensively utilized PCa screening instruments. Even though serum PSA facilitated the diagnosis of PCa at initial disease stages but high false-positive sounds as a significant disadvantage (2). Hence, a more specific PCa biomarker would be helpful in improving PCa screening. Other biomarkers, including PCA3 score, Prostate Health Index (PHI), and 4Kscore, were recommended to ameliorate the accuracy of PSA prognosis (3). Moreover, cancer-related microRNAs (miRNAs) appeared as promising candidates in the diagnosis, prognosis, and treatment of cancer (4).

MiRNAs are single-stranded noncoding RNA molecules (18-24 nucleotides) that bind to the 3′-untranslated region (3′-UTR) of target mRNA to repress gene expression post-transcriptionally (5). MiRNA expression signatures are dissimilar between cancer and normal tissue and distinct cancer subtypes (6). More importantly, well-provided evidence has emphasized the anomalous expression of miRNAs in the malignancy of cancers (7, 8). Therefore, miRNAs as cancer-specific biomarkers can be used to alleviate diagnosis, prognosis, and outcome of the therapy (9).

MiR-138 were attended as a tumor suppressor in numerous malignancies, including prostate, pancreatic, nasopharyngeal, colorectal, osteosarcoma, non-small cell lung cancers, and other malignant tumors (10-15). MiR-138 has the tumor suppressor function via diverse mechanisms such as apoptosis motivation, suppression of proliferation, metastasis, invasion, and modification of chemosensitivity in tumor cells. Distinct mechanisms imply heterogeneous targets of miR-138 in different cancer types (16-18). Considering various functions and heterogenous targets in different types of cancers, miR-138 could be regarded as a propitious remedial strategy for cancer (19).

MiR-138 negatively regulated FOXC1 to suppress the malignant progression of PCa (20, 21). MiR-138 was also incorporated in the suppression of the Wnt/β-catenin pathway in PCa (22). Furthermore, miR-138 and *CCND1* expression exhibited a reverse correlation in nasopharyngeal carcinoma (NPC), but this association has not been examined in PCa and other cancer types (14). High *CCND1* expression was also correlated with progressive malignancy in PCa (23, 24). *CCND1* (located on chromosome 11q13) functions as an essential coordinator in cell cycle progression from G1 (growth) to the S phase (synthesis) (25). Overexpression of *CCND1* was discovered in several types of cancer as an oncogene. This oncogenic behavior might be through diverse mechanisms such as disturbing cell cycle, proliferation, and neoplastic cell transformation (26-30).

In the present research, we intended to discover the target genes for miR-138 and corroborate the importance of miR-138 in tumorigenesis in PCa. Bioinformatics analysis was carried out to explore the target genes of miR-138 and discover their expression pattern. We assigned seven target genes for miR-138: *ABL1*, *CCND1*, *CCND3*, *VIM*, *TWIST1*, *HIF1A*, and *TERT*. Among them, *CCND1* emerged as a possible contributor to miR-138-regulated tumorigenesis due to its higher expression in PCa. As such, we characterized the expression profile of miR-138 in PCa cell lines, a non-cancer cell line, PCa, and benign prostatic hyperplasia (BPH) clinical samples. Moreover, the expression profile of *CCND1* in PCa cell lines, a non-cancer cell line was evaluated. The inverse regulatory function of miR-138 on *CCND1* was examined by luciferase assay. We also investigated the impact of transient overexpression of miR-138 on the cell proliferation and expression of *CCND1* in PCa cell lines to exhibit whether miR-138 is capable of hindering the proliferation of PCa cells via moderating *CCND1*.

Materials and Methods

MiRNA prediction

MiR-138 complementary sequences located within the 3′-UTR of target mRNAs were regarded as target sequences. PicTar (http://pictar.mdc-berlin.de), TargetScan (www.targetscan.org), Mirtargetlink2 (https://ccb-compute.cs.uni-saarland.de/mirtargetlink2) and mirdb (http://www.mirdb.org/) were used for this purpose. These programs combine seed matches, conservation analysis, the thermodynamic stability of miRNA-mRNA duplexes, and site accessibility, among other characteristics, to maximize the target prediction specificity (31). Total scores computed by each tool were calculated, and genes with the maximum score were considered as target genes. The number of target sites, GC content of the seed site, and nearness of any base pairing to promote the accessibility of miRNA to the mRNA response element were examined manually in the computational programs to predict the best-presumed mRNAs. Based on previous reports, genes that were substantially upregulated in the other cancer types were also considered.

Samples

Thirty-five specimens of prostate cancer and 15 specimens of BPH were taken during radical prostatectomies. Clinical samples were provided by the Department of Pathology, Hashemi Nejad Hospital (Tehran, Iran) between 2014 and 2015. All volunteers were informed and filled out

the consent form. The research was approved by the ethical committee of the Pasteur Institute of Iran (#825 and #1010 and #7967). Gleason score, pathological stage, and histological prognosis were evaluated based on the guidelines of the Union for International Cancer Control (32). These samples were used in our previous work, where we described the patient demographics and clinicopathological features in detail (33).

Cell culture

PC3 and DU145 as human PCa cell lines were supplied by Leibniz-Institute DSMZ (Germany). DU145 cells were grown in RPMI 1640 medium reinforced with 10% FBS (Gibco, USA). PC3 cells were maintained in a 1:1 mixture of Ham's F12 and RPMI 1640 medium (10% FBS). HUVEC (Human umbilical vein endothelial cell line) was provided by the National Cell Bank of Iran and was maintained in a 1:1 mixture of Ham's F12 and DMEM medium (20% FBS). The cells were cultured in 5% CO2 and 37 °C.

RNA extraction and reverse transcription

RNA extraction of PC3, DU145, HUVEC cell lines, and clinical samples was conducted based on previously described protocols (33). The ratio of absorbance at 260 nm and 280 nm was considered as the purity index of the extracted RNA. After that, 1.5 µg of each extracted RNA was applied for cDNA synthesis by PrimeScript RT reagent kit based on the manufacturer's instruction (Takara Bio, Japan). Reverse transcription of miRNA was the same as mRNA except for designed stem-loop RT primers which substituted for oligo dT primers.

Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

The expression of different mRNA was studied by a Rotor-Gene 6000 system (Corbett Life Science; Qiagen, Germany). Mixture for RT-qPCR with the ultimate volume of 20 µl included 10µl SYBR Green Master Mix (Takara, Japan), 0.5 pMol of the target specified primers (Table 1), and 1 µL of cDNA. The protocol of RT-qPCR included an initial denaturation at 95 ºC for 30 seconds, followed by 40 cycles of 95 ºC for 5 seconds and 60 ºC for 30 seconds. The mixture for Real-Time PCR with the total volume of 20 µl was comprised of 10 µl qPCR Master mix, 1 µl universal reverse-primer, 1 µl specific forward primer, and 1 µL cDNA. The thermocycling protocol consisted of an initial denaturation at 95 ˚C for 15 minutes, 40 cycles of 94 ˚C for 15 seconds, 55 ˚C for 30 seconds, and 70 ˚C for 30 seconds. The expression of mRNA was normalized with *GAPDH*. The *SNORD47* (U47) and U6 miRNAs were applied to normalize miRNA expression. The normalization of the target gene with the reference standard was performed using REST © (relative expression software tool). The relative expression was founded upon the expression ratio of the target genes in contrast with the reference gene. The ratio of mRNAs to miRNAs was calculated using the $\Delta\Delta C_T$ method. The average C_T values of the target gene were subtracted from the C_T of the housekeeping gene to calculate ΔC_T. The results were analyzed in GraphPad. The proliferation efficiency of RT-qPCR was evaluated using serial dilutions of 10^1, 10^2, and 10^3 of a pooled cDNA sample (15 samples) for every primer set, besides normalizing against references.

Table 1. Specific primers were designed for seven target genes predicted by computational tools.

AACGGGAAGCTTGTCATCAATGGAAA GCATCAGCAGAGGGGGCAGAG	GAPDH
GCTGTTATCTGGAAGAAGCCCT GCAACGAAAAGGTTGGGGTC	ABL1
GACCTTCGTTGCCCTCTGTG GAGGCGGTAGTAGGACAGGA	CCND1
CTCCCCAAAGGCAGGCTC GCAAGACAGGTAGCGATCCA	CCND3
GTACAAATCCAAGTTTGCTGACCTC TTAAGGGCATCCACTTCACAGG	VIM
CTCAGCTACGCCTTCTCCGT CGAATGCATCCCAATTCCACT	TWIST1
TCCAAGAAGCCCTAACGTGT ATGTTCCAATTCCTACTGCTTGA	HIF1A
GTGCTACGGCGACATGGAGA GGGCATAGCTGAGGAAGGTTT	TERT

Luciferase assay

The probably paired sequences of 3′-UTR of the *CCND1* gene were found from miRTarBase, which sums up the interaction sites reported in previous studies and predicts specific interactions (14). Three interactional regions in 3′-UTR of *CCND1* predicted by miRTarBase, including regions 757-776, 3096-3118, and 1457-1497 (NM_053056.3), were synthesized (Biomatik, Canada), and subcloned into the XhoI/NotI restriction sites of psiCHECK™-2 Vector (Promega, USA) consecutively with 20 nucleotides in between. The final construct was named psiCHECK2-*CCND1*-3′UTR. Syncel-miR-39-3p (negative control, abm, Canada) and miR-138-5p (miR-138 mimic) sequence vectors were purchased from Qiagen, Germany. Initially, about 10^3 PC3 cells were grown in a 96-well plate. After 12-16 hours, psiCHECK2-*CCND1*-3′, miR-138-5p-mimic+psiCHECK2-*CCND1*-3′UTR, and Syn-cel-miR-39-3p+psiCHECK2-*CCND1*-3′UTR were co-transfected into the PC3 cells in triplicates. Dual-Luciferase Reporter Assay System (Promega, USA) was adopted to evaluate the luciferase activity 24 hours after transfection, based on the manufacturer's instruction as previously elucidated (34). The multi-well plate luminom-

eter Renilla luciferase activity was normalized against firefly luciferase.

Cell proliferation analysis

The viability of transfected cells with miR-138 was examined using MTT (3-(4, 5-dimethylthiazol-2-Yl)-2, 5-diphenyltetrazolium bromide, Sigma, Germany) assay. Briefly, about 10^3 PC3 and DU145 cells were grown in every well of a 96-well plate and transfected with miR-138-5p-mimic and Syn-cel-miR-39-3p. The cells that had only been incubated with Lipofectamine were used as control. The optical density was read at 540 nm against 630 nm as the reference. Cell proliferation was interpreted respective to non-transfected cells.

Statistical analysis

Statistical analysis was conducted utilizing the mean ± standard error of mean (SEM) in the GraphPad Prism version 8.01 (GraphPad, USA). The difference among the various categories was calculated using Analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. *P values* ≤ 0.05 were deemed as noteworthy. All assays were conducted at least in triplicate.

Results

Identification of miR-138 candidate target genes in PCa

As indicated in table 2, a total of seven genes were selected based on previously validated genes in papers, and the highest scores in Target Scan, Microcosm, PicTar, and miRanda: *ABL1* (ABL proto-oncogene 1, non-receptor tyrosine kinase), *CCND1* (cyclin D1), *CCND3* (cyclin D3), *VIM* (vimentin), *TWIST1* (twist family bHLH transcription factor 1), *HIF1A* (hypoxia-inducible factor 1 subunit alpha), and *TERT* (telomerase reverse transcriptase) genes.

MiR-138 overexpressed in PC3 and DU145 cells, but for HUVEC it declined

The expression of miR-138 was examined in PCa cell lines in comparison with HUVEC, as a normal cell line utilizing RT-qPCR. HUVEC was selected as a normal control in line with the earlier researches (33, 35). U6 or U47 genes functioned as normalizers. Mean ± SEM values are represented in Table 1s. As displayed in Figure 1, miR-138 obviously overexpressed in HUVEC cells in comparison with PC3 and DU145 using U6 or U47 as normalizer (*p*-value<0.0001). Interestingly, the expression of miR-138 exhibited no substantial variations between DU145 and PC3 cell lines using U6 and U47 as normalizers.

The expression of miR-138 diminished in the PCa specimens, as compared with BPH clinical samples

The expression of miR-138 in PCa and BPH clinical samples was examined via RT-qPCR. U6 and U47 genes functioned as normalizers. Mean ± SEM values of these comparisons are represented in Table 2s. The expression of miR-138 evidently diminished in PCa clinical samples in comparison with BPH tissues using U6 (*p*-value=0.0030) and U47 (*p*-value=0.0003) as normalizers (Figure 2).

Among seven candidate genes, only *CCND1* enhanced in PCa cell lines

To evaluate the influence of reduced amounts of miR-138 on PC3 and DU145 cancer cells, the expression profile of seven selected target genes, including *ABL1*, *CCND1*, *CCND3*, *VIM*, *TWIST1*, *HIF1A*, and *TERT* genes was examined utilizing RT-qPCR. PCR efficiency of every sample

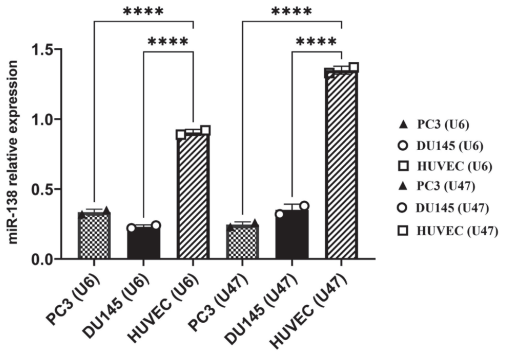

Figure 1. Results of quantitative RT-PCR on PCa and HUVEC cell lines: relative expression ($2^{-\Delta\Delta CT}$) of miR-138 in HUVEC cells compared to PC3 and DU145. U6 or U47 housekeeping genes were applied as the normalizers. **** is p-value equal or less than 0.00005.

Table 2. Selection of miR-138 target genes using four computational algorithms and considering previously validated genes in papers*.

Gene Symbol	miRDB	PicTar	Target scan	Mirtargetlink2	Validated genes in papers	Overall score
ABL1	0	0	1	0	1	2
CCND1	1	0	1	1	1	4
CCND3	1	0	1	1	1	4
VIM	1	0	1	1	1	4
TWIST1	0	0	1	1	1	3
HIF1A	1	1	1	1	1	5
TERT	0	0	0	1	1	2

* 1: MiR-138 targets a specific gene, 0: MiR-138 does not target a specific gene.

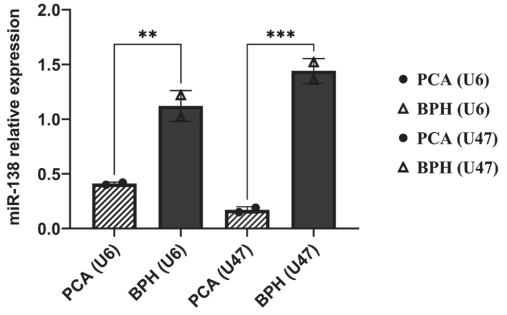

Figure 2. Results of quantitative RT-qPCR on PCa tissue samples and BPH samples: relative expression ($2^{-\Delta\Delta CT}$) of miR-138 in PCa tissue samples compared with BPH samples. U6 or U47 housekeeping genes were applied as the normalizers. ** is p-values equal or less than 0.005. *** is p-values equal or less than 0.0005.

was between 95% to 105%, based on the slope of the standard curve (Figure1s a, b). Moreover, the relative expression of *ABL1*, *CCND1*, *CCND3*, *VIM*, *TWIST1*, *HIF1A*, and *TERT* genes was compared to *GAPDH* as the control group (These target genes were previously normalized to *GAPDH* to quantify their relative expression). As elucidated in Figure 3 (a), among seven evaluated target genes, only *CCND1* was expressed at a significantly higher level (*p*-value=0.0016) in comparison with *GAPDH* as the housekeeping gene in PC3 cells. The expression of *CCND1* insignificantly raised in the DU145 cell line compared to the control group, as elucidated in Figure 3 (b). The expression of other candidate genes, including *ABL, CCND3, VIM, TWIST1, HIF1A*, and *TERT,* displayed no substantial change in comparison with *GAPDH* in PC3 and DU145 cell lines. Mean ± SEM values of the expression level of these genes are represented in Table 3s.

The expression of *CCND1* was reversely correlated with miR-138 in PCa cell lines

Pearson correlation test was performed between the expression miR-138 and *CCND1* gene in PC3 and DU145 cell lines, to explore whether the *CCND1* expression was coordinated with the level of miR-138. As displayed in figure 4 (a), the augmented level of *CCND1* was correlated to the declined miR-138 expression using U6 (R=−-0.9998, P=0.0135) and U47 (R=-0.9999, P=0.0074) as normalizers in the PC3 cell line. Moreover, *CCND1* exhibited a reverse correlation with miR-138 using U6 (R=-0.9982, P=0.0378) and U47 (R=-0.9995, P= 0.0203) as normalizers in DU145 cell line (figure 5, b). The results may propose the modulatory impact of miR-138 on the expression profile of *CCND1*.

CCND1 was corroborated as the target of miR-138 in the PC3 cell line by luciferase assay

MiRanda was utilized to analyze and predict 3′UTR sequences of the *CCND1* gene that could be targeted by miR-138. MiR-138-complementary sites located on the 3′ noncoding region of *CCND1* transcripts in miRTarBase are represented in Figure 5. To examine and corroborate the impact of miR-138 on *CCND1*, the predicted 3′UTR sequences of *CCND1* were cloned downstream of luciferase in the psiCHECKTM-2 vector. PC3 cells were transfected in three groups: psiCHECK2-*CCND1*-3′UTR plus miR-138-

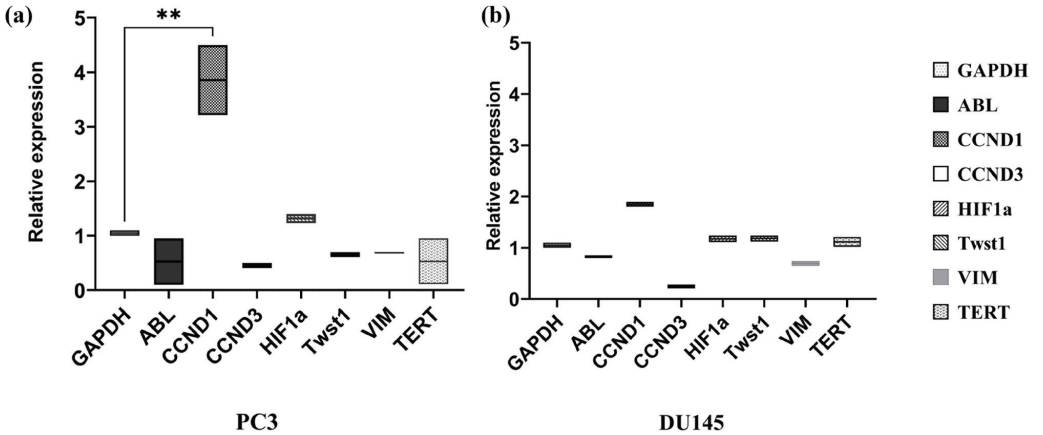

Figure 3. Results of quantitative RT-PCR on all candidate genes in PC3 and DU145 cell lines. (a) Substantial overexpression of *CCND1* in comparison with *GAPDH* as a housekeeping gene in PC3 cells. The expression profile of other candidate genes, including *ABL, CCND3, VIM, TWIST1, HIF1A, and TERT* genes exhibited no considerable change in comparison with *GAPDH* in the PC3 cell line. (b) An insignificant rise in the expression of *CCND1* in the DU145 cell line. The expression of other candidate genes, including *ABL, CCND3, VIM, TWIST1, HIF1A, and TERT* genes, revealed no substantial change in comparison with *GAPDH* in the DU145 cell line. ** is p-values equal or less than 0.005.

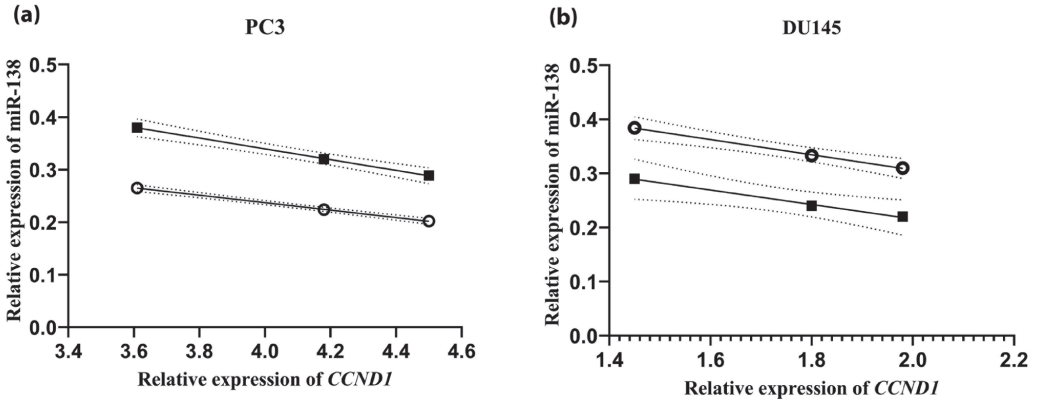

Figure 4. Pearson correlation analysis was applied to examine the correlation between the expression of miR-138 and the CCND1 gene in PCa cell lines. (a) A substantial negative correlation between the expression of CCND1 gene and miR-138 was noted using U6 (Y = -0.1026*X + 0.7500, R^2=0.9996) and U47 (Y = -0.07092*X + 0.5209, R^2= 0.9999) as normalizers in PC3 cell lines. (b) A substantial negative correlation between the expression of CCND1 gene and miR-138 was observed using U6 (Y = -0.1335*X + 0.4828, R^2=0.9965) and U47 (Y = -0.1405*X + 0.5872, R^2= 0.9990) as normalizers in DU145 cell lines.

-5p-mimic, psiCHECK2-*CCND1*-3′UTR plus Syn-cel-miR-39-3p, and psiCHECK2-*CCND1*-3′UTR alone. Mean ± SEM values of luciferase activity in transfected PC3 cells are represented in Table 4s. As elucidated in Figure 6, miR-138-5p-mimic diminished the efficacy of luciferase compared to psiCHECK2-*CCND1*-3′UTR plus Syn-cel-miR-39-3p (*p*-value = 0.0016), and psiCHECK2-*CCND1*-3′UTR alone (*p*-value = 0.0022). While no substantial variation was noted between the psiCHECK2-*CCND1*-3′UTR plus Syn-cel-miR-39-3p transfected cells, and the psiCHECK2-*CCND1*-3′UTR transfected cells. The data of the luciferase assay evidenced that transfection of miR-138-5p-mimic considerably diminished Renilla luciferase activity, and it turned out that miR-138 could modulate the *CCND1* expression through acting on its 3′-UTR in the PC3 cell line.

MiR-138-5p-mimic diminished the expression of *CCND1* in the transient transfected PCa cell lines

RT-qPCR was utilized to evaluate the expression of *CCND1* in the miR-138-5p-mimic transfected PC3 and DU145 cells to realize whether miR-138 could modulate *CCND1* by acting on its 3′-UTR in PCa cell lines. As dis-

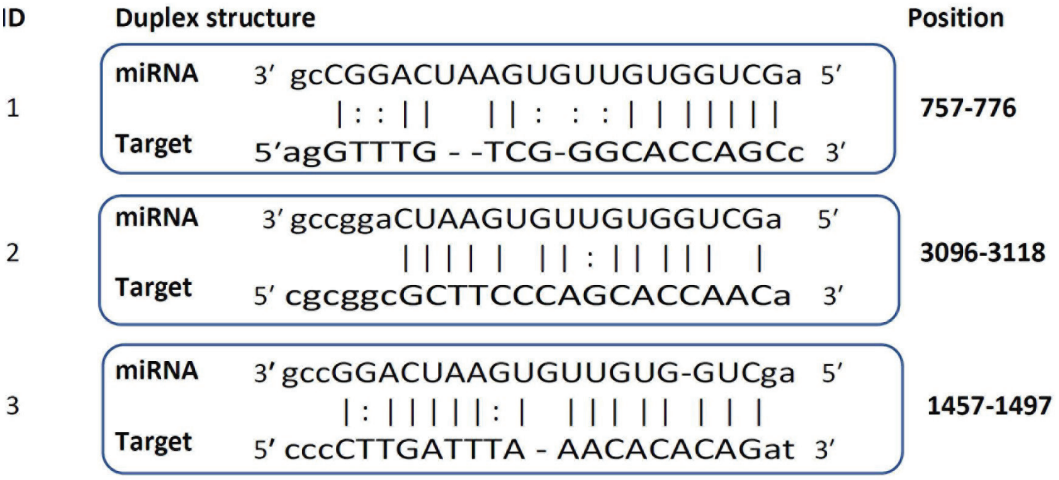

Figure 5. The complementary sequence of miR-138 located in the 3′-UTR of *CCND1* transcripts. Three interactional regions in 3′-UTR of *CCND1* were predicted by miRTarBase, including regions 757-776, 3096-3118, and 1457-1497 (NM_053056.3).

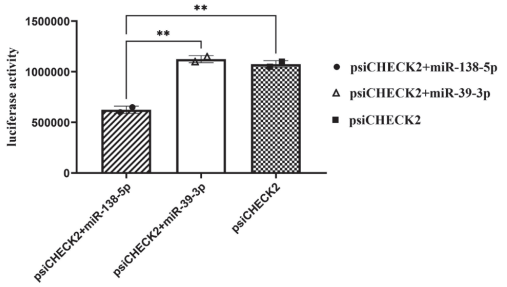

Figure 6. Impact of miR-138 on the expression of *CCND1*: PC3 cells were transfected with Renilla luciferase expression cassette, psiCHECK2 carrying the 3′-UTR of *CCND1* alone, psiCHECK2-*CCND1*-3′UTR+miR-138-5p-mimic, and psiCHECK2-*CCND1*-3′UTR+Syn-cel-miR-39-3p. MiR-138-5p-mimic transfected PC3 cells notably exhibited diminished Renilla luciferase activity. ** is p-values equal or less than 0.005.

played in Figure 7, the expression of CCND1 insignificantly diminished following transfection with miR-138-5p-mimic in DU145 cells, while *CCND1* mRNA level significantly decreased following transfection in PC3 cells (p-value = 0.0008). Mean ± SEM values of the expression level of CCND1 in transfected PC3 and DU145 cells compared with non-transfected cells are represented in Table 5s.

In conclusion, *CCND1* expression revealed a tendency to decrease in DU145 cell lines and a substantial decline in PC3 cells. Diminished expression of *CCND1* in miR-138 transfected PC3 and DU145 cell lines implies the suppressive influence of miR-138 on the expression of *CCND1* in the PCa cell lines.

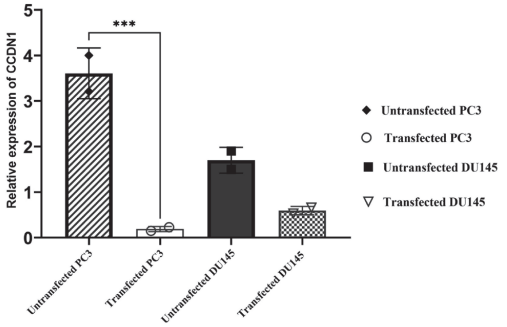

Figure 7. The impact of miR-138-5p-mimic transient transfection on the expression of *CCND1*: The expression of *CCND1* in miR-138-5p-mimic transfected PC3 and DU145 cells was examined by RT-qPCR. The expression of CCND1 in miR-138-5p-mimic transfected DU145 cells insignificantly lessened, while CCND1 mRNA level substantially reduced following transfection in PC3 cells. The results were normalized to the *GAPDH* housekeeping gene. *** is p-values equal or less than 0.0005.

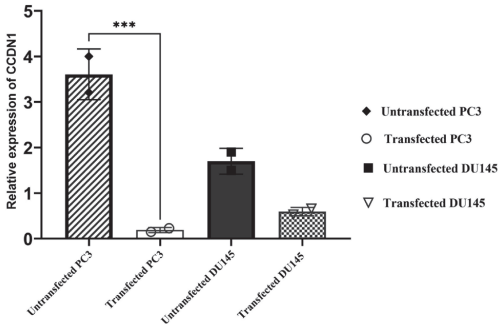

Figure 8. MTT test was utilized to examine the proliferation rate of PC3 and DU145 cells transfected in three groups, including miR-138-5p-mimic, Syn-cel-miR-39-3p, and Lipofectamine alone. MiR-138-5p-mimic transfected cell lines showed reduced proliferation in comparison with non-transfected cell lines. * is p-values equal or less than 0.05. ** is p-values equal or less than 0.005. *** is p-values equal or less than 0.0005.

MiR-138-5p-mimic could have a pivotal function in the inhibition of cell proliferation in the transient transfected PCa cell lines

The cell viability test by MTT was carried out to understand the functional activity of overexpression of miR-138 in the proliferation of PCa cell lines. Cells receiving only Lipofectamine (blank) were utilized as the negative control, and cells with no transfection functioned as the external control group. PC3 and DU145 cells were transfected in three categories containing miR-138-5p-mimic and Syn-cel-miR-39-3p in addition to the cells incubated only with Lipofectamine. Mean ± SEM values of the proliferation scale in PC3 and DU145 cells transfected in three groups are represented in Table 6s. The outcome of the MTT assay implied that the proliferation of PC3 cells 24 hours subsequent to transfection with miR-138-5p-mimic significantly declined compared to Syn-cel-miR-39-3p transfected cells (p-value =0.0006) and Lipofectamine incubated cells (p-value =0.0011). Furthermore, miR-138-5p-mimic transfected DU145 cells substantially evidenced a diminished proliferation rate compared with Lipofectamine incubated cells (p-value=0.0122), while miR-138-5p-mimic and Syn-cel-miR-39-3p transfected cells expressed insubstantial variations (Figure 8).

In conclusion, the proliferation of all miR-138-5p-mimic transfected cell lines considerably diminished compared to non-transfected cell lines.

Discussion

During the first phases of cancer diagnosis, miRNAs are being used more and more. They are also utilized for the therapy and prognosis of this disease (13). Disorganization of miRNAs is wholly proven to implicate the advance and furtherance of cancer cells (36-38). As elucidated before, expression of the microRNAs inclusive of miR-26a, miR-138, miR-1266, miR-185, and miR-30c diminished in PCa tissues and cell lines (27, 32). Moreover, miRNA expression profiles showed widespread dysregulation in primary PCa compared to normal prostate tissue.(36) As expounded earlier, miR-138 arrested the malignant advancement of several human cancers, including pancreatic cancer, colorectal cancer, and other cancer types (10, 12, 39, 40). Here, distinctive expression of miR-138 was scrutinized in PCa tissues and cell lines in comparison with non-cancerous cell lines and BPH tissues, respectively. We corroborated that the expression of miR-138 considerably lessened in PCa cell lines in comparison with the HUVEC cell line. Additionally, miR-138 obviously diminished in PCa tissues in comparison with BPH clinical samples. These findings were in line with previous studies where miR-138 evidently lessened in PCa tissues and cell lines (19, 20, 22).

Here, according to bioinformatic analysis with online software such as Target Scan, Microcosm, PicTar, and miRanda, it was assumed that *ABL1, CCND1, CCND3*, *VIM*, *TWIST1, HIF1A,* and *TERT* might be the target gene of miR-138. In accordance with RT-qPCR findings, the expression of *CCDN1* substantially augmented in PCa tissue and PC3 cell line and had an insignificant rise in the DU145 cell line. Furthermore, the correlation between the expression of *CCND1* and miR-138 was calculated in PC3, DU145 cell lines. Our data proposed that *CCND1* showed a reverse correlation with the miR-138 in the prostate cancer cell lines.

Additionally, dual-luciferase reporter assay was utilized to explore the associations between miR-138 and 3' UTR of *CCND1* and to examine whether this relationship is influential in the PCa progress. In accordance with the results of the luciferase reporter assay, overexpression of miR-138 prohibited the expression of *CCDN1* in PC3 cell lines. It was inferred that miR-138 could negatively regulate the expression of *CCDN1* in PCa cell lines. As pointed out previously, a reverse correlation was distinguished between miR-138 and the expression of *CCND1* in nasopharyngeal carcinoma (14). *CCND1* has also been associated with miRNAs in other tumorigenesis pathways in PCa. In a new signaling pathway comprising *LOXL1-AS1*, miR-541-3p, and *CCND1,* which modulates the cell cycle and proliferation of PCa cells, *CCND1* expounded as the target of miR-541 (41). Additionally, owing to the post-transcriptional regulatory function of the miR17 family, *CCND1* evidently elevated when the miR17 family was suppressed in PCa cell lines (42).

Subsequently, we scrutinized the influence of miR-138 overexpression on the expression profile of *CCDN1* in the transiently transfected PC3 and DU145 cell lines. Conclusively, overexpression of miR-138 could repress *CCDN1* expression in PC3 cells, while the expression level of the *CCDN1* gene exhibited an insignificant reduction in the DU145 cell line. MiR-138 has also shown other negative regulatory influences on PCa. As previously elucidated, overexpression of miR-138 substantially arrested the capability of the Wnt/β-catenin pathway in C4-2B and PC3 cells. Accordingly, miR-138 reversely modulates β-catenin in prostate cancer cells (22).

MTT assay was utilized to appraise the inhibitory function of miR-138 on the proliferation scale of PCa cell lines. Our findings revealed that miRNA-138 could precipitate an anti-proliferative effect on PCa cells, which was in line with preceding research that miR-138 motivated anti-proliferative influence on several cancers such as clear cell renal cell carcinoma, squamous cell carcinoma, tongue squamous cell carcinoma, and head and neck squamous cell carcinomas (38-40). Consonant with the earlier investigation, miR-138 and its target CCND1 were implicated in the modulation of the nasopharyngeal carcinoma progress. Overexpression of miR-138 also induced arrest in the G_1 cell cycle, repression of cell proliferation *in vitro,* and inhibitory effects on the tumorigenicity of mice xenografts (14). Moreover, it was previously declared that overexpression of miR-138 hindered the metastasis and proliferation of PCa through targeting and downregulating FOXC1 (20). Furthermore, earlier experiments revealed that elevated expression of miR-17 family members contributed to the lessening of *CCND1* which resulted in motivated apoptosis, suppressed proliferation capability, and colony forming potential (42). MiR-138 and PD-L1 also exhibited a negative correlation in colorectal cancer. The diminished proliferation rate in miR-138-5p mimic transfected HCT116 and SW620 cells was evidenced by MTT assay through regulating PD-L1 as the target gene (39). In the current research, the potential of miR-138 to suppress cell proliferation indicated its function as a tumor suppressor in PCa, which might be partial via regulating *CCND1* as the target gene. Our results were in line with previous research that miR-138 repressed proliferation and motivated apoptosis through modulating multiple targets (43, 44). MiR-138 also functioned in the modulation of enhancer of zeste homolog 2 (EZH2) to repress proliferation efficiency, motivate apoptosis and arrest G0/G1 cell cycle as a tumor suppressor in non-small cell lung cancer cells (45).

Therefore, miR-138 elucidated assorted biological functions through modulating the *CCDN1* gene in PCa. *CCND1* showed an inverse correlation with the miR-138 level in PCa cell lines. It implies the reverse modulatory impact of miR-138 on the expression of *CCDN1* in PCa. However, the antiproliferative effects of miR-138 could be applied through the mechanisms other than *CCDN1* regulation in the DU145 cell line. Further studies are required to identify the cooperative genes. Distinguishing these functional targets might have clinical importance hereafter. Our findings need more complementary studies, such as evaluating the repressive influence of miR-138 on the malignant advancement of PCa in vivo as well as clinicopathological features of PCa patients through analysis of the biological function of CCND1.

Here, to the best of our knowledge, we evidenced the direct association of miR-138 with the *CCND1* gene for the first time in PCa. The overexpression of *CCND1* was also negatively correlated with miR-138 in PCa cell lines. Our findings implied that miR-138 could modulate the enhanced expression of *CCND1* to some extent. Conclusively, miR-138 functions as a tumor suppressor through the moderation of the *CCND1* gene in the malignant advancement of PCa.

Conclusions

Our findings revealed an etiological relationship between miR-138 and *CCND1* gene as its target in PCa. We also corroborated the functionality of this correlation in the growth and proliferation of PCa that could function in miRNA-based diagnostic and therapeutic approaches. However, further comprehensive evaluation of the modulatory pathways and molecular mechanisms in PCa would highly facilitate the promotion of design and selection of targets for therapeutic procedures.

Declaration statements

Ethics approval and consent to participate: The study was carried out in accordance with the guidelines of the Ethics Committee of Pasteur Institute of Iran (#825, #1010, and #7967). Informed consent was obtained from all subjects involved in the study.

Consent for publication: Not Applicable.

Availability of data and material: The supporting data of the findings of this study are available by the corresponding author upon reasonable request.

Competing interest: The authors declare that they have no competing interests.

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Authors' contributions: Project administration, conceptualization, supervision, writing, review, and editing: Pezhman Fard-Esfahani. Preparation of the manuscript, interpretation, and analysis of data: Nasrin Haghighi-Najafabadi. Revision of the manuscript: Ghazal Haddad. Methodology: Shima Fayaz, Mahboubeh Berizi.

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Original article

Study on the performance of medical laboratories in Romania: microbial etiological agents identification

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Abstract

Medical laboratories must offer reliable services to the patient, and the way to guarantee this is by demonstrating their performance. External Quality Control (EQC) is a tool that helps the laboratories measure their performance, identify possible errors, and improve their activity. The aim of our research has been divided into three segments, which included the evaluation of the accuracy rate in pathogen identification, examining the unsatisfactory results, and investigating the identification methods utilized by the laboratories in Romania. As the analyses of pharyngeal exudate and urine are the most commonly requested in medical laboratories and also in External Quality Control, we have focused on the findings obtained from these programs.

Keywords External Quality Control, pathogen identification, urine cultures, pharyngeal exudate

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Introduction

An essential aspect of the microbiological diagnosis is to provide to physicians rapid and precise outcomes, as they rely on the results of the medical analyses carried out in the medical laboratory in order to confirm 70-85% of the medical diagnoses (C. POPA and G. SORESCU, 2022 [1]) Therefore, in order to achieve this desideratum, a strong connection between the patient, the physician, and the medical laboratory must be built (C. POPA and G. SORESCU, 2022 [1]). Also, medical laboratories must adopt a quality management system to operate with optimal efficiency, for the benefit of patient care (R. B. CAREY & al., 2018 [2]). External Quality Control is an important part of any quality management system. The medical laboratories regularly apply to the EQC programs to achieve continuous improvement of the system. The two main steps of an EQC program are: i) the EQC organizer provides the samples to the medical laboratory in order to be tested and ii) the laboratory examines the samples and then sends the results to the provider.

The key reason for EQC's utility is that the samples are manufactured to simulate the regular patient samples. Furthermore, the participants treat the EQC samples in the same manner as patient samples, using their routine equipment and testing methods (C. POPA and G. SORESCU, 2022 [1]).

Pharyngeal exudate analysis represents an important step to diagnose pharyngitis, which is the most common disease of the upper respiratory tract. Although viruses are the main pathogen responsible, bacteria and fungi also play a significant role in the occurrence of this disease (S. ORZELL and A. SURYADEVARA, 2019 [3]). The most significant bacteria causing upper respiratory tract infections is group A *Streptococcus pyogenes,* followed by group B, C and G streptococci (M. C. CHIFIRIUC & al., 2015 [4]).

Urinary tract infections (UTIs) are a prevalent form of infectious diseases that can occur in individuals of all ages and genders, being considered one of the most common infections worldwide (C. DELCARU & al., 2016, [10]). This kind of infection can be acquired both in the community and in hospitals and have the potential to progress towards severe forms and cause renal failure (A. FLORES-MIRELES & al., 2019 [6]; A. S. N. HAITHAM & al., 2021 [7]; V. C. CRISTEA & al., 2019, [9]). The main pathogens responsible for UTIs are *Escherichia coli* and *Proteus mirabilis* (M. C. CHIFIRIUC et al., 2015 [4]; C. DELCARU & al., 2017 [8]). Regarding immunosuppressed patients, two of the common pathogens are *Candida albicans* and *Candida glabrata* (M. C. CHIFIRIUC & al., 2015 [4]).

The purpose of this study is to evaluate the quality of the services provided by medical laboratories in Romania with respect to the microbial etiological agent identification procedures and performance.

Table 1 – The strains provided in each EQC round organized between 2017 and 2022 for Bacteriology – Urine culture

EQC Round	Strain	Percentage of satisfactory results (%)
March 2017	*Klebsiella pneumoniae*	97.93%
May 2017	*Enterococcus faecalis*	96.76%
September 2017	*Proteus mirabilis*	99.60%
November 2017	*Escherichia coli*	99.60%
March 2018	*Enterococcus faecalis*	99.20%
May 2018	*Proteus mirabilis*	99.26%
September 2018	*Enterobacter cloacae*	85.61%
November 2018	*Pseudomonas aeruginosa*	100.00%
March 2019	*Enterococcus faecalis*	98.58%
May 2019	*Serratia marcescens*	87.16%
September 2019	*Enterobacter cloacae*	91.86%
November 2019	*Escherichia coli*	99.00%
March 2020	*Serratia marcescens*	90.65%
June 2020	*Enterococcus faecalis*	99.31%
September 2020	*Proteus vulgaris*	92.33%
November 2020	Escherichia coli	99.00%
March 2021	*Enterococcus faecalis*	99.32%
May 2021	*Proteus mirabilis*	98.26%
September 2021	*Serratia marcescens*	95.44%
November 2021	*Escherichia coli*	99.70%
March 2022	*Proteus vulgaris*	97.38%
May 2022	*Escherichia coli*	100.00%
September 2022	*Escherichia coli*	99.87%
November 2022	*Escherichia coli*	99.60%

Materials and methods

We have focused our research on urine culture for bacteria and fungi identification and pharyngeal exudate for bacterial identification EQC programs. The results utilized in our study were clustered from the following three EQC schemes, with the participation of 300 to 600 Romanian medical laboratories between 2017 and 2022: Bacteriology – Pharyngeal exudate, Bacteriology – Urine culture and Micology – Urine culture. Therefore, we collected and analyzed the results to gain insight into the performance of Romanian medical laboratories in relation to pathogen identification.

Table 2 – The strains provided in each EQC round organized between 2017 and 2022 for Bacteriology – Pharyngeal exudate

EQC Round	Strain	Percentage of satisfactory resuts (%)
March 2017	*Staphylococcus aureus*	100.00%
May 2017	*Streptococcus agalactiae*	96.30%
September 2017	*Streptococcus pyogenes*	98.76%
November 2017	*Staphylococcus aureus*	100.00%
March 2018	*Streptococcus pyogenes*	99.19%
May 2018	*Streptococcus agalactiae*	96.98%
September 2018	*Streptococcus pyogenes*	99.26%
November 2018	*Staphylococcus aureus MRSA*	100.00%
March 2019	*Streptococcus dysgalactiae subsp. equisimilis*	93.12%
May 2019	*Streptococcus pyogenes*	100.00%
September 2019	Staphylococcus aureus MRSA	99.34%
November 2019	*Streptococcus pyogenes*	100.00%
March 2020	*Streptococcus pyogenes*	99.32%
June 2020	*Streptococcus agalactiae*	95.62%
September 2020	*Staphylococcus aureus MRSA*	100.00%
November 2020	*Streptococcus agalactiae*	98.96%
March 2021	*Streptococcus dysgalactiae subsp. equisimilis*	98.91%
May 2021	*Streptococcus pyogenes*	99.54%
September 2021	*Streptococcus agalactiae*	99.06%
November 2021	*Streptococcus pyogenes*	99.84%
March 2022	*Staphylococcus aureus MRSA*	100.00%
May 2022	*Streptococcus pyogenes*	99.86%
September 2022	*Streptococcus dysgalactiae subsp. equisimilis*	99.07%
November 2022	*Streptococcus agalactiae*	99.59%

Table 3 – The strains provided in each EQC round organized between 2017 and 2022 for Micology – Urine culture

EQC round	Strain	Percentage of satisfactory resuts (%)
March 2017	*Candida albicans*	100.00%
May 2017	*Candida albicans*	98.48%
September 2017	*Candida krusei*	87.31%
November 2017	*Candida albicans*	100.00%
March 2018	*Candida parapsilosis*	95.71%
May 2018	Candida parapsilosis	98.84%
September 2018	*Candida albicans*	99.42%
November 2018	*Candida albicans*	98.90%
March 2019	*Candida albicans*	100.00%
May 2019	*Candida albicans*	98.48%
September 2019	*Candida parapsilosis*	97.52%
November 2019	*Candida parapsilosis*	100.00%
March 2020	*Candida glabrata*	82.56%
June 2020	*Candida parapsilosis*	97.41%
September 2020	*Candida tropicalis*	89.58%
November 2020	*Candida albicans*	99.48%
March 2021	*Candida albicans*	99.50%
May 2021	*Candida glabrata*	97.54%
September 2021	*Candida tropicalis*	93.87%
November 2021	*Candida parapsilosis*	99.56%
March 2022	*Candida albicans*	100.00%
May 2022	*Candida parapsilosis*	99.80%
September 2022	*Candida tropicalis*	97.57%
November 2022	*Candida albicans*	99.38%

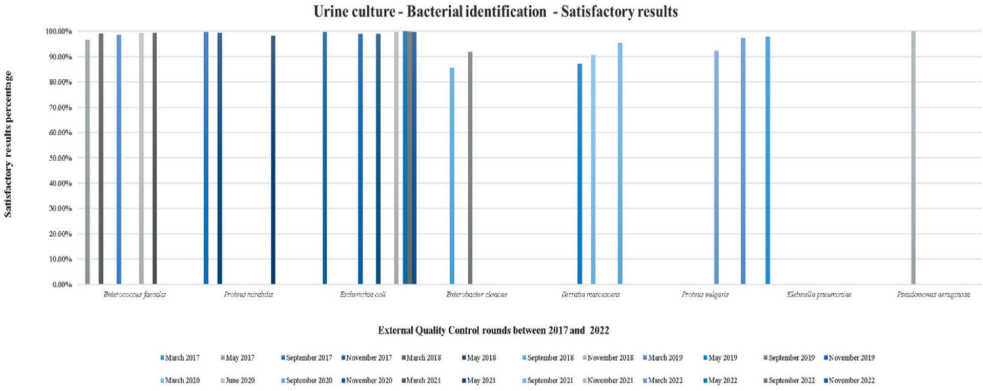

Figure 1 – The centralization of the percentages of satisfactory results obtained from urine culture sample testing

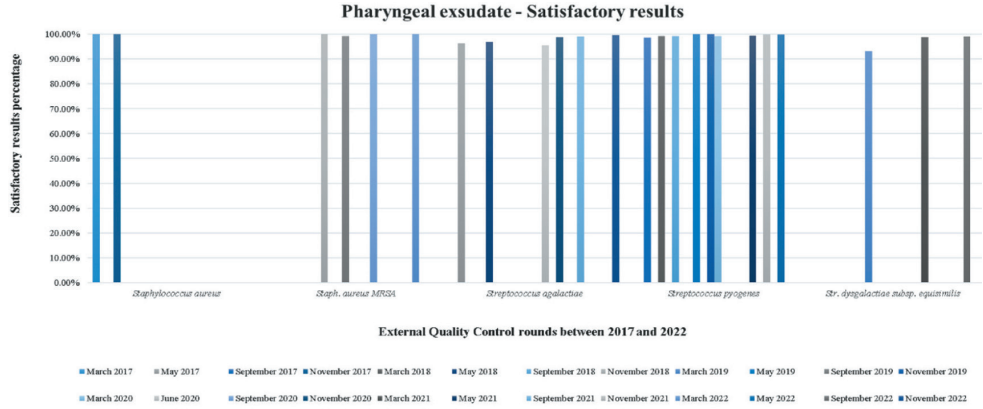

Figure 2 – The centralization of the percentages of satisfactory results obtained from testing pharyngeal exudate samples

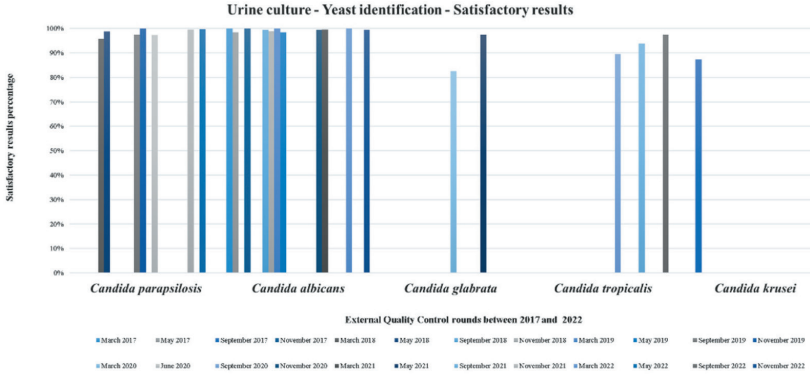

Figure 3 – The centralization of the percentages of satisfactory results obtained from testing urine culture samples – yeast identification

Results

For the analysis of the satisfactory results obtained by testing the strains provided from 2017 to 2022, the study was initiated by gathering the outcomes from the EQC rounds that were conducted from 2017 to 2022. Every round had an approximate number of 300 medical laboratories participating between 2017 and 2020 and an approximate number of 600 medical laboratories participating in 2021 and 2022. Between 2017 and 2022, the External Quality Control organizer supplied different strains for the three EQC schemes (tables no. 1, no. 2, and no. 3).

By centralizing the percentages of satisfactory results registered between 2017 and 2022 (Figure 1), we have observed that *Enterobacter cloacae* and *Serratia marcescens* strains registered the lowest number of satisfactory results. On the other hand, *Pseudomonas aeruginosa, Escherichia coli, Enterococcus faecalis,* and *Proteus mirabilis* registered the highest number of satisfactory results.

The testing of pharyngeal exudate samples revealed that the minimum percentage recorded was 93.12% for *Str. dysgalactiae subsp. equisimilis* strain and the highest (100%) was registered for *S. aureus, S. aureus MRSA,* and *Str. pyogenes* (Figure 2).

The lowest percentage for yeast identification was registered for *C. glabrata* (82.56%), and the highest was 100% for *C. albicans* and *C. parapsilosis*.

Upon analyzing the Urine culture – Yeast identification scheme, it was observed that numerous *Candida sp*. outcomes were frequently obtained, indicating that the laboratories often do not report the species to the patients.

Therefore, we chose to conduct a more detailed analysis on this topic.

Regarding the yeasts identification, the first stage of the analysis revealed that there are more than 150 medical laboratories that usually report "*Candida sp.*" (Table 4).

Going further, we focused on the medical laboratories that reported "*Candida sp.*" results in order to correlate this results with the method used for identification.

The analysis showed that there are three medical laboratories that, in spite of using an automated system for identification, could not identify the *Candida* species provided in the samples (Table 5).

The next step of the study was to analyze the unsatisfactory results registered and the methods used by the laboratories. The total number of unsatisfactory results registered

Table 4 – An overview of the results obtained in urine culture samples from *C. albicans*, *C. parapsilosis*, *C. tropicalis*, and *C. glabrata* strains in 2021 and 2022

EQC Round	Strain	Number of results
May 2021	*Candida glabrata*	*Candida glabrata* = 121
		Candida sp. = 156
September 2021	*Candida tropicalis*	*Candida tropicalis* = 254
		Candida sp. = 175
May 2022	*Candida parapsilosis*	*Candida parapsilosis* = 320
		Candida sp. = 186
November 2022	*Candida albicans*	*Candida albicans* = 329
		Candida sp. = 154

Table 5 – An overview of the results obtained in urine culture samples from *C. albicans*, *C. parapsilosis*, *C. tropicalis*, and *C. glabrata* strains in 2021 and 2022

EQC Round	Strain	Number of *„Candida sp."* results	Number of medical laboratories that use manual method	Number of medical laboratories that use automated system
May 2021	*Candida glabrata*	156	153	3
September 2021	*Candida tropicalis*	175	173	2
May 2022	*Candida parapsilosis*	186	183	3
November 2022	*Candida albicans*	154	154	0

Table 6 – Unsatisfactory results obtained from testing urine culture samples – bacteria identification

Strain	Round and year	No. of unsatisfactory results registered	Total no. of results registered	Unsatisfactory results obtained using manual method for identification	Unsatisfactory results obtained using automated systems for identification
Enterococcus faecalis	May 2017	5	247	5	-
Enterococcus faecalis	March 2018	2	249	2	-
Enterococcus faecalis	March 2019	4	282	4	-
Enterococcus faecalis	June 2020	2	288	2	-
Enterococcus faecalis	March 2021	2	292	2	-
Serratia marcescens	May 2019	38	296	37	1
Serratia marcescens	March 2020	29	310	28	1
Serratia marcescens	September 2021	30	658	30	-
Escherichia coli	November 2017	1	251	1	-
Escherichia coli	November 2019	3	300	3	-
Escherichia coli	November 2020	3	300	3	-
Escherichia coli	November 2021	2	661	2	-
Escherichia coli	September 2022	1	772	1	-
Escherichia coli	November 2022	3	743	3	-
Proteus mirabilis	September 2017	1	247	1	-
Proteus mirabilis	May 2018	2	270	2	-
Proteus mirabilis	May 2021	7	402	7	-
Enterobacter cloacae	September 2018	38	264	38	-
Enterobacter cloacae	September 2019	25	307	25	-
Proteus vulgaris	September 2020	23	300	22	1
Proteus vulgaris	March 2022	15	573	15	-
Klebsiella pneumoniae	March 2017	5	241	5	-

Table 7 – Unsatisfactory results obtained from testing pharyngeal exudate samples

Strain	Round and year	No. of unsatisfactory results registered	Total no. of results registered	Unsatisfactory results obtained using manual method for identification	Unsatisfactory results obtained using automated systems for identification
Staph. aureus MRSA	September 2019	2	302	2	-
Streptococcus agalactiae	May 2017	8	243	8	-
Streptococcus agalactiae	May 2018	8	265	8	-
Streptococcus agalactiae	June 2020	12	274	11	1
Streptococcus agalactiae	November 2020	3	288	3	-
Streptococcus agalactiae	September 2021	6	640	6	-
Streptococcus agalactiae	November 2022	3	729	3	-
Streptococcus pyogenes	September 2017	2	242	2	-
Streptococcus pyogenes	March 2018	2	246	2	-
Streptococcus pyogenes	September 2018	2	269	2	-
Streptococcus pyogenes	March 2020	2	293	2	-
Streptococcus pyogenes	May 2021	2	438	2	-
Streptococcus pyogenes	November 2021	1	635	1	-
Streptococcus pyogenes	May 2022	1	709	1	-
Str. dysgalactiae subsp. equisimilis	March 2019	19	276	19	-
Str. dysgalactiae subsp. equisimilis	March 2021	3	276	3	-
Str. dysgalactiae subsp. equisimilis	September 2022	7	753	7	-

Table 8 – Unsatisfactory results obtained from testing urine samples – yeast identification

Strain	Round and year	No. of unsatisfactory results registered	Total no. of results registered	Unsatisfactory results obtained using manual method for identification	Unsatisfactory results obtained using automated systems for identification
Candida krusei	September 2017	17	134	17	-
Candida albicans	May 2017	2	132	2	-
Candida albicans	September 2018	1	173	1	-
Candida albicans	November 2018	2	181	2	-
Candida albicans	May 2019	3	198	3	-
Candida albicans	November 2020	1	193	1	-
Candida albicans	March 2021	1	200	1	-
Candida albicans	November 2022	3	486	3	-
Candida parapsilosis	March 2018	7	163	7	-
Candida parapsilosis	May 2018	2	173	2	-
Candida parapsilosis	September 2019	5	202	4	1
Candida parapsilosis	June 2020	5	193	5	-
Candida parapsilosis	November 2021	2	451	2	-
Candida parapsilosis	May 2022	1	507	1	-
Candida glabrata	March 2020	34	195	34	-
Candida glabrata	May 2021	7	284	7	-
Candida tropicalis	September 2020	20	192	20	-
Candida tropicalis	September 2021	28	457	26	2
Candida tropicalis	September 2022	13	536	13	-

Table 9 - An overview of the percentage of laboratories that use automated systems between 2017 and 2022 for the EQC scheme Bacteriology – Urine culture

Round and year	Total no of results registered	No. of results obtained using utomated systems	Percentage (%) of results obtained using automated systems
September 2017	247	17	6.88%
September 2018	264	26	9.84%
September 2019	307	40	13.02%
September 2020	300	45	15.00%
September 2021	658	92	13.98%
September 2022	772	97	12.56%

was 241 and only in three cases these were obtained using automated systems (Table 6). Total number of unsatisfactory results registered was 83 and only 1 of them was obtained using automated systems (Table 7).

Total number of unsatisfactory results registered was 154, and only three of them were obtained using automated systems (Table 8).

A total of 478 unsatisfactory results were obtained by centralizing all unsatisfactory results obtained between 2017 and 2022 in the three EQC programs. Only seven of them were obtained using automated systems, the rest of the 471 were obtained using manual methods (Figure 4).

For the analysis of the results provided by the medical laboratories that use automated systems for pathogen identi-

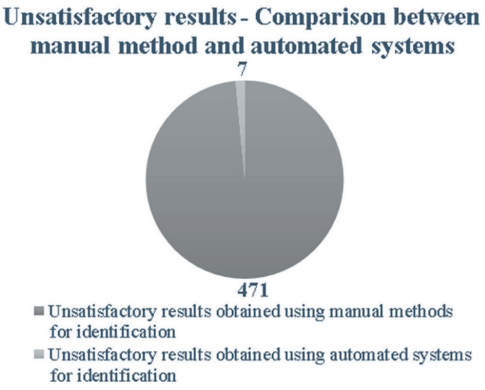

Figure 4 - Comparison between manual method and automated systems

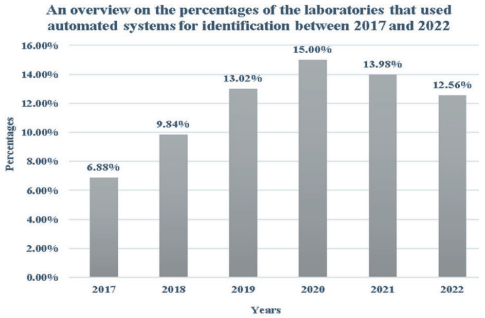

Figure 5 – An overview of the percentage of laboratories that use automated systems between 2017 and 2022

fication, we utilized the outcomes obtained for the urine culture – bacteria identification program, which had the greatest number of participating laboratories and rounds organized in September of each year from 2017 to 2022 (Figure 5).

Two primary concepts are uncovered in this phase of the investigation, i.e.: the percentage of laboratories that use automated systems is significantly low in Romania; moreover, it can be observed that it has not changed considerably during the last 4 years (from 2019 to 2022). However, the percentage has increased compared to 2017.

Conclusions

In Romania, most of the medical laboratories use manual methods when it comes to pathogen identification in patient samples. However, this study shows that in spite of not using automated systems, the laboratories manage to provide reliable services to the patients, as most of the EQC rounds concluded with satisfactory results percentages greater than 90% throughout the 6 years of the study.

Using the data from the EQC provider, we could observe that the participants encountered difficulties in identifying *S. marcescens*, *E. cloacae*, *Str. dysgalactiae subsp. equisimilis*, *P. vulgaris*, *C. glabrata,* and *C. tropicalis*. On the other hand, *S. aureus*, *Str. pyogenes*, *E. coli*, *P. aeruginosa,* and *C. albicans* sample testing registered the highest percentages of satisfactory results.

The study revealed that the percentage of medical laboratories using automated equipments in Romania is very low. It has increased from 2017 to 2020, but unfortunately, it has slightly decreased until 2022.

Most of the unsatisfactory results were obtained using manual methods.

Many laboratories do not usually report the *Candida* species to the patients. This situation brings up the matter of providing the patient with a correct medical prescription.

Acknowledgments

This document complies with the General Data Protection Regulation and the requirements of the international standard applicable to the accreditation of Proficiency Testing Schemes providers regarding the confidentiality of data provided by medical laboratories participating in External Quality Control programs.

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Original article

Torque teno viruses implications on chronic myeloid leukemia

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Abstract

Chronic myeloid leukemia (CML) is a hematological malignancy characterized by the presence of BCR-ABL+ and Ph+. According to recent studies, CML occurrence and evolution are influenced by a series of risk factors, including viruses. Multiple studies suggested that Torque teno viruses (TTVs) could modulate the treatment response and the evolution of hematological diseases. This study focuses on identifying the prevalence and clinical significance of TTVs in CML patients. The main aim was to determine if TTVs presence can be correlated with the onset of disease. We performed a retrospective study (2018-2022) that included 72 blood samples from patients diagnosed with CML. All the 3 anelloviruses were detected using hemi-nested PCR. The overall frequency of TTVs in blood samples was 93%. In our study group, most patients were carriers for the *Torque teno virus* (TTV), *Torque teno midi virus* (TTMDV) and *Torque teno mini virus* (TTMV) in 88%, 57% and 63% of samples. The largest group of carriers was represented by patients with all 3 anelloviruses (51,38%), followed by TTV (22,22%). In our study group represented by CML patients at diagnosis, the prevalence of TTVs is correlated with the molecular load of BCR-ABL. Further research and follow-up of patients with TTV are needed in the future, as well as the identification of new factors that can help to personalize treatment.

Keywords Chronic Myeloid Leukemia, Philadelphia chromosome, BCR-ABL fusion gene, Torque teno virus, hemi-nested PCR

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Introduction

Chronic myeloid leukemia (CML) is a hematological malignancy characterized by the presence of the Philadelphia chromosome and BCR-ABL fusion gene. CML represents approximately 15% of newly diagnosed cases of leukemia in adults (Miranda-Filho et al. 2018; Jabbour and Kantarjian 2022). Philadelphia (Ph) chromosome that results from t(9;22)(q34;q11), is pathognomonic for CML (Guo et al. 1994; Washburn et al. 2021), (5-8%) of patients complex rearrangements can occur (Jones et al. 2009; Jabbour and Soverini 2009; Hakim et al. 2021). BCR-ABL1 fusion gene encodes 3 types of proteins (p210, p190, p230), known for their tyrosine kinase activity (Melo et al. 1993; Kim et al. 2018). The p210 protein is specific for CML through b2a2 and b3a2 transcripts. Most cases of CML have breakpoints in ABL intron 1 or intron 2 and in BCR exons b2 or b3. After translocation, fusion gene variants (such as b2a2 and b3a2) are formed. The p210 fusion protein will be translated from both variants(Pane et al. 2002).

CML treatment was revolutionized with the discovery of tyrosine kinase inhibitors (TKI). Validation of treatment efficacy, such as hematological, cytogenetic and molecular responses, improved treatment management for CML patients (Baccarani et al. 2013). Most chronic phase patients can achieve those responses during TKI therapy. Advances in molecular biology have presented the opportunity to monitor residual disease at the molecular level (Cortes et al. 2011; Tibes and Mesa 2012).

The introduction of TKIs (Imatinib, Nilotinib, Dasatinib, Bosutinib, Ponatinib, Asciminib) as standard treatment in chronic phase of CML has improved the patient‘s prognosis, overall survival and life expectancy (Hochhaus et al. 2017). In a few cases, TKIs can lead to mutations in the BCR-ABL kinase domain and resistance to therapy (Amarante-Mendes et al. 2022).

According to recent studies, CML occurrence and evolution are influenced by a series of risk factors: physical agents (ionizing radiation), chemical pollutants (chemotherapy, benzene, chemical compounds from cigarette smoke), certain biological agents (some viruses), other diseases and age (Lim et al. 2014; Bispo et al. 2020).

Torque teno virus (TTV), *Torque teno mini virus* (TTMV), and *Torque teno midi virus* (TTMDV) are common in the general population (1-12% of blood donors) and in 4% of patients without parenteral risk factors (Prescott et al. 1998; Okamoto et al. 1999; Pineau et al. 2000; Kodama et al. 2011). TT viruses were identified in patients with hemophilia (68%), and 17.8% of those with hematological malignancies (Maeda et al. 2000; Bagheri 2012). Multiple tissues have been found to carry these viruses, including bone hematopoietic cells, peripheral blood mononuclear cells (PBMCs), and polymorphonuclear cells (PMNs) (Okamoto et al. 1999; Gallian et al. 2000; Ishimura et al. 2010). Co-infections with multiple variants of *Torque teno* viruses (TTVs) or with other human viruses may contribute to the evolution of some diseases (Maeda et al. 2000; Ninomiya et al. 2007; Spandole et al. 2009; Rocchi et al. 2009) including acute and chronic hematologic malignancies (Hino and Miyata 2007; Bagheri 2012; Shaheli et al. 2015). Also, TTVs can modulate the treatment response (Pineau et al. 2000; Kincaid et al. 2013). Mixed infections of the TTVs may cause more clinical complications in patients with leukemia(Bagheri 2012; Shaheli et al. 2015).

Consequently, it is currently speculated that TTVs, if it does not contribute to the occurrence of pathological conditions, could influence their evolution. Although there are a few studies regarding the correlations of TTVs with different pathologies (Sarairah et al. 2020), molecular pathways and interactions of these viruses associated with leukemia, remain unknown.

This study focuses on identifying the prevalence and clinical significance of TTVs in CML patients. The main aim was to determine if TTVs presence can be correlated with the onset of disease.

Materials and methods

Sample selection

Our study included a group of 72 patients with CML, ranging in age from 17 to 97 years (median age of 56 years). Being a retrospective study (2018-2022), peripheral blood samples of CML patients at diagnosis were used and it was preferred since the presence of TTVs can change during the evolution of the disease. The group of interest was selected based on the cytogenetics and molecular results (Ph+ and BCR-ABL+). This study was conducted in compliance with the principles of the Helsinki Declaration and approved by the ethics committee of the Fundeni Clinical Institute. Prior to inclusion in the study, informed written consent was obtained from all patients for the scientific use of their data.

DNA extraction

DNA was extracted from peripheral blood, using Pure Link™ Genomic DNA Mini Kit (Invitrogen, USA), according to the manufacturer’s instructions. DNA concentrations of the obtained samples were measured using a NanoDrop spectrophotometer (Spectro-photometer ND-1000).

PCR amplification

A two-steps hemi-nested PCR protocol was optimized in order to determine the presence/absence of all three

anelloviruses (TTV, TTMDV, TTMV) according to the Ninomiya et.al. technique with an additional step for hot-start enzyme. Target regions were amplified using HotStarTaq® DNA Polymerase (Qiagen, Germany) with the mixed primers NG779/NG780 (sense) and NG781/NG782 (antisense) in a reaction volume of 10 µL using 0.04 µL of each primer (Ninomiya et al. 2008). The first PCR amplification program was represented by: the initial step at 95°C/15 min, 2 cycles (94°C/2min, 55°C/30 sec, 72°C/30sec), 35 cycles (94°C/30 sec, 55°C/30 sec, 72°C/30sec) and a final extension step at 72°C/ 1 min.

All three anelloviruses genomes were amplified individually using 1 µL of each of the viral amplicons from the first PCR. We performed a second round of PCR with DNA-specific primers for the detection of TTV- NG779/NG780 (sense) and NG785 (antisense), TTMDV – NG795 (sense) and NG796 (antisense), and TTMV - NG792/ NG793/ NG794 (sense) and NG791 (antisense).

The second PCR amplification program was the following: initial step at 95°C/ 15 minutes, 2 cycles (94°C/ 2 minutes, 55°C/ 30 seconds, 72°C/ 30 seconds), 30 cycles (94°C/ 30 seconds, 55°C/ 30 seconds, 72°C/ 30 seconds) and a final extension step at 72°C/ 1 minute. Information about the primer sequences can be found in Ninomiya et.al. research. (Ninomiya et al. 2008) The PCR final amplicons were migrated in 2% agarose gel to detect a band specific to each anellovirus species.

Statistical analysis

Statistical analysis was performed using the R studio (3.4.4 version). This software was used to determine statistical significance (p-value <0.05). Also, for obtaining the phylogram of TTVs, we used Clustal X software. The basic line plot was created using https://www.bioinformatics.com.cn/en, a free online platform for data analysis and visualization.

Results

Demographic and clinical profile of study subjects

According to Table 2, there were no significant differences regarding sex or age. In terms of molecular findings, the BCR-ABL transcripts were represented by b3a2 and b2a2. In 18 patients, the coexistence of both transcripts was observed and in one case, transcripts e1a2 and b2a2 were observed.

Table 1. The clinical and paraclinical characteristics of subjects selected for this study

Variable	No. of patients
Female	37
Male	35
Mean age (years)	56
Smokers	2
Patients with leukocytosis	55
Patients with hepatosplenomegaly	30
Transcript type	
b3a2	27
b2a2	25
b3a2 + b2a2	18
b2a2 + e1a2	1

Molecular evidence of the TTV genome in CML patients

After hemi-nested PCR was performed, the PCR final amplicons were migrated in 2% agarose gel to detect a band specific to each anellovirus species. Amplification products length measured 112 to 117 bp (TTV DNA), 88 bp (TTMDV DNA), and 70 to 72 bp (TTMV DNA). It can be observed from Figure 1, we identified co-infections of two or three anelloviruses in the same sample.

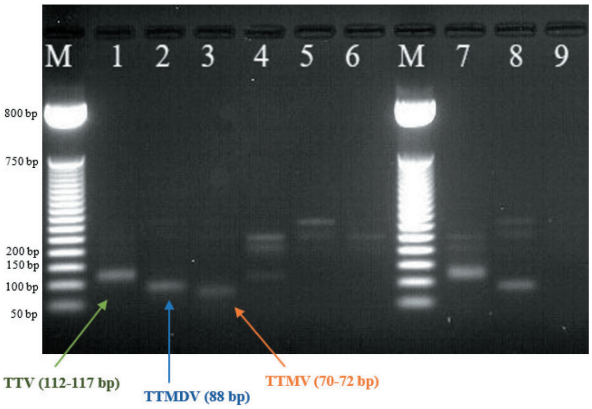

Figure 1: Electrophoresis (2% agarose) for detecting TTV, TTMDV and TTMV (M: Molecular weight marker 50bp DNA Step Ladder (Promega); Lines 1-3: patient presenting TTV-TTMDV-TTMV coinfection; Lines 4-6: patient with a positive result for TTV and negative results for TTMDV and TTMV; Lines 7-9: patient with TTV-TTMDV coinfection and negative for TTMV).

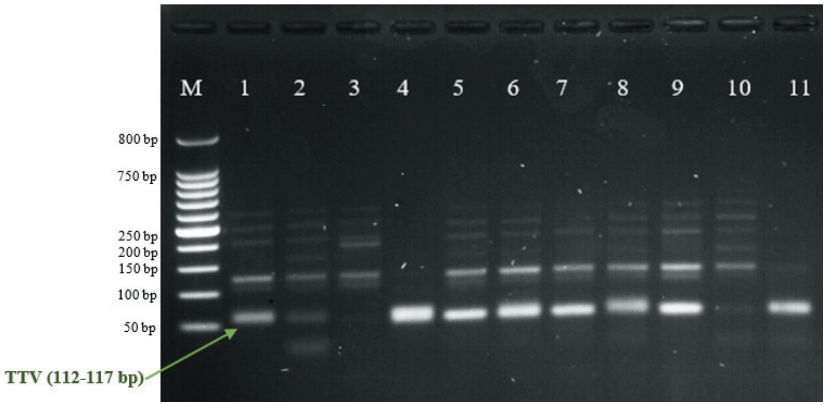

Figure 2. Electrophoresis (2% agarose) for detecting TTV (M: molecular weight marker 50bp DNA Step Ladder (Promega); 1-11: patients presenting TTV infection).

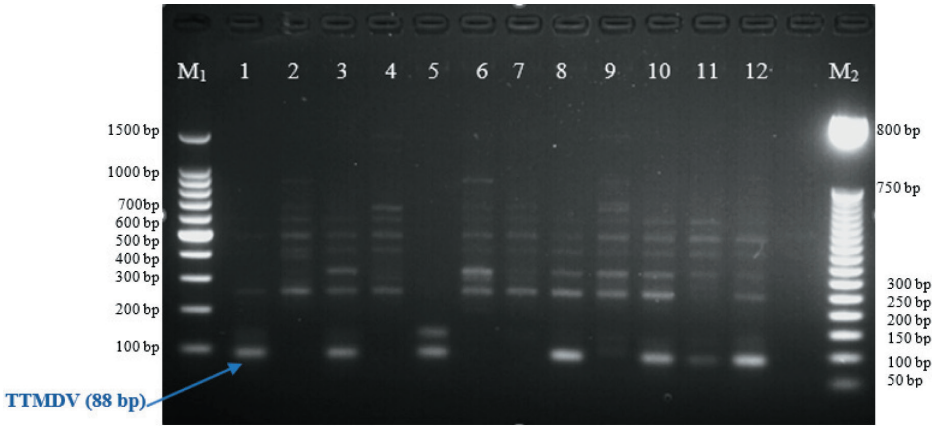

Figure 3. Electrophoresis (2% agarose) for detecting TTMDV (M_1: molecular weight marker BenchTop 100 bp DNA Ladder (Promega);1-12: patients presenting TTMDV infection; M_2: Molecular weight marker 50bp DNA Step Ladder - Promega).

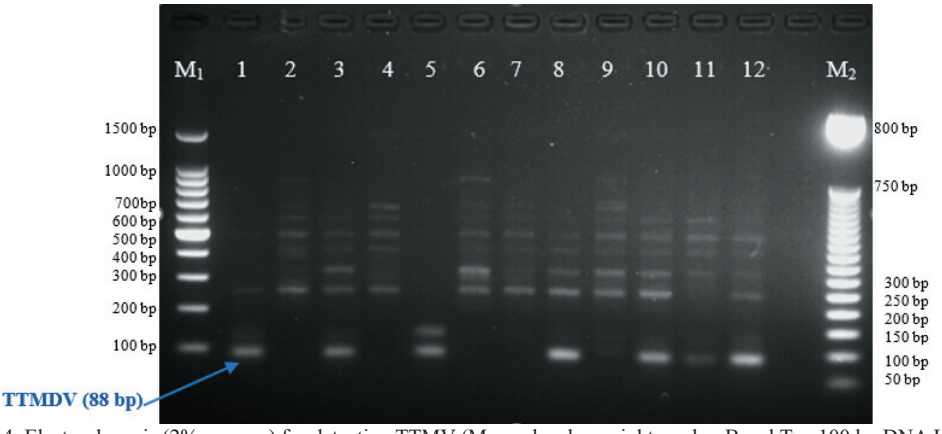

Figure 4. Electrophoresis (2% agarose) for detecting TTMV (M_1: molecular weight marker BenchTop 100 bp DNA Ladder (Promega);1-12: patients presenting TTMV infection; M_2: Molecular weight marker 50bp DNA Step Ladder - Promega).

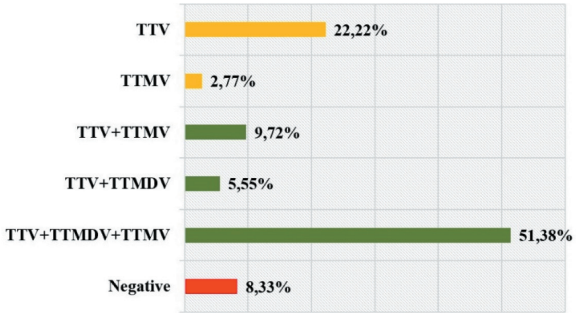

Figure 5. The variants of Torque Teno viruses identified in patients with CML (completely negative – red; single anelloviruses identified – yellow; coexistence of 2 or 3 anelloviruses – green)

Data analysis

After hemi-nested PCR was performed, we observed that the overall frequency of the 3 variants in blood samples was remarkably high (93%). Also, the majority of patients were carriers of TTV (88%), followed by TTMV (63%) and TTMDV (57%). Furthermore, an important aspect is given by the coexistence of 2 or 3 anelloviruses in the same sample. Regarding the distribution of carriers, the largest group was represented by patients for whom the presence of all 3 anelloviruses was identified (51,38%), followed by TTV (22,22%) and association of TTV and TTMV (9,72%). As can be seen from Figure 5, except for TTV, the singular presence of other viruses is rarely present in TTMV (2.77%). Also, in 8.33% of the subjects, the presence of none of the anelloviruses was identified.

As we can observe in Figure 6, the highest distribution of TTVs is registered in the case of b2a2 and b3a2, regarding the carriers of all the anelloviruses (n=13, respectively n=12). The next major category is represented by the association of both transcripts (b2a2 and b3a2) in the case of the coexistence of TTV and TTMDV (n=11). Surprisingly, although according to Figure 6, the coexistence of the 3 TTVs is found in a majority way (51.3%), we did not identify this situation in the case of any subject who was diagnosed with two BCR-ABL transcripts (b2a2+b3a2 and e1a2+b2a2).

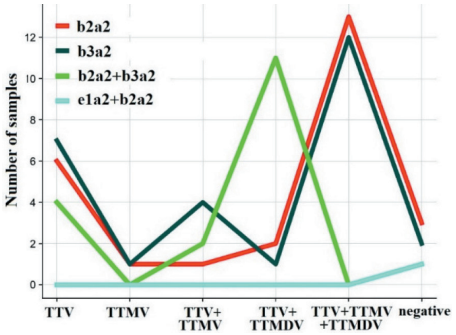

Figure 6: Distribution of TTVs based on BCR-ABL transcript type

Discussion

To our knowledge, these findings are among the few studies involving the association between TTV anelloviruses and CML. In the last decades, the study of TTVs has been full of controversies regarding their role and importance in various pathologies, especially hematological ones. Even though it has been observed that these viruses are frequently found in the general population, they seem to have a role in modulating the therapeutic response, as well as in the evolution of the disease(Spandole-Dinu et al. 2018).

In a recent study, it was observed that the presence of TTV is directly proportional to the molecular response of CML. Thus, the absence of TTV was correlated with disease-optimal molecular response (Galimberti et al. 2020). Most patients at the onset of CML were carriers of at least one of the TTVs variants. These data are consistent with those presented above, according to which the TTV load is proportional to the molecular response of patients diagnosed with CML. Since in numerous studies TTVs have been associated with bone marrow transplantation and blood transfusions, these anelloviruses seem not to be suitable as markers for immunosuppression or as prognostic markers for clinical events in patients after allogeneic transplantation (Shiramizu et al. 2002; Schmitz et al. 2020). Although there is not enough evidence to support the impact of TTV on health issues, multiple hypotheses suggest that TTVs represent a key factor in the pathogenesis of several diseases, such as acute respiratory and liver diseases, AIDS, autoimmune pathologies, and cancer (Vasilyev et al. 2009).

Due to the high variability of TTV genotypes, the distribution of TTV in different populations and pathologies is still a controversial topic (Vasilyev et al. 2009). Few studies in literature have assessed the distribution of TTVs in the global population. Most studies claim that the prevalence of TTVs depends on the PCR conditions used for detection and the amplified DNA fragment. Moreover, the prevalence of TTV genotypes might also vary based on the ethnic group of

the subjects. The TTMDV variant has also been estimated to occur with a prevalence of approximately 40% in the global population. Other research suggests that some TTV genotypes occur with higher frequency than others (Peng et al. 2015)(Spandole et al. 2015). Populational studies revealed that TTVs occurrence is higher (more than half) in Asia (China 93.3%(Peng et al. 2015); Pakistan 90% (Hussain et al. 2012); Qatar 75% (Al-Qahtani et al. 2016); India 72% (Magu et al. 2015) and Northern and Central European countries (Russia 94% (Vasilyev et al. 2009); Finland and Poland 84-88%, Czechia 52.6% (Saláková et al. 2004)). Also, it was observed a decrease in TTVs incidence in countries with warmer climates (Turkey 52% (Erensoy et al. 2002); Brazil 48% (Niel et al. 1999; Devalle and Niel 2004)) (Spandole et al. 2015; Spandole-Dinu et al. 2018; Giacconi et al. 2020).

Our data suggested that the overall incidence of TTVs in the Romanian population is higher than 90%. Considering that Romania belongs to the Central Europe region, this aspect follows the data obtained from the literature, according to which the incidence in this geographical area is higher (over 50%). Before our findings, other studies were conducted regarding the involvement of TTVs in various pathologies (2015 – diabetic nephropathy (Spandole et al. 2015); 2018 – breast cancer, chronic periodontitis, arterial hypertension, obesity, etc.(Spandole-Dinu et al. 2018)), but also regarding the incidence in the healthy population (2013)(Spandole et al. 2013). However, none of these included patients diagnosed with hematological diseases. A common term found in all these studies is the majority incidence of TTV compared to the other viruses. Also, the coexistence of all 3 viruses in the same patients was identified in high percentages. According to Figure 7, the distribution of the 3 viruses is proportional in previous studies on a group with different pathologies (TTV>TTMDV>TTMV). However, our new data suggest a different distribution of the viruses (TTV>TTMV>TTMDV), similar to the population of healthy subjects (2013) (Spandole et al. 2013). This may be due to both the differences in the pathologies studied, as well as the sizes of the groups.

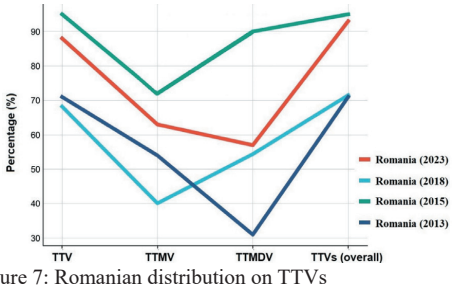

Figure 7: Romanian distribution on TTVs

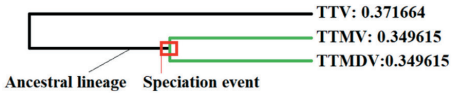

Figure 8:Phylogram of TTV viruses

Human TTV viruses (TTV, TTMDV, TTMV) are included in the Anelloviridae family. Due to the great genomic identity, the impact of these viruses on health is similar. However, it is assumed that the ancestor is TTV, from which, through evolution, TTMV and TTMDV emerged. Using Clustal X software, we obtain a phylogram, according to which, the hypothesis above is confirmed (Figure 8). Thus, the higher prevalence of TTV compared to TTMDV and TTMV (regardless of pathology and population) seems to be a matter of course. These field studies are important because they can provide answers and another perspective regarding the distribution of TTVs in different populations and pathologies.

Conclusions and future directions

Although the state of knowledge regarding the involvement of TVVs in various pathologies can be considered outdated, the impact of these viruses on hematological malignancies remains relevant.

Since our study group is represented by patients at CML diagnosis, the high prevalence of TTVs is in accordance with the high BCR-ABL load. In the future, we propose to develop new groups of patients that reflect optimal, warning and failure as responses to TKI in CML patients. According to the literature, TTVs could have an impact on therapeutic management and also on risk stratification, being able to become markers with clinical involvement in CML.

Further research and follow-up of patients with TTV are needed in the future, as well as the identification of new factors that can help to personalize treatment.

Author contributions: All authors have read and agreed on the final version of the article.

Conflict of interest: The authors have no conflict of interest to disclose.

Founding: This work received no funding.

Ethical approval: The study presented in this article was conducted following the recommendations of the Declaration of Helsinki of 1975, revised in 2013 and the Declaration of Taipei of 2016 (https://www.wma.net/what-we-do/medical-ethics/declaration-of-helsinki/). This study was approved by the Ethics Committee of the Fundeni Clinical Institute 54222/23.10.2020.

Informed consent: All participants included in the study signed the informed consent.

Disclaimer: The views expressed in the submitted article belong to the authors and are not an official position of an institution.

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Review

Insights into the roles of microbiome in non-sterile cavities cancers

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Abstract

The human microbiome represents the entire genome assembly of microorganisms colonizing the human body and comprises more than three million genes that encode thousands of metabolites, which cover several functions essential for the host health condition. Anatomical sites colonized by microorganisms include the skin, gastrointestinal, respiratory, urogenital, and reproductive tract, establishing commensal, mutual, or pathogenic relationships with the organism. The human microbiota is considered a dense 'organ' with multiple roles in nutrition, gastrointestinal tract development, and innate immunity training. Depending on the genetic predisposition, type of diet, health status, and lifestyle, this 'organ' seems to have a specific, unique signature, maintained quasi-stable, establishing symbiotic relationships with the host organism. The disruption of the dynamic balance is established between the human body and its microbiota leads to dysbiosis, which in its turn, could be the origin of a comprehensive spectrum of diseases, ranging from inflammatory, infectious, and cardiovascular diseases to cancer. In this review, we will present several types of malignancies (e.g., head and neck cancers, esophageal, colorectal, cervical, lung, bladder, and skin cancers) and the appearance of the resistance to antitumor therapies. In this minireview we present some insights regarding the implication of human microbiota in non-sterile cavities cancers.

Keywords Human microbiome, eubiosis, imbalance, cancer

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Introduction

The human microbiota comprises the totality of microorganisms that colonize the body, interact with some components, and influence its physiology. The human body is colonized by at least 2000 known microorganisms, 100 being pathogenic [WANG & al [1]), hosting 10^{13}–10^{14} microbial cells (SAVAGE [2]; BACKHED & al [3]; GILL & al [4]; URSELL &al [5]; SENDER & al [6]), more than or being close to the number of human body cells (3×10^{13}). The entire genome assembly of microorganisms colonizing the human body is called the microbiome and comprises more than three million genes that encode thousands of metabolites, which cover several functions essential for the host health condition. By other estimates, the microbiome probably exceeds 150 times the total number of human genes, estimated at 23,000 (VALDES & al [7]). The totality of effectively transcribed microbial genes forms the microbial transcriptome, the translated microbial proteins the proteome, and the metabolites produced by the microbiota the metabolome.

Being maintained in a sterile environment, the human embryo and fetus do not interact with the maternal microbiota before birth; then, during the first three years of life, the young infant experiences a dynamic evolution of the microbial communities, and by the fourth year, the composition of human microbiota becomes stable and is maintained, within limits, throughout life (PALMER & al [8]; DEKABORUAH & al [9]). Anatomical sites colonized by microorganisms include the skin, gastrointestinal, respiratory, urogenital, and reproductive tract, establishing commensal, mutual, or pathogenic relationships with the organism (OGUNRINOLA & al [10]; DEKABORUAH & al [9]). Although numerous anatomical sites vary in providing the conditions for good colonization with microorganisms, the most hospitable being the gastrointestinal tract, where 0.1–1 trillion microbial cells can coexist (PALMER & al [8]). The human microbiota is considered a dense 'organ' with multiple roles in nutrition, gastrointestinal tract development, and innate immunity training. Depending on the genetic predisposition, type of diet, health status, and lifestyle, this 'organ' seems to have a specific, unique signature, which is maintained quasi-stable, establishing symbiotic relationships with the host organism (ZOETENDAL & al [11]; ECKBURG & al [12]; WANG & al [1]). The gastrointestinal microbiota influences physiological processes such as intestinal absorption, metabolism of carbohydrates, proteins, vitamins, and other nutrients, energy supply, pathogen defense function, and early development of the immune system in newborns (SHARON & al [13]). A dynamic balance is established between the human body and its microbiota, which allows them to coexist and have mutually beneficial effects. However, at the same time, its disruption leads to dysbiosis, which in its turn, could be the origin of a comprehensive spectrum of diseases, ranging from inflammatory, infectious, and cardiovascular diseases to cancer (OGUNRINOLA & al [10]).

Particularities of human microbiota

Depending on the anatomical site, health status, personal hygiene, hormonal status, local biology, local environment, lifestyle, and type of diet of human individuals, microbial communities have different phyla and genera composition (REDINBO & al [14]). The integumentary microbiota is composed of *Actinomycetota* (*Actinobacteria*), *Bacteroidetes*, *Cyanobacteria*, *Bacillota* (synonym *Firmicutes*), and *Proteobacteria* phyla, the oral microbiota of *Bacillota*, *Proteobacteria*, *Bacteroidetes*, *Actinomycetota* and *Fusobacteria*, the intestinal microbiota of *Actinomycetota*, *Bacteroidetes*, *Bacillota*, *Verrucomicrobia* and *Enterobacteria*, respiratory tract microbiota of *Actinomycetota*, *Bacillota*, *Proteobacteria* and *Bacteroidetes*, the vaginal microbiota, predominantly *Bacillota* phylum (HOU & al [15]), and the urinary microbiota, of *Bacillota*, *Actinomycetota*, *Fusobacteriota* and *Pseudomonadota* phyla (in women) (PEARCE & al [16]), and of *Bacillota* (predominantly), *Actinomycetota*, *Fusobacteriota*, *Proteobacteria*, and *Bacteroidetes* phyla (in men) (NELSON & al [17]).

Skin microbiota

Human skin is composed of the dermis (inner layer) and epidermis (outer layer), the latter comprising layers of differentiated keratinocytes, of which the outer layer (*stratum corneum*) consists of enucleated, differentiated, squamous, interconnected cells that contribute to the barrier function of intact skin. The skin provides different types of habitats for microbial communities: oily, sebum secreting microenvironments (on the face, chest and back), which are colonized by *Propionibacterium*, *Staphylococcus*, *Corynebacterium* and *Streptococcus* bacterial and *Malassezia*, *Aureoumbra*, *Tilletia*, *Pycnococcus*, *Gracilaria*, *Pyramimonas*, *Parachlorella* and *Leucocytozoon* fungal genera; moist microenvironments, with sweat secretion (armpit, elbow crease, popliteal space, groin space, spaces between toes), which are colonized by *Corynebacterium*, *Staphylococcus*, *Propionibacterium*, *Micrococcus* and *Enhydrobacter* bacterial and *Malassezia*, *Tilletia*, *Pyramimonas*, *Parachlorella*, *Aspergillus*, *Zymoseptoria*, *Nephroselmis*, *Trichophyton*, *Gracilaria* and *Cyanophora* fungal genera; and dry microenvironments (forearm and palm), which are colonized

by *Propionibacterium*, *Corynebacterium*, *Streptococcus*, *Micrococcus*, *Staphylococcus*, and *Veillonella* bacterial and *Malassezia restricta*, *Aspergillus*, *Candida parapsilosis*, *Zymoseptoria*, *Epidermophyton*, *Pyramimonas* and *Nannizzia* fungal genera; in all these niches some viruses can be also temporarily found, but do not penetrate the skin and do not cause infection. However, the presence of fungi can cause infections (e.g., oral or vaginal candidiasis) (BYRD & al [18]) (Figure 1).

Oral microbiota

The oral cavity is a complex structure that provides connectivity to the outside and a moist environment suitable for the development of a large number of microorganisms in a dynamic balance, the disruption of which leads to dysbiosis and the development of oral or systemic diseases. The *Corynebacterium*, *Rothia*, *Actinomyces*, *Prevotella*, *Capnocytophaga*, *Porphyromonas*, *Streptococcus*, *Granulicatella*,

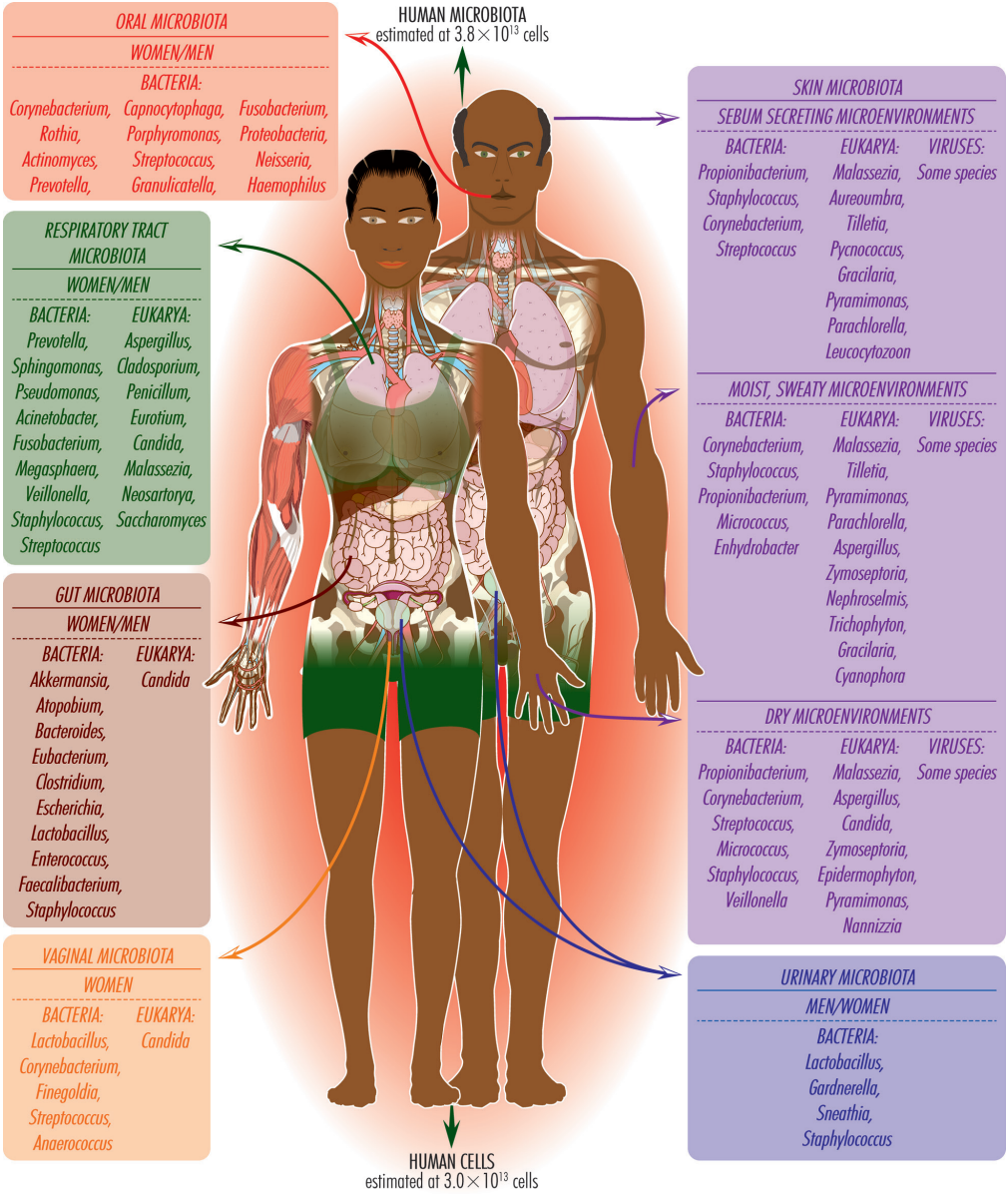

Fig.1. Main genera that colonize different anatomic niches of the human body.

Fusobacterium, *Proteobacteria*, *Neisseria*, and *Haemophilus* are among the genera most often found in the oral cavity of healthy individuals (LI & al [19]) (Figure 1).

Gut microbiota

The surface area of the human gastrointestinal tract is between 250 and 400 square meters, representing one of the most extensive interfaces between the human body's internal environment, microbiota, the immune system, and environmental factors, such as materials resulting from food digestion. During a human's lifetime, approximately 60 tons of material laden with numerous microorganisms pass through the gastrointestinal tract (THURSBY & JUGE [20]). These begin to colonize shortly after birth and include bacteria, archaea, and eukaryotic microorganisms, which establish relationships of commensalism and symbiosis in a balance that benefits both parties [BACKHED & al [3]). Of the more than 2000 species of commensal microorganisms, the majority (including at least 800 species of bacteria) (EL-SAYED & al [21]) are present in the gastrointestinal tract, where they form a real 'organ", integrated into the so-called 'superorganism' together with the human body [LI & al [19]). The commensal bacterial genera found in the gut microbiota are mainly from *Akkermansia*, *Atopobium*, *Bacteroides*, *Eubacterium*, *Clostridium*, *Escherichia*, *Lactobacillus*, *Enterococcus*, *Faecalibacterium*, and *Staphylococcus* (KHO & LAL [22]) genera, and among the fungi, *Candida albicans (*PEREZ [23]) (Figure 1). The bacterial concentration increases in the small intestine from the jejunum, where it is about 102–103 cells/gram, to the ileum, where it is about 107–108 cells/gram, to reach about 1011 cells/gram in the cecum and ascending colon and to carry out most of the metabolic reactions in the human gastrointestinal tract (NEISH [24]). In the transverse and distal colon, the concentration of microbiota decreases as it is eliminated with feces (Figure 2).

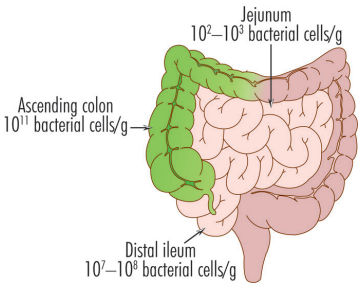

Figure 2. Increase in bacterial concentration in the intestines, from the jejunum, where the concentration is reduced, on average, to 10^2–10^3 bacteria/gram, to the ileum, where it reaches 10^7–10^8 cells/gram, and up to the cecum, ascending colon and proximal part of the transverse colon (illustrated in green), where the highest concentration of microorganisms is reached, on average 10^{11} bacteria/gram. This is where the main metabolic reactions occur, such as the fermentation of cellulose and the synthesis of short-chain fatty acids, with the microorganisms acting synergistically or antagonistically with anti-tumor therapies. From the proximal part of the transverse colon onwards, the number of bacteria/gram decreases, due to elimination with faces.

Respiratory tract microbiota

The respiratory tract is a complex anatomical entity that exchanges gases between the internal and external environment. The upper respiratory tract directs, heats, filters, and humidifies the inspired air, providing, in its compartments, varied environmental conditions for the bacterial genera *Prevotella*, *Sphingomonas*, *Pseudomonas*, *Acinetobacter*, *Fusobacterium*, *Megasphaera*, *Veillonella*, *Staphylococcus* and *Streptococcus*, and the fungal genera *Aspergillus*, *Cladosporium*, *Penicillium*, *Eurotium*, *Candida*, *Malassezia*, *Neosartorya* and *Saccharomyces* (Figure 1), which colonize them and prevent the growth of pathogenic microorganisms (SANTACROCE & al [25]).

Vaginal microbiota

The vagina is a cavitary organ, lined by the vaginal mucosa and providing specific conditions for the growth of bacteria of the *Lactobacillus*, *Corynebacterium*, *Finegoldia*, *Streptococcus,* and *Anaerococcus* genera (Figure 1), which limit the growth of pathogenic microorganisms, and *Candida* fungi, frequently associated with candidiasis (CHEE & al [26]).

Urinary microbiota

The microbiota of the human urinary tract is poorly investigated. The few studies conducted on it in healthy women categorize it into urotypes based on the relative abundance of the *Lactobacillus*, *Gardnerella*, *Sneathia*, and *Staphylococcus* genera, and individuals of the *Enterobacteriaceae* family (PEARCE & al [16]), with the genus *Lactobacillus* being involved in bladder health and frequently predominant, in women with urinary incontinence individuals of *Actinobaculum schaalii*, *Actinomyces neuii*, *Aerococcus urinae*, *Arthrobacter cumminsii*, *Corynebacterium coyleae*, *Gardnerella vaginalis*, *Oligella urethralis*, and *Streptococcus anginosus* are abundant, lactobacilli being rare (MARTINEZ & al [27]). The microbiota of the healthy male urinary tract is predominantly represented by *Bacillota*, *Actinomycetota*, *Fusobacteria*, *Proteobacteria*, and *Bacteroidetes*, with a very low abundance of *Tenericutes* and *TM7*, and is similar to that of the female urogenital tract or the integumentary or colonic microbiota (NELSON

& al [17]). The seminal microbiota of infertile men is enriched in *Aerococcus* and depleted in *Collinsella*; infertility appears to influence the rectal microbiota, which manifests decreased abundance in *Anaerococcus* and increased abundance in *Lachnospiraceae*, *Collinsella*, and *Coprococcus* in parallel with enrichment of the urinary microbiota in *Anaerococcus* (LUNDY & al [28]) (Figure 1).

Eubiosis *versus* dysbiosis

The colonizing of the body shortly after birth allows microorganisms to form simple communities, dominated by a few major bacterial groups, in different sites (predominantly in the gastrointestinal tract) and establish commensal and symbiotic relationships with it. As new species of microorganisms colonize the organism, simple communities evolve into a diverse (ROGIER & al [29]) and balanced ecosystem. Since ancient times, scientists have recognized the importance of a balance between gut microbiota components, dominated by beneficial species from the phyla *Bacillota* and *Bacteroides* (a state termed *eubiosis*). In contrast, potentially pathogenic species from the phyla *Proteobacteria* are present in a reduced proportion. In this regard, the most famous physician of ancient Greece, Hippocrates of Kos, stated over 2400 years ago that poor digestion is at the origin of all ills in the body, and death resides in the intestines (LICHTENSTEIN [30]). In the 19th century, the Russian zoologist and immunologist Ilya Ilyich Mechnikov recognized the importance of the microbiota in the gastrointestinal tract in maintaining health and triggering the disease. He said that most diseases stem from the inability of beneficial bacteria to control harmful ones, a condition he called *dysbiosis* (IEBBA & al [31]).

Eubiosis and human health

The eubiosis state is built through long-term cooperation between the host and the colonizing microbiota of the gastrointestinal tract. This is highly diverse and balanced; it includes a large number of microorganisms (KHO & LAL [22]; EL-SAYED & al [21]) and is involved in the metabolism of indigestible compounds in dietary fibers, such as cellulose, hemicellulose, pectin, xyloglucans, fructo-oligosaccharides, and oligosaccharides, making them available for intestinal absorption, providing about 10% of the body's energy and contributing to some extent to the maintenance of energy homeostasis (CHASSARD & al [32]; FLINT & al [33]). In addition, microorganisms in the gastrointestinal tract also release essential nutrients to the human body, including vitamins, can contribute to the detoxification of the body, occupy niches that opportunistic pathogenic microorganisms can colonize and affect their growth, enhance the activity of the immune system, and play an essential role in defining the intestinal architecture and maintaining the integrity of the colon (HOOPER & GORDON [34]; ROUND & MAZMANIAN [35]).

Cellulose is a polysaccharide present in plant cell walls. It consists of a linear chain of hundreds or thousands of (1→4)beta-glucose molecules linked to D-glucopyranose (CUMMINGS [36]) and fermenting microorganisms of the genera *Ruminococcus*, *Clostridium*, *Butyrivibrio*, and *Enterococcus*, as well as the species *Bacteroides cellulosilyticus*, present in the human gastrointestinal tract (CHASSARD & al [32]; FUJIMORI [37]), digest it to cellodextrin (WEIMER [38]), which can subsequently be hydrolyzed to glucose or to short-chain fatty acids, including acetic, propionic and butyric acids, hydrogen, carbon dioxide and methane (CUMMINGS [36]). With the help of microorganisms in the gastrointestinal tract, the digestibility of cellulose ingested from natural sources, such as fruits, vegetables, and cereals, is about 70-80% (PRYNNE & SOUTHGATE [39]). Pectin is a generic term for a series of polysaccharide polymers of galacturonic acid that enter the structure of plant cell walls and are digested almost entirely (CUMMINGS [40]) by the activity of microbiota in the human gastrointestinal tract by intervening in starch digestion and blood glucose regulation (by increasing the viscosity of colonic contents and inhibiting amylase activity), in the physical stimulation of the colon and the growth and balancing of the intestinal microbiota, while promoting, through fermentation, the production of short-chain fatty acids (BAI & GILBERT [41]). Xyloglucans are a group of branched polysaccharides ubiquitously present in the plant cell wall. They are metabolized by some microorganisms, such as the species *Bacteroides ovatus*, present in the gut microbiota (LARSBRINK & al [42]). Fructo-oligosaccharides and oligosaccharides, for which the human body has no intrinsic degradation mechanisms, are metabolized by species of the commensal and probiotic bacterial genera *Lactobacillus* and *Bifidobacterium* (GOH & KLAENHAMMER [43]). By metabolizing these compounds, microorganisms in the gastrointestinal tract produce 50-100 mmol·L–1 of short-chain fatty acids, which are rapidly absorbed through the colon wall, serving as precursors for the colonic mucosal lipids themselves or as a source of energy. They may also regulate intestinal motility, stimulating epithelial cell growth, inflammatory processes, and glucose homeostasis (WANG & al [1]; OGUNRINOLA & al [10]; FLINT & al [44]).

Gut microbiota (enterobacteria and species of the *Bifidobacterium* and *Bacteroides* genera) (OGUNRINOLA & al [10]) synthesize and supply the host with several essential

vitamins, including riboflavin (vitamin B2), biotin (vitamin B7/B8/H), folic acid and folate (vitamin B9), cobalamin (vitamin B12), vitamin K and other vitamins (WANG & al [1]). Riboflavin is involved in the breakdown of carbohydrates into glucose. Biotin is involved in the metabolism of carbohydrates, lipids, and proteins; folic acid and its salts play an essential role in cell regeneration, nucleic acid synthesis, and the production of red blood cells and leukocytes, cobalamin is synthesized from delta-aminolevulinate (KANG & al [45]) and serves as a cofactor for some biochemical reactions, and vitamin K is involved in the formation of proteins required for hemostasis, including prothrombin, by carboxylation of glutamic acid residues.

Food can become contaminated with toxic compounds and elements, including cadmium, mercury, chromium, lead, arsenic, etc., in small amounts but sufficient to create imbalances and induce various diseases (MONACHESE & al [46]). Metals introduced with food are sequestered by intestinal microorganisms to 40–60%, except for methylmercury, with a sequestration rate of about 10% (MONACHESE & al [46]). The divalent cadmium, Cd(II), a carcinogenic and toxic transition element, is retained in the gut by *Enterococcus faecium* resistant to this metal, which can also bioaccumulate divalent lead, Pb(II) (TOPCU & BULAT [47]; CHENG & al [48]. *Lactobacillus fermentum* and *Bifidobacterium longum* can reversibly bind these two metals (TEEMU & al [49]), and the *Lactobacillus plantarum* strain CCFM8610 has been shown to be protective against acute cadmium poisoning in mice (ZHAI & al [50]) or humans (ZHU & al [51]). Mercury is a transition metal with poorly soluble but highly toxic compounds that reach the intestine in the inorganic form, Hg(II), more soluble and with higher toxicity, or Hg2(II), less soluble and less toxic, or in the form of methylmercury, both forms affecting its microbiota. Several microorganisms resistant to mercury ions and present in the human gastrointestinal tract, *Sutterella parvirubra* and *Acidaminococcus intestini*, are involved in the degradation of mercury compounds (WATSON & al [52], especially monomethylmercury, which demethylates and reduces its toxicity (GUO & al [53]), especially in the presence of proteins. Hexavalent chromium, Cr(VI), in the form of chromate, CrO42–, and dichromate, Cr2O72– ions, which has the highest toxicity of all chromium ions, is bioaccumulated by living (23.8 mg Cr/g dry weight) and dead (39.9 mg Cr/g dry weight) cells of *Bacillus coagulans*, which can colonize the human gut only under artificial, controlled conditions, and produces lactic acid (SRINATH & al [54]). Arsenic is also a highly toxic and carcinogenic element, both in the trivalent form, As(III), interacting with the sulfhydryl (–S–H) groups present in polypeptide chains, which affect their functionality (MONROY-TORRES & al [55]), as well as in the pentavalent form, As(V), its ingestion altering the structure of the gut microbiota, favouring the development of arsenic-resistant bacterial genera, including *Bilophila*, *Desulfovibrio*, and *Bacillus* (BRABEC & al [56]), or arsenic-tolerant species, including *Escherichia coli* (WANG & al [57]). The gut microbiota plays a very important role in arsenic metabolism, influencing its oxidation states, degree of methylation, bioavailability, and excretion (CORYELL & al [58]). *Faecalibacterium prausnitzii*, a commensal species in the human gastrointestinal tract, may provide some protection against arsenic compounds (CORYELL & [59]). *Pediococcus acidilactici*, *Lactobacillus helveticus*, *and Streptococcus thermophilus* naturally present in the gut contribute to reducing the concentration of toxic organic molecules, such as polycyclic aromatic hydrocarbons, including benzopyrenes, and heterocyclic aromatic amines from fried, roasted or smoked meat products. *Lactobacillus sakei* and *Pediococcus pentosaceus* synthesize bacteriocins active against the opportunistic species *Pseudomonas aeruginosa* and *Escherichia coli* (STIDL & al [60]; BARTKIENE & al [61]).

Studies in animal models raised under sterile conditions that have never come into contact with microorganisms and studies manipulating the microbiota using selective antibiotics have provided evidence that the microbiota plays an essential role in immune homeostasis and autoimmunity (WU & WU [62]). Thus, antigen-presenting cells in Peyer's patches located in the intestinal wall synthesize higher levels of IL10 (interleukin 10) than antigen-presenting cells in the spleen (IWASAKI & KELSALL & al [63], and macrophages close to the gut microbiota develop a noninflammatory phenotype and do not produce proinflammatory cytokines when encountering microbial stimuli under homeostatic conditions (SMYTHIES & al [64]). Gut microbiota is involved in the regulation of neutrophil numbers (WU & WU [62]), IL22+NKp46+ NK cell differentiation (SANOS & al [65], and mast cell migration by expressing CXCR2 ligands on gut epithelial cells in a MyD88-dependent manner, an adaptor in the TLR signaling pathway (KUNII & al [66]). Commensal microorganisms in the gastrointestinal tract promote the residence of phagocytes, which concentrate bacterial antigens in gut-associated lymphoid tissue, activating T and B lymphocytes (YOO & al [67]). Naïve CD4+ T lymphocytes thus activated can be differentiated into four major subtypes, T helper 1 (Th1), Th2, Th17, and regulatory T cells (Treg), which produce different transcription factors and cytokines, and on CD8+ T lymphocytes, the gut microbiota plays a regulatory role, with the gut microbiota modulating the activity of plasmacytoid dendritic cells, invariant natural killer T cells, and marginal zone B lymphocytes. Present in Peyer's

patches in the intestinal wall, B lymphocytes mainly secrete immunoglobulin A (IgA) (WU & WU [62]).

Dysbiosis and human diseases

Imbalance of the gut microbiota, caused by various factors (e.g., antibiotic treatment, surgery, immunodeficiency associated with HIV1 infection), with the development of dysbiosis, underlies the development of many human diseases, including intestinal symptoms, infections, inflammatory diseases, allergies, liver disease, heart disease, metabolic disorders, psychiatric diseases and neoplasia (Table 1).

Frequently caused by antibiotic treatment, which kills or inhibits the growth of susceptible strains and favors the multiplication of resistant strains, including pathogenic ones, intestinal dysbiosis causes the appearance of clinical symptoms, including bloating (associated with *Anaerotruncus colihominis*, *Ruminococcus callidus*, *Lachnospira pectinoschiza*) (JALANKA-TUOVINEN & al [68]; BELIZARIO & FAINTUCH [69]), abdominal pain, associated with reduced abundance of bifidobacteria and diarrhea, correlated with increased abundance of *Anaerotruncus colihominis* and *Ruminococcus callidus* species (JALANKA-TUOVINEN & al [68]) and significant reduction of streptococci, especially *Streptococcus alactolyticus* species (HERMANN-BANK & al [70]; ZHANG & al [71]). Along with surgical interventions, the antibiotic treatment causes the multiplication of *Clostridioides difficile* (formerly designated *Clostridium difficile*) species (WEI & al [72]), whose toxins produce pseudomembranous colitis, and of *Escherichia coli*, *Enterococcus faecalis*, and *Enterococcus faecium*, which can cause septicemia, and *Bacteroides fragilis*, which induces intra-abdominal infections and abscesses (WILCOX [73]; ZHANG & al [71]). Periodontal disease, driven by the great multiplication of *Porphyromonas gingivalis*, *Prevotella intermedia*, *Fusobacterium nucleatum*, and *Treponema denticola* species, simultaneously with decreasing numbers of *Aggregatibacter actinomycetemcomitans* species, favors *Helicobacter pylori* infection (HU & al [74]). HIV1 infection is favored by vaginal inflammation produced by microorganisms such as *Prevotella bivia*. Colonization of the body with tenofovir-breaking microorganisms (e.g., *Gardnerella* sp.) leads to the failure of this otherwise highly effective treatment (COHEN [75]). The combination between the antiretroviral drug and a microbicidal gel leads to destroying bacteria. The microbicidal gel acts non-selectively on a broad spectrum of microorganisms, favoring uncontrolled multiplication of species from the *Bacillota* or *Bacteroidetes* phyla and disrupting, in the long term, intestinal eubiosis (COHEN [75]; LING & al [76]).

Inflammatory bowel diseases affect large numbers of people in developed regions of the world and can have several causes, including (1) interaction between commensal bacteria and the host; (2) aggressive Th1 lymphocyte-mediated cytokine response to the presence of gut microorganisms; (3) errors in recognition of commensal bacteria by macrophages; (4) defects occurring in some of the 163 loci involved in bacterial detection and clearance, which sensitize hosts and cause them to emit an exacerbated immune response to the commensal microbiota (HOENTJEN & al [77]; JOSTINS & al [78]). There are two types of idiopathic inflammatory bowel disease: (1) ulcerative colitis, localized to the colon, favored by the bacterial genera *Yersinia*, *Shigella*, *Salmonella*, *Campylobacter*, *Clostridium* and *Aeromonas*, associated with significantly reduced lactobacilli population and increased numbers of *Escherichia coli* and bacteria of the order *Clostridiales* in the active inflammatory phase, and with the presence of *Lactobacillus salivarius*, *Lactobacillus manihotivorans* and *Pediococcus acidilactici* species in the remission phase [CUMMINGS & al [79]), and (2) Crohn's disease, originally considered an autoimmune disease, but favored by a decrease of *Dialister invisus*, *Faecalibacterium prausnitzii*, *Bifidobacterium adolescentis* and *Clostridium* cluster XIVa species, and an increase of *Ruminococcus gnavus* (JOOSSENS & al [80]), or, in the pediatric form, by increasing abundance of *Enterobacteriaceae*, *Pasteurellacaea*, *Veillonellaceae* and *Fusobacteriaceae* families and decreasing abundance of *Erysipelotrichales*, *Bacteroidales* and *Clostridiales* orders representatives (GEVERS & al [81]).

The gut microbiota structure influences the development of cow's milk allergies; the abundance of *Clostridia* and *Bacillota* in the gut microbiota of 3–6-month-old infants is associated with the resolution of these allergies by the age of 8 years (BUNYAVANICH & al [82]).

The liver and the intestine interact closely, forming the gut-gut axis (GIANNELLI & al [83]). In this interaction, the liver releases the secreted compounds into the intestine, which is absorbed in the body and receives about 75% of its blood from the intestine *via* the portal vein, enriched in cellular and humoral immune components (MOROWITZ al [84]). On the other hand, through fermentation of food debris in the colon, the microbiota generates alcohol, ammonia, and acetaldehyde, which influence liver function and metabolism, and endotoxins, which influence Kupffer cell activity and cytokine production (NARDONE & ROCCO [85]). Dysbiosis of the small intestine, with impairment of the *Pseudomonadota*, *Actinomycetota*, *Bacteroidetes*, and *Bacillota* phyla and amplification of the phyla *Proteobacteria* (*Escherichia* species and other species of

the *Enterobacteriaceae* family), *Actinomycetota*, and *Bacteroidetes* (*Bacteroides* and *Prevotella* genera), the latter appearing decreased in some studies (MANZOOR & al [86]), and of the genera *Veillonella*, *Streptococcus* and *Clostridium* (CHEN & al [87]; QIN & al [88]), may contribute to the onset and progression of non-alcoholic liver disease (non-alcoholic steatohepatitis) (WU & al [89]); gut microbiota suppression after antibiotic treatment contributes to the onset of liver inflammation and Concanavalin A lectin-induced hepatitis (KAJIYA & al [90]), which can asymptomatically progress to liver cirrhosis. In cirrhotic individuals, the multiplication of pathogenic bacteria from the *Enterobacteriaceae*, *Veillonellaceae*, and *Streptococcaceae* families (CHEN & al [87]; ZHANG & al [91]; QIN & al [88]), which produce lipopolysaccharides leads to a relative decrease in indigenous commensal taxa, including *Lachnospiraceae*, *Ruminococcaceae*, and *Clostridia* (phylum *Bacillota*). Also, a decrease in *Bacteroidetes*, short-chain fatty acid-producing bacteria (BETRAPALLY & al [92]; CHEN & al [87]; BAJAJ & al [93]; MASLENNIKOV & al [94]) and of *Lachnospiraceae*, *Ruminococcaceae*, and *Blautia*, which hydroxylate primary bile acids and convert them to secondary bile acids (RIDLON & al [95]; KAKIYAMA & al [96]) has been observed in these individuals. Reduced bile acid concentration favors colonization of the small intestine with oral commensal bacteria of the species *Veillonella* sp. and *Streptococcus salivarius* (ZHANG & al [91]; CHEN & al [97]), which produce urease and break down urea into carbon dioxide and ammonia, likely contributing to endotoxemia in people with cirrhosis. The breakdown of urea due to dysbiosis and the inability of the liver to convert toxic ammonia to non-toxic urea due to cirrhosis lead to ammonia accumulating in the blood and crossing the blood-brain barrier, triggering the main complications of cirrhosis, cerebral edema, and hepatic encephalopathy (MANZOOR & al [86]).

Food-derived choline is converted to trimethylamine N-oxide in the liver, which is released into the intestine. Intestinal *Escherichia coli* converts trimethylamine N-oxide to trimethylamine, which is absorbed into the blood and can promote atheroma plaque formation and chronic heart failure (SANDEK & al [98]; DE GOTTARFI & MCCOY [99]). Intestinal dysbiosis, with reduction of *Faecalibacterium*, *Subdoligranulum*, *Roseburia*, *Eubacterium rectale*, and *Bacteroides fragilis* taxa, which regulate T-lymphocyte functions and secure an anti-inflammatory and protective response of the intestinal barrier, and colonization of the intestine with *Streptococcus*, *Escherichia*, *Shigella* and *Enterococcus* species, which adhere to the intestinal wall, disrupts intestinal microcirculation, increase intestinal permeability, favor the production of proinflammatory cytokines and amplify inflammation, with increased risk of chronic heart failure (MASENGA & al [100]). Further permeabilization of the intestinal barrier is favored by colonization with *Escherichia coli*, *Klebsiella pneumoniae*, and *Streptococcus viridians*. In contrast, the presence of *Lactobacillus brevis* has the opposite effect, inhibiting the activation of proinflammatory cytokines (ZHANG & al [91]). Colonization of the gut with *Lactobacillus plantarum* strains CECT 7527, 7528, and 7529, which assimilate cholesterol from the environment and produce bile salt hydrolysis, may help reduce cholesterol and coronary heart disease risk (BOSCH & al [101]). Increased cholesterol can trigger other cardiovascular diseases significantly when associated with hypertension. In hypertensive individuals, the gut microbiota is patchily distributed, its reduced abundance and diversity favoring the overgrowth of *Klebsiella*, *Desulfovibrio*, and *Prevotella* genera and decrease of *Blautia*, *Butyrivibrio*, *Clostridium*, *Enterococcus*, *Faecalibacterium*, *Oscillibacter*, *Roseburia*, *Bifidobacterium*, and *Lactobacillus* genera (WANG & al [102]).

With a branched-chain amino acid release, gut dysbiosis is involved in the pathogenesis of metabolic diseases, including obesity, insulin resistance, and type 2 diabetes. In obesity, the intestine is overpopulated with bacteria of the *Bacillota* phyla (e.g., *Dorea formicigenerans*, *Dorea longicatena*, *Lactobacillus reuteri*, *Staphylococcus aureus*, and some species of the class *Mollicutes* (TURNBAUGH & al [103]; COMPANYS & al [104]; HU & al [105]; GENG & al [106]) and *Actinomycetota* (*Collinsella aerofaciens*) [TURNBAUGH & al [107]; COMPANYS & al [104]), which occupy the niches vacated by *Akkermansia*, *Faecalibacterium*, *Oscillibacter*, and *Alistipe* (THINGHOLM & al [108]). Translocation of some species (*Proteus mirabilis* and *Escherichia coli*) from the gut to specific tissues induces inflammation, which affects the serum metabolome and induces insulin resistance and its progression to type 2 diabetes, *via Prevotella copri* and *Bacteroides vulgates* (MARTINEZ-LOPEZ & al [109]).

The close link between the gut and the brain has been recognized for many decades and is called the gut-brain axis. Since the gut microbiota is essential for normal brain function and involves the nervous, endocrine, immune, and metabolic systems, the concept of the gut-brain axis is extended to that of the microbiota-gut-brain axis (CRYAN & DINAN [110]). The microbial translocation into the intestinal wall and mesenteric lymphatic tissue trigger a complex immune response, releasing proinflammatory cytokines and the involvement of the vagus nerve and afferent spinal nerves. Autism spectrum disorders appear to be associated with relative decreases in the mucolytic bacteria *Akkermansia muciniphi-*

Table 1. Summary of pathological conditions in which quantitative alterations of human microbiota (dysbiosis) have been reported

Type of condition	Microorganisms with exacerbated multiplication	References	Microorganisms with decreased multiplication	References
bloating	*Anaerotruncus colihominis*, *Ruminococcus callidus*, *Lachnospira pectinoschiza*	(JALANKA-TUOVINEN & al [68]; BELIZARIO & FAINTUCH [69])		
diarrhea (incl. post antibiotic treatment)	*Anaerotruncus colihominis*, *Ruminococcus callidus, Clostridioides difficile*, *Escherichia coli*, *Enterococcus faecalis*, *Enterococcus faecium*, *Bacteroides fragilis*	(JALANKA-TUOVINEN & al [68]; WEI & al [72]; WILCOX [73]; ZHANG & al [71]).	*Streptococcus alactolyticus*	(HERMANN-BANK & al [70])
periodontal disease	*Porphyromonas gingivalis*, *Prevotella intermedia*, *Fusobacterium nucleatum*, *Treponema denticola*	[HU & al [74])	*Aggregatibacter actinomycetemcomitans*	[HU & al [74])
ulcerative colitis	*Yersinia*, *Shigella*, *Salmonella*, *Campylobacter*, *Clostridium*, *Aeromonas*	CUMMINGS [36])	Lactobacilli	(CUMMINGS [36])
Crohn's disease (adult)	*Dialister invisus*, *Faecalibacterium prausnitzii*, *Bifidobacterium adolescentis*, *Clostridium* cluster XIVa	(JOOSSENS & al [80])	*Ruminococcus gnavus*	(JOOSSENS & al [80])
Crohn's disease (pediatric)	*Enterobacteriaceae*, *Pasteurellacaea*, *Veillonellaceae*, *Fusobacteriaceae*	(GEVERS & al [81]).	*Erysipelotrichales*, *Bacteroidales*, *Clostridiales*	(GEVERS & al [81])
non-alcoholic liver disease	*Proteobacteria (Enterobacteriaceae), Actinomycetota*, *Bacteroidetes* (*Bacteroides, Prevotella*), *Veillonella*, *Streptococcus*, *Clostrisdium*	(CHEN & al [87]; QIN & al [88])	*Pseudomonadota*, *Actinomycetota*, *Bacteroidetes*, *Bacillota*	CHEN & al [87]; QIN & al [88]), MANZOOR & al [86])
cirrhosis	*Enterobacteriaceae*, *Veillonellaceae*, *Streptococcaceae, Akkermansia muciniphila*	(CHEN & al [87]; QIN & al [88]); ZHANG & al [91])	*Lachnospiraceae*, *Ruminococcaceae*, *Clostridia* (relative), *Bacteroidetes*, *Blautia*	(CHEN & al [87]; BAJAJ & al [93]; MASLENNIKOV & al [94]); (RIDLON & al [95]; KAKIYAMA & al [96])
reduced bile acid concentration	*Veillonella* sp., *Streptococcus salivarius*	(CHEN & al [87]; ZHANG & al [91])		
inflammation and increased risk of chronic heart failure	*Lactobacillus*, *Streptococcus*, *Escherichia*, *Shigella, Enterococcus*	MASENGA & al [100])	*Faecalibacterium*, *Subdoligranulum*, *Roseburia*, *Eubacterium rectale, Bacteroides fragilis*	MASENGA & al [100])
permeabilization of the intestinal barrier	*Escherichia coli*, *Klebsiella pneumoniae*, *Streptococcus viridians*	(ZHANG & al [91])		
hypertension	*Klebsiella*, *Desulfovibrio*, *Prevotella*	WANG & al [102]).	*Blautia*, *Butyrivibrio*, *Clostridium*, *Enterococcus*, *Faecalibacterium*, *Oscillibacter*, *Roseburia*, *Bifidobacterium*, and *Lactobacillus*	WANG & al [102]).
obesity	*Bacillota* (*Dorea formicigenerans*, *Dorea longicatena*, *Lactobacillus reuteri*, *Staphylococcus aureus*, *Mollicutes*), *Actinomycetota* (*Collinsella aerofaciens*)	TURNBAUGH & al [103]; COMPANYS & al [104]; BACKHED & al [3]; HU & al [105]; GENG & al [106])	*Akkermansia*, *Faecalibacterium*, *Oscillibacter*, *Alistipe*	(THINGHOLM & al [108])
type 2 diabetes	*Prevotella copri*, *Bacteroides vulgates*	(MARTINEZ & al [27])		
autism			*Akkermansia muciniphila, Bifidobacterium* spp.	(WANG & al [111])
schizophrenia	*Lactobacillus fermentum*, *Alkaliphilus oremlandii*, *Cronobacter sakazakii/turicensis*, *Enterococcus faecium*; significant multiplication of *Succinivibrio*, *Megasphaera*, *Collinsella*, *Clostridium* (*Clostridium coccoides*), *Clostridioides difficile*, *Klebsiella*, *Methanobrevibacter*	(MUNAWAR & al [112])	*Coprococcus*, *Roseburia*, *Blautia*, *Bifidobacterium*, *Lactobacillus*, *Escherichia coli*	(MUNAWAR & al [112])

la and *Bifidobacterium* spp. (WANG & al [111]), schizophrenia, with an abundance of facultatively anaerobic bacteria, including *Lactobacillus fermentum*, *Alkaliphilus oremlandii*, *Cronobacter sakazakii/turicensis*, *Enterococcus faecium*, with significant multiplication of *Succinivibrio*, *Megasphaera*, *Collinsella*, *Clostridium*, *Klebsiella* and *Methanobrevibacter* genera, and *Clostridium coccoides* and *Clostridioides difficile* species. Meanwhile, there was a reduction of *Coprococcus*, *Roseburia*, *Blautia*, *Bifidobacterium* and *Lactobacillus* genera, and *Escherichia coli*. *Clostridioides difficile* produces and releases phenylalanine derivatives, which control catecholamine levels; in schizophrenia, catecholamines, especially dopamine, have elevated levels (MUNAWAR & al [112]).

Nonsterile anatomic sites and cancer

The cancer etiology is multifactorial, including (1) genetic causes (hereditary factors related to inherited genetic predispositions, including specific mutations, weakening of loci, deletions, activation of proto-oncogenes and inactivation of tumor suppressor genes), (2) external factors, including the introduction of carcinogens or procarcinogens; (3) internal factors, related to the metabolism of procarcinogens into carcinogens, alteration of the local microenvironment and stress conditions, with the production of reactive oxygen species and attenuated or abnormal functioning of the immune system; and (4) microbial factors, especially when microbiota becomes altered (dysbiosis) and enriched in microorganisms producing compounds with a genotoxic effect..

The human microbiota is associated with several malignancies that often occur at sites colonized by microorganisms (e.g., skin, head and neck, digestive and genitourinary tract). Thus, human papillomaviruses (HPV) are involved in oral, oropharyngeal, and cervical neoplasia etiology. In addition, Helicobacter *pylori* causes gastric and probably esophageal cancer and altered gut microbiota structures are associated with colorectal cancer. Thus, adenoma and colorectal cancer are characterized by an abundance of potentially pathogenic bacteria from the genera *Pseudomonas*, *Helicobacter*, and *Acinetobacter* and decreased butyric acid-producing bacteria. In addition, the periodontal pathogen *Fusobacterium nucleatum* and the species *Bacteroides massiliensis*, *Bacteroides ovatus*, *Bacteroides vulgatus*, and *Escherichia coli* are present in high numbers during the progression from adenoma to colorectal cancer, promoting inflammation and influencing the tumor microenvironment (WANG & al [1]).

Head and neck cancers

Head and neck cancers occur in the upper aerodigestive tract (nasal cavity, oral cavity, pharynx, and larynx), more than 90% originating in squamous cells lining the mucosa (CONSTANTIN [113]) and are the sixth most common cancer worldwide (ARGIRIS & al [114]). In 2020, the number of new cases of head and neck cancers was estimated to be over 900,000 [International Statistical Classification of Diseases and Related Health Problems [115]) or 931,931, of which 699,840 new cases were in men and 232,091 new cases were in women (FERLAY & al [116]; SUNG & al [117]). Among the risk factors for head and neck cancers are: heavy smoking (active and passive) and alcohol consumption, which contribute to about 72% of cases, chewing betel quid (Areca nuts), poor oral hygiene with the colonization of the oral cavity by pathogenic microorganisms, consumption of fried, smoked or roasted meat, which introduces carcinogens and procarcinogens into the body, inhalation of chemical compounds and asbestos dust, genetic factors (TRIZNA & SCHANTZ [118]; FOULKES & al [119]; ARGIRIS & al [114]), and HPV (human papillomavirus) and EBV (Epstein-Barr virus) infections, involved in the etiology of about 25% of cases (MEHANNA & al [120]; CONSTANTIN [113]). Head and neck cancers rarely metastasize, in about 10% of cases, but are highly locally invasive, strongly affecting the physiology and functionality of the active region. Therefore, when diagnosed at early stages, they can be successfully treated by including cytoreductive surgery and/or radiotherapy/local chemotherapy treatment, with a complete cure and no long-term impairment of functionality. Most head and neck cancers are diagnosed at advanced stages when they invade and involve several anatomical structures, including locoregional lymph nodes, with impaired functionality of the area and reduced treatment options. In these cases, standard therapeutic approaches include cytoreductive surgery, radiotherapy and chemotherapy, and sometimes innovative therapies such as photodynamic therapy, immune checkpoint inhibitor therapy, oncolytic virus therapy, use of therapeutic vaccines, chimeric antigen receptor T-cell therapy, targeted therapies to treat head and neck cancers (targeting EGFR/ERBB1 (epidermal growth factor receptor), VEGF (vascular endothelial growth factor), VEGFR (vascular endothelial growth factor receptor), MET (mesenchymal-epithelial transition factor), RET, CDK4/6, FGFR, RAS, RAF, MEK, ERK, PI3K-AKT-mTOR, JAK-STAT, NOTCH, aurora kinases, cellular inhibitors of apoptosis and epigenetic modifications) and therapies using antibody-drug conjugates. Despite so many therapeutic approaches, head and neck cancers have a very high risk of recurrence,

high mortality of around 50% at five years after diagnosis (especially for primary laryngopharyngeal/hypopharyngeal tumors) and, although cured, if risk factors (e.g., smoking, alcohol consumption and consumption of Areca nuts in the form of betel quid) are present, they maintain a high lifetime risk of death. In the prognosis of head and neck cancers, oral and oropharyngeal microbiota play an important role, with *Fusobacterium nucleatum* being present at early stages and associated with good prognosis and prolonged survival (CHEN & al [121]), *Veillonella* being associated with favorable prognosis,and *Stenophotromonas*, *Staphylococcus*, *Centipeda*, *Selenomonas*, *Alloscordovia* and *Acinetobacter* being associated with poor prognosis and reduced survival (GRANATO & al [122]). HPV infection, on the other hand, generally confers a favorable prognosis and reasonable response to treatment (FELDMAN & al [123]).

Esophageal cancers

Esophageal cancers affect the upper digestive tract between the hypopharynx/laryngopharynx and the stomach, including two significant subtypes, i.e., esophageal squamous cell carcinoma, which occurs predominantly in the proximal portion of the esophagus, and esophageal adenocarcinoma, which usually occurs in the distal esophagus (YANG & al [124]). Esophageal squamous cell carcinoma originates in the lining of the esophageal squamous cell epithelium and is prevalent (more than 90% of cases) in China, Japan, and southeastern African countries (LIN & al [125]; HE & al [126]) and southern Europe [HUANG & YU [127]), and esophageal adenocarcinoma originates from glandular cells near the stomach, is more closely related to gastroesophageal junctional carcinomas or gastric cancer, and is predominant in the United States, Australia, and Western Europe (VIZCAINO & al [128]; CASTRO & al [129]; ISLAMI & al [130]). Worldwide, esophageal cancers are the eighth or ninth (if all head and neck cancers, including in the sixth position, are added together) most common cancer type, with 604,100 new cases in 2020, including 418,350 new cases in men and 185,750 new cases in women (FERLAY & al [116]; SUNG & al [117]; MORGAN & al [131]). Esophageal cancers are aggressive and have a low five-year survival rate of only 10-30%, with 24% in Australia and 36% in Japan (MORGAN & al [131]), ranking sixth in mortality (YANG & al [132]). The main risk factors for esophageal adenocarcinoma are Caucasian race, male gender, gastroesophageal reflux disease, smoking (active or passive, current or history of smoking), and obesity (HUANG & YU [127]), with neoplasia advancing in the order gastroesophageal reflux disease – Barrett's esophagus – esophageal adenocarcinoma (SHORT & al [133]; WANG & al [1]), and for esophageal squamous cell carcinoma, smoking, alcohol consumption, and achalasia (SHORT & al [133]). In both cases, esophageal microbiota may be a predisposing factor, with *Lactobacillus* overgrowth being detected in esophageal adenocarcinoma and contributing to acidification of the esophageal environment. In contrast, esophageal *Helicobacter pylori* is considered a risk factor for esophageal squamous cell carcinoma [ZHOU & al [134]). Although esophageal cancer cases have improved only slightly recently, innovative treatments may offer some favorable outcomes ((YANG & al [132]).

Gastric cancer

Gastric cancer affects the lining of the stomach and ranks fifth most common cancer worldwide, with 1,089,103 new cases in 2020, including 719,523 new cases in men and 369,580 new cases in women (FERLAY & al [116]; SUNG & al [117]), and fourth in cancer-related deaths. Thus, in 2020, 768,793 deaths in people with gastric cancers were estimated, of which 502,788 in men and 266,005 in women (FERLAY & al [116]). More than 95% of gastric cancers are adenocarcinomas, diffuse, infiltrating the gastric wall in the desmoplastic stroma and associated with hereditary genetic abnormalities, or intestinal, with the formation of mass lesions and predominantly associated with *Helicobacter pylori* infection (AJANI & al [135]). In addition to genetic factors and bacterial infection, gastric cancer is also favored by smoking, alcohol consumption, high salt intake, salted, smoked and processed meat and fish, spicy food consumption, HPV infection, sedentary lifestyle, vitamin C deficiencies (YUSEFI & al [136]). Since gastric cancer is diagnosed in advanced stages, the prognosis is guarded, with a low five-year survival rate in European and North American countries of 10-30%, but relatively good in Japan of 90%, where endoscopic digestive tract monitoring programs are implemented (SITARZ & [137]).

Colorectal cancer

Colorectal cancer affects the distal digestive tract and is the third most common type and the second most deadly worldwide. In 2020, there were an estimated 1,931,590 new cases, of which 600,896 in men and 547,619 in women, and 576,858 deaths (FERLAY & al [116]; SUNG & al [117]), and statistics indicate an increasing trend in both variables, particularly in the elderly (HOSSAIN & [138]). Risk factors for the occurrence of colorectal cancer include age (77% of people diagnosed with colorectal cancer are between 50 and 77 years of age (STEELE & [139]), family history of familial adenomatous polyposis and Lynch syndrome (hereditary nonpolyposis colorectal cancer), obesity, physical inactivity, regular alcohol consumption, active or passive

smoking, red or processed meat, colon dysbiosis with a multiplication of bacteria from the genera *Pseudomonas*, *Helicobacter*, *Streptococcus*, *Bacteroides* and *Acinetobacter*, and hormonal changes associated with advancing age [WANG & al [1]; KEUM & GIOVANNUCCI & al [140]; HOSSAIN & [138]). Colon cancer occurs in both sexes in similar proportions, metastasizes less, is easier to treat, does not require a permanent colostomy, and is prone to complete cures more easily than rectal cancer. The latter is more common in men, metastasizes intensely, predisposes to colostomy, is challenging to treat, and offers little chance of cure (KRASTEVA & GEORGIEVA [141]). Because nearly 25% of colorectal cancers are diagnosed in an advanced stage, and cytoreductive surgery alone leaves room for the development of metachronous metastases in about 20% of cases, the current primary treatment consists of tumor resection and systemic chemotherapy, along with pre- or post-prep radiotherapy, to stabilize the tumor. However, in metastatic tumors, this combination offers poor prognosis and low survival rates [MESSERSMITH [142]; KEUM & GIOVANNUCCI & al [140]). As with other cancers, cell clones resistant to chemotherapeutic agents or radiation action become selected from colorectal tumors, producing recurrences and/or metastases, necessitating the introduction of new adjuvant therapies in their therapeutic strategies. Among the usable therapeutic modalities are immunotherapy, therapies targeting VEGF/VEGFR, EGF/EGFR, HGF (hepatocyte growth factor), MET, IGF/IGF1R (insulin-like growth factor/insulin-like growth factor 1 receptor), TGF (transforming growth factor), and Wnt/beta-catenin, Notch and hedgehog signaling pathways (XIE & al [143], but also nanoparticles (KRASTEVA & GEORGIEVA [141]).

Cervical cancer

Among cancers of the female genital tract, cervical cancer is the most common. Cervical cancers can originate in the exocervix's squamous epithelium or the endocervix's glandular epithelium. However, most of them occur between the two types of epithelia in the transformation zone (AMIN & al [144]. Cervical cancer affects many women, especially in countries where prevention of HPV infection, one of the main risk factors for this time of cancer, is not in place. Globally, cervical cancer ranks seventh or eighth (in statistics that include head and neck cancers as sixth) among the most common cancers, with 604,127 new cases and 341,831 deaths in 2020 (FERLAY & al [116]; SUNG & al [117]). Risk factors for cervical cancer include HPV16 and HPV18 infections (COHEN & al [145]), HIV infections (ADLER & al [146]), sexual promiscuity, a high number of sexual partners (REMSCHMIDT & al [147]), early start of sexual life, early pregnancy (LOUIE & al [148]) and oral contraceptive pills (ASTHANA & al [149]; ZHANG & al [150]).

Lung cancers

Worldwide, lung cancers rank second in terms of the number of cases, totaling approximately 2,206,771 new cases in 2020 (1,435,943 men and 770,828 women), and first in terms of the number of deaths, with 1,796,144 deaths (FERLAY & al [116]; SUNG & al [117]). Depending on the cells in which they originate, lung cancers are categorized into small-cell and non-small-cell cancers, the latter divided into several subtypes. According to the 2015 WHO classification, the most common are adenocarcinomas, followed by squamous cell cancers and neuroendocrine tumors, including small cell carcinoma, large cell neuroendocrine carcinoma, and carcinoid (TRAVIS & al [151]). Risk factors for lung cancer include tobacco smoking (active or passive), marijuana smoking, asbestos exposure, radon exposure (dense gas resulting from the radioactive decay of uranium), air pollution with polycyclic aromatic hydrocarbons, arsenic exposure, inflammation and respiratory tract infections including tuberculosis, chronic obstructive pulmonary disease, family history of lung cancer, which increases by 1. 7 times the risk of lung neoplasia (for first-degree relatives of lung cancer patients, the risk is 2-4 times higher, even in non-smokers), older age (around 70 years and older), gender (men are twice as exposed as women) (THANDRA & al [152]). The mortality of people with lung cancers is very high (LEMJABBAR-ALAOUI & al [153]).

Bladder cancers

Bladder cancers are the most common type of cancer of the urinary system, of which urothelial carcinoma has the highest prevalence (DOBRUCK & OSZCZUDLOWSKI [154]). Approximately 75% of newly diagnosed cases have tumors that do not invade muscle tissue, with the remainder being invasive neoplasms (PENG & al [155]). Worldwide, bladder cancer ranks 11th in number of cases, with 573,278 new cases (440,864 cases in men and 132,414 cases in women) and 212,536 new deaths in 2020 (FERLAY & al [116]; SUNG & al [117]), and its development is favored by age over 55, gender (men are more likely to develop bladder neoplasia), tobacco smoking, which causes about 30-40% of urothelial carcinoma cases and up to two-thirds of all bladder cancer cases, genetic predisposition, infections (gonorrhea – *Neisseria gonorrhoeae*, , other bacterial infections and parasitosis, such as schistosomiasis – *Schistosoma haematobium*) (Figure 5), and occupational exposure to various compounds, such as 4-aminobiphenyl, 2-naphthylamine and benzidine, which accounts for 5-10% of cases (HALASEH & al [156]).

Skin cancers

Skin cancers, including melanomas and non-melanomas, including basal cell carcinoma and squamous cell carcinoma, affect the integument and are caused primarily by unprotected exposure to ultraviolet radiation. Worldwide, melanomas accounted 324,635 cases (173,844 cases in men and 150,791 cases in women) in 2020, with 57,043 deaths, and non-melanomas of the skin ranked fifth with 1,198,073 cases, of which 722,348 in men and 475,725 in women and 63,731 deaths (FERLAY & al [116]; SUNG & al [117]). Skin cancers have a multifactorial etiology, the most important risk factor being exposure to natural or artificial ultraviolet radiation during tanning sessions. Thus, for melanomas occurring on exposed portions of the skin, these appear to be the main risk factor, while for those occurring on skin not exposed to the action of UV radiation, stimulation and physical pressure of the palms and feet. For basal cell carcinoma, the most common form of skin cancer in humans, ultraviolet B radiation with wavelengths of 290-320 nm is the main risk factor, and for squamous cell carcinoma, ultraviolet radiation and long-term use of immunomodulatory drugs (OH [157]). In addition to ultraviolet radiation, the etiology of skin cancers may involve the microbiota colonizing the integument (WOO & al [158]).

Resistance to antitumor therapies

The mechanisms by which antitumor drugs manifest their therapeutic potential include inducing damage to genetic material, binding to DNA and blocking its transcription and replication, inhibiting topoisomerases, inducing antitumor immune responses, blocking receptors and ligands involved in initiating the transmission of biological signals, etc. Usually, tumor cells activate mechanisms to overcome these effects, and the resulting clones proliferate, invade neighboring tissues, and metastasize. Resistance to antitumor therapies is a significant issue, which can lead to treatment failure and further tumor progression and this phenomenon can be influence by human microbiota composition. Among the mechanisms involved in acquiring treatment resistance in tumor cells are drug efflux (which is also a main menchanism of multidrug resistance in pathogens), evasion of apoptosis (also used as a pathogenic feature by intracellular pathogens), epigenetic changes (that can be also influenced by the metabolic activity of human microbiota), DNA damage repair (trigereed by the activation of the SOS response, also active in bacteria), and altered gene expression (SEVCIKOVA & al [159]). Drug efflux is commonly encountered and occurs *via* ABC (ATP-binding cassette transporters), also present in bacteria. These represent a 48-member family, of which only ABCB1, ABCC1, and ABCG are actively involved in the transport of chemotherapeutic agents (TOWNSEND & TEW [160]). For example, ABCB1, expressed in colorectal, liver, and lung tumor cells, clears daunorubicin, doxorubicin, paclitaxel, vinblastine, and vincristine (BOGMAN & al [161]), ABCC1 is involved in anthracycline efflux, camptothecin-epipodophyllotoxins, methotrexate, mitoxantrone, and vinca alkaloids (YIN & ZHANG [162]), ABCG2 is involved in the elimination of anthracyclines and mitoxantrones from breast tumor cells (KOMATANI & al [163]). Chemotherapy- and radiotherapy-induced breaks in genetic material can be repaired by homologous recombination, mismatch repair, non-homologous splicing of ends, and nucleotide excision repair (encountered in the repair of breaks produced by platinum-based agents (REARDON & al [164]), some leading to accumulation of mutations. Thus, lesions induced by platinum-based therapeutic agents are repaired by overexpression and enhancement of ERCC1 (DNA excision repair protein) and XPF (DNA repair Endonuclease) activity (ROSELL & al [165]. Epigenetic and histone modifications, which package DNA, play an essential role in developing resistance to antitumor therapy, including altered gene expression, DNA repair, anticancer drug efflux, and the bypass of apoptosis (WANG & al [166]). They are silenced by hypermethylation of tumor suppressor gene promoters (e.g., TP53, silenced in many tumor types but not in HPV-infected tumors). By hypomethylation of oncogene promoters and MDR1 (Demethylated promoter of multidrug resistance gene 1), they are reactivated, promoting tumor progression (KANTHARIDIS & al [167]; JIN & al [168]; HOUSMAN & al [169]). Changes in the tumor microenvironment may also contribute to the acquisition of chemotherapy resistance. Thus, cancer-associated fibroblasts shape the extracellular matrix, limiting the contact of chemotherapeutic agents with tumor cells (FU & al [170]) and activation of mesenchymal stem cells, which can become tumor cells, can overcome the effects of drugs by reducing caspase three activity (VIANELLO & al [171]; TENG & al [172]). In addition to these factors, gut or intratumoral dysbiosis may have a protective role for tumor cells, conferring resistance against chemotherapy.

Capecitabine (N4-pentyloxycarbonyl-5'-deoxy-5-fluorocytidine) is an orally delivered prodrug, which, in the body, is converted to the cytotoxic 5-fluorouracil form *via* thymidine phosphorylase, predominantly active in liver and tumor cells (HUANG & al [173]). Further, uridine phosphorylase converts 5-fluorouracil to 5'-fluorouridine and then to 5-fluorouridine monophosphate (YAN & al [174]), a compound highly toxic to *Caenorhabditis elegans*. In this way, uridine phosphorylase reduces antitumor efficacy. Furthermore, it

induces the toxic effects of fluoropyrimidines (ROSENER & al [175]), while its abrogation reduces the systemic toxicity of 5-fluorouracil (CAO & al [176]). On the other hand, uridine phosphorylase converts, by phosphorolysis, the prodrug 5'-fluorouridine into 5-fluorouracil, the antitumor active form (WAN & al [177]).

Human microbiota has been shown to influence the activity of cyclophosphamide, capecitabine, oxaliplantin doxorubicin, 5-Fluorouracil, memcitabine and even immunotherapy. In some cases, the effect is synergic (e.g., due to microbial enzymes such as nitroreductase activity, stimulation of ROS release from myeloid cells by normal microbiota with the increase of the antitumoral immunity - TNF production), while in other cases microbiota acts antagonistically (e.g., increasing resistance through increasing autophagy, drug inactivation or degradation less active derivatives, upregulation of apoptosis) (CHIFIRIUC & al, 2022 [178]).

Conclusions

The dynamic balance established between the human body and its microbiota allows them to coexist and have mutually beneficial effects, while its disruption leads to dysbiosis. Imbalance of the gut microbiota, caused by various factors (e.g., antibiotic treatment, surgery, immunodeficiency associated with HIV1 infection), with the development of dysbiosis, underlies the development of many human diseases, including intestinal symptoms, infections, inflammatory diseases, allergies, liver disease, heart disease, metabolic disorders, psychiatric diseases, and neoplasia.

Investigating causal and molecular interactions between commensal microbes in mucosal body sites is expected to shed new light on human variability in cancer development, progression, and treatment responsiveness. The main challenges faced in such research are related to sample allocation, processing, sequencing, and data analysis, in addition to the need to evolve from a correlative to a causative understanding of microbial influences on cancer. Consequently, microbial contributions to cancer biology will likely take the first place in the next decade of cancer research while increasing cancer diagnosis, patient stratification, and treatment.

Author contributions

M.C., C.B., G.M. conceived and corrected the manuscript. O.C.V., S.T., M.M.M., R.E.C., I.C. contributed to the literature survey and revised the manuscript. M.C. and O.C.V. drafted the manuscript. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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