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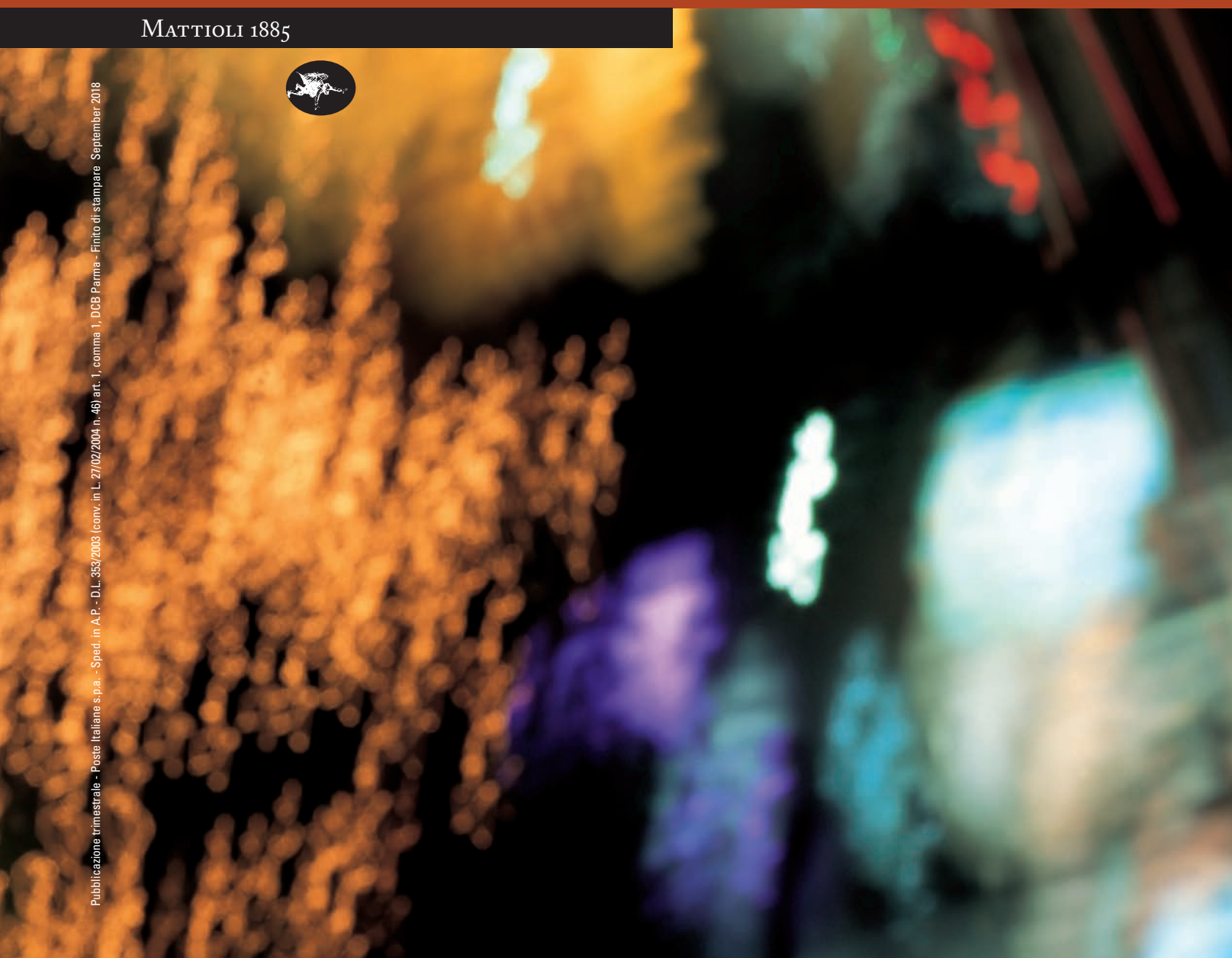

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Review

Sofosbuvir/Velpatasvir for the treatment of Hepatitis C Virus infection

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Summary. Hepatitis C Virus (HCV) infection is major health problem worldwide, with 150 million infected people according to recent epidemiologic estimations. The introduction of direct-acting antivirals made a revolutionary change in the management of HCV infected patients with surprisingly high rates of antiviral response, improved tolerability and reduced time of treatment. Sofosbuvir, in combination with different partner drugs, has been the molecule that led this incredible change. The last generation of SOF-based regimens, namely Sofosbuvir/Velpatasvir, represents a single tablet, once a day, pangenotypic and pan-fibrotic combination, demonstrated to be safe and effective in almost all type of HCV infected individuals. This review overviews the main clinical data of SOF/VEL registration trials, underlying the key features of this combination in terms of efficacy, safety and Patients Reported Outcomes obtained in more than 1800 HCV chronically infected subjects. (www.actabiomedica.it)

Key words: HCV, Sofosbuvir, Velpatasvir, Single tablet regimen, PI-free

Introduction

150 million people are infected worldwide by the Hepatitis C Virus (HCV), which is a single stranded RNA virus from the Flaviviridae family with 6 major genotypes (GTs) (1, 2). Progressive liver fibrosis is caused by chronic HCV infection, which can induce cirrhosis, hepatic decompensation, and hepatocellular carcinoma. It is estimated that the annual mortality rate of half a million people is due to liver disease associated with chronic HCV infection (3).

An estimated 35% of global HCV infections are caused by HCV GTs 2 and 3, which affect roughly 58 million people (4). Contrary to GT1, GTs 2 and 3 are diffused in low-income regions such as Latin America, Asia, sub-Saharan Africa and Eastern Europe. HCV GTs 2 and 3 were categorized together in treatment guidelines and were classified as easy to treat genotypes before the introduction of direct-acting antiviral agents (4). According to recent studies, HCV GT3 is linked to rapid disease progression and has lower rates of response to treatment compared to GT2, as particularly demonstrated in patients with cirrhosis and in patients who have not reacted to earlier treatment (5, 6).

Patients with decompensated cirrhosis caused by HCV chronic infection is set to rise in the next decade (3). Liver transplantation was the only treatment option available to these patients until recently.

An additional challenge for clinicians is the eradication treatment in the HCV/HIV co-infected population (7). In fact, HCV/HIV-coinfected patients suffer from higher rates of cirrhosis and liver decompensation disease than their mono-infected counterparts (8).

HCV treatment has recently undergone a transformation with the development of drugs that directly impede HCV replication. Effective combinations of direct-acting antiviral agents are currently available. Clinicians must consider the patient's treatment history, HCV GT and subtype, stage of fibrosis, and pat-

terns of antiviral resistance in specific cases in order to select a suitable regimen.

Regimens which include ribavirin (RBV) show a higher rate of side effects, mainly hematologic and RBV-free combinations would allow a better management of a wider range of patients, including those with a low tolerance to RBV. This would in turn minimize the necessity for pretreatment testing and monitoring during therapy, aspects that could be especially beneficial in low-income countries.

Sofobusvir (SOF) is a nucleotide analogue inhibitor of the HCV NS5B polymerase approved for HCV treatment in conjunction with other agents, which include NS5A inhibitors, NS3/4A protease inhibitors (PI), RBV, and peginterferon-RBV. Velpatasvir (VEL) (also formerly known as GS-5816, Gilead Sciences) with antiviral activity against HCV replicons in GTs 1 to 6, is a last generation, pan-genotypic HCV NS5A inhibitor.

The SOF/VEL is a single tablet, once a day regimen that combines two pan-genotypic, high potency and high genetic barrier antiviral molecules, providing >95% of SVR across all GTs with favourable safety and tolerability across a broad patient population even for decompensated cirrhotic subjects.

The SOF/VEL pill is PI, gluten, and lactose free and can be used without RBV to address unmet needs in the HCV treatment paradigm.

ASTRAL studies

An evaluation of efficacy and safety on the combination of sofosbuvir and velpatasvir was reported in different patient populations by a series of Phase III clinical trials entitled ASTRAL (ASTRAL-1, ASTRAL-2, ASTRAL-3, ASTRAL-4, and ASTRAL-5) (9-12) (Figure 1).

The ASTRAL studies demonstrated that SOF/VEL is highly effective across all GTs and different stages of liver damage, and can therefore be defined as a pan-genotipic and pan-fibrotic regimen. The following ASTRAL studies were focused on particular patient settings, providing information with regard to the efficacy and safety of SOF/VEL in subpopulations of HCV-positive subjects, which were considered as difficult to treat until now.

ASTRAL populations and study design

ASTRAL-1 included patients infected with HCV GTs 1 to 6 with different stages of liver damage up to compensated cirrhosis, with the exclusion of GT3 infected patients (9). In the current DAA therapy era, GT3 infection has been relatively difficult to treat compared to other GTs, especially in subjects with cirrhosis or prior HCV treatment failure; therefore, a dedicated clinical trial study was set-up for those infected with GT3.

Patients were enrolled at 81 sites in North America, Europe, and Hong Kong. The study was double-blinded and placebo-controlled. Patients were randomized 5:1, with the exclusion of 35 patients infected with GT5, who only underwent SOF/VEL therapy, which was attributed to the low number. A total of 624 patients received at least one dose of the drug (116 patients received a placebo), 121/624 had compensated cirrhosis and 201/624 had experienced treatment.

The results of ASTRAL-2 and ASTRAL-3 studies are reported in the same manuscript (10), focused on HCV GT2 and HCV GT3 infected populations respectively. As mentioned in the introduction section, these two GTs, previously considered as easy to treat in the IFN-era, showed lower SVR rates for DAA-based therapies (5, 6).

ASTRAL-2 and ASTRAL-3 studies shared identical inclusion/exclusion criteria, and about 20% of patients with compensated cirrhosis were enrolled. Patients who underwent previous treatment were also included (20%/total). Subjects with decompensated cirrhosis and those who interrupted previous therapy as a result of adverse events were excluded. Patients were randomized 1:1 in both of the studies, in order to receive different SOF/VEL-based regimens (12 weeks with or without RBV in ASTRAL-2, 12 weeks or 24 weeks without RBV in ASTRAL-3). ASTRAL-2, enrolled 266 patients to initiate treatment from 51 sites in the United States while in ASTRAL-3, 552 patients from centers in North America, Europe, and Australia initiated therapy.

ASTRAL-4 was dedicated to naïve and experienced HCV patients with decompensated cirrhosis (CHILD-Pugh-Turcotte class B) (11). The study enrolled patients who did not receive a liver transplanta-

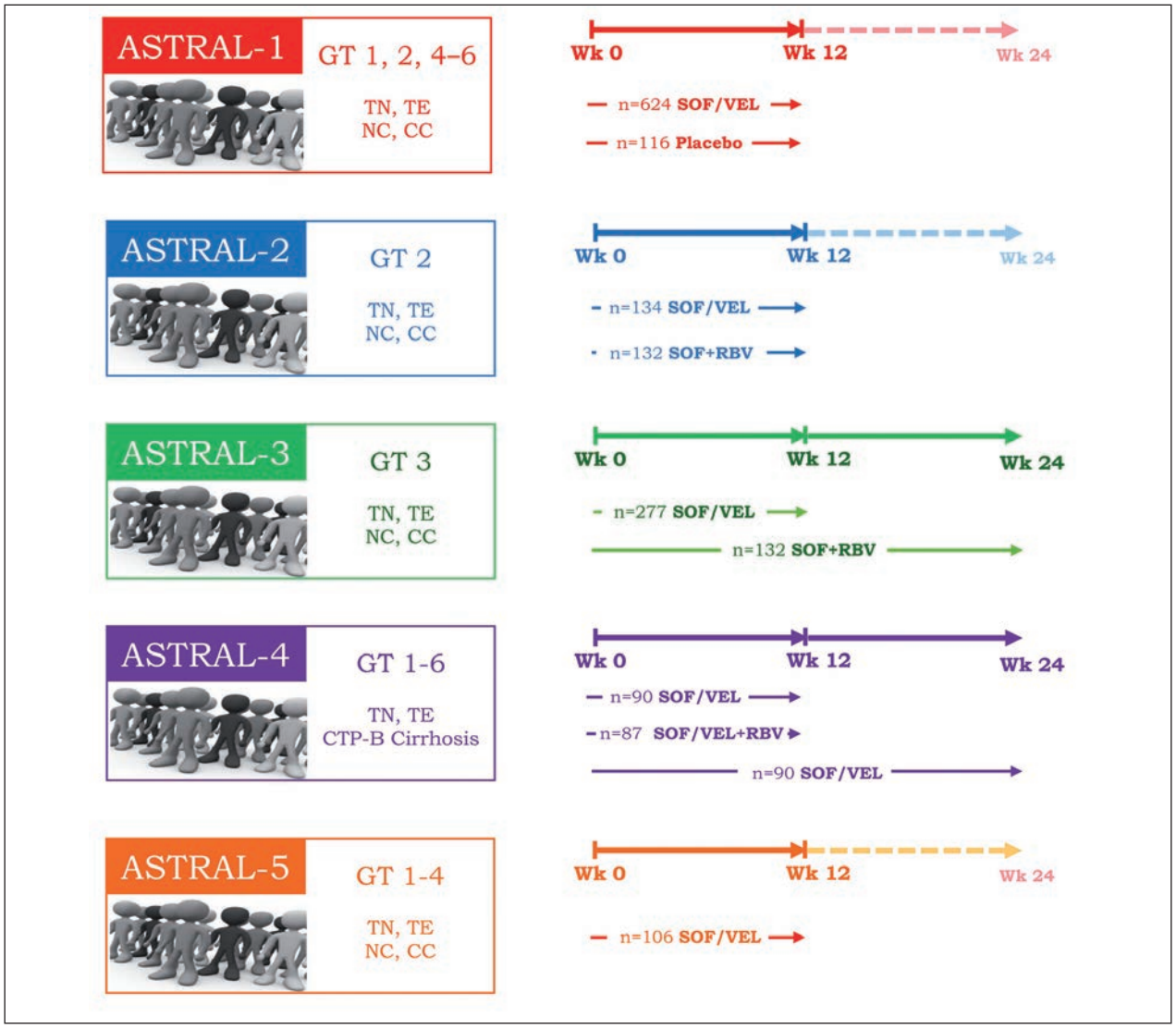

Figure 1. ASTRAL study design.
Legend: SOF: Sofosbuvir; VEL: Velpatasvir; RBV: Ribavirin; GT: genotype; TN: Treatment Naive; TE: Treatment Experienced; NC: Non Cirrhotic; CC: Compensated Cirrhosis; CTP-B Cirrhosis: Child-Turcotte-Pugh B Cirrhosis.

tion, or undergo antiviral treatment with any NS5A or NSB inhibitors, with a platelet count higher than 30,000/mm^3 and a creatinine clearance higher than 50 ml/min (Cockcroft-Gault equation). A total of 267 patients, recruited from 47 sites in the United States, initiated treatment with the following randomization: 90 patients received SOF/VEL for 12 weeks, 87 received SOF/VEL plus RBV for 12 weeks and 90 received SOF/VEL for 24 weeks. All of the HCV GTs except for GT 5 were represented.

Finally, to assess SOF/VEL efficacy and safety in HCV patients coinfected with HIV-1, ASTRAL-5, an open-label, single arm study, was performed (12). Patients were enrolled from 17 centers in the United States, and were required to be treated with an approved antiretroviral regimen, to acquire a HIV-1 viremia lower than 50 copies/mL and a CD4+ T-cell count higher than 100 cells/mL. Patients with compensated cirrhosis were also included, as well as experienced patients (excluding prior NS5A and NS5B inhibitors).

One hundred and six co-infected subjects initiated therapy consisting of a single pill of SOF/VEL once a day for 12 weeks (with the identical regimen and length for all the enrolled patients).

Efficacy across ASTRAL studies

The primary efficacy endpoint was common in all the ASTRAL studies, and was the rate of sustained virological response (SVR), defined as viremia lower than 15 IU/mL 12 weeks after therapy cessation in all the patients who received at least one dose of the drug after randomization. The secondary endpoints were different across the ASTRAL studies and depended on the specific enrolled populations and randomization.

ASTRAL-1 showed HCV infection and liver damage up to compensated cirrhosis in patients with GTs 1-6 (excluding GT3), and a SVR rate of 99% in patients who received SOF/VEL for 12 weeks, which is a significantly higher rate than the 85% value which was the pre-specified performance target. None of the subjects who received a placebo obtained an SVR (9).

The SVR rate was comparable among the different GTs (98% for GT1a, 99% for GT1b, 100% for GT2, 4, and 6, and 97% for GT5).

120/121 (99%) cirrhotic patients reached a SVR including 99.5% of experienced patients (9). Among non-cirrhotic patients 496/501 (99%) experienced a SVR (9).

ASTRAL-2 was a specifically required study by the Food and Drug Administration as a separated trial (10). The results showed a SVR rate of 99% in patients who received SOF/VEL for 12 weeks compared to 94% in those who underwent SOF plus RBV for 12 weeks. At the time the ASTRAL-2 study was performed, standard therapy showed a significant improvement in efficacy (10).

As reported in ASTRAL-3, HCV GT3 infected patients treated with SOF/VEL for 12 weeks reached a 95% SVR rate compared to 80% as shown by those receiving SOF plus RBV for 24 weeks, which is a highly significant difference in efficacy ($p<0.001$) (10). Considering non-cirrhotic GT3 patients, SOF/VEL led to SVR in 191/197 subjects (97%, while SOF plus RBV determined an SVR in 163/187 subjects (87%).

The SVR rate with all oral DAAs in decompensated cirrhosis was lower than in patients with less advanced liver disease (10).

The phase 3 ASTRAL-4 study aimed to evaluate the efficacy of SOF/VEL in the difficult-to-treat HCV-infected population, showing an eradication rate of 83% after 12 weeks of SOF/VEL, 94% after 12 weeks of SOF/VEL plus RBV, and 86% after 24 weeks of SOF/VEL (11). The SVR rate obtained from the different SOF/VEL based regimens did not show any significant differences. However, in decompensated cirrhosis caused by HCV GT3 infection, a SVR rate of 71% was previously reported (13) due to the fact that SOF/VEL plus RBV for 12 weeks resulted in 85% of the SVR.

The benefits of IFN-free therapy in advanced liver disease are still unclear. The secondary efficacy endpoints of ASTRAL-4 were linked to the improvement of liver damage, as the CPT and MELD scores changed at week 12 after therapy cessation (11). The analysis of CPT and MELD scores was performed on 250/267 patients; an improvement of CPT, compared to the baseline value, was observed in 47% of patients, and an improvement of MELD in 51% of those with a baseline value of less than 15, and in 81% of subjects with a MELD higher than 15. In general, such an improvement is due to a decrease in bilirubin levels and an increase in albumin levels (11). However, the long-term benefits on hepatic functions remain to be assessed.

Two efficacy endpoints were established in the ASTRAL-5 study, which was dedicated to the special population of HCV/HIV co-infected subjects (12). The first efficacy endpoint was common in the other ASTRAL studies and showed 95% of SVR in 106 HCV/HIV patients who underwent SOF/VEL for 12 weeks. All of the patients with cirrhosis reached a SVR (100%) along with 94% of the black patients and 94% of the experienced patients.

The secondary endpoint was the assessment of the percentage of real virological failures in patients who had viremia lower than 15 IU/mL during treatment. In fact, among the 5/106 patients not included in the SVR group, only 2 patients were virological failures (at week 4 of post-treatment), while 2 were lost during the follow-up, and 1 withdrew consent (12).

An integrated post-hoc analysis on antiviral efficacy considering the main Astral trials (Astral-1, -2 and -3) has been recently performed (14). The SOF/VEL treatment for 12 weeks in 1035 patients showed an overall SVR rate of 98% with an intention-to-treat analysis (Figure 2). The high efficacy was consistent across all genotypes, with only 2 virological relapse in GT1 and 11 in GT3 patients.

A retrospective analysis of efficacy results of SOF/VEL for 12 weeks for GT1–6 in phase 3 trials stratified by fibrosis stage has been recently proposed (15). The authors pooled patients data from SOF/VEL registration trials (ASTRAL-1 - NCT02201940, ASTRAL-2 NCT02220998, ASTRAL-3 NCT02201953) and SOF/VEL/VOX Polaris phase 3 studies (POLARIS-2 NCT02607800, and POLARIS-3 NCT02639338), where SOF/VEL treatment was considered as comparative arm.

Authors identified 1567 patients enrolled in the ASTRAL and POLARIS programs and a METAVIR category was assigned according to the FibroTest score (16). Demographics of the patient population stratified according to fibrosis score are summarized in Table 1 and Table 2.

The F0-F2 population was largely represented (n=887), with a mean age of 49 yrs, younger than F3 and F4 groups (57 and 58 yrs, respectively), as expected. GTs distribution was homogeneous between the groups with GT1 as the most prevalent except for the cirrhotic subjects where 37% of patients was infected by GT3. F4 group showed a higher proportion of experienced patients (40%) when compared to patients with milder fibrosis (20% and 28% for F0-2 and F3, respectively).

In addition to the Intention-to-treat (ITT) analysis, that considered all patients who were randomized and received ≥1 dose of assigned study drug, the Completer analysis was also performed, evaluating all patients who were randomized, completed assigned study treatment, and had HCV RNA data observed at post-treatment week 12 or imputed from a later timepoint.

SOF/VEL for 12 weeks was highly effective across all GTs regardless of degree of fibrosis as shown in Figure 3 and Figure 4 (15). Considering the Completer analysis, in the F0-F2 group almost all patients achieved an SVR (99.6%) with only 3 GT3 infected patients who relapsed out of 874 treated individuals (Figure 3). Similar high rates of response were registered also in patients with advanced fibrosis (F3: 232/234 SVR, 99.1%) and with cirrhosis (F4: 431/443 SVR, 97.2%) (Figure 4). In this latter group, high rates

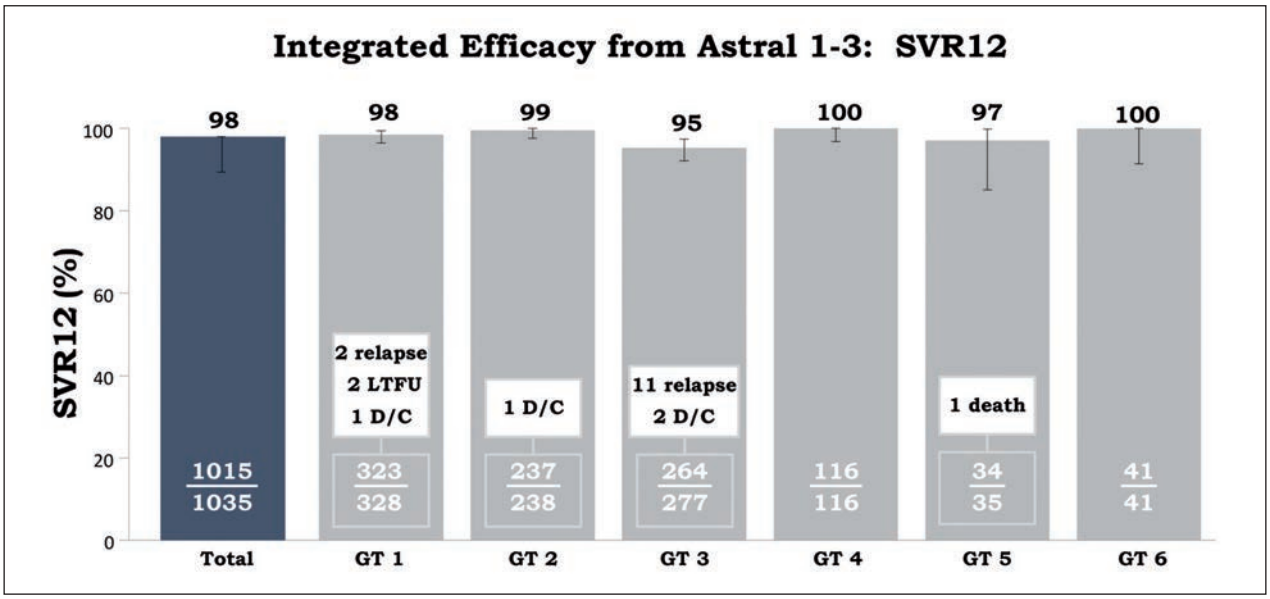

Figure 2. Integrated Intention To Treat Analysis of Efficacy from Astral 1-3: SVR12. SVR12: Sustained Virological Response 12; GT: Genotype; LTFU: Lost at Follow-Up; D/C: Discontinuation. Modified from (14).

Table 1. Demographic features of F0-F2 patients, treated for 12 weeks, from the Integrated analysis (ASTRAL and Polaris studies)

Total n of F0-F2 patients		887
Mean age, y (range)		49 (18-79)
Male, n (%)		421 (47)
Mean BMI, kg/m^2 (range)		26 (17-48)
HCV GT, n (%)	1a	205 (23)
	1b	102 (11)
	2	196 (22)
	3	227 (26)
	4	101 (11)
	5	24 (3)
	6	31 (3)
Baseline HCV RNA $\log_{10}$ IU/mL, mean (range)		6.3 (1.1-7.8)
Treatment Experienced, n (%)		177 (20)
F0, n (%)		337 (38)
F1, n (%)		160 (18)
F2, n (%)		390 (44)

Legend: BMI: Body Mass Index; GT: genotype; IU: International Units

Table 2. Demographic features of F3 and F4 patients, treated for 12 weeks, from the Integrated analysis (ASTRAL and Polaris studies)

		F3	F4
Total n of patients		236	444
Mean age, y (range)		57 (33-81)	58 (34-82)
Male, n (%)		162 (69)	355 (80)
Mean BMI, kg/m^2 (range)		28 (18-57)	28 (17-47)
HCV GT, n (%)	1a	67 (28)	107 (24)
	1b	26 (11)	48 (11)
	2	34 (14)	58 (37)
	3	77 (33)	164 (37)
	4	21 (9)	49 (11)
	5	5 (2)	5 (1)
	6	6 (3)	13 (3)
Baseline HCV RNA $\log_{10}$ IU/mL, mean (range)		6.3 (4.0-7.4)	6.2 (4.1-7.5)
Treatment Experienced, n (%)		66 (28)	176 (40)

Legend: BMI: Body Mass Index; GT: genotype; IU: International Units

of SVR were obtained also in GT3 patients (154/163, 94.4%) without the need for Ribavirin.

The ITT analysis showed minor differences, since only 16 patients out of 1567 were excluded from the Completer analysis (15).

Safety across ASTRAL studies

Rate of adverse events (AEs) and treatment discontinuation because of AEs was the secondary end point of the ASTRAL-1 study (9). Treatment was in-

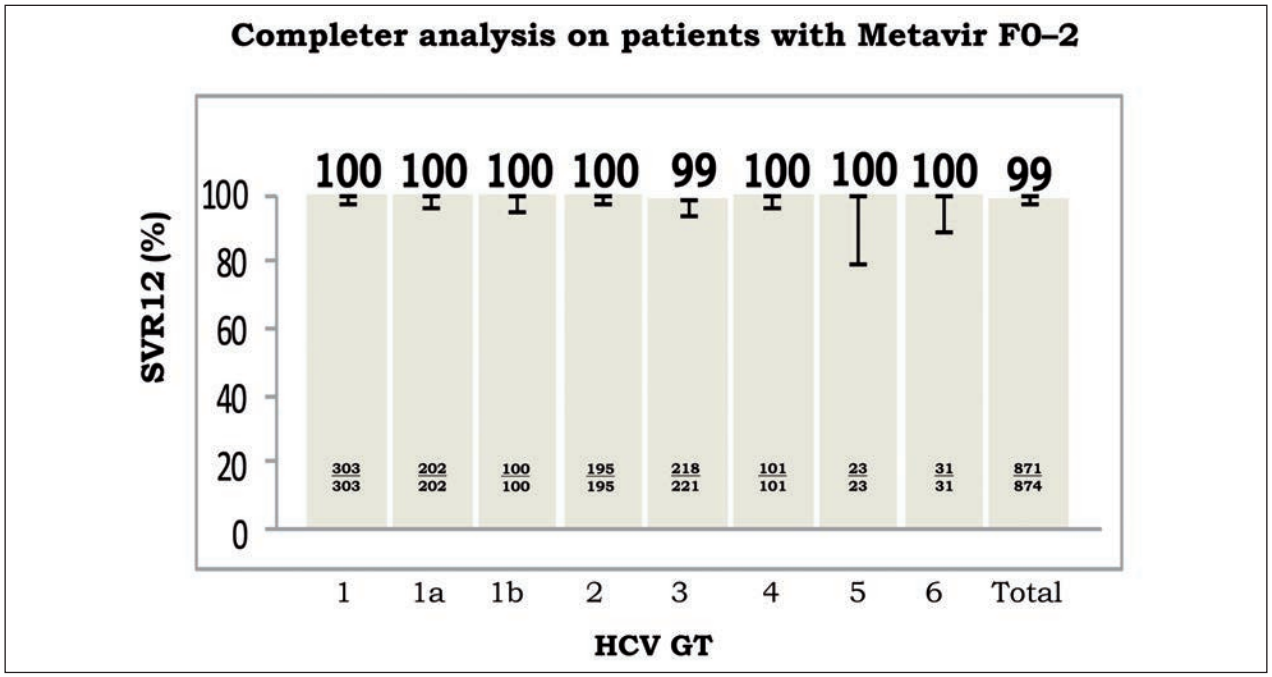

Figure 3. ASTRAL-1, -2, -3 and POLARIS-2, -3 combined retrospective analyses of efficacy in patients with METAVIR F0-F2, treated with Sofosbuvir/Velpatasvir for 12 weeks. Patients were treatment naïve and treatment experienced (including PI-failure); SVR: Sustained Virological Response; GT: Genotype. Modified from (15).

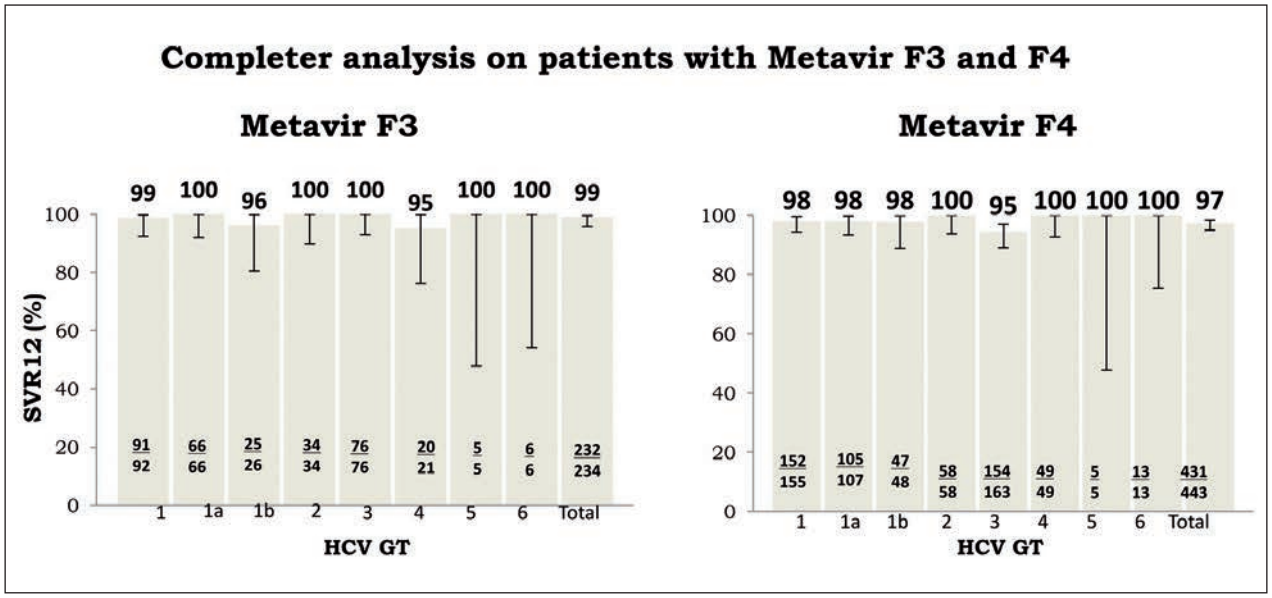

Figure 4. ASTRAL-1, -2, -3 and POLARIS-2, -3 combined retrospective analyses of efficacy in patients with METAVIR F3 and F4, treated with Sofosbuvir/Velpatasvir for 12 weeks. Patients were treatment naïve and treatment experienced (including PI-failure); SVR: Sustained Virological Response; GT: Genotype. Modified from (15).

terrupted by 1 patient (<1%) in the SOF/VEL group and by 2 patients (2%) in the placebo group. Serious AEs occurred in 15 patients (2%) treated with SOF/VEL and in none of the patients who received a placebo. Overall, AEs (mostly headache, nausea, fatigue and nasopharyngitis) were recorded in 78% of sub-

jects who underwent SOF/VEL therapy, and in 77% of those in the placebo group, without any significant difference (9).

In the ASTRAL-2 and ASTRAL-3 studies, a patient included in the ASTRAL-2 study interrupted treatment after the first pill due to anxiety and a headache (10). In ASTRAL-3, RBV was only discontinued by 9 patients (3%) as a result of AEs. In the ASTRAL-2 study, the percentage of serious AEs was the same in those who received and those who did not receive RBV (1%); in the ASTRAL-3 study, 2% of subjects who did not receive RBV experienced serious AEs compared to 15% of those who received RBV. Considering both studies, AEs were generally frequent in patients who underwent RBV-including regimens and the types of AEs were typical of RBV (anemia, insomnia, irritability and coughing). Two ASTRAL-2 patients died during the post-treatment follow-up and 3 ASTRAL-3 patients died during treatment. All the deaths seemed to be due to causes unrelated to therapy or were categorized as unknown (10).

As expected for the severe condition of the study population in terms of liver damage, the serious AEs rate was higher in the three groups of the ASTRAL-4 study with hepatic encephalopathy and sepsis being the most frequent and serious AEs (11). For the same reason, the nine deaths that occurred during the study were thought to be unrelated to treatment and were possibly ascribable to the end-stage of liver disease. Anemia was very common in 30% of patients who received RBV, and was experienced at a different level of severity.

ASTRAL-5 had a proportion of patients who interrupted treatment due to AEs as a primary safety end-point (12). In fact, 71% of patients experienced at least one AE, which was serious in only 2 cases (2%) and led to therapy discontinuation in one case. Another patient interrupted therapy as a consequence of a mild adverse event (a single vomiting episode) at day 48 and reached SVR12 regardless. None of the patients died and in none of the cases, the ARV was modified (12).

Pruritus was not observed in any patient of the ASTRAL studies among the AEs (9-12).

Overall, the SOF/VEL regimens demonstrated a very good safety profile in all the ASTRAL studies, which covered a wide range of the different features that are typical of HCV-chronically infected patients. Nevertheless, in the case of other concomitant treatments, caution is required in order to avoid drug-to-drug interactions (DDI) (17). SOF/VEL is not recommended for patients treated with amiodarone due to the risk of severe symptomatic bradycardia if taken together (17). Other drugs reduce SOF/VEL efficiency (antacids and proton pump inhibitors, some anticonvulsants, antimycobacterials, and chemotherapy topotecan) (17-19). However, the SOF/VEL regimen presents a very good DDI profile, which represents the best option in multi-treated patients with co-morbidities, in women of child-bearing potential, and in active drug users or in opioid substitution therapy (19-21). This makes SOF/VEL regimen suitable also for patients using recreational drugs, generally not mentioned during the anamnestic evaluation.

SOF/VEL can also be administered in patients with mild or moderate renal impairment, even if it is not recommended for patients with more severe renal damage (eGFR\30 mL/min/1.73 m^2).

The tolerability of SOF/VEL for 12 weeks was retrospectively assessed by an integrated safety analysis in more than 1000 patients treated in the ASTRAL-1, ASTRAL-2, and ASTRAL-3 studies (22). As showed by Table 3, SOF/VEL was well tolerated in HCV-infected patients with similar incidence and severity as in placebo treated subjects (Table 3). As reported in Table 4, the most common AEs emerging in SOF/VEL group from the integrated analysis were headache, fatigue, nausea, and nasopharyngitis, whose incidence was similar in placebo undergoing patients (Table 4).

Health-related quality of life and work productivity analysis in the ASTRAL studies

Patients with chronic HCV infection, usually have a poor health-related quality of life and impaired work productivity (23, 24). The patients reported outcomes are directly described by the patient and pertain to the patient's health, quality of life, or functional status associated with health care or treatment. The effect of SOF/VEL on PROs in HCV-patients included in the ASTRAL studies was performed (25, 26), and a

Table 3. Retrospective integrated analysis of data from 1,035 SOF/VEL for 12 Weeks patients and control/placebo patients in ASTRAL-1, -2, and -3

Patients, n (%)	SOF/VEL 12 weeks N=1035	Placebo 12 weeks N=116
AEs	821 (79)	89 (77)
Grade 3 or 4 AEs	33 (3)	1 (<1)
SAEs	23 (2)*	0
AE leading to treatment D/C	2 (<1)^	2(2)
Death	3 (<2)**	0)

Legend: SOF: Sofosbuvir; VEL: Velpatasvir; AEs: Adverse Events; SAEs: Severe Adverse Events; D/C: Discontinuation; *No SAE was assessed as related to SOF/VEL; **None were assessed as related to study treatment; ^Two subjects D/C SOF/VEL for AEs; (1 D/C day 1 due to difficulty concentrating, headache, and anxiety and 1 D/C day 13 of due to anxiety)

Table 4. More frequent adverse events from the retrospective integrated safety analysis of data from 1,035 SOF/VEL for 12 Weeks patients and control/placebo patients in ASTRAL-1, -2, and -3

Patients, n (%)	SOF/VEL 12 weeks N=1035	Placebo 12 weeks N=116
Headache	296 (29)	33 (28)
Fatigue	217 (21)	23 (20)
Nausea	135 (13)	13 (11)
Insomnia	87 (8)	11 (9)
Nasopharyngitis	121 (12)	12 (10)
Cough	57 (6)	4 (3)
Irritability	49 (5)	4 (3)
Pruritus	33 (3)	5 (4)
Dyspepsia	33 (2)	4 (3)

Legend: SOF: Sofosbuvir; VEL: Velpatasvir; severe adverse events were rare in SOF/VEL-treated patients, with headache, anxiety, and acute myocardial infarction occurring >1 patient (both cases of acute myocardial infarction were assessed as not related to SOF/VEL treatment by the investigators)

comparative analysis between patients with and without cirrhosis was also conducted (27, 28).

The analysis performed on the ASTRAL-1 patient groups showed that patients treated with SOF/VEL experienced a significant improvement in PROs during treatment and after SVR. In the placebo group, only one PRO improved by week 4 of treatment, and no further improvements were noted (25).

ASTRAL-2 and ASTRAL-3 populations were analyzed with regard to PROs in a dedicated study with a total of 818 HCV patients (25, 26). As previously mentioned, the overall rates of all adverse events were lower in the RBV-free SOF/VEL group (all $p<0.03$) and, therefore, patients who received RBV-free SOF/VEL regimens, reported significantly higher PRO scores during treatment compared to those who received the RBV-containing regimen (SOF plus RBV) (25, 26). At post-treatment week 12, changes from baseline levels were no longer different between the two treatment arms (25, 26).

Finally, a comparative analysis of PROs during and after SOF/VEL treatment in HCV patients with and without cirrhosis, from ASTRAL studies (1 to 4) was performed by Younossi and co-workers (27, 28). As expected, baseline PROs were lower in patients with cirrhosis, but, during SOF/VEL treatment and after reaching the SVR, subjects with and without cirrhosis experienced a significant improvement in the scores (27, 28).

In general, the administration of SOF/VEL produced a significant improvement in patients' quality of life, resulting in a benefit for the patients going beyond the SVR, as demonstrated by the PROs analysis of patients' perception of the treatment (25-28).

Conclusions

Data from phase III clinical trials on Sofosbuvir/Velpatasvir demonstrated that this antiviral combination addresses many unmet medical challenges. SOF/VEL make HCV treatment easier as the same therapy schedule are suitable for all the genotypes, irrespective of the fibrosis stage, making SOF/VEL a pan-genotypic and pan-fibrotic regimen. The presence of SOF warrantees high efficacy and minimal DDI and the combination with VEL, a last generation NS5A inhibitor, makes this regimen the standard of care for the treatment of chronic HCV infection.

The single-pill, once-a-day posology improves the adherence to the therapy and the absence of lactose and gluten make it suitable to patients intolerant or allergic to these substances.

SOF/VEL is a RBV-free regimen and in naïve non-cirrhotic patients attains SVR rates up to 100% in all genotypes. In decompensated cirrhotic patients, SOF/VEL, with the addition of RBV, resulted in 94% of SVR.

Actually, SOF/VEL is safe and effective on all-stages of liver disease, including decompensated cirrhosis, thanks to the absence of protease inhibitors

As a pangenotypic and pan-fibrosis regimen, it is conceivable that SOF/VEL will simplify, or perhaps eliminate, the pre-treatment assessments and on treatment monitoring that represent a barrier to treatment access in several countries. Considering the characteristics of SOF/VEL, this regimen can be considered the ideal partner in the path to HCV eradication.

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Review

Flow and volume response to bronchodilator in patients with COPD

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Summary. The response to a bronchodilator is considered as crucial to diagnose COPD and to distinguish COPD from asthma. COPD is characterized by progressive airflow obstruction that is only partly reversible, whereas asthma is associated with airflow obstruction that is often reversible either spontaneously or with treatment. In spite of the partly reversible airflow obstruction, patients with COPD may show a significant bronchodilator response both in terms of an increase in forced expiratory volume in 1 second (FEV1) or in forced vital capacity (FVC) after an adequate dose of an inhaled bronchodilator. Changes in FEV1 or FVC characterize, respectively, flow or volume response after bronchodilator administration. This overview will deal with the reversibility testing characteristics and its clinical significance in COPD patients. (www.actabiomedica.it)

Key words: COPD, airflow obstruction, bronchodilation

Introduction

The assessment of the bronchial response to drugs with bronchodilator activity plays an important role in the characterization of airflow obstruction in order to ascertain whether or not its reversibility can occur (1). From the clinical point of view, the response to a bronchodilator is considered as crucial to diagnose COPD and to distinguish COPD from bronchial asthma. COPD is characterized by progressive airflow obstruction that is only partly reversible (2), whereas asthma is associated with airflow obstruction that is often reversible either spontaneously or with treatment (3). It is of note that there is a group of individuals having characteristics of both COPD and asthma. Patients with asthma-COPD overlap syndrome (ACOS) include patients with COPD and eosinophilia, smoking asthmatics, long-standing asthmatics with airway remodeling and steroid-resistant asthmatics with neutrophilic inflammation (4). In spite of their clinical heterogeneity, patients with ACOS show a significant response to the bronchodilator (5).

Interestingly, in the general population, the bronchodilator response is not a nominal, all-or-nothing type, response, but has a continuous, Gaussian-type, distribution. This distribution was proved in the Eclipse study both in smoking subjects with or without bronchial obstruction and in healthy subjects (6). Given this normal distribution, some patients can experience a paradoxical response to β2 agonist, by showing a bronchoconstriction effect. Interestingly such a response, defined as a decrease in forced expiratory volume at 1st second (FEV1) and/or forced vital capacity (FVC) of 12% from baseline and 200 ml in absolute terms in the post-bronchodilator spirometry, was found in 5% of cases of a large smoking population with or without bronchial obstruction (7). The pathophysiological mechanisms underlying the paradoxical response to the bronchodilator are not known, even if the study by Bhatt et al (7) showed a higher prevalence

of this response in the Black ethnic group and in patients with a higher rate of comorbidities.

This brief overview will deal with the reversibility testing characteristics and the clinical significance of the test in COPD patients.

The reversibility test

The bronchodilation test consists in a inhalation of a dose of a bronchodilator drug-acting after baseline spirometry, followed by a second forced expiratory maneuver to record any change in FEV1, FVC and in FEV1/FVC ratio, which is considered as the index of airflow obstruction. Changes in FEV1 or FVC characterize, respectively, flow or volume response after bronchodilator administration. The most commonly used drug for the test belongs to the class of short acting β2 agonist and is represented by Salbutamol at a dose of 400 mcg (1). However, this test has been also performed by using lower doses of Salbutamol (200 mcg) or other drug classes, such as antimuscarinics (i.e., Ipratropium bromide, 80 mg), alone or in combination with β2 agonists. The amount and type of drug used can influence the results. Notably, in subjects suffering from COPD it has been highlighted a better response when the β2 agonist is associated with the antimuscarinic (8). Thanks to the synergistic interaction between β2 agonists and antimuscarinic agents, when these drugs are given in combination, a more effective bronchial smooth muscle bronchodilation can occur (9). Therefore, a first important variable in the bronchial reversibility assessment is represented by drug class and dosage used during the test (Tab. 1).

Baseline condition may also influence the bronchodilator response. Post-bronchodilator percentage change is related to the baseline FEV1; low baseline FEV1 value is associated with more relevant percentage gain. For this reason, ATS/ERS guidelines added also an absolute increase in the amount of 200 ml, as a requirement for the positive test (8). Alternatively, to minimize this aspect, the choice of an absolute increase equal to 10% of predicted FEV1 has proved equally as effective (10). According to the ATS/ERS guidelines, bronchial reversibility test is considered as positive whether a 12% increase from baseline in FEV1 and/or FVC and 200 ml in absolute value in post-bronchodilator curve is reached (1) (11). Other criteria to consider the bronchodilator response as positive are a post-bronchodilator FEV1 percentage increase greater than 15% of baseline, an increase in post-bronchodilator FEV1 greater than 10% of the predicted value (10) or an absolute 400 ml increase in FEV1 (6). Evidence has shown how the ATS/ERS threshold tends to exceed normal variability values and placebo inhalation response (10) (12).

Table 1. Drugs used in the reversibility testing

Drug class	Drug name	Dose	Pharmacology
β_2 agonist	Salbutamol	200 mcg 400 mcg	The binding to the β_2 receptor on smooth muscle cell membrane activates the adenylate cyclase enzyme leading to an increased cAMP synthesis. The following PKA (protein kinase A) activation brings to myosin light chains phosphorylation with transition in the inactive form and bronchodilation. The onset of action is within 15 minutes and lasts 3-4 hours.
Anti muscarinic	Ipratropium bromide	80 mcg	This drug has antagonistic action on muscarinic M_2 and M_3 receptors. M_3 receptor binding blocks phospholipase C action which normally activates the cascade of inositol triphosphate (IP3) and diacylglycerol (DAG); the first one is involved in calcium release from the sarcoplasmic reticulum, the second one in the opening of calcium channels with subsequent contraction of smooth muscles. The stimulation of these receptors on glandular epithelial cells surface increases mucus and secretions production. The onset of action is within 5 minutes and lasts 6 hours.

Table 2. Procedures relating a Bronchodilation test

1.	Assess lung function at baseline
2.	Administer 400 mcg Salbutamol through a spacer
3.	Re-assess lung function after 15 minutes
4.	An increase in FEV1 and/or in FVC ≥12% and ≥200 mL constitutes a positive bronchodilator response

Measurements of lung volumes before and after bronchodilators add sensitivity when examining for bronchodilator responsiveness. Notably, in hyperinflated patients, the measurement of FVC before and after bronchodilator administration identifies a response that may not be uncovered by the FEV1 measurement (13). Bronchodilators reduce hyperinflation and FVC improvements after bronchodilator administration are related to the reduction in residual volume (RV), functional residual capacity (FRC) and total lung capacity (TLC), which also results in an increase in inspiratory capacity (IC), a parameter linked to the improvement in exercise tolerance and dyspnea perception (10).

Daily FEV1 and FVC variability in healthy subjects, regulated by sympathetic rather than parasympathetic nervous system prevalence on bronchial caliber, is approximately 150-180 ml in absolute terms (8% as a percentage) (1). It has been also estimated that in obstructive disease this variability is about 2-3 times higher than in normal subjects (14). It was also shown that the minimum clinically appreciable FEV1 change amounts to approximately 100-140 ml (15). In any case, the use of threshold values for distinction between responders and non-responders subjects involves a certain degree of arbitrariness, since the bronchial response to a bronchodilator is a variable which is continuous and normally distributed (8) (10).

COPD and bronchial reversibility

According to the GOLD statement, reversibility testing plays a key role in the diagnosis of COPD, that is made in case of FEV1/FVC post-bronchodilator <70%; again, according to the statement, the severity degree classification is evaluated by considering the post-bronchodilator FEV1 value (11). Importantly in COPD patients, the lack of bronchodilator response in the lung function laboratory does not preclude a clinical response to bronchodilator therapy (1).

Although it is essential for the diagnosis of COPD, the bronchodilator response does not seem to be a constant and reproducible characteristic in patients with COPD. In a cohort of patients subdivided into 3 groups (COPD, active smokers and non-smokers), the bronchial reversibility (only evaluated as a response in terms of FEV1) was studied on 4 occasions during a year (6). It has been shown that only 16% of patients considered to be reversible according to the ATS/ERS criteria during the first visit kept this feature in all subsequent checks, while 66% of patients who were irreversible in the first control maintained that characteristic during the subsequent visits (6). Between permanently bronchodilator responders and non-responders patients no difference was found between the main clinical outcomes, such as mortality and severe exacerbations. However, in the same study, irreversible patients in at least 3 out of 4 occasions were characterized by a worst lung function, more severe emphysema revealed by low-dose CT and at greater risk of exacerbation. Another interesting finding of the study (6) concerned the FEV1 increase after bronchodilation, which was of comparable extent, when active smokers and COPD subjects were considered, but it was lower when non-smokers were considered.

In any case, given the intra-individual variability in reversibility tests, it is conceivable that a single test is not a reliable indicator nor any long-term response to inhaled bronchodilating therapy nor can be used as a parameter for distinguish patients into responders and non-responders (6). On the other hand, it has been shown as a negative response to bronchodilator test does not affect a long-term response to bronchodilator therapy in patients with COPD (16).

Clinical and functional features of COPD patients, who show a significant response in terms of flows with improvement in FEV1 (flow responders), of volume with increase in FVC (volume responders) or both of them are still under investigation (Figure 1). In a large population sample of patients with bronchial obstruction and severe parenchymal hyperinflation (TLC>133% predicted), only one third of the subjects improved significantly in terms of FEV1 after admin-

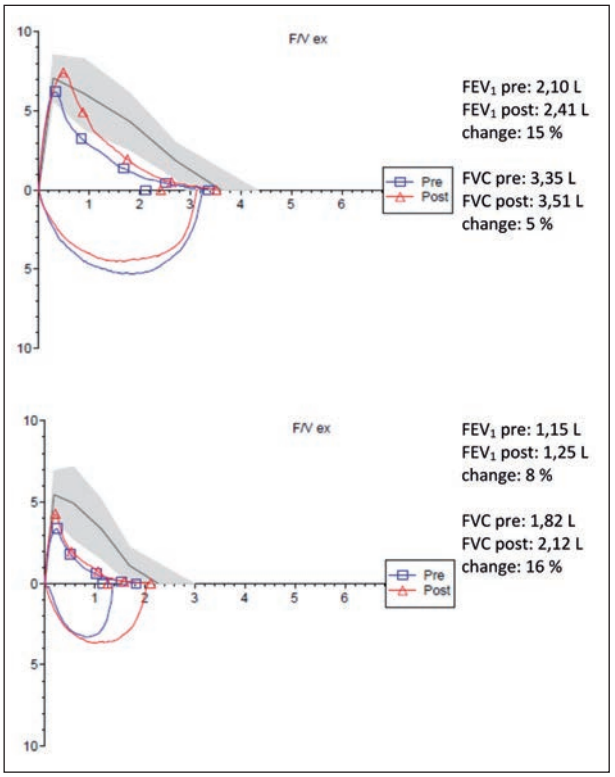

Figure 1. Flow volume curves in a flow responder patient (above) and in a volume responder patient (below)

istration of salbutamol (13). However, in the same study, significant bronchodilator percentage response was up to three-quarters of the subjects if were also considered changes in terms of FVC (13). When the bronchodilator response in terms of FEV1 and FVC was considered in relation to the four GOLD classes distribution (17), it has been shown that patients belonging to the first GOLD classes (that is, patients with mild moderate COPD) tend to be more responsive in terms of flows, a feature that is lost in severe forms, where a volume response prevails.

In patients with COPD, the impulse oscillometry system (IOS) application has also brought to light a relationship between response to the bronchodilation test and small airways dysfunction. In a large cohort of COPD patients, the small airway dysfunction, expressed as an increase in peripheral airway resistance by IOS, was related to the bronchodilator responsiveness in terms of FVC, but not in terms of FEV1 (18). The small airways dysfunction was also associated with the worst airflow obstruction degree, with a major degree of hyperinflation and worse clinical conditions (18).

Taken together these studies (13, 18, 17) show that in COPD patients the volume responder pathophysiological trait is characterized by severe obstruction with marked hyperinflation, emphysema phenotype, small airway dysfunction and poorer health status.

Since FVC correlates with static volumes, such as VR, FRC and TLC, the response in terms of volumes can reduce patient's hyperinflation, ensuring an improvement in inspiratory capacity and consequently a better exercise tolerance and a lower dyspnea perception (10). In such patients, characterized by long time constants, bronchodilator allows a better lung emptying reducing air trapping and the residual volume with displacement on the chest-lung curve to a more favorable level and consequent reduction in the work of breathing.

Conclusions

The reversibility testing is unavoidable to confirm the diagnosis of COPD in at risk patients, such as patients who smoke or have exposure to pollutants, patients who have symptoms, such as cough, sputum or dyspnea or patients who have a family history of chronic respiratory disease (2).

The bronchodilator response in COPD is a function of baseline FEV1 and increases in case of β2 agonist and an antimuscarinic agent are administered in association. Furthermore, this response in COPD, as in general population, is a normally distributed continuous variable and the categorization in responders and non-responders is based on arbitrary criteria. Indeed, this response cannot be used to phenotype patients, since it has been demonstrated that there is considerable intra-individual variability for that parameter. Also for this reason, a negative response does not preclude a long-term treatment with bronchodilators.

Any bronchodilator response can be evaluated in terms of a significant change in flows or volumes. Flow response prevails in COPD patients with mild to moderate degree of airflow obstruction, while volume response prevails in patients with severe one and/or with small airways dysfunction.

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Review

Anesthesia protocols in laboratory animals used for scientific purposes

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Summary. *Background:* A suitable, effective and free of complications anesthetic protocol is very important in experimental studies on animal models since it could bias the outcome of a trial. To date there is no universally accepted protocol for induction, maintenance and recovery from anesthesia. The endotracheal intubation with the use of inhalation anesthesia is used very especially in the from of large size laboratory animals, because it is a secure and easy control mode. However, it is not common for small laboratory animals because of the high technical skills required. *Aim:* The aim of this paper is a review of the main methods of induction of anesthesia in laboratory animals. *Materials and methods:* We performed an electronic search of MEDLINE (PubMed interface), ISI Web of Science and Scopus using the keywords "anesthesia" and "animal (s)" or "protocol (s)" or "surgery", without the data or the language restriction. We consider only the most common laboratory animals (rats, mice, rabbits, pigs). We identify all the scientific articles that refer to the use of anesthetics for studies on laboratory animals in all areas: experimental surgery, CT, MRI, PET. All documents identified the search criteria are subject to review only by identifying relevant studies. *Conclusions:* There is a strong need for application of existing guidelines for research on experimental animals; specific guidelines for anesthesia and euthanasia should be considered and reported in future studies to ensure comparability and quality of animal experiments. (www.actabiomedica.it)

Key words: experimental surgery, laboratory animals, induction and maintenance of anesthesia, inhalation anesthetic, anesthetical drugs

Introduction

Laboratory animals are sometimes used in experimental clinical studies such as pre-marketing of a drug or a medical-surgical device or in regenerative medicine and surgery. The anesthesia protocols influence the survival of laboratory animals and can also greatly affect the experimental data results. To date, there is no anesthetic protocol widely used for single laboratory animal species. The murine species (rats and mice) is the most used model in various research fields, such as for organ transplantation, regenerative medicine and imaging. The pigs are animals that are used for the search, since their cardiorespiratory physiology is very similar to humans (1). The pig animal model, however, is extremely sensitive, so the primary objective is therefore to provide a quiet environment without causing anguish and stress and it should be also adequately sedated for transport also ensuring normothermia (2). The lagomorphs model is instead an animal model of medium size useful, for example, in studies in which the murine model is too small and pig model too big. Four aspects are of paramount importance for a correct management of the trial: a correct inhalation anesthetic, effective anesthesia, the duration of the entire experimental procedure and a correct protocol of endotracheal intubation (3).

In general, anesthesia can affect some physiological parameters, such as pressure, blood oxygen saturation, cerebral blood flow and many other factors that may affect the postoperative follow-up. The majority of anesthetic agents decrease the cerebral metabolism and they often affect the neurotransmission of nerve impulses, for which, the body temperature and other physiological parameters, should be monitored during anesthesia (4).

Methods

Search strategy

We performed an electronic search of MEDLINE (PubMed interface), ISI Web of Science and Scopus using the keywords "anesthesia" and "animal (s)" or "protocol (s)" or "surgery" in

"Title/Abstract/Keywords", without the data or the language restriction, to identify all the scientific articles that refer to the use of anesthetics for studies on laboratory animals in all areas: experimental surgery, CT, MRI, PET. All documents identified the search criteria are subject to review only by identifying relevant studies.

Overall, 27 publications were identified, 8 of them have been excluded according to our study criteria. Each experimental study on animal model we tested was approved by the "Organismo Preposto al Benessere Animale" (OPBA), as required by current regulations. In total they have been taken into account and analyzed 19 scientific studies regarding the use of anesthesia in laboratory animals for different surgical procedures and not.

Premedications

The anesthesia is commonly used in laboratory animals, and can be induced by different methods depending on the type of study and the type of animal taken into account.

Konno et al., for the sedation in rats, used a closed glass chamber, where inside is fed a mixture of isoflurane (Escain®) at a concentration of 5% with airflow used as a carrier gas for 1 min (5).

After sedation and intubation of the subject, it is used by Konno et al. a mixture, called «M / M / B: 0.3 / 5.4» described by Kawai et al (6) e Kirihara et al. (7) as an anesthetic injected intraperitoneally at a dose of 0,3 mg/kg b.w. of medetomidine (Domitor®), 4,0 mg/kg b.w. of midazolam (Dormicum®) e 5.0 mg/kg b.w. of butorphanol (Vetorphale®) as premedication (5).

Hedenqvist et al. suggest to use sufentanil-midazolam combination as premedication in rabbits (8) and medetomidine like anesthesia protocols in small laboratory animals. Parenteral anesthetic combinations such as ketamine and xylazine are suggest like the agents of choice for anesthesia in the rabbit, because they are effective, easily administered and inexpensive (9). The ketamine/xylazine/acepromazine combination is also a useful regimen for normovolemic animals when anesthetic duration greater than that produced by ketamine/xylazine alone is required (9).

Re et al. creating a mixture of lidocaine, ketamine (10) and an opioid (0.6 mg ketamine /kg/h and lidocaine 3 mg/kg/h combined with morphine 0.24 mg/kg/ or fentanyl 0.0045 mg/kg/h) administered during premedication, they have noticed no significant decrease in the minimum alveolar concentration of volatile anesthetics administered in pig models (11). However, the effectiveness of this combination shows marked variations and opioids are likely to be less effective in pigs than in other species (11).

Induction and maintenance of anesthesia

The induction of anesthesia in small animals is carried out, in most cases, using anesthetic gas.

According Risling et al. the open-drop delivery of isoflurane or sevoflurane is an effective tool to anesthetize mice and small animals. The volatile concentration needed to induce anesthesia in mice following the application of 0.5 ml of anesthetic in an induction chamber volumes of 725 mL to 87.6 kPa and 20˚C, measured by a gas analyzer of precision. Anesthesia was induced with isoflurane at concentrations of 6,80±0,57% after 35.70±6.95 s while using sevoflurane induction is significantly longer (45.50±9,96 s) and requires concentrations of gas greater than (7.41±00:57%). Animals taken into the study had a rapid recovery both by using isoflurane than with sevoflurane (12).

The twelve Wistar rats studied by Konno et al., after premedication and induction of anesthesia intraperitoneally, were intubated with endotracheal tube and connected to the circuit for inhalation anesthesia with isoflurane maintained at a concentration 3.0% for males and 2.5% for females for a period of 5 min. Subsequently, the concentration of isoflurane is reduced to 2.5% for males and 2.0% for females up to the interruption of anesthesia (13).

During anesthesia, all rats should be heated on a hot plate. All intubations have ended successfully within 1 minute, and the values of vital signs measured up to 30 minutes after the monitoring were normal and stable. Moreover, the histopathological observation of the trachea and the lungs carried by Konno et al. showed no trauma despite endotracheal intubation is not easy in small animals and requires technical skill and special equipment (13). These results suggest that endotracheal intubation is a reliable, safe and environment with regard to the welfare of rats (14).

On a study reported by Imai et al. of 8 experimental models lagomorphs (white New Zealand rabbits), it was used an anesthetic gas line that provides for the administration of halothane or isofluorane. In this study it is seen that it is preferable to use the halothane as it gives a less respiratory depression during anesthesia than using isofluorane (15, 16).

Hedenqvist et al. have evaluated the possibility of finding an alternative to using isoflurane to maintain anesthesia in rabbits (8). In the study published in 2015 they have made 18 compared Himalaya rabbits divided into two groups of equal number: they were premedicated with 0.1 mg kg (-1) medetomidine and 5 mg/kg of carprofen subcutaneously, followed by the induction of intravenous sufentanil (2.3 mg mL) and midazolam (0.45 mg mL). After endotracheal intubation, anesthesia was maintained with sufentanil-midazolam in 9 subjects and Sevoflurane in the remaining 9. There were no significant differences between the two groups. In rabbits treated with sevoflurane, however, mean arterial pressure decreased in the pre-surgical phase, the heart rate increased by 25% during and after surgery, and body weight decreased by 4% after surgery (8).

For bigger animal models such as the pig, Pehböck et al. recommend starting the anesthesia by injection of ketamine and propofol followed by endotracheal intubation during spontaneous breathing (3). It is therefore necessary the presence of a specialist in anesthesia for a correct management of the airway of the animal in order to avoid dangerous complications such as death. The vascular access can be provided by a cut-down (skin incision for insertion needle-venous cannula) or ultrasound-guided techniques in the groin or in the neck region (17).

Jalde et al. of nine pigs premedicated with a bolus intramuscular injection of ketamine 10 mg/kg intravenous dose of propofol 2mg/kg injected prior to intubation have noted that very high doses of propofol caused sudden arrhythmias and refractory with circulatory collapse in some animals in the studio. Therefore, it is recommended, according to the authors, infusing low doses of ketamine intravenously in order to reduce the total amount of propofol. Anesthesia was maintained with sevoflurane which promotes a low Vt (Tidal volume) and less influence of propofol the neuro-ventilatory efficiency (18).

After intubation, maintenance anesthesia is performed by Pehböck et al. on the pig model with morphine or piritramide, propofol and rocuronium (3). Normothermia (38.5˚C) must be guaranteed (19).

Mikkelsen et al. use propofol and remifentanil infusion but have noticed, especially after a single bolus of remifentanil, a lowering of cerebral oxygenation levels although within normal limits (20).

There are few centers that perform a check of the subject during the study procedures in laboratory animals. According to the study carried out by Uhlig et al. no control during anesthesia were described in 439 cases out of 732 (60.0%) of interventions involving the use of anesthesia. In the remaining procedures 293/732 (40.0%) involving anesthesia, the use of monitoring techniques have been described during the only anesthesia 114/293 (38.9%), the experimental procedures 26/293 (8,9%) or in both cases in 113/293 (38.6%) of the interventions. In 40/293 interventions (13.7%) is no monitoring was specified (21).

Post-anaesthesia monitoring

Post-anesthetic monitoring is very important in the recovery phase of the laboratory animals. It is im-

portant to control the side effects that might be from general data, such as heart rate, body temperature and the concentration of gases and electrolytes in the blood, as well as it is important to assess the reflex responses. According Fleischmann et al. mice should be awakened in their cages and evaluate the heart rate, body temperature and the degree of pain for at least 24 hours (22).

There are many methods tried to assess pain in rats that holds the account or vital signs and can rely on an accurate animal inspected (23). Arterial blood gases exam, recommended postoperatively, can reveal acidosis, hypoxia, hypercapnia and an increased concentration of glucose (22). To induce waking in rats, as well as set a pain relief, it is appropriate to use anesthetic antagonists to ensure a faster awakening. Fleischmann, using naloxone-flumazenil-atipamezole, noted that rats regained consciousness after 110±18 s and are quickly returned to the physiological basal values. Without antagonist instead mice showed marked hypothermia (22±1.9°C) and bradycardia (119±69 beats/min) to several hours (22). The effect of anesthesia induced in rabbits is antagonizzabile in 25-25 min with rapid animal's recovery time. Fundamental becomes the monitoring of body temperature, heart rate and oxygen saturation in the blood according to Flecknell studies (24).

In bigger animals models, such as pigs, it is good to have an adequate observation center for a few days in the frame of reference so that you can transfer the animal immediately after surgery and avoid a second sedation for transportation. The recovery phase in the large animals is slower and requires support and continuous monitoring in the later stages upon awakening (25).

Discussion

Anesthetic agents which are most frequently used (ketamine, propofol, isoflurane/halothane) to induce and maintain anesthesia in laboratory animals influence the carbon dioxide tension in arterial blood (PaCO2) or exhaled (as ETCO2) and can cause respiratory acidosis. They must therefore be carefully monitored all the vital parameters of the animal and restoring fluid and electrolyte balance in the event that it were altered.

Ketamine typically increases cerebral blood flow and indirect sympathetic mimetic effects on the metabolism of the brain (26) increasing the plasma concentration of norepinephrine and, being an antagonist of NMDA receptors, it can also determine neuronal damage known as Olney lesions (27). All these combination of drugs used in rabbits xylazine-ethyl-(1-methyl-propyl) malonyl-thio-urea salt (EMTU), ketamine-EMTU, xylazine-pentobarbital, xylazine-acepromazine-ketamine (XAK), ketamine-chloral hydrate and ketamine-xylazine can induce a depression of respiratory rate. Although rectal temperature values were reduced to some degree in each group, the most profound hypothermia was induced by XAK (28). Propofol is a short-act anesthetic drug that readily crosses the blood-brain barrier; its effect starts after a minute. It is rapidly cleared from plasma, and the consciousness returns more quickly with propofol that with other anesthetic drugs. Propofol allows a better cerebral autoregulation most other anesthetic agents (29). Isoflurane and halothane allow ga ood control of anesthesia duration and deepness (30). The anesthesia can also affect the blood glucose levels and lipid concentration that may indirectly affect brain metabolism (31).

The cerebral metabolism may also be affected by changes in body temperature, and in particular that hypothermia is common during prolonged anesthesia in small animals. Hyperglycemia, for example, can greatly increase the risk of global cerebral ischemia (32) since the fluctuations in blood glucose levels can greatly affect brain function by modulating the mechanisms and neuroprotective properties of the blood-brain barrier. Blood glucose levels should be monitored carefully during maintenance of anesthesia in order to avoid both hyper- and hypoglycemia (4). Medetomidine commonly used to sedate laboratory animals can cause hypotension and respiratory depression, especially at low doses, while not reduce cerebral blood flow (33). The drugs mainly used in different anesthesia protocols in the literature can cause numerous side effects that could change the success of a clinical trial and damages the animal model "quod vitam". Monitoring of vital signs and animal welfare must be safeguarded during all study procedures (34).

Conclusions

This systematic review revealed insufficient reporting of methods of anesthesia in experimental studies. The studies are always with a low number of laboratory animals. In addition, this review shows that there is a strong need for guidelines in research on experimental animals; specific guidelines for anesthesia and euthanasia should be considered and reported in future studies to ensure comparability and quality of animal experiments. This is very important to translate experimental results in (future) clinical applications.

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Thomas Percival. Discussing the foundation of Medical Ethics

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Summary. *Background and aim of the work:* In 1803, the English physician Thomas Percival published *Medical Ethics*, a work destined to become a milestone in the development of modern codes of medical ethics, starting from the first edition of the American Medical Association's ethical code. Notwithstanding the undeniable influence that this book has exerted upon the codification of the principles of medical ethics, researchers and experts foster different and opposing points of views on its real nature. They question whether *Medical Ethics* truly belongs to the literary genre of codes of medical ethics or, better yet, to that of medical etiquettes. *Methods:* This debate is crucial in the field both of medical history and of medical ethics, with regard not only to Percival's work, but also to the ethical value of the current codes of medical ethics and deontology. *Results:* The lack of a rigorous philosophical-moral analysis of the current medical codification is reflected in its mere loyalty to the legal regulation, in substantial continuity with the past. However, the constant challenges proposed by the biomedical development, require the need to rethink the traditional conceptual tools of the current codes of medical ethics, with the purpose to achieve new schemes and innovative solutions. *Conclusions:* On this perspective, when the codes of medical ethics are worked out by physicians, they could be considered as wrongly titled medical etiquettes. This consideration could regard current codes of medical ethics, that remain faithful to tradition and that would more probably be codes of medical etiquette with a wrong title. (www.actabiomedica.it)

Key words: history of medicine, history of medical ethics, medical ethics, code of medical ethics, medical etiquette

Introduction

As the eighteenth century drew to an end, Europe experienced the effects of urbanisation as a consequence of the industrial revolution. A great part of the population moves into big cities for economic opportunities, but they also often face diseases, misery and indigence. Thanks to the doctrine of the Enlightenment that breathes new life into the principle of tolerance, an emerging sense of help and support toward lower classes starts to enliven the modernisation of hospitals, which are now seen as medical institutions devoted to the social dimension of public health.

This notwithstanding, soon hospital institutions turn out to be difficult to manage. The main causes of the problem seem to stem from the medical practice itself. The presence of compulsory regulations and statutes, characterised by strict policies on available resources undermines the prescriptive authority of physicians and surgeons. Furthermore, several inner contrasts overshadow the medical profession, which is divided into three main groups: physicians, surgeons and pharmacists. Periodically, these tensions result in hostilities and threaten the *esprit de corps* and unity of the medical profession and as a consequence its stability and development.

Within this difficult context, the English physician Thomas Percival conceived his *Medical Ethics* (1), that has successively inspired modern codes of medical ethics (the first example was the Code of ethics by the American Medical Association, dated 1847), which organically collect the rules of conduct for physicians. However, another mainstream of thought states that it is not possible to address to Percival the foundation of medical ethics' codification in Western society (2, 3). If this second perspective would be the correct one, how could we qualify modern codes of medical ethics? Should these codes be discussed with regard to their belonging to the field of medical ethics? Or differently to Percival's code, modern codes should be treated as part of medical ethics on the basis of their upgrade?

In any case, Percival's work is crucial both in the field of study of history of medicine and in that of medical ethics, in that it reconstructs the history of the codification of medical ethics' evolution. From this perspective, the recognition of its importance is widely shared, even if its knowledge within the field of history of medicine, medicine and moral philosophy is, especially in Europe, not so widespread as it would deserve.

1. Medical Ethics or Medical Jurisprudence?

Together with the problems that are oppressing medical facilities, in 1789 Manchester is swept away by an epidemic of typhus, which seriously endangers the operational efficiency of its hospital.

In order to solve the organisational problems, the managers of the hospital double the number of the staff members. Unfortunately, the increasing number of the personnel causes several internal quarrels, which in 1791, as the epidemic is still flaring up, lead even to the closure of the ward dedicated to the care fever. The subsequent scandal forces the managers to designate one of the hospital's most authoritative members, Thomas Percival, to work out a code of conduct with the aim of disciplining the behaviours of the staff (4).

Many similar works precede Thomas Percival's *Medical Ethics*. Amongst others: *Lectures on the Duties and Qualifications of a Physician* by John Gregory, "On the Duties of a Physician" in *Enquiry into the Duties of Men in the Higher and Middle Classes of Society in Great Britain* by Thomas Gisborne and *Statuta Moralia* by the *Royal College of Physicians* in London. Notwithstanding this, Percival states that such works do not contain the specific references to the competences and tasks of medical professionals. A gap that he wants to fill in by detecting the rules of conduct directly within the field of medical practice, thus taking into consideration several medical statutes and regulations already existing.

With the aim to create a work, which would have stuck to the real needs of physicians in their professional practice, Percival has long discussions on its main contents with friends and colleagues. On this purpose, in 1792 the first chapter of the book is published and in 1794 the entire work is edited for private distribution. It is worth noting that the first draft of the work is titled *Medical Jurisprudence*, as it is originally conceived as a neat collection of those regulations which are already ruling the medical conduct of the professionals who work at Manchester hospital. The title *Medical Ethics* appears only in 1803 with the final edition dedicated to his son who has decided to undertake the medical profession. The choice of the title seems to be suggested by some friends, who may have persuaded Percival to substitute the term "jurisprudence" with that of "ethics", as the latter mainly enshrines the duty to respect both legal and ethical rules.

Anyway, the first title cannot go unnoticed, as it introduces the controversy on the work's real nature. Can the contents of Percival's work be actually considered as the foundation of medical ethics and the related reflection on the principles of medical morality? Or better yet, is the book just a collection of rules of conduct, which comes into being and dies within the mere practice of the physicians who worked in an English hospital where the main problem was that of mutual respect and the living up to the medical profession's good name?

As a matter of fact, the formal office to which Percival was entrusted coincides with his declared purpose: writing down a sort of guide for health professionals (physicians, surgeons, pharmacists) who work in his hospital, which is first of all useful to define their roles and related responsibilities, together with the rules of good fellowship. It is worth noting the wide interest of this work in the legal aspects of medical practice. In fact, the book devotes a whole chapter (the

fourth) to the regulation of the physician's behaviour in the cases «which require a knowledge of law».

Considering the task entrusted to Percival, his own purpose and the book' broad interest in the legal aspects, the first title ("Medical Jurisprudence") could apparently seem the most appropriate in order to define the work. However, even if this book was specific part of the regulations issued for Manchester hospital, it cannot be included in the body of Manchester (or England) health and medical laws. As a consequence, Percival's friends were right to propose him to change the previous title "Medical Jurisprudence". But was the decision by Percival to substitute this title with that of "Medical Ethics" actually the correct one?

2. Medical Ethics or Medical Etiquette?

In the course of the eighteenth century, even though several physicians still inspire their professional conduct to individual conscience and good sense by following a personal "code of honour", some efforts to systematise physicians' duties and bans into specific lists start to appear. These first approaches to the codification of what is fair and unfair in medical practice can be considered as evident examples of *medical etiquette*. The literary genre of medical etiquette stems from the reflection of a single physician, often famous and authoritative, who proposes himself as an ideal model for his readers. Hence, being it the result of a formulation, which is not shared by all colleagues, its guidelines do not provide sanctions in case of inobservance.

Medical etiquettes seem to be inappropriate to face the new challenges brought about by the medical profession, such as the struggle against several quack physicians and the social control of public health through the claims of exclusive competences, the defence of dignity and unity of professional (5), the warrant of a trusty relationship with untutored patients who are then unable to evaluate the services they receive (6). In this light, to fulfil the task of working out shared documents, which are able to identify the profession also from outside, the newly born medical associations or Orders formulate for their subscribers some codes of conduct, which are occasionally endowed with disciplinary enforceability. In mid-Southern Europe, these documents are called "codes of medical deontology", whilst in the Anglo-Saxon culture they are known as "codes of medical ethics" (7).

The matter which is here called into question is the attribution of modern medical ethics to Percival and his work *Medical Ethics*.

First of all, the term itself "medical ethics" (or "professional ethics" or "practical ethics") can not be addressed to Percival. In fact, this expression existed in the English literature long before Percival's work was published (8). Some examples could be the works by Charles Davenant (9), David Fordyce (10), the abovementioned Thomas Gisborne, who used the definition of "applied moral philosophy" and David Hume who analyses the notion of "practical morals" to distinguish it from more abstract speculations.

Beyond the question referring to the invention of the definition "medical ethics", it is worth noting the controversy, which derives from the interpretations of the real nature of Percival's work. The crucial point is whether it should be enlisted among the works on medical ethics (defining Percival as a moral philosopher) and consider it as the first European code of medical ethics (11), or appreciate it as a mere collection of rules on medical etiquette.

As regards the substance, the basic difference between medical ethics and medical etiquette lays in the fact that the former concentrates on a wide-ranging reflection, which covers various fields from the intra-professional conduct to the doctor-patient and society relationship. The latter merely regulates the behaviour among physicians, on the basis of the principle of mutual courtesy.

According to some researchers, Percival's *Medical Ethics* would be composed of sensitive and profound reflections, which make it not only a timeless work amongst the greatest classics (12), but better yet a milestone in Western medical ethics (13, 14). Behind the misleading concept of professional *decorum*, in its innermost essence the work enshrines a solid and definable moral theory, the *ethical theory of virtue*, chosen and applied by Percival to medicine after years of studies on moral philosophy (15, 16). Hence, *Medical Ethics* would have brought about a definitive separation between the old Ippocratic ethics and modern ethics (17, 18). becoming the first modern Code of medical

ethics (19, 20), whose precepts would have remained unchanged up to the present (21).

After all, according to other authors, it seems more convincing the stance of those who state that Percival's work merely represents a set of maxims and aphorisms of intra-professional etiquette, which aims only at regulating good fellowship (22). In the work, there is no evidence of moral-philosophical analyses, which aim at exploring the general aspects of ethics, since the main purposes of the book are only those of perpetuating the paternalistic spirit of medicine and propagandizing the monopolistic tendency and cooperative system of the medical profession (13). Because of its being "withdrawn", this book seems to side with a corporatist sense with the main purpose to safeguard the medical team's interests. In fact, we should not forget that Percival, as a conservative man, wrote his work in a time in which the English medical corporations, that had an elitist structure, were undergoing a democratic strike, especially by the liberal economic conception. To face it, he opposed a model of profession as a whole of unity and integrity in front of society.

To sum up, unless we do not opt for a special meaning of "ethics", such as that of «morally permissible standards of conduct governing members of a group simply because they are members of that group» (23), Percival's book cannot be evaluated as a work of medical ethics. But, if we agree that *Medical Ethics* is a wrongly titled work of medical etiquette, how should we assess modern codes of medical ethics, which have their background in Percival's book? In fact, if that of Percival is not a work of medical ethics, how could current codes on medical ethics be considered as such?

3. Medical Ethics or Paternalistic Medical Ethics?

Come abbiamo detto, *Medical Ethics*' wide spreading, that exceeded the expectations of its Author, is undeniable (24). In the decades following the publication of *Medical Ethics*, both in England and in Scotland several hospitals and medical associations, amongst which the *Manchester Medico-Ethical Association* and the *British Medical Association*, take inspiration from its language and contents to self-regulate the medical profession. The fame of *Medical Ethics* spread overseas, in particular in the United States, where several professional bodies based their own ethical codes on Percival's work, even sometimes copying word by word some procedures contained in the book, as in the case of the *Boston Medical Society.* Moreover, in 1847, a group of American physicians, amongst others Benjamin Rush and Isaac Hays, took Percival's Code as the backbone for the development of the ethical code of the newly born *American Medical Association*, which had been crafted one year before by Nathan Smith Davis with the purpose of promoting high levels of quality in medical education and professional practice.

As a result of such influence that Percival had on modern codes of medical ethics, we should legitimately expect a certain continuity between Percival's book and modern codes. But if, as we have already pointed out, Percival's Code is a work of medical etiquette, how is it possible that modern codes are documents of medical ethics?

One could say that current codes, even if they are based upon the (togliere) Percival's work, show evident differences to it. In particular, they would reflect the moral maturation of contemporary society, by marking the conversion from the Hippocratic approach, steeped in medical paternalism and corporatism, to contemporary medical ethics that recognizes the patient's central value in the care relationship.

Indeed, Percival's work just represents the umpteenth effort to reassess the Ippocratic medical ethics (25), as it lacks in significant originality if compared to its original source (17). Indeed, Percival's perspective is evidently conservative with regard to the traditional ethical paradigm, which he however tries to adapt to the hospital practice of his times (26), with the principal aim to maintain the classical division into physicians, surgeons, and pharmacists to stress the duty of mutual respect of their own competences and related hierarchical roles.

However, is it also true that modern codes have outdated the Hippocratic and paternalistic tradition, showing a full sensitivity towards medical behaviour which aims at safeguarding not only professionals, but also patients?

In order to answer this question, we look at what happens when the principles of medical ethics are recognised by and translated into Codes of conduct, which

one should not forget, are worked out by medical associations composed almost exclusively by physicians. So far, the noble moral reflection on the ethical implications of medical practice is often replaced by a list of behaviours according to which the hierarchy relating to the good of the patient (and society) and the good of physicians (and their category) is not always clear. Besides, as we have seen, the main historical reason which leads to the formulation of codes of medical ethics is not the defence of a person's interests, but rather the will to create a kind of "internal contract", however recognised at a social level, aimed at claiming and defending the medico-centric perspective of the healthcare organisation, together with the monopolistic interest and paternalistic model of the profession. Then again there is a conflict of interests, obviously: if this is really about reaching a new foundation of medical ethics on a social basis, the authors of the professional regulations should not be exclusively physicians. They should pretty include representations of patients, or at least provide a consultation with associations of health care services' consumers.

In addition to the presence of a practical "openness" to the external dimension (that should not be only stated by general principles, but also achieved within the rules of conduct), one thinks to what other fundamental features are needed to identify a code of conduct as a document of medical ethics. These features emphasise, on the one hand, the presence of a conscious and organic moral-philosophical reflection on the ethical theory choosen the code, and, on the other hand, the use of a method of rational justification for the selection of the rules of conduct.

In the long run, also these features will not be part of modern codes of medical ethics. In order to fill this gap, experts in moral philosophy (at present a small part of the commissions responsible for the elaboration of codes, should be included among the codes' authors.

To conclude, we argue that if the critical overview set on Percival's work is correct, the same logic should be applied to current codes of medical ethics.

Conclusion

Percival's *Medical Ethics* is not a work on moral philosophy applied to medicine, but rather a book inspired on the one hand to the method of positive law and, on the other hand to the principles, which could be also religious, of traditional ethics. Its twofold purpose is to establish harmony among the conflicting factions at the hospital of Manchester and defend the corporatist interests of the medical profession. To this extent, amongst the various literary genres to which the codes of conduct for physicians could be attributed, the one that better fits Percival's work is the medical etiquette. Anyway, *Medical Ethics* has undoubtedly left an indelible mark in the history of medical ethics, as well as in the history of medicine, and it should be considered as a fundamental reference for those who study this subject. Again, this aspect reinforces the difference between medical ethics and its related codification by a medical hand.

Reflections on medical ethics have no boundaries. The subject is stimulated from within by several moral theories and paradigms, which are developed on the basis of issues evaluated by rational justifications. Medical ethics is moral philosophy when it examines every sensitive issue that is directly or indirectly raised by medicine from its focal nucleus: the patient not only as an object but also as a subject of medical care (27). In the age of Percival, it is worth mentioning John Gregory (28), whose reflection, based on the moral philosophy of David Hume, stems from the idea that the doctor-patient relationship should be the core interest of medical ethics, and that society should be free to decide for its own good-health, even though that means to discourage the privileges of the medical class. Alternatively, we could hold up as an example Rev. Thomas Gisborne (29), whose work, less fortunate than those of Percival and Gregory, aims at giving priority to patients, rather than to physicians.

The codification of medical ethics consists in the declension of the principles of medical ethics by self-regulating the medical conduct in which, to every professional duty corresponds a right. In this light, it is more likely that the codes of medical ethics remain faithful to tradition, instead of accepting new moral interpretations, according to which a patient increasingly gains a central role (30). In other terms, if the codes of medical ethics are worked out by the bodies, which represent the profession rather than society, these would more probably be codes of medical

etiquette with wrong titles. This is exactly the case of Thomas Percival's *Medical Ethics*.

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Some observations on the signs of certain death in the 18th century

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Summary. In 1783, the work of Ferdinand de la Boissiere, *Letters above the certainty of death signs with various observations and experiences over the drowned*, was published in Rome. Manuscript is a translation of the French work of Antoine Louis, *Lettres sur la certitude des signes de la mort*, printed in France in 1752 and in which the surgeon discussed on the certain diagnosis of death. The Louis's work represented an important contribute of medicine especially because in the 18th century only the appearance of the first putrefactive processes was recognized as the indisputable sign of death. In the debate of the time, the treaty of Antoine Louis (1723-1792) overtaken the believed that the only indisputable sign of death was the appearance the first putrefaction processes. Our paper is limit to offering some author's account around the characteristic signs of death, which dispense from putrefaction of the bodies or the rigidity of the limbs and the collapse and softness of the eye. The book boasted a great meaning in the medical association of the time, especially because it discussed with contemporary criteria the signs of certain death. (www.actabiomedica.it)

Key words: Ferdinand de la Boissiere, Antoine Louis, signs of certain death

Lettera sopra la certezza de'segni della morte con varie osservazioni ed esperienze sopra gli annegati. Versione francese. Letters above the certainty of death signs with various observations and experiences over the drowned. French version. Ferdinand de la Boissiere published the book in Rome in 1783 (1). It is a very interesting work of 250 pages, composed by "six letters", "a memory", "a notice", "a reasoned examination", and "a conclusion". Three small chapters of surgical observations of the author (*On the way to bring together the lip Leptorrino, above cancerous tumours. On the usefulness of Forceps it 'difficult parts*) followed the translated book. Even if the translator never revealed the author's name, it was not difficult for us to discover the paternity of the original work. It was the translation of the manuscript of Antoine Louis "*Lettres sur la certitude des signes de la mort*", published in France in 1752. The book boasted a great meaning in the medical literature of the time, especially because it discussed with modern criteria the signs of certain death. The immobility, the absence of respiration and the cardiac arrest were considered the major indicators to ascertain death. Our historiography between the 18th and the 19th century boasts several titles on the subject of the signs of certain death. A good review of the relative bibliography appeared few years ago in interesting essay of Claudio Milanesi (2).

However, no certainty of guidance had been outlined yet during the 18th century the medicine discussed with updated criteria around the concept of apparent death. Physicians of the past often discussed on the certain signs of death, distinguishing those "spontaneous" with several observations. The most important were: the pulse, the absent of respiration, the *facies ippocratica*, the *rigor mortis* and the eyes flaccidity. They recorded among the "traditional evidences": sternutatori, herbs stinging, the glass of water placed on the sternum, while among the "surgical evidences" they listed: the scarification, the needles introduced under

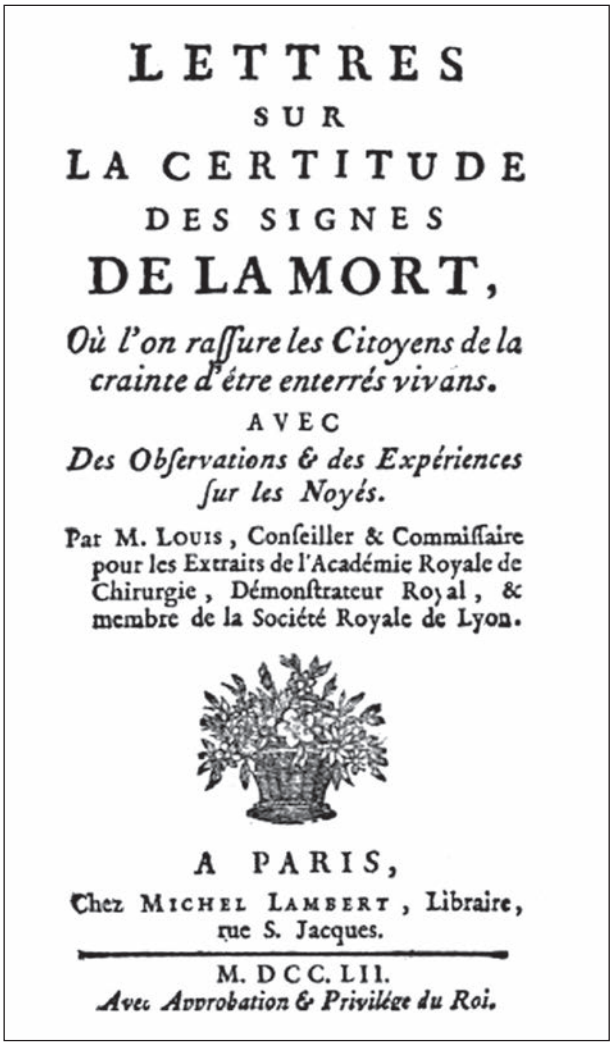
LETTRES
SUR
LA CERTITUDE
DES SIGNES
DE LA MORT,
Où l'on raſſure les Citoyens de la crainte d'être enterrés vivans.
AVEC
Des Obſervations & des Expériences ſur les Noyés.
Par M. LOUIS, Conſeiller & Commiſſaire pour les Extraits de l'Académie Royale de Chirurgie, Démonſtrateur Royal, & membre de la Société Royale de Lyon.
A PARIS,
Chez MICHEL LAMBERT, Libraire, rue S. Jacques.
M. DCC. LII.
Avec Approbation & Privilége du Roi.

Figure 1.

the nails, the etching of the foot sole. In the past, it was very high the risk of making incorrect diagnosis of death, especially because some signs were confused with the symptomatology of some diseases. The scientific community generally described that the insensibility and the manifestation of death appearances occurred especially in cases of suffocation, apoplexy, fainting hysterical, of frostbite by lightning. These arguments were reported also in some medical texts of several specialties and in some philosophical treatises. In this regard, the Benigne Winslow's work, published in 1740, *An mortis incertae signa minus incerta a Chirurgicis, quam ab aliis experimentis?*, was indubitably among the important treatises of the time. The thesis, presented by Leandre Peaget in the Medical Faculty of Paris, discussed on the problematic related the uncertain surgical evidences that traditional were used to diagnose death. The thesis work was translated and expanded by Jean Jacques Bruhier (1658-1756). The author showed the statistics of incidents of the time, trying to testify the frequent risk of being buried alive when the burial occurred before that the signs putrefaction appeared (3). It was also necessary to rely on certain aspects of anatomical research that in those years instructed the preservation of corpses (4).

Winslow's thesis and Bruhier's dissertation highlighted several stories and legends about the uncertain diagnosis of death, which undoubtedly contributed to spread the loss of credibility of the medical science. In the debate of the time, the treaty, in the form of letters, of Antoine Louis (1723-1792) raised the controversy on the believed that the only indisputable sign of death was the appearance the first putrefaction processes (4). The author in the fourth letter claimed the ability to identify the certain symptoms to reach an indubitable diagnose of death reporting in broad examination. It is evident that Louis sought to counteract the distrust of medicine that, despite being included in the domain of experimental sciences, included several doubts, controversial from different points of view and often opposed to each other. The ideas of the French surgeon were disclosed in the Italian scientific literary circles thanks to the translation of Ferdinand de La Boissiere, as mentioned above. The Italian edition, dedicated to Monsignor Rommualdo Braschi Onesti, the nephew of Pope Pius VI, was enriched with the addition of other surgical observations of the translator. Here we don't' tell about the each letter of the work but we limit to offering some author's account around the characteristic signs of death, which dispense from putrefaction of the bodies or the rigidity of the limbs and the collapse and softness of the eye. For example, the author talk about the inflexibility of the joints, which could depend from the convulsive syncope of the body and that it could be confuse with a death status. The author also described the difference between the cadaveric rigidity and a transient muscular restriction. In the second case, the degree of flexibility of the affected muscles and their antagonists is different. In addition to this, the alteration of the ocular bulb was a decisive sign to

refute any doubt in a diagnosis of death. It is undeniable that the spreading fear of premature burial, spread by the Bruhier and Winslow thesis, was excessive in relation to the effective danger. Moreover, the dogmatism that animated the minds of their opponents, despite the irrational spread of such a fear, could hinder the progress of scientific research. The ideas presented in Louis's work and the translated work did not obtain the comfort of being accepted, even if throughout the 19th century Legal Medicine continued to affirm that the only sure sign of death was the beginning of the putrefactive phenomena (5). We find significant traces of scientific interest in these themes also in the medical teaching of the University of Pavia, when in 1825, the student Marcoantonio Germani, discussed his degree thesis with the professor of legal medicine Camillo Platner, dealing the theme of the signs of death (6). Pavese's academic reality, where J.P Frank's great medical teaching was still dominant, was one of the most favorable environments to examine these issues. Indeed, in his dissertation, the student did not forget to talk about the establishment of the mortuary chambers that Frank's dictates had recently imposed, in response to the requests for prolonging the time of exposition of the corpse. Moreover, Germani discussed the legislative arguments, referring to the most significant features of the decree, which regulated the burial of the corpse and which required to doctors the *sacred duty to visit the dead and to write the death certificate to avoid the danger of burying a person asphyxiated* […]. Echoes of these discussions were also present in the second half of the 19th century (8, 9) To conclude, Ferdinando de la Boissiere did not make any personal contribution to the medical diagnosis of a certain death. The authentic fruit of his thoughts are just the definitive pages of the work in which he expressed himself with the skills of the surgeon of the time.

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Historical evolution of the concept of health in Western medicine

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Summary. "Health" is a positive multi-dimensional concept involving a variety of features, ranging from ability to integrity, from fitness to well-being. According to the first principle of the constitution of the World Health Organization "Health is a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity". This constitution was adopted by the International Health Conference held in New York in June-July 1946 and became operative in April 1948. This classical, seventy-year old definition of the World Health Organization is nowadays considered a historical one and it stands as a fundamental milestone of a diachronic track beginning, in Western medicine, with the definition of health proposed by Hippocrates and his School. For Hippocrates health was the state of equilibrium of four humours. This philosophical-naturalistic definition has been flanked in the history of Western medicine by various concepts of health and disease, alternatively based, according to different scientists and in different medical contexts and periods, on epidemiological, anatomical, physiological, functional, social and molecular perspectives. Since biomedical definitions are always prone to integration and updating, depending on the continuous achievements of medical science and bioethics, the fascinating journey through the concepts of health and disease, the fundamental milestones of which are here briefly proposed, is still in progress. (www.actabiomedica.it)

Key words: health, disease, history of medicine, bioethics, anatomy, physiology, epidemiology, methodology

"Health" is a positive multi-dimensional concept involving a variety of features, ranging from ability to integrity, from fitness to well-being. According to the first principle of the constitution of the World Health Organization (WHO) "Health is a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity" (1). This constitution was adopted by the International Health Conference held in New York in June 1946; it was signed in July 1946 by the representatives of 61 States and became operative in 1948. This classical, seventy-year old definition of the WHO is nowadays considered historical and it stands as a fundamental milestone in the diachronic track beginning, in Western medicine, with the definition of health proposed by the Hippocratic School (2).

The Greek physician Hippocrates of Cos (460-ca.377) is considered the father of medicine in the Western world and the founder of a school according to which the human body was retained to be a container of four liquids, the so-called humours. Blood, phlegm, black bile and yellow bile were these humours, and health was constituted by the state of equilibrium of these substances. In turn, disease was the condition of their imbalance (3). Health, as formalized in the conceptual framework of the Hippocratic School, was a philosophical-naturalistic conceit, nonetheless retaining relevant and long-lasting practical consequences. In effect, given that blood, because of its location and composition, was the only humour that could be safely collected, blood-letting became in the V-IV centuries before Christ the universal "therapeutic" intervention implemented to restore good health in the case of potentially every disease. This practical fall-out of the classical Greek theoretical idea of health has remained de-

cisively active for many centuries; indeed, at the beginning of the XX century in many prestigious European hospitals blood-letting was still considered the cornerstone of the treatment of various pathologies, ranging from pneumonia to tuberculosis. Even the consolidated Western medical tradition of administering purges and emetics to the sick finds its conceptual motivation in the Hippocratic humoural theory (4).

In the course of the Renaissance (XIV-XVIII centuries) other concepts of health were proposed by illustrious physicians and scholars. According to the German-Swiss physician and alchemist Philippus Aureolus Theophrastus Bombastus von Hohenheim, generally known as Paracelsus (1493-1541), human health resided in the harmonic correspondence between the microcosm of the human being and the macrocosm of the entire universe. Paracelsus not only ascribed the causes of diseases to different entities - the ideal, the spiritual, the natural, the poisonous and the planetary ones - as he wrote in his "Opus Paramirum" (1531), but also to the principles identified in sulphur, mercury and salt (5). In the case of pathologies, and so to re-establish the philosophical-physical state of health, Paracelsus prescribed remedies derived from alchemy and suggested the ample implementation of the concept of similarity as a curative principle (the famous idea of "similia similibus" – "things should be treated with similar things") (6). Still in the XVI century, the Italian physician and astronomer Girolamo Fracastoro (1478-1553) put forward an innovative view of health and disease. On the basis of the observation of the many and serious infectious diseases of his time, Fracastoro hypothesized that pathologies were determined by the transmission of "seminaria" ("seeds" of disease) that propagated from sick people to healthy ones through direct contact or by means of personal items (7). The idea of these "seminaria", to all effects and purposes precursors of modern germs and microbes, was specifically elaborated by this Italian doctor and geologist through his ample consideration of the frequent and devastating occurrence of syphilis (8). The concepts of health and disease identifiable in the works of Fracastoro anticipate, according to some medical historians, the modern "epidemiologic" assessment of (infectious) pathologies in large populations.

In the XVIII century the concepts of health and disease were developed and enriched by other notable scholars privileging, in different European countries and in various cultural settings, "anatomical" or "physiological" views of the matter (9). The Italian physician and anatomist Giovanni Battista Morgagni (1682-1771) retained good health to be the status of clinical-anatomical integrity of the human organism. An illustrious clinician and pathologist, he considered disease the anatomical alteration of one or more organs of human bodies, that he had accurately described as consequence of hundreds of dissections personally performed (10). On "physiological" grounds, the Scottish physician John Brown (1735-1788), propounder of the "excitability" theory of medicine, was of the idea that human health depended on the sound interaction between the internal excitability typical of the body and the numerous external stimuli, that he named "exciting powers", to which human organisms are subjected and to which they had to respond (11). As consequence, Brown subdivided diseases according to their ability to exert an over- or an under-stimulating influence on the human body (12). The Swiss professor of medicine and biologist Albrecht von Haller (1708-1777), considered one of the founders of experimental physiology, and his 1766 masterpiece "Elementa physiologiae corporis humani" should be remembered (13). In the context of human health and pathology that he investigated in physiological terms, he furnished a complete description of the perceptive faculty characteristic of nervous fibres, which became his famous concept of "sensibility", and he provided a description of the contractile muscular capacity prompted by irritation ("irritability") (14, 15).

In the XIX century the ideas of health and disease based on physiological and anatomical research were further pursued. "Physiologically" speaking, the French philosopher and physiologist Claude Bernard (1813-1878), considered a pioneer of the application of the principles of experimentation to life sciences, elaborated the concept of "internal environment" ("milieu intérieur") of organisms, leading to the later understanding of human homeostasis (16). Bernard did not consider health and disease as rigidly separated entities but, on the contrary, as two of the components of a continuous spectrum, merging one into the other (17). "Anatomically" speaking, it was in the eighteen hundreds that Morgagni's organic level of investigation was fur-

ther elaborated through the study of the constituents of organs, namely, the tissues and, more significantly, by the research on the singular components of the tissues, namely, the cells. It was precisely in the context of cells that the prestigious German anatomical school collocated, in the XIX century, the roots of the concepts of human health and pathology, identifying in altered cells the triggering points of diseases (18). One of the major representatives of this school, the pathologist Rudolf Virchow (1821-1902), should be remembered for his pioneeristic studies on a number of pathological processes, scientifically investigated at the cellular level.

In the course of the XX century, on the one hand the study of normal and pathologic cells left space to the consideration of sub-cellular components, and medicine became more and more molecular and submicroscopic; on the other hand, a renewed global attention to human beings, both healthy and sick, gave origin to synergic, multi-faceted definitions of health (and disease). An example is precisely that of WHO presented at the beginning of this text, which shows how account was taken not only of physical-anatomical features, but also of mental-psychological and social-functional ones.

The progress of medical sciences in the nineteen hundreds was explosive, with many new, original achievements leading to a change of classical paradigms in a number of biomedical areas (19,20), among which the ample epistemological one dealing with the concepts themselves of health and disease may be remembered. As a consequence, even the historical WHO concept of health has in turn become the object of scientific and bioethical discussion, demonstrating that the fascinating journey through the notions of health and disease, whose fundamental milestones have been briefly proposed, is still in progress.

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Original article

Novel approach for quality assessment and improving diagnostic accuracy in cell-based infection imaging using ^{99}mTc-HMPAO labeled leukocytes

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Summary. *Background and aim:* Labeled leukocytes with ^{99m}Tc-HMPAO are routinely used for infection imaging. Although cell labeling with ^{99m}Tc-HMPAO represents an imaging probe to detect infection sites, the diagnostic efficiency of the probe is largely influenced by cell manipulation, multidisciplinary interventions (i.e., biologist, technicians) and available technology (i.e., SPECT, SPECT/CT). The aim of the study was to assess in vitro and in vivo accuracy of a comprehensive approach for quality assessment (QA) of all steps of the procedure. *Methods:* Radiochemical purity (RCP), pH, labeling efficiency (LE) were measured in 320 procedures. White Cell Viability Factor (WVF) was determined in consecutive blood samples. Images (490 studies) were scored using a 5-point scale. Training program was evaluated using a Learning Questionnaire and a score system. *Results:* Pre/post-labelling WVF was 0.99% (max value 1%) in all blood samples. LE (mean value 72%) and RCP (>80% until 55 minutes) yielded considerably high values. The vast majority of images were scored as diagnostic by three independent observer (90% with score ≥4). *Conclusions:* This method appears highly reproducible and easy to use in clinical routine for leukocyte labeling, especially when standardized training and total QA system are implemented. (www.actabiomedica.it)

Key words: labeled leukocytes, ^{99m}Tc-HMPAO, radiopharmaceutical, biomedical imaging, quality assessment, leukocytes

List of abbreviations

ACD: Acid-citrate-dextrose
CT: Computed Tomography
EANM: European Association of Nuclear Medicine
HAES: Hydroxyethyl starch sodium chloride solution
HGB: Erythrocyte Hemoglobin
HMPAO: Hexamethylpropyleneamine Oxime
ITLC: Istant Thin Layer Chromatography
LE: Labeling efficiency
LQ: Learning Questionnaire
MR: Magnetic Resonance
PET: Positron Emission Tomography
PI: Propidium Iodide
QA: Quality assessment
QC: Quality Control
RBC: Red Blood Cells
RCP: Radiochemical Purity
SPECT: Single-photon Emission Computed Tomography
WVF: White Cell Viability Factor

Introduction

Leukocytes radiolabeling with ^{99m}Tc-HMPAO is the most common approach for infection imaging, thus allowing reliable detection of white blood cells (WBC) accumulation at the site of infection (1). This

process of cell migration can be visualized by planar and SPECT imaging, which enable differentiating sites of sterile inflammation from foci of pathogens infection . Infection imaging by means of labeled leukocytes also allows reliable monitoring of treatment efficacy (2-4).

Many cell types other than leukocytes can be labeled with ^{99m}Tc-HMPAO, thus including platelets (5-6), dendritic and endothelial cells (7-8), spermatozoa (9-10), but this approach can also be applied to labeling of liposomes (11), dendrimers (12) and nanoparticles (13).

Labelling of WBCs is an extemporaneous preparation of radiopharmaceuticals involving multiple steps in vitro (blood manipulation, dispensing) and in vivo (sampling, administering, etc.). This procedure is performed according to specific rules and recommendations, which require a classified environment and qualified personnel (14-16), since inadequate quality assurance of the compounding processes, involvement of inexpert personnel for carrying out compounding and inappropriate environmental conditions may all generate an unfavorable impact on the final product.

Some European countries have adopted specific guidelines and regulations for production of extemporaneous radiopharmaceuticals, especially for labeling of autologous cells, since these cannot be efficiently sterilized after the labeling procedure.

The European Association of Nuclear Medicine (EAMN) has performed a recent survey, concluding that WBC labeling is a well-established technique in Europe, which is mainly performed by trained personnel under sterile conditions in a laminar flow cabinet or cell isolator (class A), installed according to local regulations (17-18).

In 2005 the Italian standards of good preparation were approved by the National Healthcare System ("Roles of Good Preparation of the radiopharmaceuticals") .

According to this specific regulation, labeling procedure and staff training must be validated for guarantying patient safety as well as diagnostic efficacy and accuracy. Some critical issues during the labeling procedure may compromise imaging results and ultimately generate and adverse clinical impact, as clearly proven by the current scientific literature. Detrimental effects of intracellular radiolabelling on leukocytes include viability problems related to DNA damage (19-27), maintenance and over time reproducibility of the method ,which can hence impair clinical efficacy, complexity and stability of the labeling technique (28). The recent introduction in clinical practice of hybrid diagnostic technology for producing images with different 3D modalities (CT/MR for morphology, SPECT/PET for metabolic and functional information) necessitates a highly efficient detection rate of the target biological process, so improving localization within fused images, along with characterization and over time monitoring of disease.

The current evidence hence suggests that accurate infection imaging with labeled leukocytes requires accurate techniques and adequate training/experience (29-30). Both these factors were main aspects of our study, based on a comprehensive approach to assess the quality of infection imaging, including all the different steps of the whole process (in vitro, in vivo, learning). The quality of acquired images was also assessed in the fused setting of SPECT/CT in our investigation.

Materials and methods

Infection imaging is a routine diagnostic tool performed in our institution (University Hospital of Parma). The local method is based on a standardized multi-step procedure entailing leukocytes isolation and labeling, quality control of prepared radiopharmaceutical, acquisition and post-processing of images. All phases of this process need appropriate learning and adequate training of the staff, both conducted with a standardized approach.

The purposes of this study were scoring and measuring single step data obtained throughout the entire procedure, without impacting routine activity.

In vitro quality assessment was performed by assessing the effect of ^{99m}Tc-HMPAO on blood cells and leukocyte viability, the number of cells efficaciously labeled using ^{99m}Tc-HMPAO at the lowest level of radioactivity and within the smallest possible volume, the maintenance of high radiolabeling yield and complex stability. The high over time reproducibility of labeling method obtained with standardized operator training

and high quality diagnostic images were defined as *in vivo* quality indicators of infection imaging. Written informed consent was obtained from all subjects.

Isolation of leukocytes

Peripheral venous blood (40 ml) was drawn from patients using a 19 gauge i.v. line into a sterile syringe containing 12 ml of acid-citrate-dextrose anticoagulant solution (ACD; formulation A according to the European Pharmacopoeia, consisting of 0,73 g of anhydrous citric, 2,2 g of sodium citrate dihydrate and 2,45 g of dextrose monohydrate in 100 ml of water for injection) and 6 ml of hydroxyethyl starch sodium chloride solution (HAES-sterile according to the European Pharmacopoeia, consisting of 100 g of Poly (0-2-hydroxyethyl) starch and 9 g of sodium chloride) (16).

The isolation of leukocytes from other blood components was performed by centrifugation at 1000x g at room temperature for 10 min, thus finally yielding a leukocyte pellet ready to be labelled.

Radiopharmaceuticals and cell labeling

Hexamethyl propylene amine oxime (exametazime) was supplied as a ready-for-labeling kit (Ceretec®; GE Healthcare). The lipophilic primary ^{99m}Tc-HMPAO complex was obtained after resuspension of Ceretec® with freshly eluated sodium ^{99m}Tc-pertechnetate, according with the manufacturer's instructions (2).

Quality control (QC) of the ^{99m}Tc-HMPAO preparation was performed in agreement with the procedure described by the manufacturer with the accompanying leaflet.

Freshly prepared ^{99m}Tc-HMPAO (750-1000 MBq) in 1 ml of saline was added to the mixed leukocytes suspension (or purified granulocytes) and incubated for 10 minutes at room temperature. The labeling process was stopped by adding 5 ml of NaCl 0,9% (w/v) in the solution. Labelled cells and unbound ^{99m}Tc-HMPAO were then separated by centrifugation. The radioactivity of both supernatant and cell pellet was measured in a dose calibrator, and the efficiency of the labeling method (labeling efficiency, LE) was estimated as the percentage of residual radioactivity in the cells.

The pellet containing the labelled mixed leukocytes was resuspended in 5 ml of NaCl 0.9%(w/v) and the dose (recommended dose 370-740 MBq) was administered to the patient.

In vitro quality measures

As regards the quality control of ^{99m}Tc-HMPAO-labelled WBC, several methods have been described (16), although only a few of them are used in routine clinical practice. In our QC laboratory the quality of each ^{99m}Tc-HMPAO preparation is regularly checked, as for manufacturer's guideline.

A visual inspection of the final product is needed shortly after Ceretec® resuspension, thus enabling to identify aggregates, clumps or clots.

Radiochemical purity (RCP) of ^{99m}Tc-HMPAO has been assessed with istant thin layer chromatography (ITLC) on iTLC-SG glass microfiber chromatography paper impregnated with silica gel (2.5 cm* 2.0 cm, Agilent Technologies) as stationary phase and using two solvent systems as mobile phases. 0.9% NaCl produces a tiny little pick of unincorporated pertechnetate $^{99m}TcO_4^-$ at the front (R_f=0.8-1.0), whilst methyl-ethyl ketone yields a little pick of $^{99m}TcO_2$ at the origin (R_f=0-0.15). RCP, that is the proportion of a radionuclide present in the desired chemical form, must be ³80% (2).

The pH of the ^{99m}Tc-HMPAO preparation was measured using pH test strips, and must be comprised within 9.0-9.8.

LE (%) was assessed after each production, by measuring the amount of radioactivity of both supernatant (soluble ^{99m}Tc-compounds) and pellet (cell-associated ^{99m}Tc) of the labeling solution obtained after centrifugation. LE was calculated using the following formula:

$$\left(\frac{\text{Activity of cell pellet}}{\text{Activity of cell pellett + Activity of supernatant}}\right) \times 100$$

A LE values comprised between 40-80% is advisable. When LE is <40%, further quality controls (e.g., microscopic inspection and trypane blue exclusion test for cell viability) should be performed.

In vitro assessment of blood cells and leukocytes viability

Leukocytes viability was assessed with a dye-exclusion assay on three different blood samples before and after labelling with ^{99m}Tc-HMPAO. In this assay, dead cells are stained with a small-molecule dye which can only permeate cells with compromised plasma membranes, whereas the die is not incorporated by live cells with intact membranes, which hence remain unstained. Propidium iodide (PI) is a membrane impermeant dye, which is generally excluded from viable cells, binds to double-stranded DNA by intercalating between base pairs and finally becomes fluorescent.

The fluorescence intensity obtained by staining the nuclear DNA with propidium iodide is expressed as the White Cell Viability Factor (WVF; max value, 1), which represents the fraction of viable leukocytes.

The effect of the labeling process on blood cells was assessed *in vitro* with the fluorescence flow cytometer CELL-DYN Sapphire, a multi-parameter, fully-automated hematologic analyzer.

Quality assessment of operator training

The training process of the local radiopharmacy is scheduled according to the guidelines for safe preparation of radiolabelled blood cells (16, 29-31). This consists of theoretical instructions (local rules and recommendations, available guidelines and pharmacopoeia, guidelines for working in aseptic conditions, including the use of a Class IIa safety cabinet, equipment maintenance), trainee observation (1 wk), supervised practice (2-3 wk) and proficiency assessment (at least three test sets) by personnel certified for cells labelling and performing in vitro quality controls.

Training scheduling and competency assessment were standardized, following the Quality Assurance Manual of the local radiopharmacy.

Before the personnel is qualified for routine activity without supervision, each trainee undergoes competency assessment.

Training program was evaluated using a Learning Questionnaire (LQ). The main objectives of the program were converted into a list of items aimed to capture information about the extent of being comfortable with each of the key objectives (rules, safety cabinet, equipment maintenance). Learning was assessed using a score system from 6 ("a lot") to 1 ("nothing") for each operator (n=3).

In vivo quality assessment by imaging analysis

Lung uptake was evaluated at 5 and 30 min after labeled leukocytes reinjection on planar image of the thorax, to detect aspecific accumulation of activated neutrophils or cell clumps due to the labeling process. Three independent readers reviewed all planar, SPECT and SPECT/CT images obtained from January 2012 and November 2013 and between February and June 2017, and graded image quality using a 5-point scale (1=non-diagnostic; 2=poor, diagnostic confidence significantly reduced; 3=moderate, but sufficient for diagnosis; 4=good, diagnostic, and 5=excellent).

Statistical analysis

Data were reported as mean ± standard error of the mean (SEM). PCR values were compared using Students paired *t*-test. The statistical analysis was performed with SPSS software.

Results

Preparation and stability of ^{99m}Tc-HMPAO labeled at high specific activity

Ceretec® was supplied in amounts of 0.5 mg (1.85 μmol/L) of exametazime per vial, reacting upon reconstitution with $NaTcO_4$ (3.4-3.7 GBq) to a final volume of 3 ml, in a one to one molar ratio to form the ^{99m}Tc-HMPAO complex. According to manufacturer's guidelines, pertechnetate may be added to Ceretec® in amounts not exceeding 1100 MBq, and the preparation must be used within 30 min after resuspension. Larger amounts of pertechnetate (3-5 GBq) may be added with decreased tenability of preparation (2, 32).

Radiochemical purity control assays of ^{99m}Tc-HMPAO on ITLC-SG strips in 0.9% NaCl displayed showed a modest pick of unincorporated pertechnetate $^{99m}TcO_4^-$ at the front (R_f=0.8-1.0), which significantly increased after 60 min. The radiochemical purity con-

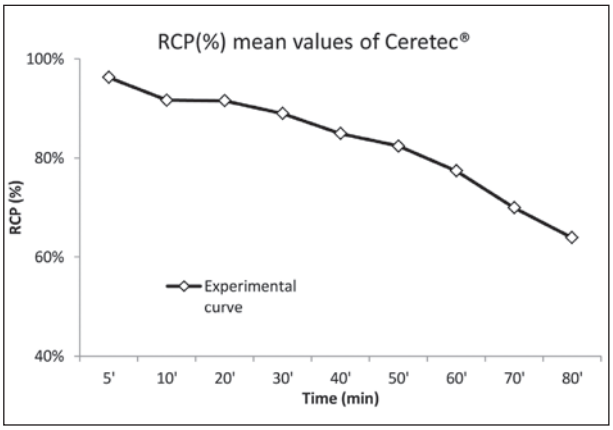

Figure 1. Radiochemical purity of ^{99m}Tc-HMPAO. Mean values over time (3 measures for each time interval from the labeling until 80 minutes)

trol assays on ITLC-SG strips in methyl ethyl ketone solvent showed displayed a modest pick of $^{99m}TcO_2$ at the origin (R_f=0-0.15), which significantly increased after 50 minutes.

Final radiochemical purity was maintained as hisg as 80% up to 55 min (Fig. 1).

Effect of the labeling process on blood cells

Effect of the labeling process on blood cells is summarized in Fig. 2.

WVF was 0.99% in all blood samples, both before and after the labelling process.

The comparison of pre- and post-labeling data (Fig 3 - a,b) shows that the mononuclear component (lymphocytes and monocytes) exhibits a more compact cluster, probably attributable to coarctation of cytoplasmatic membrane, which then generates a reduction of cell volume for adhesion to nuclear membrane.

This morphological alteration is attributable to the method used for cell treatment (i.e., centrifugation) and not to the labeling reaction, as shown by the initial absence of morphological alarms.

After treatment, the appearance of these reflects the presence of immature granulocytes (7.2%) with high intensity (0.74%).

The comparison between pre- and post-centrifugation data (Fig. 3 - c,d,e,f) is suggestive for displace-

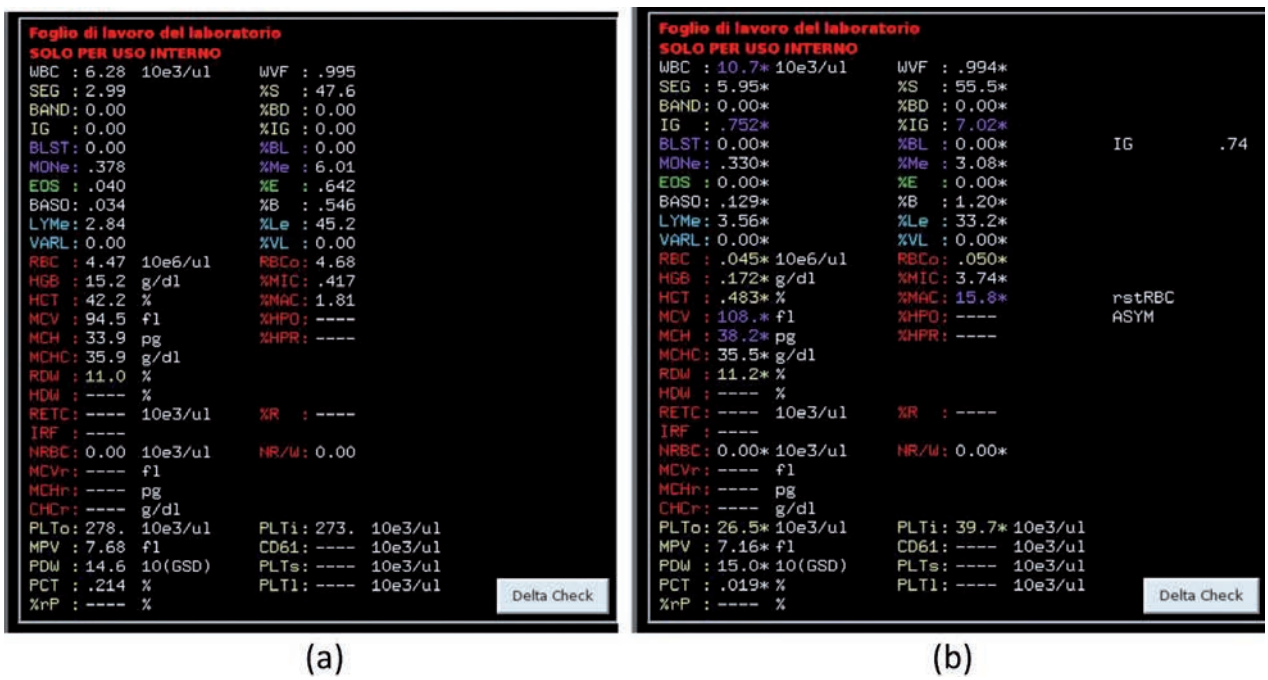

Figure 2. Effects of the labeling process on blood cells. Data acquired by CELL-DYN Sapphire. (a) Data analysis of the blood sample before labeling with ^{99m}Tc-HMPAO. (b) Data analysis of the blood sample after labeling with ^{99m}Tc-HMPAO

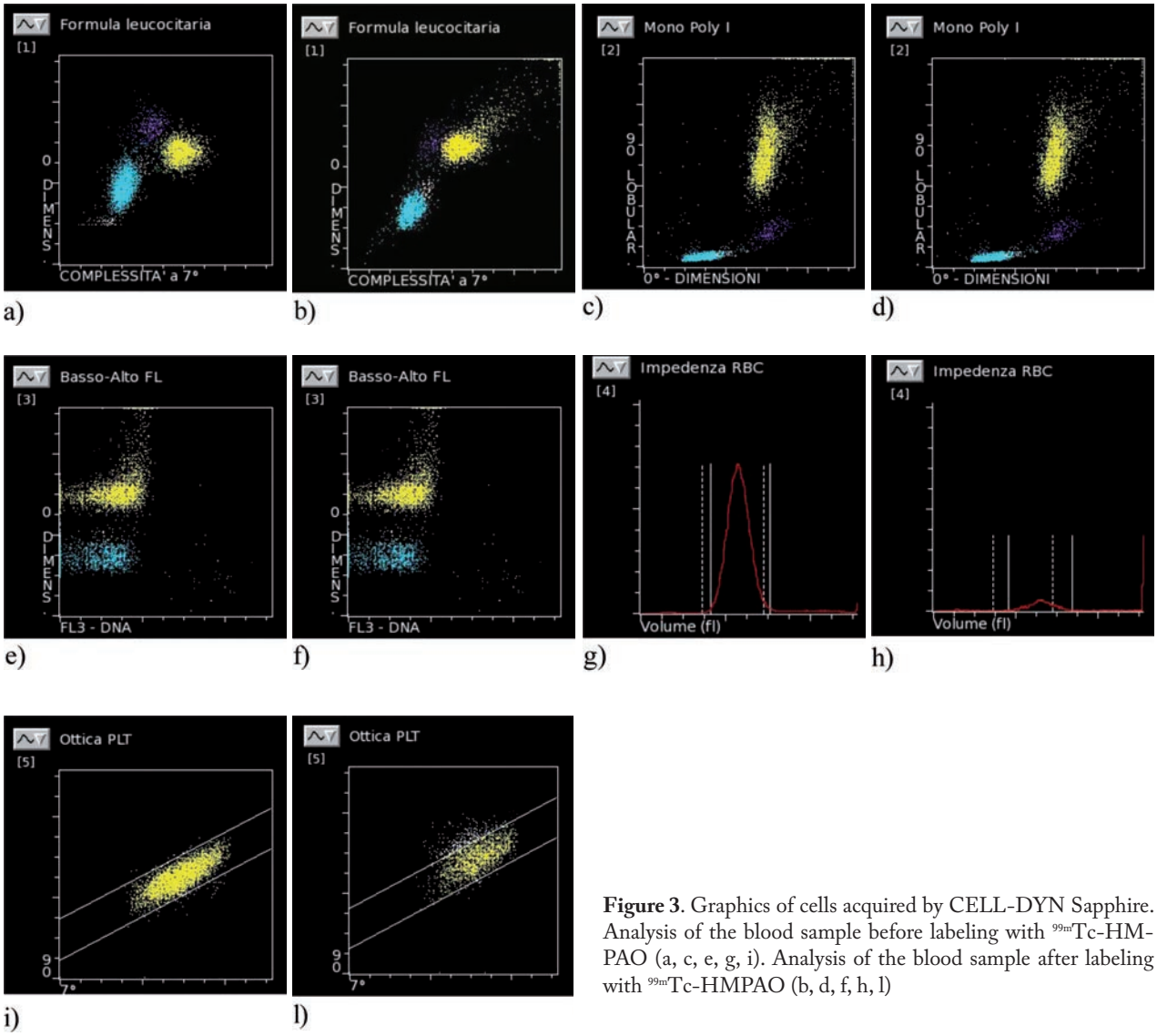

Figure 3. Graphics of cells acquired by CELL-DYN Sapphire. Analysis of the blood sample before labeling with ^{99m}Tc-HMPAO (a, c, e, g, i). Analysis of the blood sample after labeling with ^{99m}Tc-HMPAO (b, d, f, h, l)

ment of cell clusters due to decrease of lymphocytes/monocytes volume and dispersion of neutrophils and eosinophils for plasma swelling.

Sample hemolysis ($4.47x10^{-6}$/µl vs $0.045x10^{-6}$/µl; $p<0.001$) and hemoglobin content (152 g/l vs 1.75 g/l; $p<0.001$) were decreased after labeling (Fig. 3 - g,h).

The red blood cell distribution width (RDW) did not differ before and after the labeling process (11.0% vs 11.2%).

Platelet count assessed with the optical method was significantly reduced after labeling ($26.5x10^{-9}$/L vs $278x10^{-9}$/L; $p<0.001$) (Fig. 3 - i,l).

Imaging results and assessment of training

A mean number of 160 studies with labeled leukocytes are performed each year in the local facility (1203 from January 2009 to June 2017) for diagnosing infection and identifying the. Overall, bone infection or orthopedic implant infection (27% hip, 23% knee) was the reason for ordering the test in 94% of cases, whilst the test was performed for other causes in the remaining 6% of patients (i.e., for prosthetic vascular graft infection, endocarditis, cardiac device infection, abdominal abscess or fistula, fever of unknown origin).

Planar images were recorded from the segments involved in all patients; SPECT imaging and SPECT/CT was performed in 26% and 10% of them, respectively (Fig. 4).

For the purpose of this investigation, we reviewed 490 studies with labeled leukocytes (planar, SPECT and SPECT/CT images) obtained from January 2012 and November 2013, and those review between January and March 2017.

Lung uptake of labeled leukocytes could not be detected in any patient at the end of operator training.

In two cases lung uptake was identified during the phase of new operator validation (LE of 68% and 66% respectively; RCP of ^{99m}Tc-HMPAO 96.54%).

An optimal absolute agreement in image quality was found among the three independent observers and all the images were score as diagnostic (90% of observation with score ≥4).

SPECT post-processing was performed with iterative algorithm (8 iterations, 4 subsets) and in all cases the reconstructed images were scored as diagnostic (i.e., score comprised between 3-5), thus allowing localization of the leukocyte accumulation foci in the fused images. The training and learning programs were scored by key objective areas, with a mean value of 5 (4.8, 5.8 and 4.8 for each area, respectively), a good result also considering operator turnover (5 operators were changed from 2012).

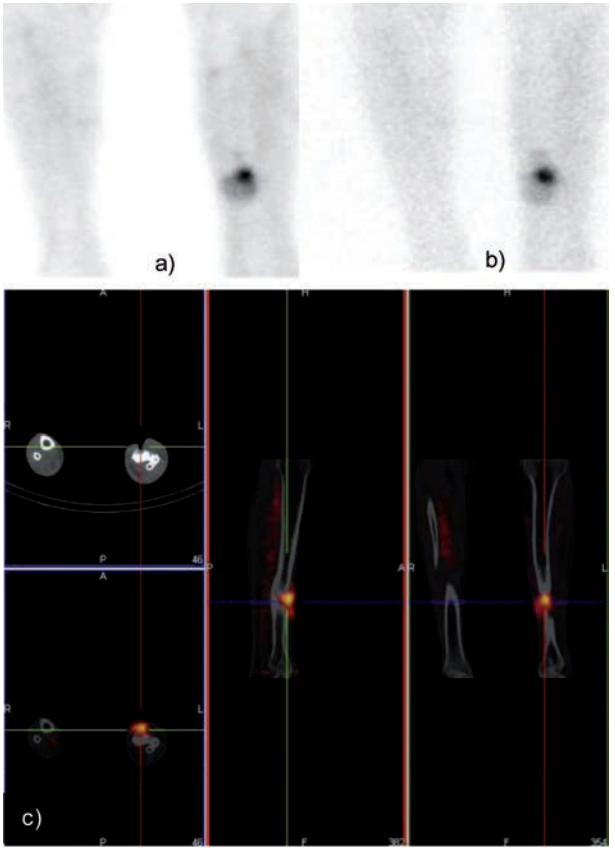

Figure 4. Imaging of bone infection with labeled leukocytes. M, 49 yrs, tibia/fibula fracture: from 1990 recurrent infections. ^{99m}Tc-HMPAO labeled leukocytes scintigraphy detecting leukocytes accumulation in the left tibial plateau.
Planar images at 3 (a) and 24 hrs (b) and SPECT/CT images (c).

Efficiency of leukocytes labeling

The comparison of mean LE (%) values during six months of two consecutive years (i.e., averaging 188 measures) also indicated that the labeling method used in this study was effective to produce a high leukocytes labeling efficiency over time, with an average yield of approximately 72% (Fig. 5).

Moreover, no statistical significance was found (p=0.95) comparing data distribution of LE (%) mean values during the different semesters of the two years, thus confirming the reliability of the labelling technique.

Discussion

The radiolabeling of white blood cells has been introduced in 1976 as an imaging procedure, and has

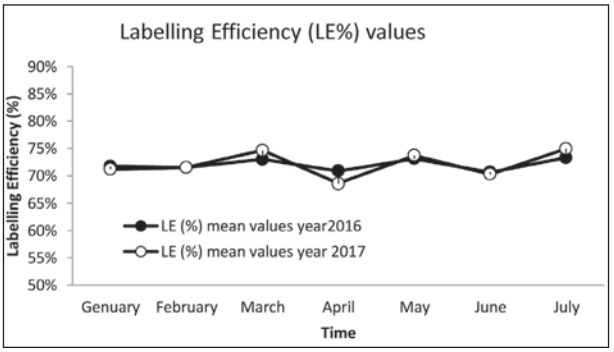

Figure 5. Mean values of radiolabelling yields.
Mean values of LE (%) during six months of two consecutive years 2016-2017 (188 measures)

been used as routine technique in nuclear medicine for detecting infection and inflammation (2-4).

Since the time when Peters et al. described the possibility of labeling granulocytes with ^{99m}Tc-HMPAO, this compound has been considered the preferred labeling agent and has then been commercially available for nearly 30 years (33). Nevertheless, several studies described that radiotoxic effects can be expected with intracellular labeling of leukocytes due to Auger electrons originating from decay of ^{99m}Tc. Detrimental effects of intracellular radiolabeling on leukocytes have also been described, such as response to blastogenic stimulation, chromosomal aberrations, structural changes and DNA damage (19-27).

Taken together, the results of our study provide clear evidence that ^{99m}Tc-HMPAO-labelling do not alter leukocytes viability. Our labeling procedure hence allows maintaining all leukocytes vital. The White Cell Viability Factor, which aimed to assess leukocyte viability (max value, 1), was 0.995% before labeling and remained virtually identical (i.e., 0.994%) afterwards. Our results also demonstrate that the observed morphological alterations were exclusively due to the centrifugation process and not to the labeling reaction.

The centrifugal treatment of the cellular elements caused a substantial reduction of red blood cells (over 99.9%) and erythrocyte hemoglobin (approximately 99%). Although the platelet number was also contextually decreased by approximately 90%, this is an expected outcome during separation of cellular elements by centrifugation. Regardless of these results, WVF did not vary from the baseline, thus confirming the effectiveness and efficiency of the whole labeling process.

The results emerged from our study also indicate that a specific standardized training modality combined with our labeling technique ensure high reproducibility over time, facility of implementation in routine clinical practice despite operator turn-over. We could also document preservation of high leukocytes labeling efficiency (average yield 72%) and high stability (55 minutes) because the agent (Ceretec®) remains bound to labeled leucocytes without decreasing their vitality.

The education program was well received by the operators and made it possibile to achieve the main goals of the labelling technique.

Finally, such a reproducible and stable method allows to obtain high-quality imaging of infection sites also with tomographic (SPECT) and hybrid (SPECT/CT) technology (score 3-5), ultimately enabling an accurate localization of leukocytes accumulation foci in routine clinical practice.

Conclusions

In conclusion, our results shows that cell-based infection imaging with ^{99m}Tc-HMPAO-labeled leukocytes can be easy implemented in routine clinical practice using a standardized approach for training and learning. This can hence allow high reproducibility and establishment of a quality assessment system for reducing vulnerability in lab activity or images acquisition, especially in the challenging context of an increasing turnover of the staff.

This technique also enable to maintain labeled leukocytes vital, is reproducible and stable over time. Finally, this technique allows obtaining high-quality imaging of infection sites also using SPECT and SPECT/CT technology in the daily practice. ^{99m}Tc-HMPAO (Ceretec®) may be used as an efficient and safe tool to study leukocytes turnover and activity in inflammation/infection diseases.

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Authors' contributions

SM and AS carried out the labeling procedures, participated in the data analysis and drafted the manuscript. CC, GB, AS and MS carried out the imaging studies and participated in the images scoring. CG and GS participated carried out technology assessment and performed the statistical analysis. SP and GL performed the cell viability assessment, AS carried out the labeling procedure and collaborated in collecting data, LR conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Authors' information

Authors of this paper are competent in many different disciplines from basic science to clinic. Cell. based procedures are complex and require a multidisciplinary approach and different skills related to radiochemistry (SM, AS), medical physics (CG and GS), biochemistry (GL and SP), nuclear medicine (CC, GB, MS, AS, LR).

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Original article

Could decorin be a biomarker of coronary artery disease? A pilot study in human beings

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Summary. *Background and aim:* Nowadays there is a strong necessity in identifying patients who may be exposed to the risk for future cardiovascular events like progressive atherosclerotic disease. Biomarkers are valuable tools for this purpose. Coronary artery calcification (CAC) is utilized as an important tool for the global risk assessment of cardiovascular events in individuals with intermediate risk. Decorin (DCN) is a small leucine-rich proteoglycan that induces calcification of arterial smooth muscle cell and localizes to mineral deposition in human atherosclerotic plaque. The main purpose of this clinical study was to find out the correlation between Decorin serum concentration and CAC in human for the first time. *Methods:* In this study 84 patients with coronary artery disease who fulfilled inclusion and exclusion criteria, entered the study. For all patients a questionnaire consisting demographic data and traditional cardiovascular risk factors were completed. CT-Angiography was carried out to determine coronary artery calcium score and ELISA method was used for measuring DCN serum concentrations. *Results:* No significant correlation between DCN serum concentration and total CAC score and also CAC of left anterior descending, right coronary artery, left main coronary artery and circumflex was found in the study population (P>0.05). *Conclusions:* On the basis of our results DCN serum concentration is not a suitable biomarker of coronary artery disease. However, more studies with higher sample size are necessary for its confirmation. (www.actabiomedica.it)

Key words: coronary artery calcification, decorin- tumor necrosis factor β, glycosaminoglycan, proteoglycans, biomarker

Introduction

Vascular calcification is a threatening the survival complication of cardiovascular disease and is an independent risk factor for high morbidity and mortality (1).Vascular calcification is an important feature of atherosclerosis and cardiovascular diseases, and it is an inevitable process particularly in the advanced stages of atherosclerosis which can create break in the vessels and cause the plaque rupture. Coronary artery calcification (CAC) is a surrogate marker for subclinical atherosclerosis and is known to reflect atherosclerotic burden. Increased coronary artery calcium score (CACS) correlate with the risk of cardiovascular dis-

ease (2). CAC determined by electron beam-computed tomography (EBCT). EBCT was recently determined a strong predictor that comforts the prediction of future cardiovascular events particularly in intermediate risk subjects while in the past CAC has been a poor prognosis for vascular disease (3).

Recent studies have provided impetus to shift from cellular interaction based calcification models to models emphasizing on the important role of extracellular matrix (ECM) in calcification. The ECM contains number of non-collagenous matrix molecules such as proteoglycans, which is important regulator of bone mineralization, because this regulates collagen fibril formation and tendency and directly controls hydroxyapatite crystal growth (4). Proteoglycans, especially those belonging to the small leucine-rich proteoglycan family which DCN is an exponent example, have a significant role in calcification. The study of in vitro and in vivo animal models suggest an important role of DCN in arterial calcification, however this role is not very clear. The proteoglycans are the ingredient of superfamily leucine-rich repeat (LRR) (>300 members) (5). Proteoglycans consist of one or more glycosaminoglycan (GAG) side chains bound to a core protein. According to the type of the GAG, the proteoglycans classified into, dermatan sulfate, heparan sulfate, keratan sulfate and chondroitin sulfate proteoglycans (6,7). DCN is composed of a 38 Kdalton core protein with 12LRRs containing one dermatan sulfate or chondroitin sulfate chain, and is expressed in skeletal tissues, the adventitia of blood vessels and the skin (8,9). DCN overexpression increases calcification arterial smooth muscle cells (SMCs) and aggregates to areas of atherosclerotic plaques in arteries involved in calcification (10).The DCN core protein binds to TGF-β isoforms by means of GAG chain which control TGF-β/ECM interactions. Decorin GAG chain has a very important function as an inducer of VSMC bio mineralization which actives TGF-β signaling and Ox-LDL-induced SMC mineralization. Overexpression of decorin increases TGF-β activation and regulates TGF-B bio activity considerably; In addition to, decorin-induced TGF-β signaling expedites osteogenic differentiation of VSMCs. TGF-β and DCN alike were implicated in promoting vascular calcification (10-12).The TGF-B is one of the influential factors which acts via expressing the properties of osteoblastic on the arteries calcification in atherosclerotic plaques. In addition, TGF-B with high concentration can be fined in atherosclerotic plaques and increases the formation speed of mineralized nodules (13). From the other point of view, overexpression of decorin induces collagen gel stiffness and accumulation and promotes collagen synthesis, enlarges fibronectin fibrillogenesis, and results in the formation of a dense collagenous matrix in the intima of injured arteries in vivo. Also, DCN directly binds to hydroxyapatite that might be involved in the effect of decorin on calcification (14, 15). Having examined the previous studies in this field, all of investigations were done based on the in vitro and yet, any studies have not been done on humans.

According to this, we evaluated the DCN as a diagnostic biomarker in human to determine the extent of vascular calcification and subsequent coronary disorders such as CAC.

Methods

Patients

Eighty-four patients with diagnosis of coronary artery disease are enrolled in this study between November 2015 and March 2016. Patients were recruited from Cardiology ward of Razavi Hospital, Mashhad, Iran. This study was accepted by ethics committee of Mashhad University of Medical Sciences (code: 931459). Patients with calcium and Phosphor metabolic disorder, parathyroid disease, renal dysfunction, history of osteoarticular disorders, zero calcium score, and were excluded from the study. A questionnaire containing demographic data, laboratory data, drug history, medical history, familial history of cardiovascular risk factors was completed for all patients. All patients signed the consent form prior to entry in the study.

Determination of decorin serum concentration and CAC

Whole blood was collected from patients and centrifuged at 2500 rpm for 10 min. The plasma frac-

tion was isolated and stored at -70°C until required for analysis. Routine biochemical measurements such as plasma glucose, total cholesterol (TC), triglycerides, low density lipoprotein Cholesterol (LDL-c), high-density lipoprotein cholesterol (HDL-c), and serum calcium and phosphorus level were carried out by routine laboratory methods. Serum level of soluble DCN was measured with an enzyme-linked Immunosorbant assay (ELISA) -kit (Zellbio, Germany); each assay was calibrated using BGN standard curve following the manufacturer's instructions. Coronary Artery Calcification was determined by CT-Angiography.

Statistical analysis

Statistical analysis was carried out by SPSS16, All measured values are presented as mean ± SD.

Correlation between Serum Concentration of DCN with CAC was analyzed using spearman correlation test. To compare serum concentration of DCN between different groups, Independent-sample T Test was used. Results were considered significant at $p<0.05$.

Results

Characteristics of the study population

The study population consists of 84 patients, male (77%) and female (23%). The mean age of population was 56.80±10.73 years. Patients' characteristics, laboratory tests including biochemical parameters, traditional cardiovascular risk factors and mean decorin serum level are summarized in Table 1.

Table 1. Patients' characteristic, laboratory data, traditional cardiovascular risk factors and mean decorin serum level of patients

Patients' characteristics	Mean±SD
Age (year)	57.13±10.7
BMI (kg/m^2)	28.36±4.78
Female/male ratio	0.29
Laboratory tests	**Mean±SD**
HDL-C (mg/dl)	41.92±9.97
LDL-C (mg/dl)	90.81±29.14
Total cholesterol (mg/dl)	163.30±33.32
FBS (mg/dl)	104.56±24.00
Traditional risk factors	**Frequency (%)**
Hypertension (%)	45.88
Dyslipidemia (%)	63.52
Positive family history (%)	51.76
Diabetes (%)	20.58
Current Smoking (%)	35.29
Concentration of DCN (pg/mL, Mean±SD)	34.45±19.78

BMI: Body Mass Index, HDL-C: High Density Lipoprotein-Cholesterol, LDL-C: Low Density Lipoprotein-Cholesterol, FBS: Fast Blood Sugar

Correlation between DCN serum level and coronary artery calcification agatson score

There was no significant correlation between DCN serum level and total coronary artery calcification score and also CAC score of LAD, LMCA, RCA and CX ($P>0.05$) (Table 2).

Table 2. Correlation between DCN serum concentration with LAD, RCA, LMCA, and CX coronary artery calcification score

Coronary artery Calcium score	Mean ±SD	P value Spearman Correlation Test	r s Correlation coefficient
Total calcification of coronary vessels (agatson score)	357.29±590.81	0.28	-0.121
Calcification in coronary LAD (agatson score)	184.60±304.46	0.763	0.044
Calcification in coronary RCA (agatson score)	63.37±101.86	0.125	-0.22
Calcification in coronary CX (agatson score)	44.86±99.00	0.064	-0.264
Calcification in coronary LMCA (agatson score)	34.11±116.00	0.460	-0.107

LAD: Left Anterior Descending, RCA: Right Coronary Artery, CX: Circumflex, LMCA: Left Main Coronary Artery, DCN: Decorin

Discussion

In this study, the correlation of the DCN serum level with CAC was evaluated in patients with coronary artery disease. As can be found from aforementioned results, there was no significant correlation between DCN serum level and total CAC and CAC of RCA, LAD, LM and CX (P>0.05). Until now, the relationship between the DCN serum concentration and CAC has been investigated in vitro or only in animal models. In accordance with the obtained results from the previous studies, it is entirely apparent that the calcification is stimulated by Decorin by the means of TFG-β.

In two studies conducted in vitro, it was shown that decorin induces the osteogenic differentiation of the smooth muscle cells. Decorin affects this differentiation via TGF-β signaling pathways. The decorin central protein connects to TGF-β isoforms via side chain. The oxidized LDL adjusts the synthesis of decorin side chain. The difference in the composition of the glycoseaminoglycon chain may occur in various cases such as the spread of atherosclerosis and progressive calcification and vascular rearrangement. High sulfate level of chondrotin sulfate stimulates osteoblastic mineralization and increases the XT-I level which is the enzyme responsible for the synthesis of the chain. The side chain acts as the inductor of bio mineralization of smooth muscle cells of vessels and activator of the signaling pathways TGF-β and adjusting MSX-2 with support of mineralization induced by oxidized LDL. Therefore, the increase in decorin expression significantly increases the TGF-β activity and TGF-β induces the calcification of smooth muscle cells of the vessels. Also, the signaling induced by decorin accelerates osteogenic differentiation in the smooth muscle cells of the vessels (16, 17).

In an in vitro study, the increase of the expression of decorin in retroviral induces the collagen gel contractions and stimulates collagen synthesis. In an invivo study, the increased synthesis of fibrin fibronectin leads to the formation of dense collagenous matrix within the damaged intima of the artery. So, it is possible that the decorin connects the matrix components such as collagen and fibronectin as the onset or the nucleus for the growth of hydroxyapatite crystals. In another study, it has been shown that decorin increases the hydroxyapatite formation for mineralization of collagen gel (16, 17).

Moreover, an in vitro study revealed that DCN prevents of matrix mineralization with unknown mechanism. DCN binds via leucin-rich chain to collagen and thereafter, the GAG component is exposed at the surface of collagen fibrils. This enlarged GAG, possibly with partially exposed protein core, may inhibit hydroxyapatite development in sides of collagen fibrils. Followed by, regulating the assembly and stableness of collagen fibrils may lead to the inhibition of matrix mineralization. Further studies are necessary to clearly elucidate the inhibitory mechanisms (18). According to the results mentioned above, there was no significant relationship between the coronary artery calcification and decorin serum concentration.

It is possible that by increasing the studied population size, -as there is enough data about calcium score in different sub-groups- we can understand the relationship between this biomarker and coronary artery calcification much better. On the other hand, the coronary artery calcium score of the studied patients in the sub-groups was not distributed uniformly. Perhaps if the calcium score distribution was balanced, a significant relationship could be found.

Conclusion

In this study, the correlation of the DCN serum level with CAC was clinically evaluated for the first time that there was no significant correlation between DCN serum level and total CAC, and CAC of RCA, LAD, LM and CX (P>0.05).

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Original article

Relation of the frequency and mortality of pulmonary thromboembolism with meteorological parameters

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Summary. *Objective:* The objective of this study is to find the relationship between incidence rate and mortality of acute pulmonary thromboembolism (PTE), and seasonal and meteorological factors. *Materials and methods:* The data from 234 patients who were hospitalized due to acute PTE in the emergency service or policlinics between 2001 and 2008 were investigated retrospectively. Cases that developed APE (acute pulmonary embolism) in the hospital were excluded. Seasons and months in which acute PTE was diagnosed were recorded. Mortality rates by months and seasons were evaluated. The mean pressure, temperature and humidity values were evaluated for periods of three days, seven days and one month before the day of presentation. The effects of meteorological factors on the severity (massive or non-massive) and mortality of APE were investigated. *Results:* The incidence rate of acute APE showed a significant difference according to seasons (p=0.000). APE was diagnosed most commonly in spring and winter. The mean pressure values for three days, seven days and one month and the mean humidity values for three days for the dead patients were found to be significantly lower than those of the survived ones (p<0.05). The mortality rate for patients admitted in summer was significantly higher than the rates for other seasons (p=0.02). There were no seasonal differences among the massive APE incidences. Mortality rates were higher in summer because of the nonmassive APE patients rather than the massive patients. *Conclusion:* Acute PE is a disease whose incidence and mortality rates are affected by meteorological factors. (www.actabiomedica.it)

Key words: pulmonary thromboembolism, meteorological parameters

Introduction

Barometric pressure is the pressure that is formed by gravity acting upon the atmosphere. Normal pressure at sea level, at a temperature of 15°C, is 760 mmHg or 1013.25 millibars (1). Barometric pressure is affected by changes in altitude and temperature. When altitude and temperature increase, barometric pressure decreases, and it increases at sea level and when temperature decreases (2).

It has been found that thromboembolic events such as pulmonary embolism, acute myocardium infarct, temporary ischemic attack, paralysis and retinal vein occlusion show chronobiological differences and occur more frequently in specific seasons and months (3-5). An autopsy study revealed that deaths from PTE are more common in some seasons (6). Other studies in the literature show no associations between meteorological factors and PTE (7, 8). When we looked closer at the literature we saw that studies to identify the relationship between PTE and meteorological factors are not numerous, and the case has not yet been made clear.

We observed clinically that the number of patients with acute PTE increases in some periods. We planned to find out whether this observation was correct, and if so, to investigate its relationship with me-

teorological factors. Within this study we also investigated the association between meteorological factors and the severity of PTE.

Materials and methods

Data from 234 patients who were with diagnosed PTE by the Chest Diseases Clinic of Trakya University Medical Faculty between May 2001 and April 2008 were investigated retrospectively. Patients who developed acute PTE in the hospital were excluded from the study.

Patients with high probability V/P scintigraphy and low or medium probability V/P scintigraphy, together with DVT diagnosed through Doppler USG or thrombus diagnosed through spiral thoracic CT, were included in the study.

Addresses and demographic features of the patients, dates of their admissions to ER, types of PTE – massive or nonmassive – treatments applied and the results (discharge from hospital, death) about the patients, were all recorded. Months and seasons of admission dates were defined. Daily meteorological pressure, humidity and temperature values were obtained electronically from the general directorate of meteorology. Based on these data, the meteorological parameters (pressure, humidity and temperature) were defined for three days, seven days and one month before the day of presentation of the patients and the mean values were calculated.

Data were analyzed with the SPSS 15 program. Descriptive statistics, Kolmogorov-Smirnov test, X^2 test, correlation analysis and T test for the independent samples were used. Values of $p<0.05$ were deemed statistically significant.

Results

234 patients with PTE were included in the study. Of the patients 113 were female (48.3%), 121 were male (51.7%) and the mean age was 59.43±15.9, with the youngest being 19 and the oldest 93. The demographic features of the patients according to seasons and co-morbidities are summarized in Table 1.

When we examined the seasonal distribution of patients, we determined that most of the patients that treatment procedures applied were in spring (n=67, 28.6%) and the least in autumn (n=47, 20.1%). Differences among seasons were found to be statistically significant (p=0.000).

When we looked at the monthly distribution of the patients treated due to PTE, we established that most patients were followed in March (n=28, 12.0%) and the least in November (n=12, 5.1%). Differences among months were found to be statistically significant (p=0.02).

Table 1. Demographic features of the patients according to seasons and co-morbidities

	Spring (n=67)	Summer (n=56)	Autumn) (n=47	Winter (n=64)
Female / Male	37/30	24/32	21/26	31/33
Age	59±15	60±15	57±17	60±15
Undergone PTE	3	2	0	3
Cardiovascular Disease	35	28	25	33
DM	5	7	4	5
KBY	1	2	1	1
Hematological Disease	0	2	0	1
DVT	2	4	2	4
Varix	3	0	3	5
Cerebrovascular Disease	3	4	5	9
COPD	9	6	2	6
Lung Carcinoma	2	4	2	2
Extrapulmonary Malignity	2	5	4	4

Table 2. Results about patients according to seasons

Seasons	Discharged n (%)	Mortality n (%)	p value
Spring	60 (25.9)	7 (3.0)	0.47
Summer	44 (18.9)	12 (5.2)	**0.02**
Autumn	43 (18.6)	4 (1.7)	0.24
Winter	55 (23.7)	7 (3.0)	0.69

When we evaluated the rates of discharge from hospital and mortality after treatment, we obtained the figure of 232 patients out of 234. We determined that 30 (12.8%) of the patients died and 202 (%87.2) were discharged. 14 of the dead patients were female and 16 were male; 97 of the patients discharged were female and 105 were male. The mean age for the dead patients was 67.7±13.6, while for the discharged patients it was 58.2±15.9 (p=0.01).

When the mortality rates were compared according to seasons, it was found that the mortality rate increased in summer (n=12, 5.2%) although the number of patients was relatively less (n=56, 24.1%) in summer. This increase was statistically more significant than the other seasons (p=0.02). The results about patients according to seasons are shown in Table 2.

When the rates of discharge and mortality were evaluated according to months, it was found that the mortality rate increased in August (n=5, 2.1%) although the number of patients decreased (n=14, 5.9%). This increase was statistically more significant than the other months (p=0.00).

When the mean temperature, pressure and humidity values were compared, summer was the season with the lowest pressure and humidity values but the highest temperature, while winter had the highest pressure and humidity values but the lowest temperature. Seasonal differences among pressure, temperature and humidity were statistically significant (p=0.00). There was an "inverse" correlation between temperature and pressure and humidity. When we compared the mortality rates according to seasons, we found that the mortality rate was highest in summer during which pressure and humidity were lowest and temperature highest (p=0.02) (Figure 1).

When the number of patients and mortality rates were compared according to months, the mortality rate was found to be significantly higher in August during which pressure and humidity were the lowest and temperature the highest (p=0.02) (Table 3).

The results about patients and pressure, humidity and temperature values for three days, seven days

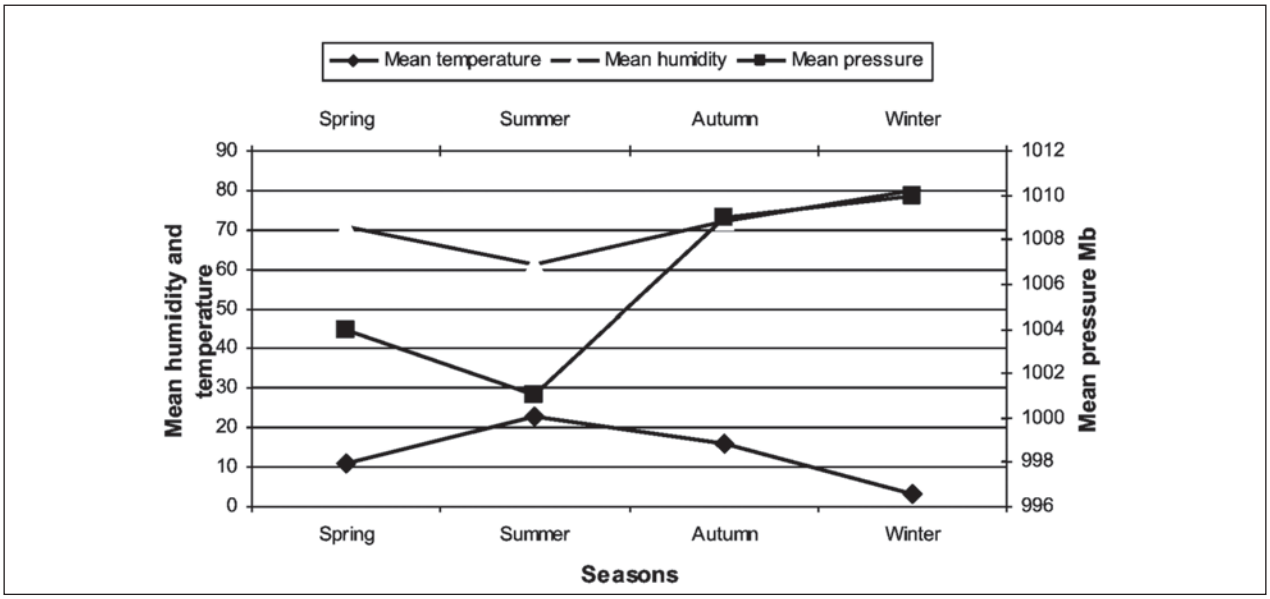

Figure 1. Mean pressure, temperature, humidity values according to seasons

Table 3. Mean pressure, temperature, humidity values, number of patients and mortality rates according to months

Month of diagnosis	Patients (n)	Mortality (n)	Mean pressure (Mb)	Mean temperature (°C)	Mean humidity (%)
January	24	2	1011.0	1.68	81.1
February	20	1	1010.1	4.8	78.2
March	28	2	1005.0	8.4	74.8
April	21	3	1003.1	11.8	69.2
May	18	2	1004.3	16.8	68.2
June	20	5	1004.6	22.2	62.5
July	20	2	999.4	24.2	56.8
August	14	5	998.1	25.0	62.1
September	16	1	1010.8	20.1	65.9
October	22	2	1007.2	16.6	72.8
November	12	1	1011.1	10.4	78.5
December	17	4	1011.0	4.3	81.1

Mb: Millibars; °C: Degree centigrade

and one month before the presentations of the patients were compared. The mean pressure values for three days (1001.4±10.9 Mb), seven days (1001.8±11.3 Mb) and one month (1002.8±102 Mb), and the mean humidity values for three days (67.8±11.9%) before the presentation of the patients who later died were significantly lower than those values of the patients who were discharged from hospital. As for the temperature, no significant effects were detected on mortality or discharge rates ($p<0.05$) (Table 4).

When patients with PTE were grouped as massive and nonmassive, then of the 234 patients 53 were massive and 181 were nonmassive. The mean age was found to be 58.6±16.3 for the massive PTE patients and 62.5±14.3 for the nonmassive PTE patients ($p=0.12$). Of 53 patients with massive PTE, 29 were female and 24 were male; of the patients with nonmassive PTE 84 were female and 97 were male ($p=0.28$). In their follow up period we determined that 17 (32%) of the massive and 13 (7%) of the nonmassive PTE patients died. The higher rate of mortality in massive PTE was statistically significant ($p=0.00$).

The mean age for the patients who expired due to massive PTE was 64.2±14.6 and that of the discharged patients was 61.3±14.3. The association between mortality due to massive PTE and age was statistically insignificant ($p=0.49$). The mean age for the patients who expired due to nonmassive PTE was 72.2±11.1 and that of the discharged patients was 57.6±16.2. We found the association between mortality due to nonmassive PTE and age statistically significant ($p=0.00$).

When we investigated the incidence of 53 patients with diagnosed massive PTE according to seasons and the results about them, we found that the

Table 4. Association between the results about the patients with pulmonary thromboembolism and meteorological factors

Meteorological feature	Deceased patients	Discharged patients	p value
Mean pressure for three days±SD	1001.4±10.9	1006.3±10.4	**0.03**
Mean pressure for seven days±SD	1001.8±11.3	1006.5±9.8	**0.02**
Mean pressure for one month	1002.0±10.2	1007.3±9.6	**0.01**
Mean humidity for three days±SD	67.8±11.9	73.2±12.6	**0.04**
Mean humidity for seven days±SD	69.1±11.2	72.7±12.1	0.16
Mean humidity for one month±SD	70.3±9.8	72.6±10.2	0.31
Mean temperature for three days±SD	13.6±9.1	12.8±8.1	0.62
Mean temperature for seven days±SD	14.2±8.4	12.5±7.9	0.33
Mean temperature for one month±SD	13.4±8.4	12.4±7.8	0.55

SD: Standard Deviation

most of the patients were treated in winter (n=16), and the fewest were followed in spring and autumn (n=11). The difference between seasons was found to be statistically significant (p=0.01). Concerning the mortality rates according to seasons, we determined that the highest mortality was in spring and summer (n=5) and the lowest in autumn (n=3), which meant a statistically insignificant difference between seasons in terms of mortality (p>0.05).

We obtained the results of 179 nonmassive patients and investigated the incidences according to seasons and the results about them. We saw that the maximum number of patients were followed in spring (n=56) and the minimum number in autumn (n=38), and the difference between the seasons was significant (p=0.00). The results about the patients according to seasons were examined, and an increase in mortality rates was observed in summer although there was a decrease in the number of patients. The highest mortality was detected in summer (n=6) and the lowest in autumn (n=1). The rise in the mortality rate in summer was statistically significant (p=0.03).

When we looked at the monthly distribution of patients with massive PTE, we saw that the maximum number of patients were admitted in January and June (n=6) and the minimum number of patients were admitted in November (n=2). There were no significant differences in this monthly distribution (p=0.58). Concerning the mortality rates according to months, it was determined that most of the deaths were in May (n=3) and the differences in mortality rates was not significant (p>0.05).

When we considered the months of diagnosis, we determined that most of the nonmassive PTE patients were followed in March (n=24) and the least were followed in November (n=10). The difference among the months was found to be statistically significant (p=0.003). With respect to mortality rates of patients according to months, we found that the maximum number of patients died in June and August (n=3). The rise in August was considered statistically significant (p=0.03).

Our investigation concerning the association between the development of massive and nonmassive PTE and the values of pressure, temperature and humidity for three and seven days and one month revealed that these meteorological parameters have no effects on the incidence of massive and nonmassive PTE (Table 5).

When we investigate the correlation between the outcomes of patients with massive PTE and the values of pressure, temperature and humidity for three and seven days and one month, we found that the mean pressure value for one month was lower in the period that patients mostly died, and this was statistically significant (p=0.04). Temperature and humidity values did not have any effects on the results for massive PTE patients.

When the results of patients with nonmassive PTE and the values of pressure, temperature and humidity for three and seven days and one month were investigated, the mean pressure value for three days was found to be lower in the period patients died and this was statistically significant (p=0.03). Temperature

Table 5. Effects of meteorological factors on the development of massive and nonmassive pulmonary thromboembolism

Meteorological feature	Massive PTE Mean±SD	Non massive PTE Mean ±SD	p value
Pressure for three days	1004.7±10.9	1006.1±10.6	0.46
Pressure for seven days	1005.0±10.3	1006.3±10.0	0.46
Pressure for one month	1005.6±9.4	1007.0±9.9	0.42
Humidity for three days	70.6±12.8	73.1±12.5	0.25
Humidity for seven days	70.7±12.3	72.7±11.9	0.34
Humidity for one month	70.6±10.0	72.8±10.2	0.21
Temperature for three days	13.4±9.1	12.6±8.0	0.59
Temperature for seven days	13.8±8.6	12.3±7.8	0.30
Temperature for one month	13.1±8.6	12.3±7.7	0.55

SD: Standard Deviation

and humidity values were established to have no effects on mortality and discharge in those patients with nonmassive PTE.

Discussion

In our study we identified that most of the patients with PTE were followed in spring (n=67), then in winter (n=64), and the least in autumn (n=47) and summer (n=56). When we investigated the two studies carried out in our country about the seasonal distribution of patients with PTE, we saw that in the study of Meral et al. (3) involving 91 patients, similar results had been obtained, with more PTE cases being observed in the spring.

Boulay et al. (9) investigated PTE and DVT cases in all the hospitals in France by using ICD codes. 62,237 patients with PTE and 65,081 patients with DVT were included in their study.

As a result they found that the number of both PTE and DVT patients markedly increased in winter and decreased in summer.

The second study made in our country about this subject was made by Ercan et al. (10) and included a limited number of patients (n=49). This study revealed a decreased number of PTE cases in winter. Similar results were obtained in the studies made by Gallerani et al. (11) and Manfredini et al. (12). The study of Gallerani et al. (11) included 22,436 patients diagnosed in the hospitals of the Emilia-Romegna region in Italy. This large-scale study established that the number of PTE patients increase markedly in winter.

In the study of Masotti et al. (13), patients were grouped into internal and surgical patients, and the incidence rates according to seasons were investigated. When all patients were evaluated in general, an increase in the number of patients in winter months was discovered and this increase was more prominent in the surgical patient group. Sharma et al. (14) evaluated 248 PTE patients and found that PTE incidences were 2.9 times higher in the autumn and winter months.

Beside the studies indicating the seasonal differences in PTE incidences, other studies show no differences. Stein et al. (15) evaluated 2,475,000 PTE and 5,767,000 DVT patients in the USA between 1979 and 1999. This large-scale study revealed no differences in the incidences of PTE and DVT according to seasons and months. Bilora et al. (16) investigated the seasonal association of nonmassive PTE and DVT. In their study, although there were differences in the winter and summer months, these differences were not statistically significant. An increase in PTE and DVT development was determined on Saturdays and in the mornings. Scott et al. (17) investigated incidences of PTE development according to seasons and barometric pressure and found that PTE development was not associated with the seasons.

When we evaluated the PTE incidences according to months, we saw that most of the patients were followed in January (n=24) and March (n=28). Masotti et al. (13) similarly determined that PTE patients were mostly admitted in January and March. Likewise, Gallerini et al. (11) and Ercan et al. (10) established in their studies that PTE cases were most common in January.

When we investigated the mortality rates of PTE patients, we determined a significant increase in mortality due to PTE in the summer, especially in August. In the literature, however, there are few studies about the subject and they lead to different results. In an autopsy study by Green and Edwards (6), a higher mortality rate due to massive PTE was established for spring and autumn. When we made an evaluation according to seasons, the rise in October was found to be statistically significant. Masotti et al. (13) evaluated PTE and mortality rates in their study. Their study concluded that there was an increase in the number of patients with PTE and mortality rates in the winter months. In their study covering a period of 18 years, Stein et al. (18) found that PTE mortality was not associated with seasons.

When we investigated PTE development and barometric pressure, temperature and humidity values, we observed an inverse correlation between temperature and values of pressure and humidity. During the period when the pressure was low the number of patients increased; the changes in temperature and humidity values did not have any effects on the number of patients. When we looked at the association between mortality rates and pressure, temperature and humidity values, we found that in the period when pa-

tients died due to PTE the mean rates of pressure for three days, seven days, one month and humidity rates for three days were low, and temperature had no effect.

Of the studies made about our subject, the one by Masotti et al. (13) established that the number of patients with PTE increased when barometric pressure was low. In their study about the development of PTE, Scott et al. (17) determined an increase in the number of PTE patients when barometric pressure decreased. Clauss et al. (19) found in their study that humidity and rain affected PTE development positively, but temperature and pressure had no effects.

In our country the study of Meral et al. (3) determined that the number of patients with PTE increased during the periods when the pressure was low; on the other hand they could not find a relation between mortality and pressure. Ercan et al. (10) found in their study, which they made with a limited number of patients, that PTE was associated with pressure and temperature.

In our study we established that seasons and meteorological parameters do not have any effects on the development of massive and nonmassive PTE. Meral et al. (3) also reached similar results in their study and reported that pressure was not associated with the development of massive and nonmassive PTE.

The most important factor restricting these two studies made in our country is the limited number of patients. Our study is the largest one made in our country about this subject.

The effects of seasonal variability on PTE development are associated with hematological reasons. It has been shown that changes in blood viscosity and coagulation (20) and the development of red blood cells and the rise in the number of platelets due to slight surface cooling (21) cause spontaneous thrombosis. This state of hypercoagulability is supported by increased fibrinogen levels. In the cold months of the year fibrinogen levels rise a lot (22). Fibrinogen level is a major risk factor for cardiovascular diseases (23). Another effect of cold weather is that it raises blood pressure levels (24). A rise in sympathetic activity and a decline in liquid and sodium loss can explain the high levels of blood pressure in winter months (25). Low temperatures increase cardiovascular and respiratory morbidity and mortality. A short term exposure to temporary air pollution is effective on cardiorespiratory mortality and morbidity (26). Cold wind alone can determine mortality more than temperature can. In some studies this subject was investigated and it was stated that moving air causes physiological changes in a few hours and increases blood viscosity at the rate of 20% (21).

Consequently, this study confirmed our observation about the increase in the incidence and mortality of pulmonary thromboembolism in some periods. Our findings indicating that low atmospheric pressure can affect the variability of mortality need to be supported by large- scale studies.

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Original article

The role of FFR in clinical decision making in patients with moderate coronary lesions: a pilot study

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Summary. *Background and Aim:* Applying fractional flow reserve (FFR) recently helped to assess borderline coronary defects and also facilitates assessment of these lesions. The present study aimed to assess cost-effectiveness of FFR in detection of these borderline lesions. *Methods:* This cross-sectional study was conducted on140 consecutive patients with 219 diseased arteries who underwent coronary angiography and suffered intermediate coronary lesions. *Results:* Of 18 patients who candidate for CABG before FFR, only one patient underwent CABG after determining FFR (P-value<0.05), while 15 patients were scheduled for PCI and 2 patients for medical treatment. Of 122 patients who candidate for PCI, 59 were programmed to underwent PCI after FFR determination(P-value<0.05), while the strategy in 63 patients (47 with one-vessel disease, 15 with two vessel diseases, and 1 with three vessel diseases) was modified to medical treatment. Considering strategy modifying from PCI to medical treatment, 101 stents were saved (P-value<0.05). Also, in change of strategy from CABG to PCI, spending has decreased as much as 77.3% (P-value<0.05). Furthermore, the change of treatment approach from PCI on much number of coronary vessels to PCI on less number of coronary lesions led to saving of 52.2% of costs(P-value<0.05). *Conclusions:* In patients with an intermediate coronary lesion, measuring FFR to guide the decision to determine treatment strategy may lead to significant cost savings. (www.actabiomedica.it)

Key words: fractional flow reserve, myocardial, coronary vessels, cost-benefit analysis, percutaneous coronary intervention, coronary artery bypass

Introduction

Management of moderate coronary lesions in coronary angiography which is defined as coronary stenosis between 50 to 70 percent by eyeball estimation is a challenging issue. Recently, applying fractional flow reserve (FFR) helped to assess moderate coronary stenosis and also facilitate assessment of these lesions in catheterization laboratory (1-3). This parameter is defined as the ratio of pressure distal to stenosis to aortic pressure (4, 5). Different studies have shown that the FFR lower than 0.75 can predict cardiac ischemia with a high level of accuracy (6-8). Furthermore, deferring PCI in the presence of FFR<0.75 can significantly diminish long-term adverse events of revascularization procedures (9). Besides helpful role of cardiovascular diagnostic procedures, the cost-effectiveness of these procedures should be considered judiciously (10-12). In this context, the cost-effectiveness of FFR is currently under survey and there are a small number of

studies on this subject. In several studies, high effectiveness of FFR for diagnosis of borderline coronary lesions has been revealed (13-16); however in some other studies, the cost of coronary pressure wire for assessment of moderate coronary lesions has been reported noticeably high (17, 18). The present study aimed to assess cost-effectiveness of FFR in detection of these moderate coronary lesions.

Materials and methods

This cross-sectional study was conducted on140 consecutive patients who were candidate for coronary angiography and had moderate coronary lesions (coronary stenosis between 50 to 70 percent in angiography). The patients were randomly assigned into two groups that undergoing CABG or PCI without considering FFR value (control group) and undergoing these procedures when FFR estimated less than 0.75 (case group). Baseline information including demographics and medical history of patients were recorded. Cost-effectiveness of the two procedures (defined as reduction in necessitating CABG or PCI and also reduction of materials and agents which are needed for revascularization) was assessed and compared between the two study groups. Results were showed as mean ± standard deviation (SD) for quantitative variables and were summarized by frequency (percentage) for clear-cut variables. Continuous variables were compared using T-Test and Mann-Whitney test. On the other hand, categorical (clear-cut) variables were compared using chi-square test. For the statistical analysis, the statistical software SPSS version 20.0 for windows (SPSS Inc., Chicago, IL) was used. P values less than 0.05 were considered statistically significant.

Results

Totally, 140 patients were included in our study. The mean age was 58.71 ± 9.88 years ranged 33 to 83 years and 64.3% were male. The most common cardiovascular risk factors were hypertension (57.9%) followed by hyperlipidemia (42.9%), diabetes mellitus (36.4%), and cigarette smoking (29.3%). Family history of coronary disease was revealed in 22.9% and 15.7% were obese. Regarding clinical symptoms, 66.4% had chest pain, 10.7% had dyspnea, and 22.9% had both symptoms. Mean LVEF was 51.19 ± 9.46 in echocardiography. In coronary angiography, 36.4% had single-vessel disease, 38.6% had two-vessel disease, and 25% had three-vessel disease. Also, the number of coronary vessels needing angioplasty was one vessel in 45%, two vessels in 37.1%, and three vessels in 12.9%.

Of 18 patients who candidate for CABG before FFR, only one patient underwent CABG after determining FFR (P-value<0.05), while 15 patients were scheduled for PCI and 2 patients for medical treatment. Of 122 patients who candidate for PCI, 59 were programmed to underwent PCI after FFR determination (P-value<0.05), while the strategy in 63 patients (47 with one-vessel disease, 15 with two vessel diseases, and 1 with three vessel diseases) was modified to medical treatment (Figure 1). In those with remained PCI strategy, PCI on two vessels was modified to one vessel in 27 cases, PCI on three vessels was modified to two vessels in 4 cases, and PCI on three vessels was modified to one vessel in 8 cases(P-value<0.05). In total, considering strategy modifying from PCI to medical treatment, 101 stents were saving (P-value<0.05). Also, in change of strategy from CABG to PCI, spending has decreased as much as 77.3% (P-value<0.05). Furthermore, the change of treatment approach from PCI on more coronary vessels to PCI on less coronary lesions led to saving 52.2% of cost spending(P-value<0.05).

In 1-year follow up, in the "PCI before FFR" arm, of 63 patients who finally underwent medical treatment after FFR, only one patient had non-STEMI who was admitted and PCI was performed for the patient; also, one mortality due to non-cardiovascular causes was recorded; of 59 patients who finally underwent PCI after FFR, only one re-admission due to unstable angina was recorded and managed conservatively. In the "CABG before FFR", of 15 patients who finally underwent PCI after FFR, only one patient re-admitted due to unstable angina and managed conservatively; those patients who finally underwent medical treatment or CABG, revealed no adverse event after 1-year follow up. All of these results were showed in the table under the diagram (Figure 1).

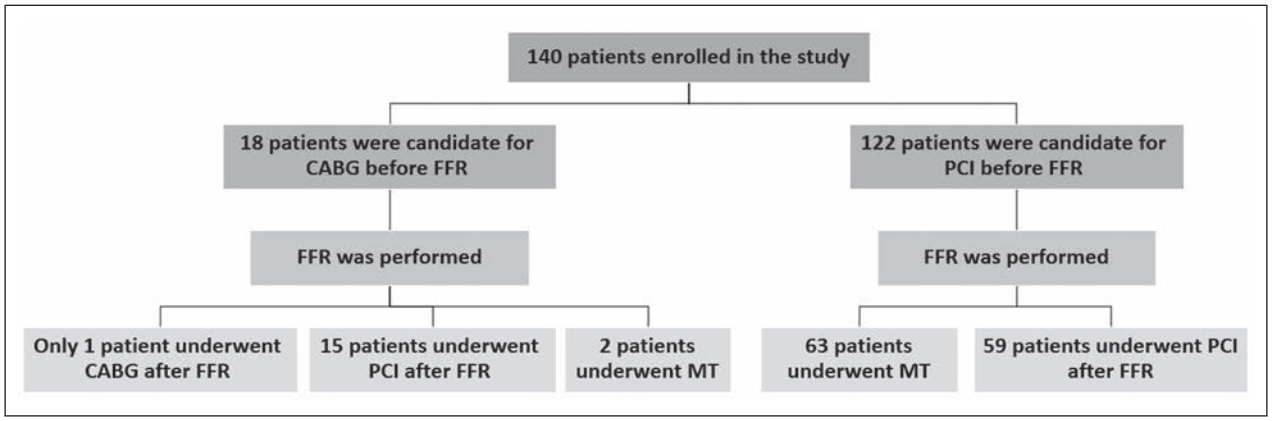

Figure 1. Patients treatment plan before and after performance of FFR; table under the diagram, shows 1-year follow up results of each groups .CABG: coronary artery bypass graft; FFR: fractional fellow reserve; MT: medical therapy; PCI: percutaneous coronary intervention

Discussion

The cost of interventions and related materials in the field of cardiology and cardiac surgery is one of the essential issues of health care systems .and all efforts are focused to lessen these costs as much as possible. The main goal of the present study was to assess cost-effectiveness of FFR in determining reasonable treatment approach in patients with moderate coronary lesions (coronary stenosis between 50 to 70 percent). In fact, decision on selecting the best treatment method in these patients had been ever challenging; thus inappropriate selection of treatment method may result in adverse consequences and also unnecessary expenditure. Thus, the current study aimed to determine the role of FFR on reduction of treatment costs and cost-effectiveness. Based on our results, assessment of FFR before therapeutic procedures can effectively reduce costs. On the other hand, in most patients scheduled for CABG, the type of protocol was changed from CABG to PCI or medical treatment leading considerably decrease of medical costs. Also, notable number of patients programmed to undergo PCI on coronary lesions were considered to undergo PCI on less lesions or even to receive medical treatment. In total, it seems that those patients with moderate coronary lesions can finally managed by medical treatment after considering FFR.

Almost all previous reports confirm high cost-effectiveness of FFR in CAD patients (19). In one study, the FFR strategy saved 1795 dollars per patient compared with the nuclear stress imaging study strategy and 3830 dollars compared with the stenting of all intermediate lesions strategy, while quality-adjusted life expectancy was similar among the 3 strategies (20). In another study of patients with multi-vessel CAD and borderline lesions, FFR measurement identified those who can be treated conservatively with a good long-term outcome and prevented unnecessary PCI (21). In another investigation; Two-thirds of those for whom PCI had appeared to be warranted, were treated conservatively; and only one quarter of the original "surgical" group underwent CABG (22). It was shown that the decision for bypass surgery was supported by FFR; in FFR above 0.75, a conservative approach was acceptable (13).

As a conclusion, in patients with an intermediate coronary lesion, measuring FFR to guide the decision to determine treatment strategy may lead to significant cost savings. Our study is a single center study with relatively small sample size and limited resources; and is considered as a pilot study for future large multicenter studies. The result of our pilot study revealed that FFR-guided interventions (including PCI or CABG) of moderate coronary lesions could be more cost-effective in comparison of conventional decision for PCI or CABG.

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Original article

Surgical management of middle ear cholesteatoma in children with Turner syndrome: a multicenter experience

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Summary. *Background and aim:* As in other syndromes characterized by craniofacial anomalies, middle ear cholesteatoma is known to have a high prevalence in Turner syndrome. The aim of this study was to review a multicenter experience with the surgical management of middle ear cholesteatoma in children with Turner syndrome. *Methods*: We retrospectively analyzed sixteen girls with Turner syndrome who underwent otologic surgery for middle ear cholesteatoma between January 2000 and December 2012. Surgery was performed in 3 tertiary care otologic centers. Four patients had bilateral disease, resulting in a total of 20 ears treated. The following data were recorded: age, history of ventilation tube insertion, status of the controlateral ear, cholesteatoma location and extension, and surgical technique involved. Cholesteatoma recidivism, stable mastoid cavity and hearing levels were the main outcomes measured. *Results:* Follow-up ranged from 3 to 15 years (mean 7 years). Fourteen ears underwent canal wall down mastoidectomy: no cases of recurrent cholesteatoma were observed in these cases; revision mastoidectomy with cavity obliteration was needed in 2 ears (14.3%) for recurrent otorrhea. In the remaining 6 ears a staged canal wall up mastoidectomy was performed: 1 child showed a recurrent cholesteatoma and required conversion to canal wall down mastoidectomy. A postoperative air-bone gap result of 0 to 20 dB was achieved in 6 ears (30%); in 9 ears (45%) postoperative air-bone gap was between 21 and 30 dB, while in 5 (25%) was >30 dB. Bone conduction thresholds remained unaffected in all cases. *Conclusions:* Cholesteatoma in children with Turner syndrome is a challenging entity for the otologic surgeon. Although not mandatory, canal wall down mastoidectomy should be regarded as the technique of choice to achieve a safe and dry ear in TS children with middle ear cholesteatoma. Intact canal wall mastoidectomy should be adopted only in appropriately selected patients such as those with limited attic cholesteatoma that can be regularly followed-up. (www.actabiomedica.it)

Key words: cholesteatoma, Turner syndrome, middle ear surgery, mastoidectomy, hearing

1. Introduction

Turner syndrome (TS), a relatively common chromosomal disorder occurring in one out of 2,000 live-born girls, is caused by complete or partial absence of one X chromosome (1). Monosomy 45,X, having only one X-chromosome in all cells, is the most frequently occurring karyotype (50%), while 30-40% of the patients have a mosaicism, with two or more chromosomally different cell lines (2). The prominent features of TS are gonadal dysgenesis, short stature, and dysmorphic abnormalities, including webbing of the neck, cubitus valgus, micrognatia, high arched palate, low posterior hairline. Several studies (3-5) reported an

increased prevalence of otologic and audiologic problems in TS. The external ear anomalies include low set ears, cupped auricles, external auditory canal stenosis, and abnormally protruding ears. Women with TS often develop a mid-frequency SNHL in their teens or adolescence; as long as the higher frequencies are still heard, this usually does not cause hearing problems (5). Literature data (6-7) show great variability in the prevalence of SNHL varying from 9 to 66%. Girls with TS are known to have a high prevalence of middle ear disease with a predisposition for the development of chronic and recalcitrant otologic disease. The predisposition to middle ear disease is most likely due to craniofacial anomalies frequently observed in TS patients, such as growth disturbances of the cranial base, downward sloping of the external auditory canal, abnormal orientation of the Eustachian tube, palatal anomalies. An interesting theory, the cell cycle hypothesis, was proposed by Barrenas et al. (4) to explain the predisposition of TS patients for acute and chronic otitis media. Because of a prolonged cell cycle and a lack of transacting growth-regulating genes such as the SHOX/PHOG gene, the up-regulation of the cell cycle in the branchial arches and in the neck region is insufficient, leading to growth disturbance of the cranial base, influencing the anatomy and the function of the Eustachian tube.

As in other syndromes characterized by craniofacial anomalies (7-10), middle ear cholesteatoma is known to have a high prevalence in TS. Verver et al. (2), in a case series of 60 girls founded cholesteatoma in 3 patients, affecting four ears. In a study on 179 patients with TS, Lim et al. (11) reported a cholesteatoma incidence of 3.9% and highlighted the need for early diagnosis and careful follow-up in order to minimize the risk of cholesteatoma progression. Although transfer technologies have been proposed to treat or prevent middle ear disease (12), surgical treatment is our only tool to avoid cholesteatoma-related complications. In the literatute, there are little and sparse data regarding the surgical treatment of cholesteatoma in children with TS. In the present study, we describe the results obtained in 3 tertiary otologic care centers, sharing the same criteria in choosing the surgical technique in the management of acquired middle ear cholesteatoma in children.

2. Material and methods

Charts of children (0-18 years) with TS and middle ear cholesteatoma who had been surgically treated at three tertiary otologic care centers (Carpi, Milano, and Parma) during the period between January 2000 and December 2012 were retrospectively reviewed. The study protocol was approved by the institutional review boards and an informed written consent was obtained from all parents of the patients. Cholesteatoma was diagnosed otoscopically and confirmed by high resolution computed tomography (HRCT) in all children. Medical reports were analyzed by age, history of ventilation tube insertion, cholesteatoma location and extension, status of controlateral ear, neuroradiological findings, surgical procedure adopted, preoperative and postoperative audiograms. Cholesteatoma recidivism, ears with canal wall up mastoidectomy (CWUM) later requiring conversion to canal wall down mastoidectomy (CWDM), postoperative complications, and hearing levels were the main outcomes measured. Hearing results were evaluated according to guidelines set forth by the Committee on Hearing and Equilibrium of the American Academy of Otolaryngology and Neck Surgery for the evaluation of results of treatment of conductive hearing loss (13). Pure-tone average (PTA) was calculated as the mean of 500, 1000, 2000, and 3000 Hz thresholds. The air-bone gap was reported as the four-tone PTA for air-conduction and bone-conduction values determined at the same time. Hearing results were determined at the last follow-up.

3. Results

A total of 16 children were identified and included in this study. Four girls had bilateral disease, resulting in a total of 20 ears surgically treated. Relevant patient demographic data were reported in Table 1. All patients had fresh primary acquired middle ear cholesteatoma. The median age at the time of first surgery was 10.9 years (range, 7-18 years). A positive history for ventilation tube insertion was found in 15 (62.5%) out of the 24 ears. On presentation, all patients reported hearing loss in the affected ear, while recurrent otorrhea was present in 15 (75%) ears. Five

Table 1. Patient demographic data, cholesteatoma type and extension, surgical technique, and postoperative complications

Patient/Ear	Age	Cholesteatoma type	Cholesteatoma extension	Surgical technique	Intraoperative findings	complication Postoperatives
1/Left	11	Attic cholesteatoma	E	Staged CWUM	Residual cholesteatoma	
2/Left	15	Attic cholesteatoma	E	Staged CWUM		
2/ Right	18	Attic cholesteatoma	E	Staged CWUM	Residual cholesteatoma	
3/Right	7	Attic cholesteatoma	E	Staged CWUM		
4/Left	13	Attic cholesteatoma	E	Staged CWUM		
5/Right	12	Attic cholesteatoma	EAM	Staged CWUM		Recurrent cholesteatoma requiring CWDM
6/Left	10	Attic cholesteatoma	EAM	CWDM		
7/Right	15	Attic cholesteatoma	ERAM	CWDM	LSCC fistula	
8/Right	9	Attic cholesteatoma	EAM	CWDM	Contracted mastoid	
8/Left	10	Attic cholesteatoma	EAM	CWDM		Otorrhea treated by medical therapy
9/Left	10	Sinus cholesteatoma	R	CWDM		
10/Left	11	Sinus cholesteatoma	R	CWDM	Contracted mastoid	
11/Right	8	Sinus cholesteatoma	R	CWDM		
12/Left	13	Sinus cholesteatoma	R	CWDM	Contracted mastoid	Otorrhea requiring cavity obliteration
13/Left	12	Sinus cholesteatoma	ERAM	CWDM		
13/Right	13	Sinus cholesteatoma	ERAM	CWDM		
14/Right	10	Sinus cholesteatoma	ERAM	CWDM		Otorrhea requiring cavity obliteration
14/Left	9	Tensa retraction cholesteatoma	ERAM	CWDM		
15/Left	14	Tensa retraction cholesteatoma	ERAM	CWDM		
16/Right	10	Tensa retraction cholesteatoma	ERAM	CWDM		

E: epitympanum; A: antrum; R:retrotympanum; M: mastoid; CWUM: canal wall up mastoidectomy; CWDM: canal wall down mastoidectomy; LSCC: lateral semicircular canal.

patients (31.2%) had evidence of sensorineural deficits including 4 with mixed and 1 with isolated sensorineural hearing loss. Preoperative otoscopic examination showed an attic cholesteatoma in 10 ears, a sinus cholesteatoma in 7 ears, and a tensa retraction cholesteatoma in 3 ears. A stenosis of the external auditory canal was observed in 13 (65%) out of the 20 ears. The controlateral ear was normal in 13 ears; 4 ears presented a cholesteatoma, 2 a retraction pocket and one a tympanic membrane perforation. The average follow-up time was 5 years (range 3 to 10 years). At surgery, the location of the cholesteatoma was as follows: lim-

ited to the attic in 5 ears; attic, antrum and mastoid involvement was evident in 5 ears; limited to the retrotympanum in 4 ears; retrotympanum, attic and mastoid involvement was present in 6 ears. In the majority of patients, HRCT findings included: stenotic external auditory canal, directed posteriorly and superiorly, and more oblique than usual; prominence of conchal cartilage obstructing the meatus; para-transverse direction and increased angle of major axis of the petrous bone (45° instead of 30°); contracted mastoid with low cellularity (Fig. 1).

In fourteen ears a one-stage CWDM was performed: no cases of recurrent cholesteatoma were observed in these cases at last follow-up examination. Three patients had recurrent otorrhea due to cavity granulation: in one case, granulation tissue formation was treated by suctioning and debriding in the office followed by short period local application of otological drops, while in 2 patients a revision surgery was performed with blind sac closure of the external auditory canal, removal of skin and tympanic membrane remnants, Eustachian tube closure with periosteum and bone wax, and cavity obliteration with abdominal fat. A planned staged CWUM with facial recess opening was used in 6 ears, in which preoperative imaging and intraoperative findings evidenced a small cholesteatoma strictly confined to the attic with minor erosion of the posterior canal wall. Among the 6 ears that underwent staged CWUM, one developed a recurrent cholesteatoma, and required conversion to CWDM. During the second-look procedure, one ear (16.6%) was found to have a residual cholesteatoma. A postoperative air-bone gap ≤20 dB was achieved in 6 ears (30%); in four of these 6 ears a planned CWUM was performed. Results comparing preoperative and postoperative hearing are shown in Table 2. No cases of bone conduction deterioration were observed. Four patients with bilateral hearing loss were rehabilitated by means of a bone-anchored hearing aid.

4. Discussion

The incidence of middle ear cholesteatoma in childhood has been reported at 3-6 per 100.000 (14) while in children with TS has been reported to be approximately 1000 times higher than in the general population (11,15). Hall et al. (16) described a population of 178 TS patients of whom 6 (3.4%) had cholesteatoma; similarly, Dhooge et al. (3) found cholesteatoma in 2 (2.3%) out of 87 ears. In the only study reporting specifically on cholesteatoma management in TS patients, Lim et al. (11) observed a cholesteatoma in 7 (3.9%) of the 179 girls reviewed; a bilateral disease was present in 2 cases resulting in a total of 9 ears treated. A CWDM was performed in 5 ears and a CWUM in the remaining 4 ears. A recurrent cholesteatoma was observed in 2 cases (22.2%), one after a CWDM, and the other one following a CWUM and requiring conversion to modified radical mastoidectomy.

Hall et al. (16) reported on result of surgery for otologic disease in TS and included patients operated on for a variety of diagnoses. Six children were found to have cholesteatoma; one patient had bilateral disease, resulting in a total of 7 ears surgically treated. Multiple interventions were required to obtain a dry and safe ear

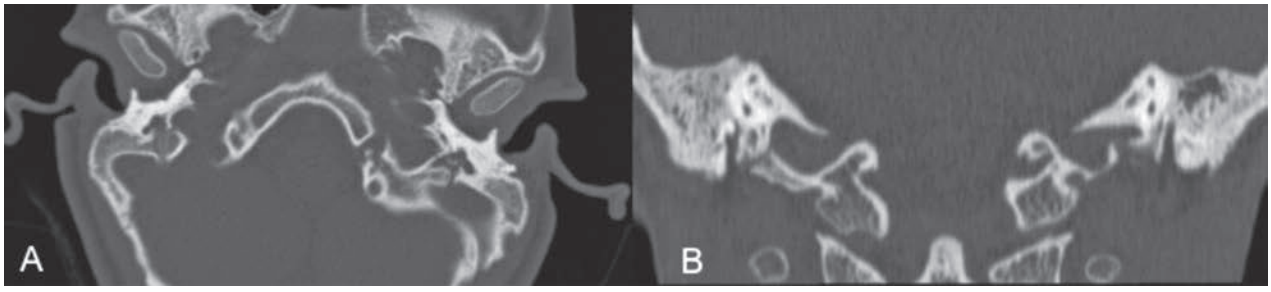

Figure 1. High resolution computed tomography (A: axial view; B: coronal view) showing typical findings in Turner Syndrome: stenotic external auditory canal, directed posteriorly and superiorly, and more oblique than usual; prominence of conchal cartilage obstructing the meatus; para-transverse direction and increased angle of major axis of the petrous bone (45° instead of 30°); contracted mastoid with low cellularity

Table 2. Pure tone averages and air-bone gap preoperatively, and at last follow-up

Patient/Ear	Preoperative BC PTA (dB)	Postoperative BC PTA (dB)	Preoperative AC PTA (dB)	Postoperative AC PTA (dB)	Preoperative ABG (dB)	Postoperative ABG (dB)	Notes
1/Left	10	10	30	10	20	0	
2/Left	10	10	20	20	10	10	
2/Right	15	15	35	30	20	15	
3/Right	10	10	40	35	30	25	
4/Left	20	20	40	20	20	0	
5/Right	10	10	35	35	25	25	
6/Left	30	30	65	60	35	30	
7/Right	10	10	45	20	35	10	
8/Right	30	30	55	55	25	25	
8/Left	30	30	50	70	20	35	BAHA
9/Left	10	10	50	40	40	30	
10/Left	10	10	40	40	30	30	
11/Right	45	45	45	45	0	0	
12/Left	10	10	30	55	20	45	
13/Left	40	40	80	80	40	40	BAHA
13/Right	30	30	75	60	45	30	
14/Right	10	10	60	60	50	50	BAHA
14/Left	10	10	40	40	30	30	
15/Left	40	40	70	65	30	25	
16/Right	20	20	60	60	40	40	BAHA

BC: bone conduction; AC: air conduction; PTA: pure tone average; ABG: air-bone gap; BAHA: bone-anchored hearing aid.

as reflected by two or more procedures required in 5 of the 7 ears. In the child with bilateral cholesteatoma, the left ear underwent 4 operations, the last of which was a modified radical mastoidectomy, while the right ear underwent 2 interventions (tympanomastoidectomy and subsequent CWDM). The authors (16) concluded that revision procedures are common and reflect the recalcitrant nature of middle ear disease in TS patients. Similarly, O'Malley et al. (10), in a study on chronic ear surgery in syndromic subjects, reported on five patients with TS who underwent 11 surgical procedures on 6 ears. Three patients were successfully managed with one surgery, one patient required three surgeries, and another patient required five surgeries. Four of the 6 ears were found to have cholesteatoma. Three of the 4 ears required a CWDM, while the remaining patient underwent a cavity obliteration procedure. Verver et al. (2), reporting on ear and hearing problems in TS, affirmed that, although they prefer to perform a CWUM in children with middle ear cholesteatoma, a canal wall down procedure was necessary in 5 of the 7 ears treated due to the recurrence of the disease. Our experience in the surgical treatment of middle ear cholesteatoma in TS, is quite similar. Although CWUM represents our preferred approach to pediatric cholesteatoma, location and extension of cholesteatoma evaluated by preoperative imaging and intraoperative findings represent the major decisional factor in the choice of the surgical technique. We do not hesitate to perform an open technique in presence of irreparable erosions of the postero-superior canal wall, dead ear, labyrinthine fistula, contracted mastoid, recurrent cholesteatoma or, if the child is judged either unable or unwilling to collaborate with adequate follow-up. In the present case series, a CWDM was performed as technique of choice in 14 ears because of extensive cholesteatoma with posterior canal wall considered unreconstructable (10 cases), contracted mastoid (3 cases), and large labyrinthine fistulae (1 case). In accordance with O'Malley et al. (10), we think that

the presence of a syndrome characterized by craniofacial anomalies, should not be considered a criterion, in itself, to perform an open technique. Certainly, in patients with craniofacial anomalies, chronic Eustachian tube dysfunction must be taken into account, since it represent the main risk factor for the development of recurrent cholesteatoma in CWUM. For this reason, we consider fundamental the reconstruction of even minimal loss of substance of the postero-superior canal wall using autogenous bone pate graft (17). Another source of recidivism is represented by epidermal debris left behind from surgery. Staging the procedure, use of otoendoscopes as an adjunct to operating microscope to better visualize and clean the blind areas in the middle ear, and chemically assisted dissection by means of mesna permit to reduce the likelihood of residual disease (18-21).

As for hearing results, Lim et al. (11) reported an improvement of air-bone gap between 7 and 40 dB in four cases, while the remaining eight had deterioration of air-bone gap between 8 and 23 dB; after surgery, no change in bone conduction occurred in any patient. Two girls required hearing aid post-cholesteatoma surgery. Also in our series, bone conduction thresholds remained unaffected in all cases; a postoperative air-bone gap between 0 and 20 dB was obtained only in 30% of the ears. As one could expect, better hearing results were obtained in patients who underwent CWUM. Five patients required hearing aids after surgery; 2 cases treated by means of CWUM benefitted from a traditional hearing aid, while the remaining 3 patients had a CWDM and received a bone-anchored hearing aid.

Surgery performed by different surgeons, next to the small number of patients, represent the main limitations of this study. That said, the results brought us to some considerations. Although the presence of a syndrome characterized by craniofacial anomalies, should not be considered a criterion, in itself, to perform an open technique, CWDM should be regarded as the technique of choice to achieve a safe and dry ear in TS children with middle ear cholesteatoma. CWUM may give excellent durable anatomical and functional results even in syndromic patients, but it should be adopted only in appropriately selected patients such as those with limited attic cholesteatoma that can be regularly followed-up.

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Original article

A three-year experience with medial-pedicle-based breast reduction for different mammary hypertrophy

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Summary. *Background and aim:* The aim of breast reduction is to reduce excessive breast volume, ensuring an adequate vascular supply and sensitivity of the nipple-areola complex, as well as to produce an aesthetically pleasing final shape. The authors report on their experience with medial-pedicle-based breast reduction combined with both vertical and inverted-T skin resection patterns for different types of breast hypertrophy. *Methods:* From January 2012 to June 2015, 27 female patients (mean age: 49 years) underwent reduction mammoplasty with the medial pedicle technique. The choices of medial pedicle base widths were: 6 cm for low-grade mammary hypertrophy (350-500 gr per breast), 6-8 cm for medium-grade hypertrophy (500-1000 gr per breast), or 8-10 cm for severe mammary hypertrophy (>1000 gr per breast). The authors chose the model of vertical skin resection for low-grade breast hypertrophy. The vertical model was used for medium-grade breast hypertrophy, and Wise skin resection was chosen on a case-by-case basis; only the Wise model was applied to severe breast hypertrophy. *Results:* The mean weight of breast excised was 540 g on the left (range, 207 to 1160 g) and 564.8 g on the right (range, 215 to 1150 g). The complications were minor and self-limiting. All patients reported relief of neck pain, back pain, and bra strap indentations after 6 months of follow-up. *Conclusions:* Breast reduction surgery must address both functional and aesthetic issue by restoring an aesthetically pleasing shape to ptotic or hypertrophic breasts, repositioning the NAC in a physiological position. Various breast reduction techniques have been attempted to combine the safety of the pedicle with aesthetic and functional results. Surgeons should tailor the best technique to each patient. We found that medial-pedicle-based reduction mammoplasty is effective and reliable because it can be applied to a wide range of breast hypertrophy, with reproducible breast weight reduction and results that are aesthetically satisfactory for both patients and surgeons. (www.actabiomedica.it)

Key words: breast reduction surgery, plastic surgery, medial pedicle, mammary hypertrophy

Introduction

Background

Breast reduction surgeries must address both aesthetic and reconstructive issues. Patients with large breasts may suffer from back, neck and shoulder pain, as well as bra strap grooving, and these patients could benefit from breast reduction surgery (1). The aim of this surgery is to reduce excessive breast volume, ensuring adequate vascular supply and sensitivity of the nipple-areola complex (NAC), as well as to produce a final shape that is aesthetically pleasing (2-7). Various breast reduction techniques have been attempted to combine the safety of the pedicle with aesthetic and functional results. All involve a pedicle design (or free nipple graft) to move the NAC, a parenchymal resection pattern, and a skin resection pattern. Surgeons should be aware of different techniques to adapt to different patient presentations. In breast reduction, the pedicle,

the skin-resection pattern and the parenchymal-resection pattern must be considered separately. Six main pedicles have been described: the superior pedicle (8), the inferior pedicle (9,10), the lateral pedicle (3), the horizontal bipedicle (11), the vertical bipedicle (12,13), and the superomedial pedicle (14). With regard to skin resection pattern, an important advance was made in 1956, when Robert Wise designed a skin resection pattern adapted from a brassiere design, which came to be known as the inverted T (15). All pedicles were then adapted to the Wise skin resection pattern. Arie[4] first described reduction mammoplasty with a vertical scar in 1957 to eliminate the horizontal scar. In 1969, Lassus (11,16) developed the superior dermal-glandular pedicle for transposition of the NAC with a central *en bloc* excision of skin, fat, and glands, as well as a vertical scar. In 1994, Lejour (17-19) modified Lassus' technique and used pre-excision liposuction to eliminate the fat contributing to breast volume. In 1999, Hall-Findlay (20-24) modified the superomedial-pedicle-based mammoplasty ideated by Orlando (14) in 1975 and combined a full-thickness medial dermoglandular pedicle with a vertical skin resection pattern to transpose the NAC. This vertical skin excision pattern reduced the incidence of boxy appearance that sometimes occurred with the inverted T (15).

The vertical or inverted-T skin resection patterns have been applied to different pedicles and parenchymal resection patterns. Articles in the literature advocate for the supremacy of one technique over another, but what has emerged is the idea that "the best breast reduction is the one that the surgeon does the best" (24).

Aim

In this paper, we report our experience with medial-pedicle-based breast reduction combined with both vertical and inverted-T skin resection patterns for different types of breast hypertrophy.

Methods

At the Cutaneous, Mini-invasive, Regenerative and Plastic Surgery Unit (Parma University Hospital, Italy) from January 2012 to June 2015, 27 female patients aged 29 to 67 years (mean age: 49 years) underwent reduction mammoplasty with the medial pedicle technique. The average Body Mass Index (BMI) was 29.13 kg/m^2 (range, 22.3-33.3 kg/m^2). The mean suprasternal notch to nipple distance was 27.4 cm (range, 23-35 cm) on the left breast and 27.6 cm (range, 23-34 cm) on the right breast.

The two main exclusion criteria for breast reduction surgery were: BMI >35 kg/m^2, heavy smokers (25, 26) or patients who would not quit smoking between the period one month before and one month after surgery. Patients receiving oral contraceptives were told to stop the therapy one month before surgery.

Patients were required to sign informed consent prior to surgery and were educated about surgical and cosmetic risks: possible loss of sensation to the NAC or the skin breast, inability to lactate, NAC loss and breast asymmetry. Patients with severe hypertrophy (>1000 gr) were anxious due to the increased risk of poor cosmetic results with the vertical scar reduction mammoplasty; thus, they were informed of the need for the Wise skin resection pattern. A single surgeon performed all the procedures under general anesthesia; the mean operative time was 122 minutes (range, 96-142 minutes).

All patients underwent a medial-pedicle-based breast reduction surgery. The choice of the medial pedicle base width was determined by the degree of breast hypertrophy. The pedicle base widths were as follows: 6 cm for low-grade mammary hypertrophy (350-500 gr per breast), 6-8 cm for medium-grade hypertrophy (500-1000 gr per breast), and 8-10 cm for severe mammary hypertrophy (>1000 gr per breast). The skin resection pattern was driven by the degree of breast hypertrophy. The vertical skin resection pattern was used for low-grade mammary hypertrophy. For medium-grade mammary hypertrophy, both vertical and Wise skin resection pattern were chosen on a case-by-case basis; only the Wise pattern was applied to severe mammary hypertrophy. No liposuction procedures were performed.

Antibiotic therapy was administered in the immediate pre-operative phase and was continued until the 10th postoperative day (POD). Social activity was limited for 4 weeks after the day of discharge.

Patients were required to wear a bra at all times for 3 weeks following surgery. Patients were discharged at

the 3rd POD, and follow-up occurred on a weekly basis for the first month; they were then observed again at the 3rd and 6th month.

The follow-up period was 6 months; early and late complications were recorded. The patients' satisfaction was evaluated with a simple survey.

Preoperative marking

Preoperative design was performed as suggested by Hall-Findlay (20). First, we identified the inflammatory fold (IMF), which was easily located by placing a tape measure horizontally in the fold under both breasts. Then, the breast meridian (generally 9-11 cm from the midsternal line) was drawn straight from the midpoint of the clavicle until the IMF, on which the new NAC was typically located (usually 1-2 cm above the IMF) by placing one hand behind the breast to the level of the inframammary crease and projecting anteriorly onto the breast. This usually resulted in a suprasternal notch to nipple distance of 20-23 cm, depending on the height of the patient, the level of the IMF, and the desired size after the mastopexy. Because the ideal nipple position is slightly below the middle position on the breast mound, in case of an "empty" upper pole breast, the NAC was located 1-2 cm lower than it would have been with a "full" one.

To delineate the vertical limbs and thus the length of the medial and lateral flaps as well as the width of the extended pedicle, the breast was rotated laterally and superiorly and the vertical axis of the meridian was transposed to the displaced medial breast tissue. In the same fashion, the breast was subsequently rotated medially and superiorly so that the meridian was transposed to delineate the lateral extent of skin resection. Through use of a keyhole template, the areolar pattern was then drawn (spreading apart its limbs so they overlapped with the medial and lateral vertical pillars) with a diameter of approximately 5 cm and a periareolar scar length of less than 16. The inferior extent of the skin resection was marked in a "U" shape with the patient in supine position. The skin resection pattern thus resulted in the shape of a snowman.

The inferior limit of the skin excision was located 4-6 cm above the inframammary crease: shorter in low- and medium-grade mammary hypertrophy and longer in severe hypertrophy.

Additionally, the Wise skin resection pattern was performed in the same fashion and employed only for the lower excision design. The medial and lateral vertical pillars were marked at 6 cm in length. The amount of skin to be resected was determined by pinching the breast between the thumb and the index finger; these two points were then drawn up to 1-2 cm above the lateral and medial extension of the IMF. Finally, the medial pedicle was drawn in a 'U' shaped pattern and extended down to within the vertical markings, stopping approximately 1 cm (at least) from the NAC to preserve the neuro-vascular plexus.

With the patient in the upright position, all of these marks were compared to assess their symmetry.

Operative technique

All incisions were made through the dermis with partial thickness and the area of the medial pedicle was de-epithelialized, leaving the deep dermis intact to prevent damage to the blood vessels traveling superficially through the pedicle. The surgical excision of skin, fat, and gland was performed *en bloc,* as outlined by the skin markings. The excision was extended down to the chest wall, leaving a layer of breast tissue over the pectoralis fascia to prevent bleeding, postoperative pain and NAC sensitivity deficiency. The lateral and inferior resection of the mammary gland was carried out with an inward cutting angle; the flaps were maintained at 2.5 cm thickness throughout their length. The excision was performed with the non-dominant hand constantly holding the pedicle in order to ensure it would not be undermined. A proper parenchymal resection was performed superiorly above the proposed nipple position to create a pocket for the auto-augmentation; an excess of breast tissue left in place could promote bottoming-out with time. A 0.5-1 cm thickness subcutaneous tissue was left at the IMF level. The pedicle was then safely rotated upwards (rotational angle between 30° and 90°) without risk of compression or torsion, and it was fixed to the upper pole. The lateral and medial pillars were sutured together by simple interrupted absorbable 3/0 subcutaneous sutures in place of Lejoure's technique, which leads to ruffling of the scar to shorten its length.

Although cutaneous wrinkling of the vertical scar associated with gathering of the skin would disappear 6 months postoperatively, the Lejoure suture technique could interfere with the blood supply of the skin edges, resulting in delayed healing. The skin was gathered beginning at the IMF. If the lengths of the vertical scar exceeded 5-6 cm, we associated a small horizontal scar, resulting in a short reverted T, to better distribute the excess skin reducing the tension over the vertical suture. All suturing of the skin was performed using a 4-0 Monocryl suture (Ethicon Inc, Somerville, NJ). Deep dermal, inverted sutures were used to inset the NAC. Intradermal, continuous sutures were used for closer approximation of skin edges of the periareolar wound.

Following the Wise pattern breast reduction mammoplasty, the surgical excision was performed *en bloc* as outlined by the skin markings. A 1-2 cm thick subcutaneous tissue was maintained underneath the wound edge at the medial and lateral portion of the IMF to reduce the occurrence of dog-ear. The pedicle was rotated as previously described. The first suture to be positioned was that at the apex of the inverted T, joining together the lowest portion of the lateral and medial pillars with the middle-point of the IMF by simple interrupted absorbable 2/0 subcutaneous suture. All sutures were performed as previously described.

One closed suction drainage was routinely placed in each breast before wound closure and was left in place until the output was less than 30 ml/day.

The wounds were dressed with dry gauze, while the NAC was dressed with paraffin gauze, followed by dry gauze. These were held in place by Hypafix (BSN Medical, Luxemburg). Wound dressings were held in place until the 3rd POD.

Results

We performed 54 breast reductions using the medial pedicle technique, from January 2012 to June 2015; 36 with a Wise pattern skin resection and 18 with a vertical one. There was no need for free-nipple graft.

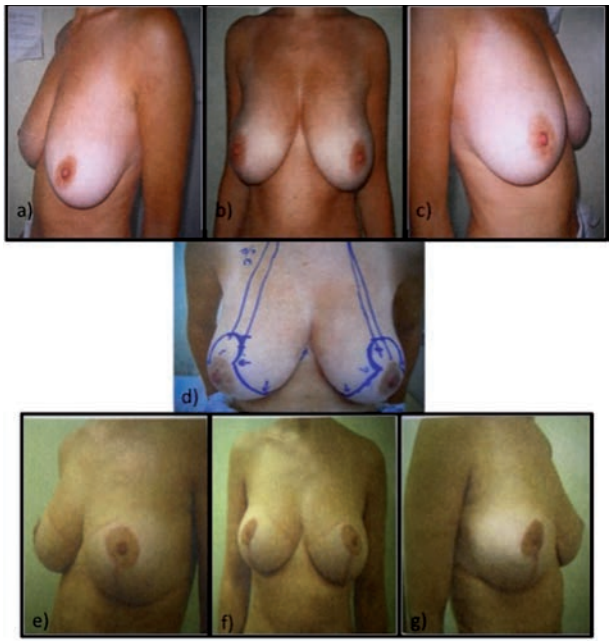

Figure 1. A 37-year old female patient, who undergone medial-pedicle-based reductive mammoplasty with vertical skin resection pattern. The base of the medial pedicle was 6-cm width. The weight of the breast excised was 250 gr on the left and 270 gr on the right. The patient is shown preoperatively (a, b, c, d) and at 6° month after surgery (e, f, g)

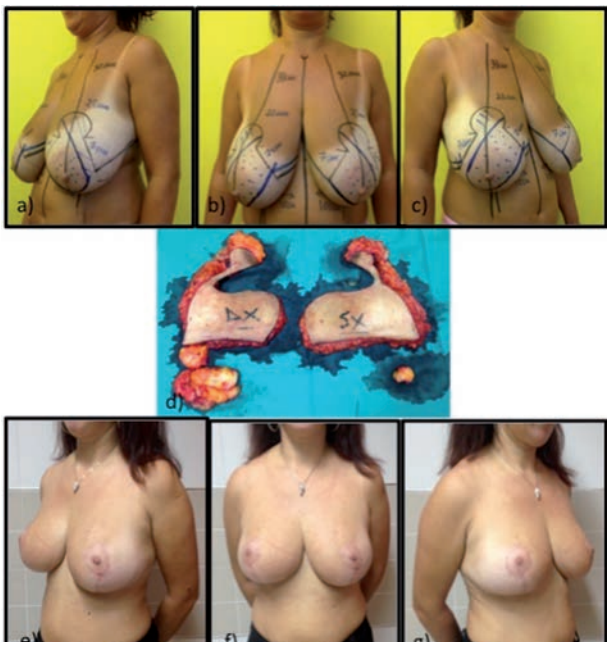

Figure 2. A 48-year old female patient, who undergone medial-pedicle-based reductive mammoplasty with Wise skin resection pattern. The base of the medial pedicle was 7-cm width. The weight of the breast excised was 630 gr on the left and 590 gr on the right. (d) The patient is shown preoperatively (a, b, c) and at 6° month after surgery (e, f, g)

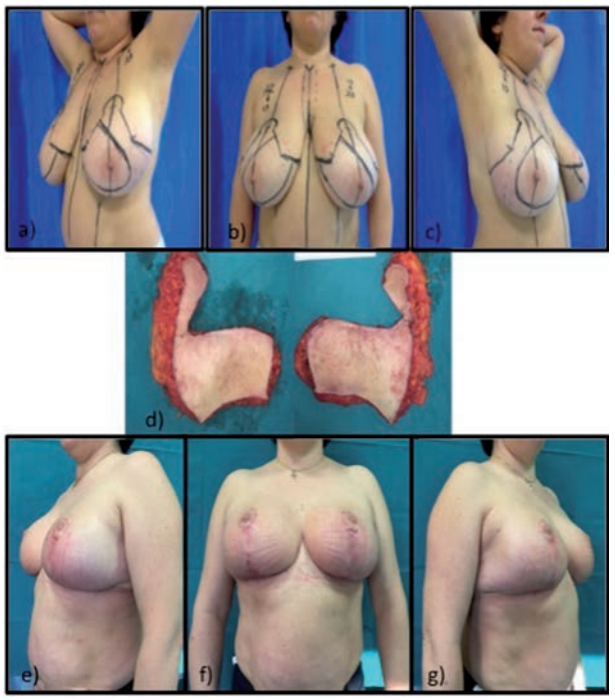

Figure 3. A 47-year old female patient, who undergone medial-pedicle-based reductive mammoplasty with Wise skin resection pattern. The base of the medial pedicle was 10-cm width. The weight of the breast excised was 1160 gr on the left and 1150 gr on the right. (g) The patient is shown preoperatively (a, b, c) and at 6° month after surgery (d, e, f)

The mean weight of breast excised was 540 g on the left (range, 207 to 1160 g) and 564.8 g on the right (range, 215 to 1150 g). At the 6th-month follow-up after surgery, the mean suprasternal notch to nipple distance obtained was 21.4 cm (range, 19-23 cm) on the left and 21.8 cm (range, 19-23 cm) on the right.

Breast and nipple projection was restored in all patients who were satisfied with nipple projection, and 25 of 27 (92.6%) patients reported satisfaction with breast shape. All patients reported relief of neck pain, back pain, and bra strap indentations.

Early complications were reported in 8 breasts (14.9%), and they were all managed conservatively. No seroma or infection was identified. After the Wise pattern skin resection, partial nipple and areola necrosis occurred in two breasts (5.5%), which healed with secondary intention without the need for revision and with repeated dressing. Hematoma formation occurred in 1 breast (2.7 %). T-junction breakdown was limited to 3 breasts (8.3%) and was treated conservatively with repeated dressings; each case healed satisfactorily.

After the vertical pattern skin resection, NAC viability was maintained in all breasts. Hematoma formation occurred in 1 breast (5.6%), and there was one incidence of wound dehiscence of the vertical limb (5.6%), which was treated with simple dressing alone and did not require further revision surgery.

Table 1. Early and late complications

	Wise Skin Resection Pattern		Vertical Skin Resection Pattern	
	Breasts	%	Breasts	%
Early Complications				
Dehiscence at T-junction	3	8.3%	1	5.6%
Nipple-areola necrosis	2	5.5%	0	0%
Hematoma	1	2.7%	1	5.6%
Infection	0	0%	0	0%
Seroma	0	0%	0	0%
Late Complications				
Nipple-areola Sensory Loss	2	5.5%	0	0%
Under-reduction	0	0%	1	5.6%
Over-reduction	0	0%	0	0%
Scar Hypertrophy	1	2.7%	2	11.2%
Nipple Retraction	0	0%	0	0%
Contour Problems	0	0%	1	5.6%
Dog Ears	2	5.5%	0	0%
Hypo-Hyperpigmentation	0	0%	0	0%

Late complications were reported in 9 breasts (16.7%).

Sensation was assessed by light touch and patient response; it was retained in 34 of 36 breasts (94.4%) following reduction mammoplasty with Wise pattern skin resection and in all breasts after vertical pattern skin resection.

There was one case of under-reduction following vertical pattern skin resection mammoplasty, but the patient refused to undergo further surgery.

Scar hypertrophy was reported in 1 breast (2.7%) following reduction mammoplasty with Wise pattern skin resection and in 2 breasts (11.2%) after the vertical pattern. These were treated with monthly topical injection of a 40 mg/ml-suspension of triamcinolone acetonide, as well as daily topical application of a silicon-based cream for 6 months. The results obtained in all three cases were satisfactory. Dog-ears were observed in two breasts following vertical pattern skin resection mammoplasty; this complication was treated with a liposuction procedure three months from the surgery day. No NAC hypo-hyperpigmentation was observed.

Conclusions

Breast reduction surgery aims to restore an aesthetically pleasing shape to ptotic or hypertrophic breasts, repositioning the NAC in a physiological position. Over the last century, several techniques have been designed to achieve this goal (3-23). These differ mainly in three features: the choice of the pedicle to ensure the viability of the NAC, the type of skin and the parenchymal resection pattern. Studies have been carried out to demonstrate the supremacy of one technique over another (15). As mentioned previously, what has emerged instead is the notion that surgeons should have several different options to apply the best technique to each patient.

The medial pedicle is a technique derived from the superomedial pedicle (14) that was modified and popularized by Hall-Findlay (20-23) with the vertical skin resection pattern.

Initially, Hauben (27) stated that the superomedial pedicle technique was suitable for breasts of "moderate to rather large size." (28). Then, Finger et al. (29) demonstrated that resections as large as 4100 g and NAC transpositions of up to 30 cm were well tolerated, with nipple viability and preservation of sensation. Other studies followed, demonstrating that the medial pedicle reduction mammoplasty was safe and reliable, even in cases of severe mammary hypertrophy, as the pedicle contains the primary blood supply to the breast and is shorter than the interior pedicle in a given breast (30). Moreover, the lateral rotation of the pedicle from its base prevents excessive traction on the NAC, as well as stem twisting reductions in huge breasts. Breast shape and projection were enhanced when compared with amputation and free-nipple graft and were equivalent to results obtained with the inferior pedicle technique.

Advantages of a medially based pedicle include reliable circulation, preservation of NAC sensation, reduction of NAC hypopigmentation occurrence, and enhancement of central breast projection. An anatomical study by Michelle le Roux et al. (30). examined the neurovascular anatomy of female breasts on 11 cadavers, it showed that the nerve supply arise from the fourth intercostal nerve and play a unique role in NAC innervations (31). They also concluded that de-epithelialization or thinning of the superficial aspect of the superomedial pedicle could lead to vascular compromise or denervation of the NAC as the blood supply coursed through the pedicle in a superficial plane. They recommended that resection should be performed from the deep surface or the base of the pedicle if needed (30). This study supports the safety of the partial-thickness superior or medial pedicle design, which we used in our technique. The use of a full-thickness pedicle may prevent the successful implementation of the nipple-areola complex, folding and compromising the vascularization of the NAC.

Nahabedian et al. (32) confirmed that NAC sensitivity loss was directly related to breast size and consequent chronic nerve traction injury, and not to the technique performed. In our study, NAC sensation loss occurred in 2 breasts (2.7%) following reduction mammoplasty with the Wise pattern skin resection. One possible explanation is that we usually perform this type of surgery in patients with moderate/severe breast hypertrophy who could have suffered from chronic nerve traction injury, as stated above.

Vertical scar reduction mammoplasty using a medial pedicle improve long-term projection of the breasts, along with less scarring than inverted-T scar. We believe it is the inferior wedge resection of the redundant breast tissue that contributed to breast ptosis and that subsequent suturing of the medial and lateral pillars that result in coning of the breast are responsible for the long-term shape (21-32). Landau (33) applied the medial pedicle reduction mammoplasty with Wise pattern skin resection to 61 patients; he designed the medial pedicle with a base width of 10 cm and had a 90°-rotational angle. Serra (34) also performed the superior-medial pedicle for serious gigantomastia (>1200 g) with Wise pattern resection. Complication rates were similar to those reported in literature (15); wound dehiscence at the inverted T apex and the occurrence of dog-ears were the main complications. We experienced the same complication in our study, probably because we performed the Wise pattern skin resection in patients with moderate/severe breast hypertrophy, which resulted in more tension at the vertical scar and more parenchymal/adipose tissue to be excised.

Breast reduction surgery must address both functional and aesthetic issues. We found the medial-pedicle-based reduction mammoplasty effective and reliable because it can be applied to a wide range of breast hypertrophy, with reproducible breast weight reduction and results that are aesthetically satisfactory for both patients and surgeons. In accordance with other studies, we demonstrated that medial pedicle reduction mammoplasty is a safe surgical option, even for severe mammary hypertrophy. In addition, the operative time is short, and the complication rate is acceptably low. While agreeing with the statement that "the best breast reduction is the one that the surgeon does the best" (24), we regard the medial-pedicle-based reduction mammoplasty with both vertical and Wise skin resection patterns as an acceptable gold standard in various degrees of breast hypertrophy.

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Original article

A clinical efficacy experience of Lacosamide on sleep quality in patients with Nocturnal Frontal Lobe Epilepsy (NFLE)

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Summary. *Background:* Nocturnal frontal lobe epilepsy (NFLE) is a focal epilepsy with seizures arising mainly during sleep and characterized by complex motor behavior or sustained dystonic posturing. First described in 1981, it was considered a motor disorder of sleep and was indicated as nocturnal paroxysmal dystonia (NPD). The debated on epileptic origin of this condition was demonstrated in 1990 and the term NFLE was introduced. Since then it has been demonstrated that the heterogeneous aspects of morpheic seizures were responsive to antiepileptic drugs (AED's) with sodium blocking action mechanism, especially the carbamazepine (CBZ). *Aim of Work and Methods:* We report a clinical experience of NFLE patients associated with sleep disorders treated with Lacosamide, AED's with a novel mechanism of action. In vitro electrophysiology studies have shown that lacosamide selectively boosts the slow inactivation of the sodium-voltage-dependent channels, resulting in a stabilization of the hypersensitive neuronal membranes. *Results and Conclusion:* On the treated patients we observed a positive clinical response to lacosamide therapy without significant side effects. In particular, the effective clinical response to the pharmacological treatment was obtained at a dose of 200 mg/day. (www.actabiomedica.it)

Key words: Nocturnal frontal lobe epilepsy, sleep disorders, Lacosamide

Introduction

Nocturnal Frontal Lobe Epilepsy (NFLE) is a form of focal epilepsy with heterogeneous clinical presentation of morpheic seizures that tend to cluster overnight (1).

Genetically the NFL is inherited in dominant autosomal manner. The genetic defect was first isolated to the gene CHRNA4 coding for the alpha4 subunit of the neuronal nicotinic acetylcho-line receptor (nAChR) and afterwards mutations in genes CHRNA2 and CHRNB2 coding for other subunits (alpha2 and beta2) of the nAChR have been identified (2, 3). Often recording EEG, both in the ictal and interictal stages, is negative for epileptiform activities. In most cases a good response to carbamazepine (CBZ) was described but not to other AEDS. Related to the clinical presentation (stereotyped dystonic/dyskinetic features of attacks), the almost absence of EEG abnormalities and the recurrence of episodes during sleep, the authors debated the epileptic origin of the NFLE, considering it as a sleep motor disorder with an unclear pathophysiology (4).

Furthermore, due to the recurrence of the motor events during sleep, NFLE patients may complain of daytime sleepiness and/or other sleep disorders (5, 6) .

Below we report our clinical experience in five patients of both sexes and ages between 26 and 43 years affected by NFLE and sleep disorders successfully treated with lacosamide (LCM).

LCM present a novel mechanism of action: the active principle, lacosamide (R-2-acetamido-N-benzyl-

3-methoxypropionamide) is an aminoacid to which other functional groups have been added. Although the precise mechanism of action by which lacosamide has an antiepileptic effect in humans has not yet been fully explained, in vitro electrophysiology studies have shown that lacosamide selectively boosts slow inactivation of sodium-dependent voltage-dependent channels , resulting in a stabilization of the hypersensitive neuronal membranes (7).

Methods

In our study, we observed five patients with multiple-frequency night-time seizures episodes, men and women aged between 26 and 43 years. In all of our patients, night-time seizures were stereotyped (ballistic upper right and chewing gait movements, explosive vocalization, scratch movements, dystonic posturing of the left toe, etc.) with multiple frequency and short duration. Only in three of the five patients described, partial epileptic seizures have been sometimes followed by secondary generalization.

Family history and brain magnetic resonance imaging (MRI) was negative in all patients, except for one patient who had a positive history for febrile seizures.

Ictal and interictal scalp EEG revealed nonspecific anterior slow discharges in everyone.

Video-polysomnography showed an increase in sleep fragmentation and high percentage of waking with a simultaneous decrease in the percentage of deep sleep and the REM phase in four out of five patients; bilateral frontal slow waves that arise during the transition phases between NREM and REM sleep, was registered on ictal video-polysomnography in only one patient.

Mutations in nAChR were undetected.

All patients were administered the Epwort Sleepiness Scale (ESS) to assess the degree of daytime sleepiness and the score was greater than 10 in all five of our patients. In particular, 3 patients scored between 11 and 15 (moderate daytime sleepiness), and 2 patients scores above 16 (severe daytime sleepiness).

At the time of enrollment in the study, patients underwent antiepileptic therapy with high doses of carbamazepine/oxacarbazepine (1200mg/600mg day), which however was ineffective in night-time seizures control or ill tolerated for many side effects.

Therefore, the patients slowly withdrew from carbamazepine/ oxacarbazepine and started Lacosamide until the dosage of 100mg BD.

After 6 months of follow-up on Lacosamide treatment, 3 patients had still about two or three nocturnal seizures per month.

After 12 months follow-up, all patients were seizures free without significant side effects, and the daytime sleepiness disappeared (ESS score<10).

Conclusion

Nocturnal Frontal Lobe Epilepsy (NFLE) is a form of focal nocturnal epilepsy that due to its heterogeneous clinical presentation, the almost absence of EEG abnormalities and the recurrence of episodes during sleep, was renamed Sleep-Related Hypermotor Epilepsy (SHE) (4).

Although epileptic seizures are non-disabling since they occur at night and controlled by CBZ, these patients showed a reduced sleep quality that is negatively reflected on the activities of daily life.

The night-time awakenings, sleep fragmentation and parasomnias, induce an excessive daytime sleepiness and paradoxical insomnia.

This report has shown how lacosamide can be effective not only in the treatment of nocturnal seizures, but also in sleep disorders associated with them in SHE, probably due to the multiple action mechanisms of this drug (slow inactivation of voltage-gated sodium channels, inhibitions of carbonic anhydrases) (7,8).

Although in the literature has recently described some cases of NFE patients successfully treated with lacosamide (9), further studies will be needed to confirm these data and to investigate the positive effects of the drug on heterogeneous sleep disorders in this category of patients.

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Original article

Face rejuvenation: a new combinated protocol for biorevitalization

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Summary. *Introduction:* The slowing of the aging process is subject of great research and attention in modern society, particularly aging of face. Processes involved are very complex. Mesotherapy. hyaluronic acid and carbon dioxide injection can be used for biorevitalization and skin rejuvenation. *Methods:* Three groups were made and 62 patients were enrolled. Patients with superficial wrinkles of the face, neck and/or décolleté, without presence of nasolabial folds and marionette wrinkles were included in group 1. Patients with superficial/medium depth wrinkles of the face, neck and/or décolleté, with moderate nasolabial folds but no marionette wrinkles were included in group 2. Patients with deep wrinkles of the face, with deep nasolabial folds and marionette wrinkles were included in group 3. Patients were treated with three different protocols that included injections of amino acids, vitamins and hyaluronic acid in association with carbon dioxide injection. We submitted the PAIS and GAIS scales and we analyzed the scores obtained with Wilcoxon's and Kolmogorov-Smirnov's tests. Statistical Product and Service Solutions (SPSS) softare was used. The p-value was considered acceptable if inferior to 0,05 ($p>0,05$). *Results:* In according with these tests, the differences of values at one week and at the end of the study are significant ($p<0,05$) for both PAIS and GAIS. No side effects were reported. *Conclusions:* Protocol treatment used in this study gave statistically valid results in the rejuvenation of face for mild, moderate and severe aging. (www.actabiomedica.it)

Key words: face rejuvenation, carbon dioxide, carboxytherapy, biorevitalization

Introduction

The slowing of aging process is interested by numerous researches; more figures (plastic surgeon, cosmetic surgeon etc) pay attention to aging of face in modern society. Processes involved are very complex but we can identify two important factor:

1. Volume loss
2. Repetitive muscle movements that cause wrinkles and folds (1, 2).

In addition there are environmental factors that must be considered, among which the most important are the photo-aging, smoking, stress and an diet (3, 4). All of these factors, combined with a genetic component (which may be more or less relevant), determine a skin aging with thinning of the dermis, subcutaneous atrophy, skin laxity and skin ptosys according to the force of gravity.

Mesotherapy is a medical procedure introduced by Pistor in 1958 and consists of intradermal injection of substances that have trophic effects such as vitamins, hormones, etc., to obtain skin rejuvenation increasing hydration and fibroblast activation (5-7).

On the other hand, hyaluronic acid and carbon dioxide injection into the skin stimulate the expression of collagen type 1, matrix metalloprotease and their inhibitors, resulting in skin rejuvenation (3, 4, 8, 9) with less pain (10-13).

Following an our previous study that evaluated the combined treatment of hyaluronic acid and carbon

dioxide for nasolabial folds (4), we decided to develop an indicative scheme as a guide for bio-revitalization and rejuvenation of the face using a modulate protocol including mesoherapy, injection of hyaluronic acid and injection of carbon dioxide.

Materials and methods

The study started in January 2015 and ended in December 2015.

We have established the following criteria for cohorts' determination:

Inclusion criteria

- Age between 30 and 70 years

Exclusion criteria

- Age not between 30 and 70 years
- Aesthetic medicine treatments performed within 12 months from the start of the study
- Patient who undergo to other beauty treatments (medical and/or surgical) during the study
- Surgery of any type within 12 months from the start of the study
- No compliance in following the treatment protocol used in the study.

Sixty-two patients were enrolled in the study. The average age was 43 years, in a range between 32 and 66 years.

We divided the patients into three groups:

Group 1: patients with superficial wrinkles of the face, neck and/or décolleté, without presence of nasolabial folds and marionette wrinkles.

Group 2: patients with superficial/medium depth wrinkles of the face, neck and/or décolleté, with moderate nasolabial folds but no marionette wrinkles

Group 3: patients with deep wrinkles of the face, with deep nasolabial folds and marionette wrinkles.

The three groups were treated according to a precise algorithm.

1. Group 1 was treated as follows: mesotherapy (14-18) with 2ml of low-dose medication of Vitamin C (D6), Vitamin B1 (D6), Vitamin B2 (D6), Vitamin B6 (D6), Nicotinamidum (D6), Acidum cis-aconitum (D6), Acidum fumaricum (D6), Acidum alpha-ketoglutaricum (D6), baryum oxalsuccinicum (D6), oxalaceticum Natrium (D6), Natrium pyruvicum (D8), Sulfur (D12), Magnesium gluconicum (D6), Manganum phosphoricum (D10), Collagen suis (D8/D30), Hyaluronidase (D8/D30), Funiculus umbelicalis suis (D10/D30), musculus suis (D20), Placenta suis (D10), Hepar suis (D20), Gland suprarenalis suis (D10), Hahnemanni Mercurius (D20), Calcium fluoratum (D30), cleavers (D6), Thuja (D6), Cutis suis (D8/D30). The drug was injected in: glabellar region, lateral periocular, before tragus, buccal side corner, the nasolabial sulcus and nasal wing corner. Reinforcements were made on the bar code if any. In the same session we practiced injections of carbon dioxide (CO_2) with class 2/b devices (European Directive 93/42 CEE) with flow of 15-20 cc/min, with temperature of 38-42 centigrade degrees, for 5-15 cc-per-area in: corner jaw, chin, under the chin, on the cheek, neck and décolleté. This treatment was repeated once a week for 4 weeks and then once a month for 2 months
2. The second group was treated with the following scheme: mesotherapy with same substance of Group1 plus a vial of Amino Acids (Glycine 25mg, L-Proline 25 mg, L-Leucine 25 mg, L-Lysine 25 mg) and Sodium Hyaluronate 30 mg/3ml plus carbon dioxide injection as Group1. This schema was performed once a months for three months and then we made a maintenance every 4 months.
3. The third group was treated as the second, except that we replaced the vial of amino acids and sodium hyaluronic acid with a 2 ml vial containing an association of polynucleotides (10 mg/ml) and hyaluronic acid (10 mg/ml). Followed protocol was: one treatment a month for three months and then we made a maintenance every 4 months.

We submitted the PAIS (patient's aesthetic improvement scale, see Table 1) after first week and at end of treatment to all patients. They have also been studied with the GAIS (global aesthetic improvement stairs, see Table 2) by a physician not involved into medical treatments.

Table 1. Patient's Aesthetic Improvement Scale (PAIS). Grade description

Grade	Description
1	worse
2	no change
3	somewhat improved
4	moderately improved
5	very much improved

Table 2. Global Aesthetic Improvement Scale (GAIS). Grade description

Grade	Description
1	worse than before treatment
2	no change
3	minimal improvement
4	good improvement
5	optimal improvement

Statistical analysis

The differences of scales scores were evaluated with two tests for non-parametric dependent continuous variables: Wilcoxon's and Kolmogorov-Smirnov's tests. Statistical Product and Service Solutions (SPSS) softare was used. The p-value was considered acceptable if inferior to 0,05 ($p>0,05$).

Results

Following this classification we found 28 patients in group 1, 22 in group 2, 12 in group three.

The values of PAIS and GAIS are shown in Tables 3 and Table 4. Differences of values were evaluated with Wilcoxon's and Kolmogorov-Smirnov's tests; according with these tests, the differences of values at one week and at the end of the study are significant ($p<0,05$) for both PAIS and GAIS. No side effects were reported.

Figure 1 shows a result obtained on a patient of the group 1: we obtained a decrease of nasolabial folds (Figure 1a: pre-treatment; figure 1b: after two treatments; Figure1c: at the end of the study) without acting on the patient's expressiveness.

Figure 2 shows a result of a patient of group 2: we have obtained an improvement of the nasolabial folds

Table 3. Percentage distribution of patients based on GAIS Score

	No. of patients					
	Group 1		Group B 2		Group 3	
	After 1 week	At end of study	After one week	At end of study	After one week	At end of study
Worse (Grade 1)	0	0	0	0	0	0
No change (Grade 2)	0	0	0	0	0	0
Somewhat improved (Grade 3)	5	2	6	3	4	0
Moderately improved (Grade 4)	16	8	10	9	2	4
					(18/20)	
Very much improved (Grade 5)	7	18	8	13	6	8
	100% (28/28)		100% (24/24)		100% (12/12)	

Table 4. Percentage distribution of patients based on GAIS Score

	No. of patients					
	Group 1		Group B 2		Group 3	
	After 1 week	At end of study	After one week	At end of study	After one week	At end of study
Worse (Grade 1)	0	0	0	0	0	0
No change (Grade 2)	0	0	0	0	0	0
Somewhat improved (Grade 3)	3	0	6	0	4	0
Moderately improved (Grade 4)	16	8	10	9	2	2
					(18/20)	
Very much improved (Grade 5)	9	20	8	16	6	10
	100% (28/28)		100% (24/24)		100% (12/12)	

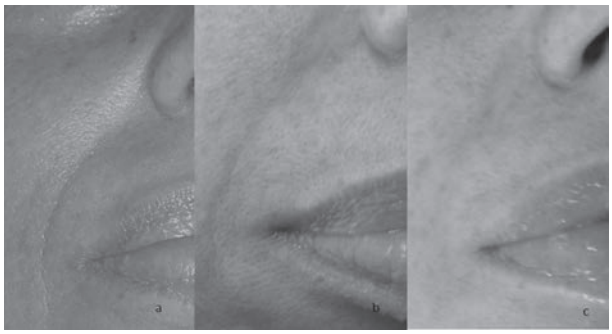

Figure 1.

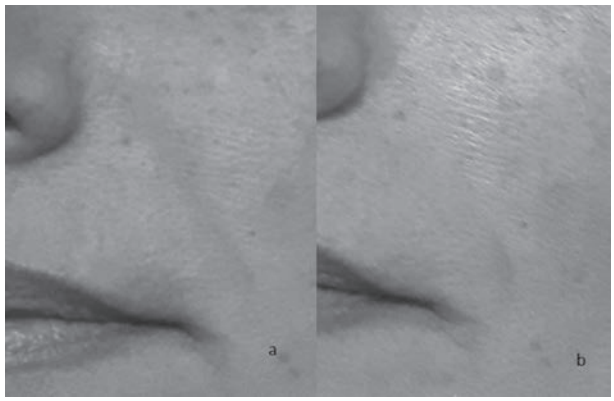

Figure 2.

and a skin improvement (Figure 2a: pre-treatment; Figure 2b: at the end of the study).

Figure 3 shows a result of a patient of group 3 with marked improvement of the nasolabial fold and the angle of the mouth (Figure 3a: pre-treatment; Figure 3b: at the end of the study).

Discussion

One of the most important causes of human skin aging is environment, so, skin aging can be considered as a consequence of envitonmental damage (2).

Environmental factors involved are ultraviolet (UV), smog, stress but there are many other factors to consider, such as lifestyle behavior as smoke, diet, etc (19, 20).

All these factors combine with each other and ultimately determine the clinical appearance of skin folds and wrinkles, pigment changes, dehydration, loss of tensile strenght (2, 21, 22).

In the approach to facial rejuvenation we must consider the importance of using antioxidants and molecules able to moisturize, improve microcirculation, determine the appearance of juvenile collagen and new elastic fibers. In this view, the use of vitamins, hyaluronic acid and carbon dioxide injections appears

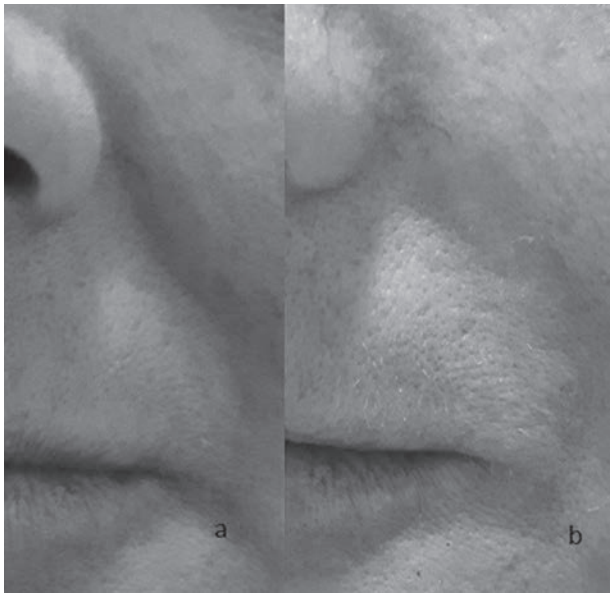

Figure 3.

a good combination of methods to achieve the purpose of rejuvenating the skin of patients.

In our study we wanted groped a treatment protocol based on the patient's aesthetic situation we must remember that the cosmetic procedures for facial rejuvenation are constantly increasing and that, in addition to mesotherapy and to the use of hyaluronic acid fillers, there are other principals that are used as neuromuscular blocking agents and the traction wires. The ability to stick to a set of indicative guidelines for the overall rejuvenation of the face is a novelty and a convenience for those who approach to facial rejuvenation.

Conclusions

Protocol treatment used in this study gave statistically valid results in the rejuvenation of face for mild, moderate and severe aging. The synergistic use of different methods (mesotherapy and injection of carbon dioxide) is advantageous, without side effects and with a high degree of patient satisfaction.

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Case report

Traumatic deep neck infection due to pulling a tooth with pliers

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Summary. Deep neck infection is life-threatening and mortal condition that requires immediate treatment. This infection is generally polymicrobial and frequently seen after upper respiratory infections, poor dental hygiene, trauma and surgery to the head and neck region. The symptoms of deep neck infections are swelling, dysphagia, pain, trismus, dysphonia and otalgia. Deep neck infections can be seen at any age and its mortality is about 20-50%. Initial management of the deep neck infection is intravenous antibiotic, protection of airway and drainage of abscess. Deep neck infections can cause severe complications even dead can be seen, so physicians should be aware of these complication. Herein, we reported a 71-year-old-woman suffering from traumatic deep neck infection due to pulling a tooth with pliers. (www.actabiomedica.it)

Key words: deep neck infection, trauma, pliers

Deep neck infection is life-threatening and mortal condition that requires immediate treatment (1). This infection is generally polymicrobial and frequently seen after upper respiratory infections, poor dental hygiene, trauma and surgery to the head and neck region and in immuncompromised patients (1).

Odontogenic infections are common reason of the fascial infections (2) The purulent material of these infections may radiate to fascial gaps like sublingual, buccal, pterygomandibular and submandibular areas (2). The symptoms of deep neck infections are swelling, dysphagia, pain, trismus, dysphonia and otalgia (1). Herein, we reported a case of traumatic deep neck infection due to pulling a tooth with pliers.

A 71 year-old-woman admitted to emergency department suffering from pain, dysphagia, dysphonia and progresive swelling of the right part of the neck and face. The patient stated that she had pulled premolar tooth from right lower jaw with pliers. The patient's vital signs were as follows; blood pressure, 100/60 mmHg; body temperature, 38.7°C; hearth rate, 115 beats/min. On physical examination, the patient was oriented, alert and conscious. In the right part of the face and neck, there was periorbital diffuse edema, fluctuating mass and hyperemia. There was no previous medical history except hypertension. In laboratory examinations, white blood cell count was 15,6 10^3 µ/L, C-reactive protein was 467 mg/L, there was no other abnormal test result. Computerized tomography of paranasal sinuses and neck region revealed subcutaneous gas and hemorrhage in the soft tissue of the right maxillary region and additionally there was gas and rise in soft tissue density in the right paraphyrangeal space (Figure 1). The abscess drained by otorhinolaryngologist and antibiotic treatment including meropenem were given. The patient who under the treatment died in the third day due to uncontrollable hypotension.

Deep neck infections can be seen at any age and its mortality is about 20-50% (2). These infections usually originate from pharynx and oral cavity afterwards spread to submandibular, paraphayngeal and retropharyngeal spaces (2). Furthermore, these zones conjoin to critical areas such as skull base, meninges,

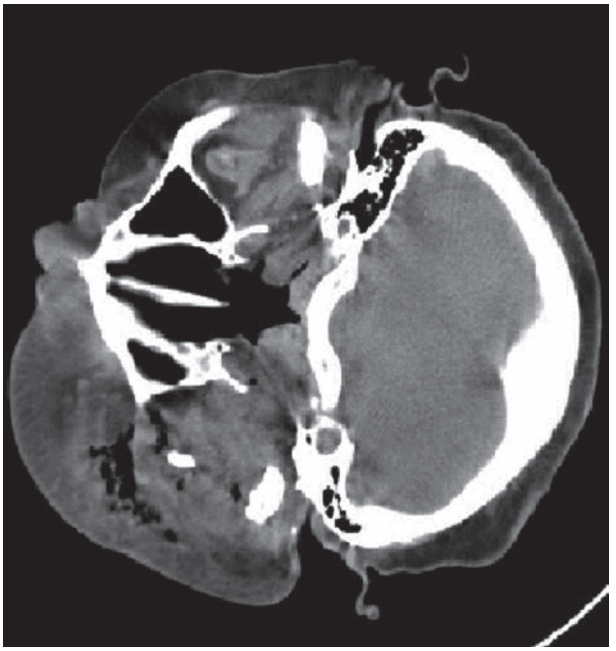

Figure 1.

mediastinum, neck and carotid sheath (2). Airway obstruction may results if infection radiates to near the pharynx and also hemorrhage and nerve injury may occur if infection spreads to the carotid sheath (3).

Initial management of the deep neck infection is intravenous antibiotic, protection of airway and drainage of abscess (1). Although the culture-guided antimicrobial treatment is suitable, empiric antibiotherapy has an important role in the progression of infection (4). In contrast with the geographical variations in microbiological specimen, several studies showed that the Staphylococcus (S.) species are main pathogens of deep neck infections (4). Even though proper treatment, severe complications with 35-50% mortality rate may occur (3). Mediastinitis, necrotizing fasciitis, gangrenes and shock are serious complications of deep neck infections (3). Ultrasound, MRI, computerized tomography and soft tissue neck X-ray are diagnostic tests for evaluation of deep neck infections (3).

In conclusion, deep neck infections can cause severe complications even dead can be seen , so physicians should be aware of these complication. Patients withdrew neck infection have to be hospitalized and intravenous antibiotheraphy and should be applied immediately.

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Case report

Linezolid-induced black hairy tongue

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Summary. Black hairy tongue (BHT) is a self-limiting disorder characterized by abnormal hypertrophy and elongation of filiform papillae on the surface of the tongue. The exact mechanism of drug-induced BHT is unknown. Several factors have been implicated and included smoking or chewing tobacco, drinking alcohol, poor oral hygiene and antibiotics such as tetracyclines and penicillins. We report a quite uncommon case of Linezolid-induced BHT in a patient with a long-lasting history of chest wall infection (www.actabiomedica.it)

Key words: black hairy tongue, lingua villosa nigra, linezolid

Introduction

Black hairy tongue (BHT), also called "lingua villosa nigra", is a benign condition characterized by elongation and hyperplasia of the filiform papillae into "hair-like" prolongations and the appearance of a brown or black discoloration of the dorsal part of the tongue (1). BHT is induced by imperfect desquamation of the dorsal surface of the tongue (2). This imperfect desquamation precludes regular debridement resulting in uncontrolled growth and thickening of the filiform papillae that accumulate debris, bacteria, fungi or other foreign substances which contribute to the discoloration. The specific way of drug-induced BHT is unknown. Different components have been thought to cause and/or predispose BHT; those components consisted of smoking or chewing tobacco, drinking alcohol, scanty oral hygiene, smoking street drugs (crack, for instance), using peroxidecontaining mouthwash, radiation therapy, using drugs that provoke xerostomia (anticholinergics, for instance), and antibiotics such as tetracyclines and penicillins (1,2). Linezolid-induced BHT is an uncommon, benign, self-limiting disorder that has been rarely previously reported (3-8). We hereby report a quite uncommon case of Linezolid-induced BHT in a patient with a long-lasting history of chest wall infection.

Clinical case

A 80 year-old non-smoking man was admitted for a long history of chest wall infection. As a result of right chronic pleural effusion, he underwent CT-guided trans-thoracic pleural biopsy with no sign of tumour. Three months later, he developed a chest wall bulge at the level of previous punctures. A spontaneous drainage of purulent fluid occurred. Microbiological examinations revealed the presence of *Staphylococcus Haemolyticus* and *Enterococcus Faecalis*. According to the antibiogram, a dual oral antibiotic therapy was started and daily dressings were performed for 2 months with no benefit. Therefore a surgical resection of the fistulous tract was performed: during the operation, a meticulous toilette and debridement of the infected area was obtained. Soon after the operation, a further infection occurred. Daily dressings were resumed for 3 months. Since further local microbiological examinations showed the persistence of both bacteria, an antibiotic therapy with linezolid 600 mgx2/die

was started. Two weeks later, the patient complained of swelling and discoloration of the tongue. At clinical examination, a black discoloration of the tongue with elongated filiform papillae was confirmed (Fig. 1). Linezolid was discontinued and his tongue return to normal within two weeks (Fig. 2).

No further antibiotic therapy was proposed. Daily dressings were continued for a few weeks until spontaneous definitive closure of the fistulous tract occurred. Six months later, no sign of recurrence was visible and the patient was asymptomatic.

Discussion

We present a case of Linezolid-induced BHT in a patient with a long-lasting chest wall infection. In this patient, chest wall infection was probably due to repeated diagnostic attempts performed by transthoracic needle biopsies (TTNB). Chest wall infection is extremely rarely reported after CT-guided TTNB (9). Even in case of major chest wall resection, the occurrence of postoperative local infection is seldom encountered (10).

Although very rarely encountered, black hairy tongue, also known as *lingua villosa nigra*, should be always taken into consideration in patients under Linezolid therapy. A very few cases of patients with BHT related to linezolid intake have been reported in the literature (3-8). BHT is a self-limiting disorder characterized by abnormal hypertrophy and elongation of filiform papillae on the surface of the tongue (1-2). At the moment, there are no clear indicators for recognizing this disorder (1). The diagnosis of BHT depends on the macroscopic visualization of discolored, elongated, and hypertrophied filiform papillae of the tongue. Although BHT could be asymptomatic, some patients may refer tickling/swelling or burning of the tongue, nausea, halitosis or a different appearance of the tongue (3-8). Our patient complained of swell-

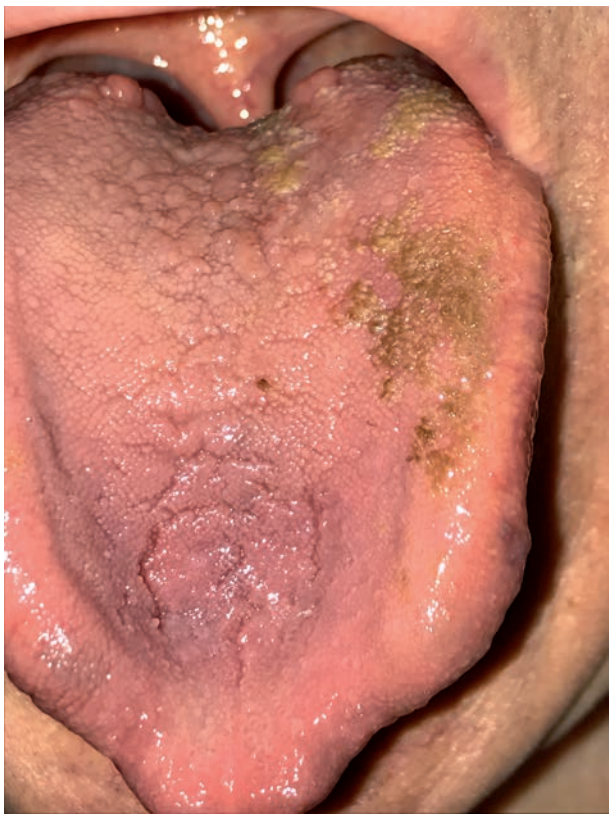

Figure 1. Black discoloration of the tongue with elongated filiform papillae occurred during linezolid therapy

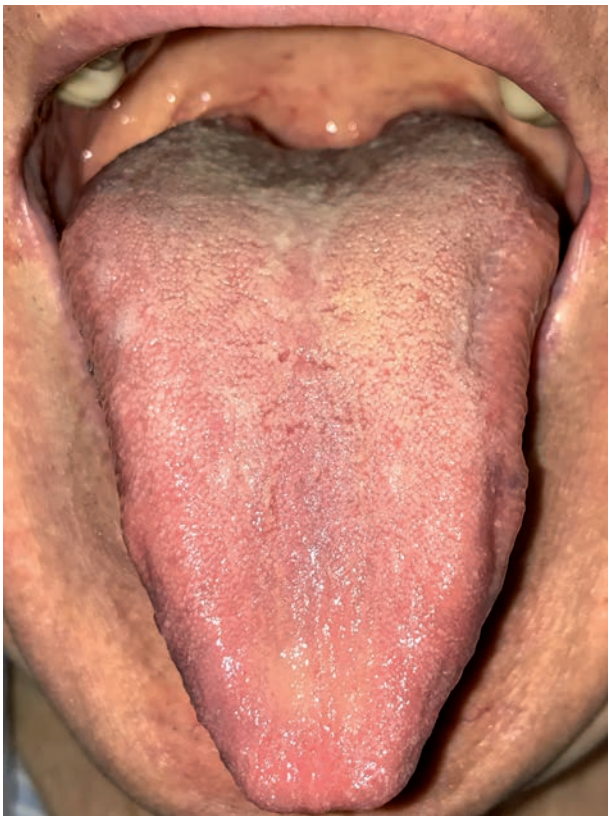

Figure 2. Two weeks after linezolid discontinuation, patient's tongue return to normal

ing of the tongue associated with a black discoloration more visible at the dorsal surface (as clearly visible in Figure 1). The "black" coloration has been historically applied to describe this disorder even though other different discolorations have been infrequently observed (such as brown, yellow and green) (1-2).

BHT might be correlated with the presence of chromogenic organisms (such as Candida Albicans) or the use of some drugs medications (doxycycline and bismuth-containing compounds are more commonly involved). The etiopathogenesis is not clear, but it might be due to proliferation of the filiform papillae of the tongue, which stain black with porphyrin-producing chromogenic bacteria or yeast (11). For this reason, bacterial or mycotic superinfection is a significant point in the management of patients with BHT. In fact, a correct diagnosis and treatment (including the discontinuation of possible predisposing factors) might prevent the development of burning mouth syndrome (11).

Acknowledgement

The patients' consent was obtained for publication of the clinical details and images in this article.

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Case report

Schwannoma of right cerebellopontine angle. A cytologic diagnosis

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Summary. Schwannomas affect mainly head and neck peripheral nerves, are benign tumors and derive from Schwann cells. Schwannoma of right cerebellopontine angle is extremely rare to diagnose by cytology. We report one such rare case presenting the cytological features in material obtained during the resection of the tumor. *Case report:* A 47-year-old female was diagnosed by MRI with a tumor of right cerebellopontine angle.. Cytologic material from the tumor was obtained intraoperatively and diagnosed cytologically as a neurilemoma. *Conclusion:* This case is presented here to focus the ability of cytology in diagnosis of schwannoma in intraoperative material of the tumor, using immunohistochemistry and confirmed by histology- immunohistochemistry. (www.actabiomedica.it)

Key words: Schwannoma, cytology, histopathology, immunocytopathology

Introduction

Neurilemomas or Schwannomas are the most common benign encapsulated neoplasms arising from peripheral nerve tissue. Usually they are encapsulated perineural tumors of neuroectodermal derivation that originate from the Schwann cells of the neural sheath of motor and sensory peripheral nerves The etiology is still unknown (1,2).

Schwannomas occur in patients with neurofibromatosis type 2 (NF2) and schwannomatosis. Most NF2 patient tumors have biallelic inactivating mutations in the NF2 tumor suppressor gene. Schwannomatosis patients do not harbor the NF2 germ line mutations, and the molecular basis of their disease remains unknown, however schwannomas from schwannomatosis patients also have biallelic NF2 mutations.

The NF2 protein, merlin or schwannomin, belongs to the ERM (ezrin-moesin-radixin) family of membrane-cytoskeleton linking proteins. There is a loss of NF2 in schwannomas. NF2 inactivation causes tumor formation through cellular changes and subsequent cytoskeletal abnormalities (13).

Neurilemomas occur in all age groups, but most frequently between 20 and 50 years. Many patients have minor symptoms from the tumor and pain after surgery can be more pronounced than it was before operation. When an excision of the lesion is considered necessary a correct diagnosis helps the surgeon to plan surgery so as to avoid neurological sequelae (3).

This case has been reported for its rare and unusual site for cytodiagnosis of a Schwannoma confirmed by histology.

Case report

A 47-year-old female patient was presented at Univ. Hospital of Heraklion Crete and diagnosed by MRI with a solid- cystic tumor of cerebellum. She

suffered from cranial pain and there was no history of trauma. Past, personal and family history was non-contributory.

The hematological and biochemical parameters were with in normal limits. The MRI signal in the center of the mass was hyperintense on T2-weighted and isointense on T1-weighted images.

Material and Methods

Cytology: The cytologic material obtained intraoperative (during the resection of tumor) was smeared on glass slides. The air dried smears were used for Giemsa stain and immunochemistry while the alcohol (80%) fixed for routine Papanicolaou stain (Fig. 1).

Immunocytology: In air dried smears immunocytochemistry was performed using the markers S-100 protein (Fig. 2) and Vimentin

Histology: In histological specimens of the tumor fixed in 10% formalin, the H-E stain was performed (Fig. 3).

Immunohistochemistry: The markers S-100 protein, Vimentin, EMA, NF, GFAP, Calretinin, PGM-1 and the proliferation index MIB-1 were used.

Results

Cytology: showed cellular smears of spindle shaped tumor cells and Schwann cell processes. Isolated cells were elongated, round to spindle shaped with elongated, slender vesicular nuclei with ill-defined cytoplasm. In the background many lymphocytes and histiocytes were found.

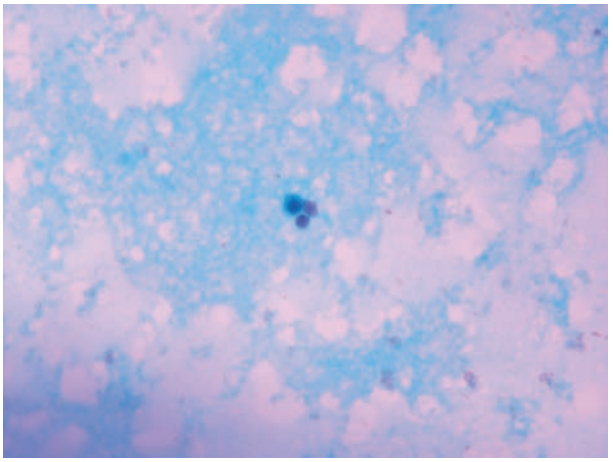

Figure 1. Schwannoma, Intraoperative smear. Isolated neoplastic cell. Papanicolaou stain X400

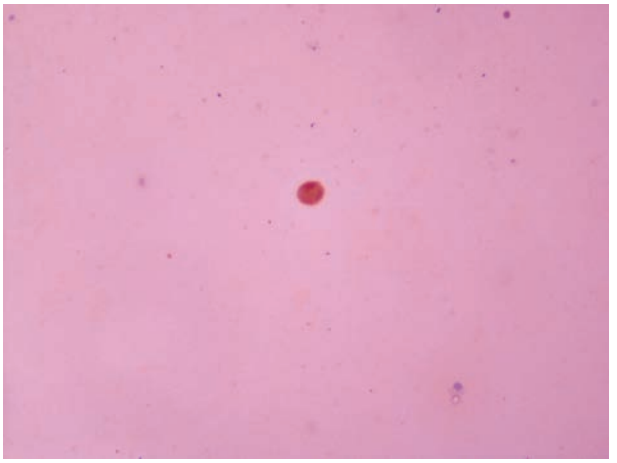

Figure 2. Schwannoma, Intraoperative smear. Isolated neoplastic cell. S-100 immunostain X400

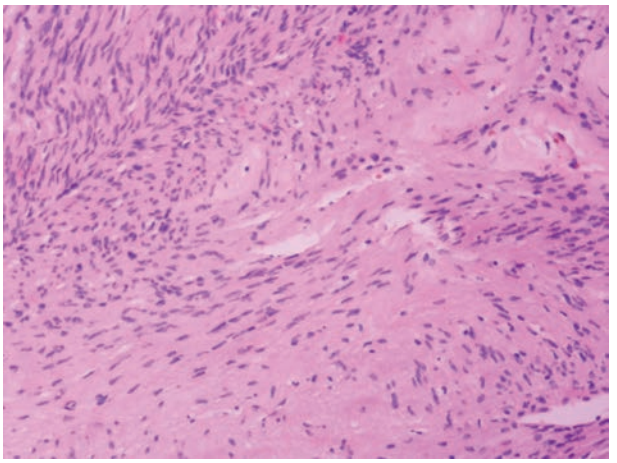

Figure 3. Schwannoma. Tumor section. Hematoxylene-Eosin(H@E) stain X 400

Histology: Abundant material with many spindle-shaped neoplastic cells with mild pleomorphism, rare nuclear atypia and nuclear inclusions. At places Verocay bodies were found and in the background many histiocytes were observed. Mitoses or necrosis were not found.

Immunocytochemistry: The majority of neoplastic cells were found to be cytoplasmic positive for S-100 protein (Fig. 4).

Immunohistochemistry: The tumor cells expressed cytoplasmic S-100 protein, Vimentin and EMA mark-

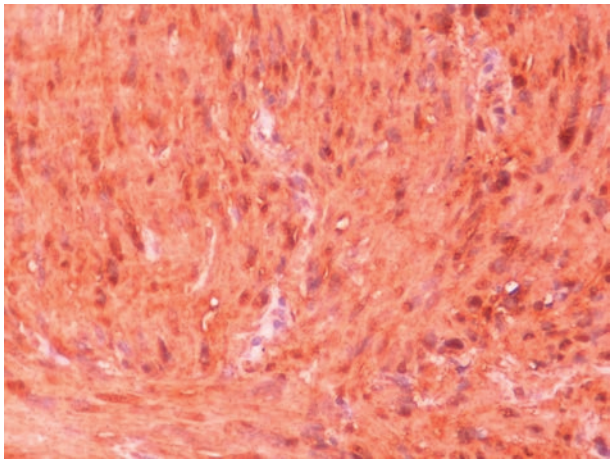

Figure 4. Schwannoma. Tumor section. S-100 immunostain X 400

ers. The neoplastic cells were found to be negative for NF, GFAP and PGM-1 and proliferating index MIB-1 was found to be positive in 1% of tumor cells.

Discussion

About 25% of the Schwannomas occur in the head and neck region (3,5), usually involving cranial nerves and sympathetic chain. The schwannoma is the most common tumor of the peripheral nerve occurring anywhere in the body (4,5), often presents as a solitary painless, and slow- growing mass of variable size.

Cystic schwannoma is thought to grow more rapidly than non- cystic (4,8,10).

Smears from schwannomas with cystic degeneration obtained preoperative by FNAB show scattered round- to- oval cells, accompanied by occasional histiocytes (3-6,8,10).

In this report the cytologic material was obtained intraoperative and was adequate characteristically composed of spindle- shaped cells, vesicular nuclei, scanty cytoplasm without mitoses and necrosis with the presence of many lymphocytes and histiocytes because of the cystic degeneration of the tumor.

Histologically, a typical schwannoma is composed of two areas (11): The Antoni A area characterized by closely packed spindle cells with occasional nuclear palisading and Verocay bodies as observed in our case. The Antoni B area is occupied by loosely arranged tumor cells which are separated by abundant myxoid stroma. An Antoni A area (cohesive cellular clusters) and an Antoni B area (loosely cohesive or poorly cellular sheets) are occasionally found in cytology (2,3).

Immunohistochemically, schwannomas are usually positive for S-100 protein,Leu-7 (CD57),and glial fibrillary acidic protein (GFAP) (7,9-12).

The immunophenotype of schwannomas is highly distinctive: S-100, Collagen IV and lamin, are expressed expecially in Antoni A areas, GFAP may be seen in a significant number of schwannomas, Neurofilament protein NFP staining is limited.

Recent markers include podoplanin, calretinin and SOX10 (14, 15).

In our case the majority of neoplastic ells were found to be cytoplasmic positive for S-100 protein but negative for Vimentin by immunohistochemistry.

In our case the schwannoma occurs in right cerebellopontine angle and in this region is a very rare entity for cytologic diagnosis of schwannoma because of FNAC aspirates have often inadequate material (2,3,7,8).

By immunohistochemistry the tumor cells expressed cytoplasmic S-100 protein, Vimentin and EMA markers but were found to be negative for NS, GFAP and PGM-1 markers.

In conclusion, hence FNAC is not helpful in achieving preoperative diagnosis in case of schwannoma, the intraoperative cytology provides a convenient and safe diagnosis confirmed by histology.

Writen informed consent was obtained by the patient to submit this case report to the journal.

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Case report

Scapular reconstructions after resection for bone tumors: a single-institution experience and review of the literature

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Summary. Scapulectomy and limb-salvage surgery are indicated for low and high-grade tumors of the scapula and soft-tissue sarcomas that secondary invade the bone. After total or partial scapulectomy there are 3 options of reconstruction: humeral suspension (flail shoulder), total endoprosthesis and massive bone allograft. Nowadays prosthesis and allograft reconstructions are the most used and humeral suspension is reserved only as salvage technique when no other surgery is possible. Several studies reported dislocations and wound infections as the most frequent complications of scapular prosthesis, account for 10-20%. Recently, in the attempt to prevent these complications, some authors have used homologous allografts to replace shoulder girdle after scapulectomy for bone tumors, avoiding common complications of scapular prosthesis. Scapular reconstruction following tumor resection is a safe procedure and can be performed with good functional, oncological and cosmetic results but in reference centres and by skill surgeons. In this paper we present three cases of scapular reconstructions following resections for scapular tumors (chondrosarcoma in all cases) performed in our Institute and we analyse the different options of reconstruction described in the current literature. The final message is to send these rare tumors to reference centres where a multidisciplinary team is able to treat these rare entities and where a group of skill oncology surgeons are able to plan this complex surgery. (www.actabiomedica.it)

Key words: scapular reconstruction, allograft, scapular tumor, resection, scapular prosthesis

Case 1

We report the case of a 68-year-old woman with grade 1 chondrosarcoma of the right scapula, treated at our Institute with total scapulectomy and reconstruction with homologous massive allograft of scapula.

The patient was admitted at our Institute on April 2007 because of occasional discovery of an osteolysis of the scapula (Fig. 1).

During hospitalization she performed Magnetic Resonance Imaging (MRI) (Fig. 2) that showed the cartilaginous nature of the tumor and a computer tomography (CT)-guided biopsy: the diagnosis was grade 1 chondrosarcoma of bone. The tumor was located in S1-S2 region according the Enneking's classification (4).

On June 2007 an intra-articular total scapulectomy [type 3 according Malawer's classification (8)] and reconstruction with homologous massive bone allograft was performed. A posterior surgical approach was used with an anterior extension (deltopectoral approach), to obtain a good view of the neurovascular structures and the posterior muscles surrounding the scapula. The acromion-clavicular joint was fixed with a transarticular Kirschner wire; coracoclavicular fixation was obtained with an artificial ligament (LARS, Ligament Advanced Reinforcement System) passed beneath the base of the coracoid and superiorly through two holes in the clavicle.

The host coracoid process was fixed to the allograft bone with a screw (Fig. 3-a-b). The joint capsule, tendon and the periscapular muscles detached during the

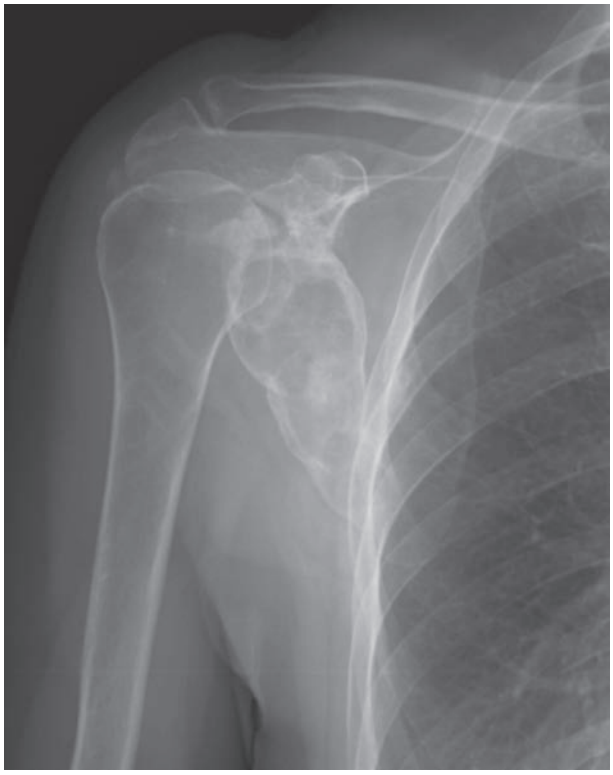

Figure 1. Radiograph shows an osteolytic lesion of the scapula with calcifications

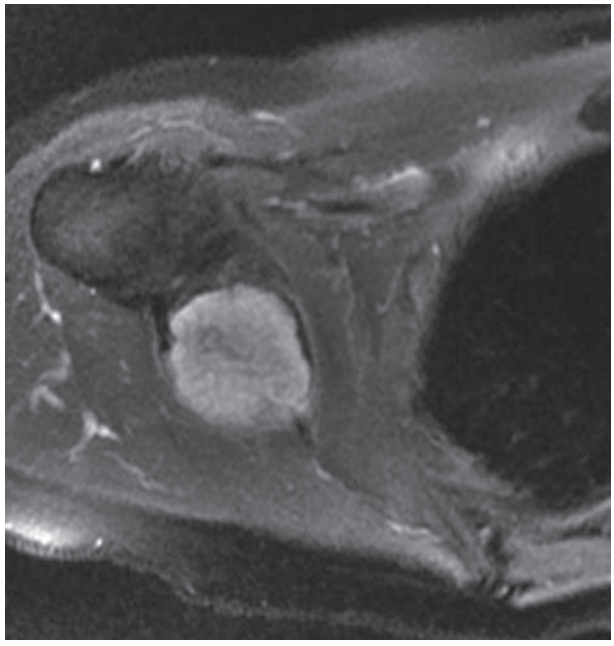

Figure 2. STIR sequences show a hyperintense lesion of scapula invading soft tissues

resection procedure, were reattached to their origins or sutured to the corresponding stumps on the allograft.

The upper limb was immobilized with abduction brace for the first 4 weeks and then passive motion of the shoulder joint was allowed for 4 weeks. Active exercises and functional rehabilitation started at 2 months postoperatively. Physiotherapy in water was preferred at the beginning to reduce stress and load to the shoulder joint.

From an oncological point of view, wide surgical margins were achieved.

During the follow-up the patient complained intolerance to fixation and underwent removal of Kirschner-wire and regularization of the graft.

On September 2009 she underwent wedge resec-

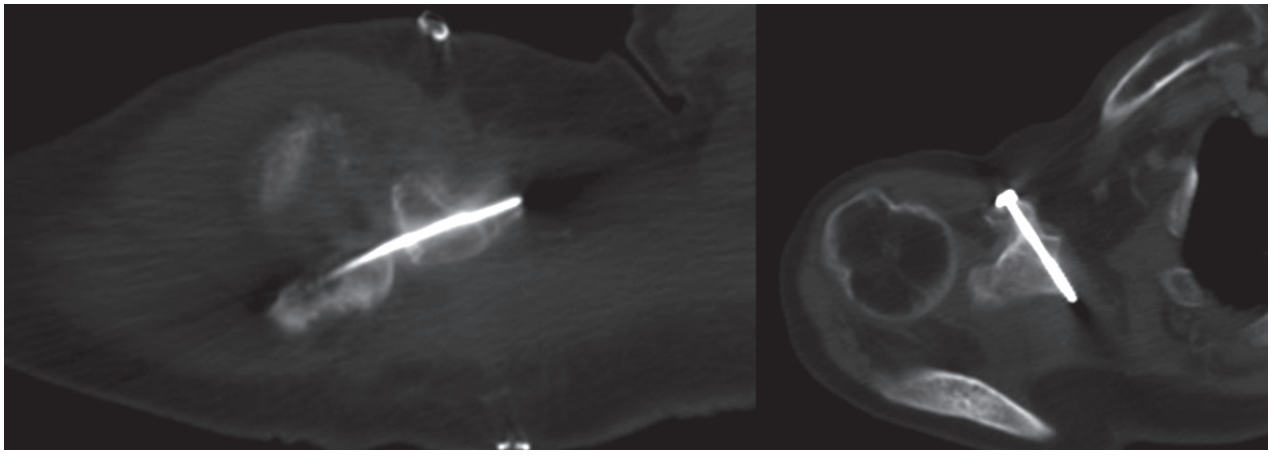

Figure 3 a-b. Postoperative CT-scan shows the internal fixation of the graft to the host bone

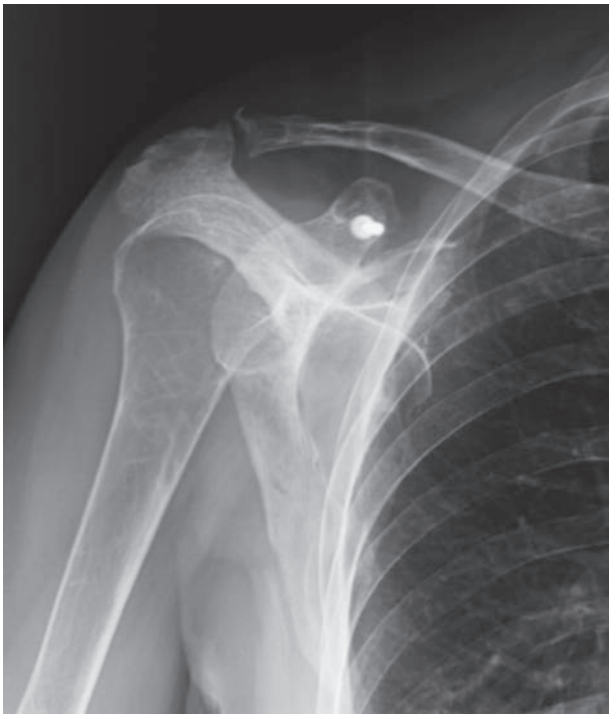

Figure 4. Follow-up at 7 years

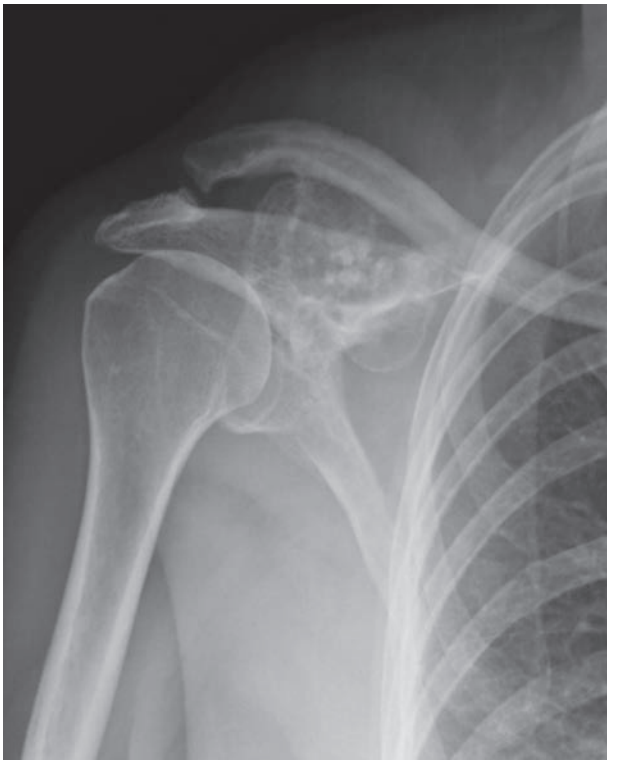

Figure 5. Osteolysis of the coracoid process with calcifications

tion of left lung because three nodules were found at CT-scan but histologically there was no evidence of disease.

At last follow-up of September 2014 the patient had no evidence of disease (Fig 4).

The functional result was good with abduction for 45° and MSTS score (5) of 82%.

Case 2

The second case is a 58 year-old woman with low-grade chondrosarcoma of the right coracoid process (Fig. 5).

The patient was admitted at our Institute on February 2010 because of pain and swelling of the anterior part of the shoulder.

In 1997, due to a shoulder trauma and subsequent radiography, a diagnosis of enchondroma of the coracoid process was made. So the patient began clinical and radiographic follow-up until 2009, when she complained pain and swelling in the right shoulder.

X-ray and TC showed an osteolysis of the coracoid process with central calcifications (Fig. 5).

CT-guided biopsy was performed and the diagnosis was low-grade central chondrosarcoma.

After staging disease, on February 2010 a resection of the coracoid process and reconstruction with homologous bone allograft was performed (Fig. 6).

A deltopectoral approach extended posteriorly was used to obtain a good view of the neurovascular structures and the posterior muscles surrounding the scapula. After resection of the tumour, the bone gap was reconstructed with an omologous bone graft from our bone tissue bank. The allograft was fixed with plate and screw to the host bone. The acromion-clavicular joint was fixed with a transarticular Kirschner wire; coracoclavicular fixation was obtained with an artificial ligament (LARS). The joint capsule, tendon and the periscapular muscles detached during the resection procedure, were reattached to their origins to the corresponding stumps on the allograft.

Wide margins were achieved.

During follow-up she complained intolerance to fixation and the K-wire was removed.

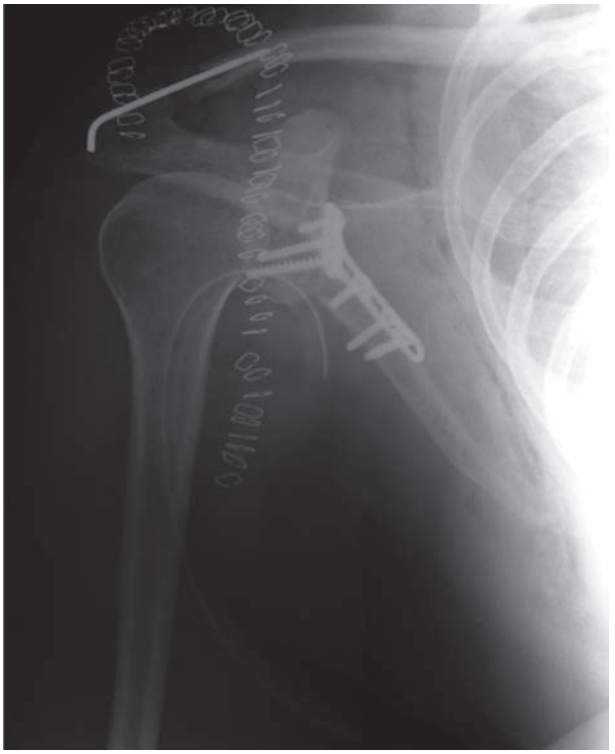

Figure 6. Postoperative radiography

At last follow-up on October 2014 there was no evidence of disease. She had good abduction and strength, without limitation in daily-life.

MSTS score was 77%.

Case 3

The last case is a 31 year-old woman with low-grade chondrosarcoma of the left scapula (Fig. 7).

The patient was hospitalized at our Institute on August 2009 for swelling and pain of the left shoulder since three months.

So she performed x-rays, CT and MRI that showed an osteolysis of the coracoid process with central calcifications.

CT-guided biopsy was performed: the diagnosis was low-grade central chondrosarcoma.

After staging the tumor, resection of the glenoid and reconstruction with custom-made prostheses was performed on December 2009. A deltopectoral approach extended posteriorly was used to perform the resection of the tumour. The specimen was then sent

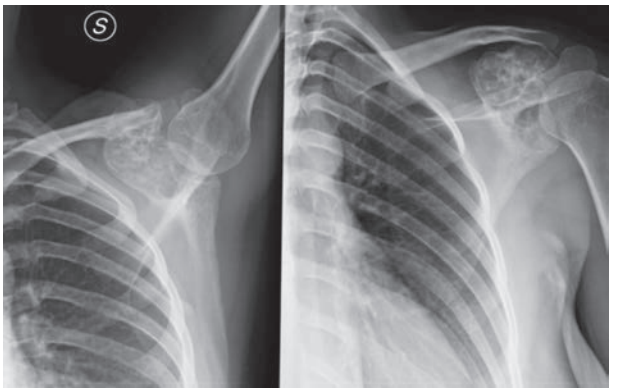

Figure 7. Preoperative radiography

to our pathologist for histological examination. The acromion-clavicular joint was fixed with a transarticular screw and K-wire (Fig. 3, 4). The custom-made prosthesis was fixed to the residual scapula with screws (Fig. 4). The joint capsule, tendon and the periscapular muscles detached during the resection procedure, were sutured to the corresponding stumps of the prosthesis.

During surgery, a fracture occurred accidentally into the body of the scapula, which was then stabilized with two opposing ribs of homologous bone and plates (Fig. 8). From an oncological point of view wide margins were achieved.

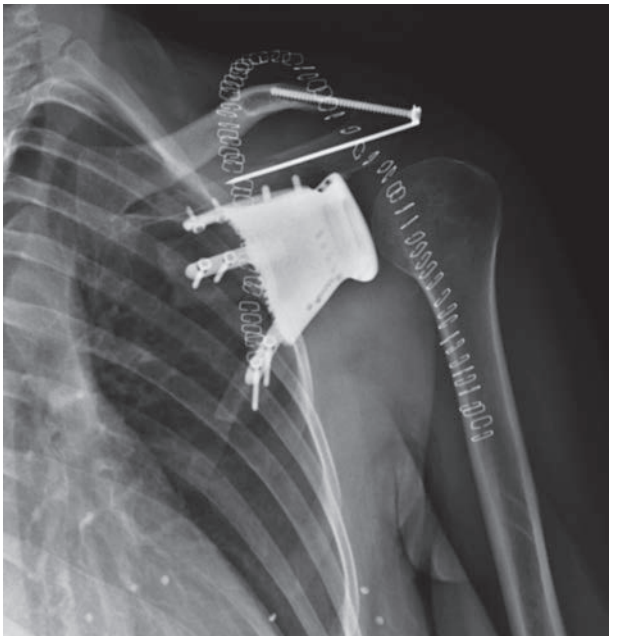

Figure 8. Postoperative radiography

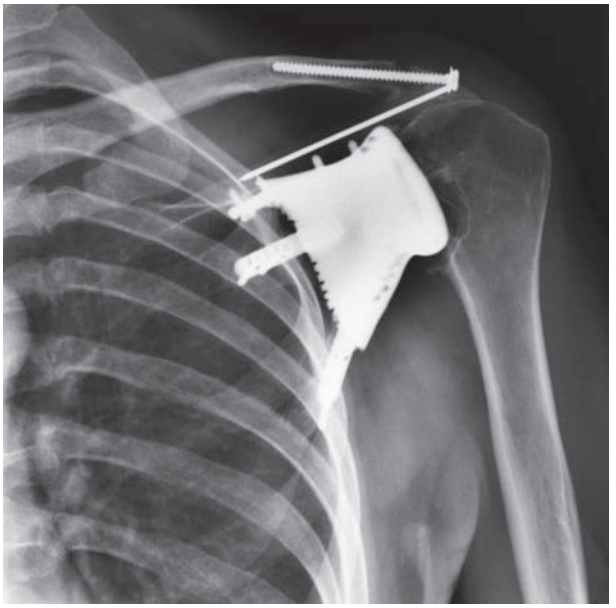

Figure 9. Five year follow-up x-ray

At last follow-up on June 2015 there was no evidence of disease; the prosthesis was stable but she complained occasional pain, she had limited extrarotation and abduction for 30°. X-ray showed osteolysis of the proximal humerus due to arthritis (Fig. 9). Despite occasional pain and discomfort, the patient refused revision surgery.

MSTS score was 62%.

Discussion

Bone tumors of the scapula are very rare. According the Rizzoli Bone Tumor database, the most common lesion is chondrosarcoma (52.4%), followed by Ewing sarcoma (27.4%) and osteosarcoma (12.9%) (14).

The Enneking classification system for bone tumors of the scapula, approved by the Musculoskeletal Tumor Society (5), divides the scapula into 2 zones (Fig. 10). The surgical options depend on the location and size of the tumor. Usually, S1 tumors can be treated with partial scapulectomy without reconstruction; S2 tumors require glenoid reconstruction. Tumors affecting S1 and S2 region require total scapulectomy and represent a challenge for the orthopaedic surgeon,

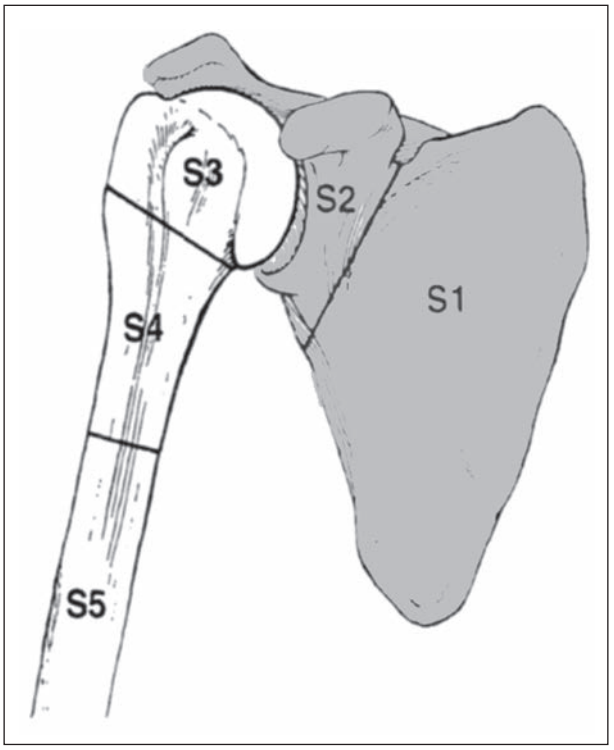

Figure 10. Segment 1 (S1): scapular blade and spine; segment 2 (S2): glenoid, coracoid process and acromion.

because of the complex anatomy of this region and the need to perform en-bloc resection with adequate margins preserving the neurovascular bundle and possibly rotator cuff and muscles of the shoulder girdle. Whenever the glenohumeral joint is preserved, good function of the arm can be expected.

Contraindications include tumour extension into the axilla with involvement of the neurovascular bundle and inability of the patient or unwillingness to tolerate limb-salvage surgery. Relative contraindications may include chest wall invasion, pathological fractures, previous infection, lymph node involvement and inappropriate placed biopsy with soft-tissue contamination (10).

The first scapulectomy was reported by Lister in 1819 (7), who described a case of an ossified aneurismal tumour. Since then, most shoulder girdle resections have been performed for low and high-grade sarcomas of the scapula and soft tissues.

Currently, the Malawer classification system (8) is the most used for shoulder girdle resections and is based on the concepts of surgical margins (intra-artic-

ular vs. extra-articular), the relationship of the tumor to anatomic compartments (intracompartmental vs. extracompartmental), the status of the gleno-humeral joint and the abductor mechanism.

After total or partial scapulectomy there are 3 options of reconstruction: humeral suspension (flail shoulder), total endoprosthesis (non-constrained or constrained) and bone allograft.

Humeral suspension was the most popular reconstructive procedure after total scapulectomy until the early 1990s. In humeral suspension the proximal humerus is simply stabilized with heavy nonabsorbable sutures or wires to the clavicle. Mayil Vahanan et al. (11) reported on the functional and oncological results of 23 patients who underwent scapulectomy for scapular tumors without reconstruction (flail shoulder). They described excellent/good results in 13 patients and fair/poor in 10 patients. Restriction of shoulder abduction was the major functional limitation, but they had normal hand and wrist functions.

Pritsch et al. (15) in 2006 performed a study with 2 groups of 16 patients each one, comparing the functional results of humeral suspension and scapular endoprosthetic reconstruction. They assessed that after total scapulectomy, scapular endoprosthetic reconstruction led to better functional and cosmetic results than humeral suspension and therefore they recommended performing this procedure whenever rhomboids, latissimus dorsi, deltoid and trapezius could be preserved. So, nowadays, humeral suspension represents a salvage technique when no further reconstruction is possible.

The aim of endoprosthetic scapular reconstruction is to form a connection between the arm and the chest wall, lateralizing the reconstructed shoulder and preserving its offset, preventing traction on the brachial plexus and filling the defect that remained after resection of the scapula (10).

Baran et al. (1) reported on the results of 7 patients who underwent partial or total scapulectomy followed by reconstruction with fibular autograft (1 case) and scapular prosthesis (6 cases) for bone or soft tissue scapular tumors. In this series there was no complication after surgery. MSTS score was 73,3%.

Tang et al. (17) described a study of 10 patients who underwent constrained prosthetic replacement after total scapulectomy for bone malignant tumor. They reported 1 dislocation, treated with open reduction, and 1 superficial wound infection, treated with surgical debridement. MSTS score was 76,7%.

Masamed et al. (9) and Schwab et al. (16) presented the biggest groups of patients who underwent scapular prosthesis, reporting on the functional results and complications of 13 and 19 patients respectively. Both authors reported dislocations and wound infections as the most frequent complications, account for 10%-20%.

Recently, in the attempt to prevent the complications of scapular prosthesis, some authors have used homologous allografts to replace shoulder girdle after scapulectomy for bone tumours (2, 13, 19). Zhang et al. (19) described scapular allograft reconstructions of 7 patients who underwent partial or total scapulectomy. They concluded that the gleonoid-saved reconstruction had better functional results than the glenoid-resected group. They had 1 deep infection that required surgical debridement and 1 case of shoulder pain throughout the follow-up period. MSTS score was 80%. More recently, Capanna et al. (2) presented the largest series of scapular allograft reconstructions after total scapulectomy: they performed 6 massive bone allografts, of which 1 was an irradiated autograft. They had 2 breakages of the osteosynthesis and 2 allograft fractures, with ISOLS score of 66.7%.

Our functional results are similar to other studies (Tab. 1), with average MSTS score of 73% (range 62%-82%) and a long follow-up (average 60 months). The main limit is the restricted number of patients, due to the rarity of scapular tumors and the selected surgical indications.

Conclusion

Limb salvage surgery of the scapula is a challenge for the orthopaedic surgeon, because of the complex anatomy of this region and the need to perform en-bloc resection with adequate margins preserving the neurovascular bundle and possibly rotator cuff and muscles of the shoulder girdle.

Scapular reconstruction following tumour resection can be performed with good functional, oncological and cosmetic results but in reference centres and

Table 1. Reported results of scapular reconstructions (prosthesis and allograft) in comparison to the current study

Author	Patients	Reconstruction	Follow-up	Complications	Functional score
Witting (18)	3	Constrained prosthesis	16 months	None	ISOLS 80%-90%
Schwab (16)	19	Constrained-noncostrained prosthesis	18 months	2 infections 2 dislocations 2 wound necrosis	ISOLS 82%
Pritsch (15)	15	Constrained-noncostrained prosthesis	90 months	2 wound dehiscences 1 dislocation	ISOLS 79%
Masamed (9)	13	Constrained prosthesis	Not available	3 dislocations 3 wound seromas	Not available
Baran (1)	7	1 Fibular autograft 6 Constrained-non costrained prosthesis	35 months	1 shoulder instability	MSTS 73.3%
Tang (17)	10	Constrained prosthesis	36 months	1 dislocation 1 wound infection	ISOLS 76.6%
Lee (6)	2	2 Glenoid allograft	33 months	None	ISOLS 90%
Mnaymneh (13)	6	5 Total scapula allograft 1 glenoid allograft	1 allograft fracture 44 months	ISOLS 82%	
Zhang (19)	3	3 glenoid allograft	26 months	1 chronic pain	ISOLS 79%
Chandrasekar (3)	2	2 irradiated scapula autograft	36 months	None	Not available
Merriman (12)	1	1 total scapula allograft	61 months	None	Not available
Capanna (2)	6	5 total scapula allograft 1 irradiated scapula autograft	66 months	2 osteosynthesis failures 1 allograft fracture	ISOLS 66.7%
Current study	3	1 total scapula allograft 1 glenoid prosthesis 1 coracoid allograft	60 months	1 intraoperative scapula fracture and proximal humerus arthtritis	MSTS 73%

by skill surgeons. The goals of scapular reconstructions are to restore shoulder stability and good elbow, hand and wrist function, adequate suspension of the humerus and meticulous soft-tissue reconstruction, without compromising oncological results.

Preservation of rotator cuff and deltoid can warranty good flexion and abduction.

The main limit of these studies is the number of patients, due to the rarity of scapular tumors. Further studies, bigger group of patients and long-term follow-up are necessary to assess the functional results of scapular allografts and prosthesis reconstructions.

Finally, this paper does not aim to help the orthopaedic surgeon to perform a shoulder girdle resection.

The final message is to send these rare tumors to reference centres where a multidisciplinary team is able to treat these rare tumors and where a group of skill orthopaedic oncology surgeons are able to plan this complex surgery.

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Case report

Unilateral twin tubal pregnancy: a case report and review of the literature

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Summary. *Background:* Unilateral twin tubal pregnancy is an extremely rare condition, occurring in 1/20.000-250.000 pregnancies and represents a major health risk for reproductive-aged women, leading to even life-threatening complications. *Aim:* We present a case of a 31-year-old woman with unilateral twin tubal pregnancy, treated with methotrexate and then surgically because of failure, followed by review of the literature. *Methods:* Researches for relevant data were conducted utilizing multiple databases, including PubMed and Ovid. *Results:* The most common type of twin ectopic pregnancy is the heterotopic (1/7000 pregnancies) in which in which both ectopic and intrauterine pregnancy occur simultaneously. Expectant, medical and surgical therapy have similar success rates in correctly selected patients. Two prospective randomized trials did not identify any statistically significant differences between groups receiving MTX as a single dose or in multiple doses. Among the 106 cases reported in literature, methotrexate was tried just in 4 patients (3 unilateral and 1 bilateral) before ours. Details are reported in the table 1. *Conclusion:* The recent shift in the treatment of singleton ectopic pregnancies to the less invasive medical therapy might apply even in the case of twin implants. (www.actabiomedica.it)

Key words: unilateral, twin tubal pregnancy, rare condition

Case presentation

Our 31-year-old patient (gravida 2, para 0) had a history of endometriosis and right tubal pregnancy treated with laparoscopic salpingectomy 7 months earlier at our institution.

On a routine ultrasound, at an estimated gestational age of 6 weeks and 2 days given her last menstrual period, a left ectopic pregnancy was suspected so she was sent to our emergency room. Vitals were all stable and the patient was well, conscious and well-oriented.

On physical examination her abdomen wasn't tender and just slight left adnexal tenderness was elicited. Vaginal bleeding was light.

Ultrasound revealed an empty uterine cavity and in the left adnexa, adjacent to the ovary, a complex mass measuring 29 x 16 mm encompassing to a further evaluation 2 thick-walled fluid-filled cystic masses measuring 5 and 16 mm (Fig. 1). No fluid in the pouch of Douglas was identified. Serum βhCG level was 13217 mIU/ml. The patient was then diagnosed with unilateral twin tubal pregnancy. As she was stable without any sign of tubal rupture and considering as well the previous right salpingectomy, we administered a single dose of methotrexate (50 mg/m^2, corresponding to 75 mg) + folinic acid rescue (5 mg once a day by mouth). 4 days later βhCG level was 23276 mIU/ml (+76%) and 7 days later 19783 mIU/ml (-15%). Ul-

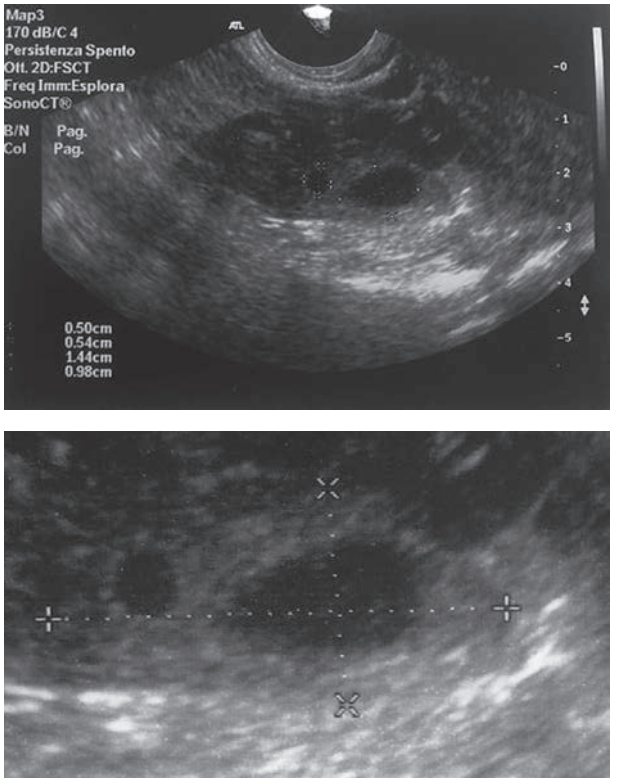

Figure 1.

trasound monitoring revealed no change. Patient was asymptomatic. A second dose of MTX (still 50 mg/mq) + folinic acid rescue was then administered.

7 days after the second MTX dose βhCG level was 16000 mIU/ml (-19%), ultrasound was unchanged and the patient asymptomatic. A third dose of MTX (still 50 mg/mq) + folinic acid rescue was administered.

4 days after the last dose, the patient came to our emergency room complaining pelvic and abdominal mass. βhCG level was 3659 mIU/ml (-77%), ultrasound revealed increasing mass dimensions as 41 x 27 mm and estimated 100 ml of blood in the pouch of Douglas.

Laparoscopic left salpingectomy was performed, total blood loss was 2500 ml and the patient underwent intraoperatively a blood transfusion. The pathology specimen confirmed the twin tubal pregnancy. The post-operative course was regular. The patient was well on 6-months follow up.

Review and comparison

Methodology

Researches for relevant data were conducted utilizing multiple databases, including PubMed and Ovid. Searches included combinations of the key terms: 'twin and ectopic pregnancy', 'twin and tubal pregnancy', 'twin heterotopic pregnancy', 'laparoscopy and twin pregnancy', 'laparoscopy and twin ectopic', 'laparoscopy and twin tubal pregnancy', 'surgery and twin pregnancy', 'surgery and twin ectopic', 'surgery and twin tubal pregnancy', 'methotrexate and twin pregnancy', 'methotrexate and twin ectopic', 'methotrexate and twin tubal pregnancy'.

Ectopic pregnancy occurs when blastocyst implants outside the uterine cavity, being this true for both singleton and multiple pregnancies.

The first un-ruptured twin tubal pregnancy was described in 1986 by Santos (1). Ectopic pregnancy develops in almost 2% while twin pregnancy counts for 1 every 80 spontaneous pregnancies (2). Unilateral twin ectopic pregnancy is though a rare condition occurring with a frequency of 1/20.000-125.000 pregnancy and 1/200 ectopic pregnancy, rarer than expected (3-5). Moreover, ectopic pregnancy has a recurrence rate of 10% for one and 25% for two or more previous (6).

Although the trend for ectopic pregnancy has been constantly increasing over the past 30 years (mainly because of Assisted Reproductive Technology and epidemiological reasons), unilateral twin ectopic pregnancies have remained anneddoctical, just approximately 106 cases described in literature, out of whose only 8 cases had live twin 1 a year.

In addition the incidence is likely to be underreported because the diagnosis is primarily surgical (<10 out of 106 were diagnosed preoperatively) and/or pathological (consider the well-known phenomenon of the vanishing twin and the deterioration of the material after medical therapy) (7).

30 years ago the mortality due to ectopic pregnancy ranged between 72 to 90% while recently, mainly thanks to early diagnosis, it dramatically dropped to 0.14% (2).

Risk factors are basically all conditions that might impaire the migration of the blastocyst/embryo to the

endometrial cavity by distorting tubal anatomy i.e. prior pelvic inflammatory disease, previous ectopic pregnancy, tubal surgery or ligation, assisted reproductive technology and also congenital anomalies.

Other factors are, although less counting, increasing age, smoking, intrauterine contraceptive device and defects of the zygote itself or in the hormonal milieu (8). The most common type of twin ectopic pregnancy is the heterotopic (1/7000 pregnancies) in which in which both ectopic and intrauterine pregnancy occur simultaneously (1).

Based on case reports from the literature, monozygotic and monoamniotic are the most frequent (95%) among unilateral twin tubal pregnancies, nonetheless a DNA analysis theorized that many of these might be dizygotic (3). The delay in tubal transport may play a role in the extensiveness of unilateral twin ectopic implantation; conversely it has been also supposed that the larger size of the twin cell mass itself causes the transport retard (9). Some authors explain the twin ectopic pregnancy as a mere result of a bilateral ovulation. Just like in singleton ectopic pregnancy, fallopian tube is the most common site.

Compared to a same sized singleton pregnancy, the chance of rupture for a twin ectopic one are lower as trophoblastic invasion may be less due to lower gestational age at presentation in the latter case. Being somehow similar and somehow different, the management of twin ectopic pregnancy can't just mirror the singleton one. The symptoms of the classic triad of amenorrhea, vaginal bleeding and pelvic pain are all presents in less than half patients.

Risk factors can prop up the diagnosis, especially a previous ectopic implant.

Serum βhCG can be much higher than the well-know discriminatory zone of 1500-2000 mIU/ml valid for singleton ectopic pregnancy (with a mean of 9846), due to the larger trophoblastic tissue (10). Interestingly, the value can thus resemble to the ones of normal intrauterine pregnancies: in the absence of an intrauterine gestational sac and normally rising βhCG the chance of a twin ectopic implant has to be considered, even if rare. The majority of tubal ectopic pregnancies are detected by transvaginal ultrasound with a sensitivity of 87.0-99.0% and a specificy of 94.0-99.9%.

In some cases the evaluation, even combining all the informations, is not discerning: the pregnancy is then classified of unknown location (PUL). The echographic signs might be, as well as in singleton ectopic implants, divided into direct and indirect. The only direct sign is the identification of a non homogeneous or solid-cystic adnexal mass encompassing two thick-walled fluid-filled cystic masses, formed by the gestational sacs (yolk sac and/or embryo are less often visualized than in singleton).

Indirect signs are the same as singleton: no evidence of intrauterine sac, fluid in the pouch of Douglas; sometimes fluid collects in the uterine cavity appearing as so-called 'pseudosac'.

Management

The primary goals are fertility preservation (being the main predictive factor the status of the controlateral tube at surgery) and avoidance of unfavorable outcome as tubal rupture (9).

As reported by Mol, "laparoscopic surgical approach in the most cost-effective method for treating ectopic pregnancy, but in carefully selected cases, the use of systemic MTX is proved to be a great alternative with similar success rates, and it is completely non-invasive (11). Moreover, some studies agreed that medical treatment doesn't impair tubal patency nor the ovarian reserve (it might spoil oocyte production but temporarily) (12-13).

Lastly, as reported by Menon, "rates of treatment failure are substantially and statistically greater if the initial values of βhCG exceed 5000 mIU/ml", simultaneously the higher the level, the higher the risk for tubal obstruction (11).

Summarizing, expectant, medical and surgical therapy have similar success rates in correctly selected patients (14).

Two prospective randomized trials did not identify any statistically significant differences between groups receiving MTX as a single dose or in multiple doses (15,16).

Among the 106 cases reported in literature, methotrexate was tried just in 4 patients (3 unilateral and 1 bilateral) before ours. Details are reported in the table 1 (6, 17, 18).

Table 1. Previous reported cases in literature

Patient, Year	Gestation Days	Day 0 Bhcg	U/B	Crl Diameter-mm	MTX -TR	DOF	Simptoms	Blood-loss	Bhcg (before surgery)	Result
1, 1993	77	3640	U	25 x 30; Crl 11 and 13	31.5 mg of MTX Into Each Gestational Sac; 2 Days Later a Systemic Dose Of 63 Mg (IM)	31	-			S
2, 1997	Unsure	539	B	Diameter 25	Single Dose 50 Mg/M2)	4	Acute Abdomen	100		U
3, 2008	Non Reported	763	U	Not Reported	Multiple Dose Regimen (1 Mg/Kg on Days 1,3,4 and 7 + 0.1 Mg/Kh of Folinic Acid on Days 2,4,6 and 8) + a Single Dose on Day 14 Th (1 Mg/Kg)	32	-			S
4, 2009	49	18780	U	CRL 11 Ans 8 Mm	Single Dose (1 Mg/Kg)	42	-			S
Ours, 2016	44	13217	U	5 and 16	Multiple Dose (50 Mg/Mg) on Day 1, 7 and 14 + Folic Rescue (5 Mg/Die Per Os)	18	Acute Abdomen	2500	3659	U

CRL: Crown-rump length; DOF: Duration of follow-up; U/B: Unilateral/Bilateral; U/S: Success/unsuccess; MTX-TR: Methotrexate Treatment Regimen

Conclusions

When making a diagnosis of ectopic pregnancy, even though rare, the chance of twin implant has to be considered. The recent shift in the treatment of singleton ectopic pregnancies to the less invasive medical therapy might apply even in the case of twin implants.

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Letter to the editor

Hypersensitivity to metallic implants: pathophysiologic and diagnostic considerations

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Metals are ubiquitous in the surrounding environment and constitute an important class of substances that can act as allergens. Consumer products such as earings, zip, jewellery, cosmetics, paints, dental and body implants as well as endovascular, intracardiac and orthopedic devices are made from metals. Metal anions eluted from various metals are common allergic sensitizers. In Europe nickel, chromium and cobalt induce allergic skin reactions in about 20%, 4%, and 7% while in the United States14%, 4%, and 9% respectively (1).

In the very interesting published in *Acta Biomedica* (2) the authors concluded that for total knee arthroplasty nowadays, the hypersensitivity to metals has to be considred as possible preoperative risk or a postoperative cause of failure and not as a "fiction". The patient's information and the medical history, associated, in suspect cases, with laboratory testings seems to be crucial . Furthermore, in today's commercial market several knee implants are available and safe for allergic patients.

Indeed, this review delineates important issues as far as metal allergy, its diagnosis and treatment:

1. All metals implanted in the human body either in orthopedics or cardiology and elsewhere undergo some kind of corrosion. Metal ions circulate with the blood stream and come in touch with the blood proteins and in total knee arthroplasty, in partcular, may form complexes with native proteins intraarticularly acting as haptens. Haptens behave as antigens and can cause immunologic responses in the human body or in the synovial joint. The implant-related hypersensitivity is generally a type IV allergic reaction, a delayed cell-mediated response, that activates specific T lymphocytes. The prevalence of metal sensitivity in patients with well-functioning implants, mostly of the hip, seems to be high (3) and it is estimated to be approximately 25%. In patients with a failed, loose, or poorly functioning implants, the average prevalence of metal sensitivity could be as high as 60% (range,13% to 71%), but is not known whether this phenomenon is a cause or an effect (4). In a recent paper (5), concerning a patient with allergy to nickel sulphate and cobalt chloride and bone cement, zirconium and titanium alloys were used during re-operation for total knee arthroplasty. However, since these alloys are not anymore regarded as inert materials (6, 7), the patient was advised that should be followed up for any consequences for more than 3 years.

2. Detection of metal or drug allergens and corroboration of any immune response and the protein interaction can be achieve by ordering skin patch testing and measuring total IgEs. In acute systemic manifestations or relapse, serum histamine, serum tryptase and eosinophil count could be helpful. Furthermore, the followings could be of additional value for metal anion hypersensitivity where and when are appropriate:

- Lymphocyte proliferation assay that measures the ability of lymphocytes to undergo a clonal proliferation when stimulated in vitro by a foreign molecule, antigen or mitogen.
- Serum specific IgE measurements for the suspected metal anion

3. We must always remember that patients are frequently sensitized to multiple metal anions. This can occur to any practice using metallic implants either in cardiology or in orthopedics and elsewhere. Concurrent sensitization, cross-reactivity, or both seem to be possible. It has been shown that sensitization to one metal anion increases the possibility of being sensitized to additional metals. Metals seem to "join forces" to sensitize individuals (8).

Therefore, as Innocenti et al (2) have suggested, hypersensitivity to metals should considered as a possible preoperative risk or a postoperative cause of failure of total knee arthroplasty and not as a "fiction" (2). Crucial is the information of patients and the medical history, associated in suspect cases to laboratory testing.

We suggest, therefore, that taking careful and detailed previous histories of diseases and adverse drug reactions as well as hypersensitivities and to apply specific laboratory testing in susceptible individuals is of paramount importance

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"Should I stay or Should I go": patient who leave Emergency Department of an Italian Third-Level Teaching Hospital

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Summary. *Background and Aim:* Patients could leave ED not receiving the desired care either Without Being Seen by a doctor (LWBS) or Against Medical Advice (DAMA). In term of care quality, LWBS may be related to inappropriate access and process of care, while DAMA may lead to increased risk of mortality and re-admissions. This study aims to identify frequency of patients who leave ED, determine their characteristics and identify associated factor. *Methods:* This was a retrospective observational study of patients that attended EDs of University Hospital Trust of Verona in 2017. Demographic and ED access associated variables were collected for LWBS, DAMA and completed-ED-treatment patients. Univariate and multivariate data analyses was based on EMUR-PS administrative data. *Results:* 5,901 of 127,180 ED accesses were uncompleted treatment (4.64%); LWBS were 4,664 (79.04%) and DAMA 1,237 (20.96%). Those who leave ED tended to be younger (39.35 vs. 45.56, p<0.01). Independent factors associated with ED leaving resulted: i) non-urgent triage category (OR: 2.941, 95%CI: 2.405-3.596) ii) non-Italian-nationality (OR: 1.695, 95%CI: 1.493-1.924) and requiring psychiatric consult (OR:6.16 95%IC 4.82-7.87); while protective factors resulted: i) female gender (OR: 0.713, 95%CI: 0.633-0.803); i) Paediatric ED (OR: 0.593, 95%CI: 0.437-0.805); ii) Obstetrics-Gynaecology ED (OR: 0.284, 95%CI: 0.193-0.416) iii) inclusion in fast track pathways (OR: 0.747, 95%CI: 0.602-0.927). Higher ED leaving rate were observed during night-time and Sunday, either overcrowding resulted not associated.*Conclusion:* Results show the necessity to implement primary care-ED integrated pathway, mainly in frail sub-population, improve awareness on healthcare service use and refine communication skills in ED-team. (www.actabiomedica.it)

Key words: Emergency Department, patients who leave emergency department, DAMA, LWBS, quality of ED care

Background

Emergency Departments need to provide adequate acute medical care in limited time frames; patients could leave ED not receiving the desired care either Without Being Seen by a doctor (Leave Without Being Seen by a doctor, LWBS) or Against Medical Advice (Discharge Against Medical Advice, DAMA). Self-discharge occurrence represents an important marker of emergency care quality; LWBS may be related to healthcare service inappropriate access and process of care, while DAMA rate may lead to an increased risk of mortality and re-admissions.

A survey analysis conducted by using the 2009-2011 National Hospital Ambulatory Medical Care Survey (NHAMCS) in the United States have shown that 2.62% of patients left ED without completing medical care; of these visits 67.7% were LWBS

and 32.3% were DAMA patients. Increased rates of LWBS patients were associated with higher ED volumes and ED crowding; these patients identified waiting times as a major reason for leaving before medical assessment. Conversely, high rates of patients DAMA have not been shown to correlate with ED overcrowding, but were older and had higher acuity visits than the LWBS patients (1). Previous research evidenced that many of these patients, who leave before completing ED care, were vulnerable, with poor overall access to care (2).

These data underline the importance to clearly identify the reasons that lead patients to leave ED, in order to optimize the quality of acute medical care. However only limited data have been published related to Italian contest. Available evidence on this topic refer mainly to American or Australian healthcare system and it cannot be directly transferred to the National Italian healthcare system. Up to our knowledge the only Italian study on this issue is limited to LWBS patients and showed a rate of 1.34% (3).

This study aims to identify frequency of uncompleted ED admission, determine characteristics of self-discharged patients and subsequently recognise patient and organization factors associated with early ED leaving compared to completed ED care episodes.

Methods

A retrospective observational study was conducted. All admissions to EDs of University Hospital Trust of Verona during 2017 were included; accesses in General, Paediatric and Obstetrics-Gynecology Emergency Departments were considered. Admissions for non-medical purposes as work-related accident certificates (I.N.A.I.L. certificates), red triage category (life threatening conditions) and people dead on arrival at ED were excluded from the analysis.

Data were collected from the information system for monitoring assistance in Emergency-Urgency (EMUR_PS) and from ED medical chart. From EMUR_PS dataset were collected the following information: gender, age, level of consciousness upon arrival, trauma or non trauma status, triage category (1-emergent/red, 2-urgent/yellow, 3-semi-urgent/green and 4-not urgent/white), time of presentation, admission and discharge and patient's nationality. From ED medical charts were collected information regarding patient's admission in a fast track pathway (orthopedic and traumatological, ophthalmological, dermatological, ENT fast track pathways), mode of referral (own decision or referred by physician), requirement of a psychiatric consult. Patients were stratified in 9 age-groups. Moreover, the daily number of ED admissions was calculated and its percentile distribution was used to classify ED crowding in five classes (from 1-very low affluence to 5-very high affluence).

Data Analysis

All analyses were performed using ED Admission as unit of analysis. Patients who left ED at their own decision were classified by EMUR_PS "outcome status" variable into LWBS and DAMA; they were compared to admission of those who completed ED-treatment with respect to all collected variables.

Categorical data were compared using the Chi-squared test while T-test or Mann-Whitney test were performed for continuous variables. Logistic univariate analyses were performed to identify variables affecting decision to leave ED before completing all requested care; significant associated variables were included in the multiple logistic regression model to calculate adjusted Odds Ratio. A level of significant of 0.05 was considered for all the performed analyses. All analyses were performed using STATA version 15.

Results

A total of 133,418 episodes of care accesses to the AOUI Verona Emergency Departments occurred during 2017, involving 90,809 individual patients. Among these admissions, 4,345 were non-medical purposes referred accesses (issuing I.N.A.I.L. certificates) and 1,893 were red triage category; they were excluded leaving a final dataset of 127,180 ED visits among 89,595 patients. 121,279 episodes of care completed ED treatment while 5,901 (4.64%) resulted uncompleted; LWBS were 4,664 (79.04%) and DAMA were 1,237 (20.96%).

Table 1 shows ED Access general characteristics for completed and uncompleted ED treatment. Those who leave ED before or against medical advice compared to those who completed ED care tended to be younger (mean age of 39.35 vs. 45.56, p<0.01). Among patient who leave ED higher frequencies were observed in the 35 to 44 years old (16.94%) and in the 55 to 64 years old age groups (16.04%). Those who leave ED tended to have an higher male proportion (54.65% vs. 47.11%, p<0.01; OR: 0.739, 95%CI: 0.701-0.778), a higher prevalence of not Italian citizens (26.44% vs. 19.23%, p<0.01; OR: 1.509, 95%CI: 1.422-1.602) and higher rate of patient referred by own decision (90.88% vs. 80.10%, p<0.01; OR: 2.475, 95%CI: 2.263-2.708).

Among ED visits characteristics, self discharged patients showed an higher prevalence of white triage category (52.11% vs 24.43%, p<0.01; OR: 17.576; 95%CI: 15.209-20.313), an higher requirement of a psychiatric consult (6.31% vs. 1.09%, p<0.01; OR: 6.116, 95%CI: 4.832-7.740) and an higher proportion of traumatic complaints (26.40% vs. 22.30%, p<0.01; OR: 0.800, 95%CI: 0.754-0.849). Self-discharged patients attended more frequently to General ED (92.17% vs. 77.74%, p<0.01), out of a fast track pathway (1.95% vs. 9.28%, p<0.01; OR:0.183, 95%CI: 0.152-0.222), during evening time - from 6.00 pm to 11.59 pm (44.28% vs. 24.69%, p<0.01; OR: 1.820, 95%CI: 1.650-2.007), with an higher proportion on Sunday and Monday (respectively 15.73% vs. 13.56%, p<0.01 and 17.88 vs. 15.40%, p<0.01; OR: 1.001, 95%CI: 0.914-1.096) and during spring and winter (respectively 29.57% vs. 25.78% and 27.37% vs. 24.83 % p<0.01). In January and April the highest self discharge frequency (10.34% and 11.15% respectively) was observed. Finally regarding "daily crowding", an increasing leaving risk trend was identified as increased the ED affluence (Table 1).

Once adjusted, according to Multivariate regression model, significant associated variables with the ED leaving resulted: lower age group, male patient, lack of inclusion in fast track pathway, General ED access, Sunday as day of presentation, triaged as non urgent or semi urgent, necessity of psychiatric consult, foreign origin and night and evening as time of presentation. Table 2 shows the results of multivariate analysis.

Discussion

Nowadays patient's dissatisfaction in the provided care is becoming a major concern for healthcare systems, that is particularly evident in service access. Whenever waiting time becomes too long in relation to perceived need to medical intervention, the Emergency department becomes the only citizen's opportunity to get immediate service's access; as a consequence, ED becomes overcrowded by low acuity pathologies (4). Reducing ED leaving occurrence represents a major goal of healthcare systems as regards to ED process quality and safety of care; identifying factor associated to higher rate of ED leaving is essential to implement targeted intervention.

Among patients who leave ED before completing care about eighty percent were LWBS, demonstrating a higher rate compared to previous studies; the reason could be found in different healthcare systems and the relative distinct patient payment participation (2).

Multivariate analysis evidences the highest risk of ED leaving among paediatric age group, up to more than three times higher than elderly; on the other hand, Paediatric ED access resulted a significant protective factor. This apparently contrasting result may be related to improper use of general rather than paediatric ED. Such result underlines the importance to realize dedicated pathway for paediatric subpopulation; moreover, awareness of the factors involved in ED service parent's perception should facilitate successful interventions for this potentially vulnerable group (5).

Patients requiring psychiatric consult, as previously evidenced by other studies, showed higher risk of self-discharge; this result refers only to DAMA patients since in LWBS patients it can't be estimated. According to literature, ED leaving among psychiatric patients is not ascribable only to patient's but also to provider's factors (6,7). The need to develop adequate communicative and relational skills is particularly important for this specific subgroup. Psychiatric patients represent a frail chronic population and an adequate management of acute state may be essential to prevent relapses; a sub-analysis on this population could be helpful to improve ED care with dedicated pathway.

Like other studies we found higher risk of LWBS and DAMA in male population and in foreign origin

Table 1. Characteristics of uncompleted and completed ED accesses

Characteristics	Uncompleted ED treatment	Completed ED treatment	Unadjusted Odds Ratio	p value	OR 95% CI
Age (yr.)	39,35 (±26.65)	45,56 (±21.31)			
Age Group (yr.)					
0-4	573 (9.73)	15,626 (12.89)	2.290	0.000	2.003-2.619
5-14	211 (3.58)	3,867 (3.19)	3.408	0.000	2.865-4.055
15-24	830 (14.09)	10,147 (8.37)	5.110	0.000	4.503-5.799
25-34	942 (15.99)	16,059 (13.24)	3.664	0.000	3.238-4.147
35-44	998 (16.94)	15,502 (12.78)	4.022	0.000	3.557-4.547
45-54	945 (16.04)	14,26 (11.76)	4.140	0.000	3.658-4.685
55-64	656 (11.14)	11,645 (9.60)	3.519	0.000	3.087-4.012
65-74	381 (6.47)	11,975 (9.88)	1.987	0.000	1.717-2.300
>74	355 (6.03)	22,179 (18.29)	1		
Gender					
Male	3,225 (54.65)	57,134 (47.11)	1		
Female	2,676 (45.35)	64,145 (52.89)	0.739	0.000	0.701-0.778
Fast Track Pathway					
Absent	5,792 (98.15)	110,025 (90.72)	1		
Present	109 (1.85)	11,254 (9.28)	0.183	0.000	0.152-0.222
Emergency Department					
General	5,439 (92.17)	94,285 (77.74)	1		
Paediatrics	402 (6.81)	15,312 (12.63)	0.455	0.000	0.410-0.504
Obstetrics-Gynaecology	60 (1.02)	11,682 (9.63)	0.089	0.000	0.068-0.114
Day of Presentation					
Sunday	928 (15.73)	16,442 (13.56)	1		
Monday	1,055 (17.88)	18,671 (15.40)	1.001	0.977	0.914-1.096
Tuesday	835 (14.15)	17,283 (14.25)	0.856	0.002	0.777-0.942
Wednesday	734 (12.44)	17,158 (14.15)	0.758	0.000	0.686-0.837
Thursday	735 (12.46)	17,331 (14.29)	0.751	0.000	0.680-0.829
Friday	766 (12.98)	17,479 (14.41)	0.776	0.000	0.704-0.856
Saturday	848 (14.37)	16,915 (13.95)	0.888	0.015	0.807-0.977
Month					
January	610 (10.34)	10,36 (8.54)	1		
February	539 (9.13)	9,398 (7.75)	0.974	0.666	0.864-0.109
March	504 (8.54)	10,554 (8.70)	0.811	0.001	0.718-0.915
April	658 (11.15)	9,748 (8.04)	11464	0.018	1.023-1.284
May	532 (9.02)	10,45 (8.62)	0.864	0.017	0.767-0.974
June	508 (8.61)	10,393 (8.57)	0.830	0.003	0.735-0.936
July	455 (7.71)	10,177 (8.39)	0.759	0.000	0.670-0.859
August	374 (6.34)	10,07 (8.30)	0.630	0.000	0.552-0.719
September	432 (7.32)	9,526 (7.85)	0.770	0.000	0.678-0.873
October	552 (9.35)	10,111 (8.34)	0.927	0.211	0.823-1.043
November	365 (6.19)	9,846 (8.12)	0.629	0.000	0.551-0.718
December	372 (6.30)	10,646 (8.78)	0.593	0.000	0.520-0.677
Season					
Winter	1,615 (27.37)	30,117 (24.83)	1		
Spring	1,745 (29.57)	31,269 (25.78)	1.040	0.261	0.970-1.115
Summer	1,277 (21.64)	30,635 (25.26)	0.777	0.000	0.721-0.837
Fall	1,264 (21.42)	29,258 (24.12)	0.805	0.000	0.747-0.868

(continued)

Table 1 ***(continued)*****.** Characteristics of uncompleted and completed ED accesses

Characteristics	Uncompleted ED treatment	Completed ED treatment	Unadjusted Odds Ratio	p value	OR 95% CI
Daily Crowding					
Very Low Affluence	1,007 (17.06)	24,328 (20.06)	1		
Low Affluence	967 (16.39)	22,449 (18.51)	1.040	0.386	.951-1.138
Medium Affluence	1,254 (21.25)	24,898 (20.53)	1.216	0.000	1.117-1.324
High Affluence	1,168 (19.79)	22,718 (18.73)	1.242	0.000	1.139-1.353
Very High Affluence	1,505 (25.50)	26,886 (22.17)	1.352	0.000	1.246-1.467
Triage Category					
2-Urgent/Yellow	198 (3.42)	31,55 (26.45)	1		
3-Semi-Urgent/Green	2,576 (44.48)	60,39 (50.62)	6.796	0.000	5.878-7.858
4-Not Urgent/White	3,018 (52.11)	27,36 (22.93)	17.576	0.000	15.209-20.313
Psychiatric Consult					
Absent	1,159 (93.69)	119,959 (98.91)	1		
Present	78 (6.31)	1,32 (1.09)	6.116	0.000	4.832-7.740
Mode of Referral					
Referred by physician	538 (9.12)	24,129 (19.90)	1		
Own decision	5,363 (90.88)	97,15 (80.10)	2.475	0.000	2.263-2.708
Nationality					
Italian	4,34 (73.56)	97,951 (80.77)	1		
Foreign	1,56 (26.44)	23,325 (19.23)	1.509	0.000	1.422-1.602
Continent for Foreigner					
Europe	731 (48.47)	10,796 (46.44)	1		
Africa	391 (25.93)	5,961 (25.64)	0.968	0.623	0.853-1.099
Asia	290 (19.23)	5,106 (21.97)	0.838	0.014	0.729-0.964
America	91 (6.03)	1,342 (5.77)	1.001	0.990	0.799-1.254
Oceania	5 (0.33)	41 (0.18)	1.801	0.216	0.709-4.571
Type of Complaint					
Trauma	1,558 (26.40)	27,05 (22.30)	1		
No Trauma	4,343 (73.60)	94,229 (77.70)	0.800	0.000	0.754-0.849
Time of Presentation					
Night 00.00-05.59	506 (8.57)	10,554 (8.70)	1		
Morning 6.00-11.59	952 (16.13)	39,391 (32.48)	0.504	0.000	0.451-0.562
Afternoon 12.00-17.59	1,83 (31.01)	41,393 (34.13)	0.922	0.115	0.833-1.019
Evening 18.00-23.59	2,613 (44.28)	29,941 (24.69)	1.820	0.000	1.650-2.007

ED: Emergency Department, yr.: years

patient's (3, 7, 10). Non-Italian citizens are at higher risk to have a limited access to primary care assistance, consequently they tend to overuse ED service for low acuity situations (8).

Finally, among patient related factors, non-urgent triage category resulted at higher risk of uncompleted ED care, as confirmed by literature.

Considering service's related factors, ED type and fast track pathway resulted strongly associated to ED leaving; specifically, Paediatric and Obstetric-Gynaecologic ED demonstrated lower leaving risk as the patient inclusion in a fast track pathway. Such result underlines how the development of a patient centre emergency organization have generalised positive impact on the quality of care and specifically may reduce LWBS and DAMA rate (9).

The highest rate of uncompleted care was observed on Sunday and during evening/night time; at

Table 2. Multivariate analysis of completed and uncompleted ED accesses

Characteristics	Adjusted Odds Ratio	95% CI
Age Group (yr.)		
0-4	3.679	2.535-5.339
5-14	3.423	2.299-5.096
15-24	3.058	2.267-4.125
25-34	2.445	1.813-3.297
35-44	3.282	2.475-4.352
45-54	3.036	2.292-4.021
55-64	2.031	1.485-2.778
65-74	1.570	1.120-2.201
>74	1	
Gender		
Male	1	
Female	0.713	0.633-0.803
Fast Track Pathway		
Absent	1	
Present	0.747	0.602-0.927
Emergency Department		
General	1	
Paediatrics	0.593	0.437-0.805
Obstetrics-Gynaecology	0.284	0.193-0.416
Day of Presentation		
Sunday	1	
Monday	0.749	0.589-0.951
Tuesday	0.798	0.643-0.990
Wednesday	0.759	0.611-0.942
Thursday	0.852	0.690-1.052
Friday	0.744	0.597-0.926
Saturday	0.805	0.653-0.993
Month		
January	1	
February	0.822	0.632-1.070
March	0.755	0.561-1.016
April	1.175	0.752-1.837
May	1.102	0.707-1.720
June	1.152	0.746-1.781
July	0.914	0.551-1.517
August	0.830	0.496-1.389
September	0.763	0.476-1.222
October	0.815	0.512-1.298
November	0.824	0.517-1.311
December	0.731	0.520-1.029
Season		
Winter	1	
Spring	0.840	0.581-1.214
Summer	0.729	0.473-1.124
Fall	0.763	0.524-1.111
Daily Crowding		
Very Low Affluence	1	
Low Affluence	1.060	0.871-1.290
Medium Affluence	1.056	0.870-1.282
High Affluence	1.014	0.826-1.246
Very High Affluence	1.163	0.938-1.442
Triage Category		
2-Urgent/Yellow	1	
3-Semi-Urgent/Green	1.906	1.586-2.289
4-Not Urgent/White	2.941	2.405-3.596
Psychiatric Consult		
Absent	1	
Present	6.161	4.821-7.873
Mode of Referral		
Referred by physician	1	
Own decision	1.004	0.845-1.194
Nationality		
Italian	1	
Foreign	1.695	1.493-1.924
Type of Complaint		
Trauma	1	
No Trauma	1.109	0.957-1.285
Time of Presentation		
Night 00.00-05.59	1	
Morning 6.00-11.59	0.673	0.547-0.827
Afternoon 12.00-17.59	0.771	0.632-0.942
Evening 18.00-23.59	0.867	0.707-1.063

ED: Emergency Department, yr.: years

these time span patient access to primary care is limited to continuity of care services as doctor on duty. This raise questions on whether territorial services are actually perceived by patients as a valid alternative to ED for low acuity care; this result confirms the need to strengthen primary care services in order to guarantee appropriate care use.

In our study the multivariate analysis did not confirm the correlation between uncompleted care and ED overcrowding observed in univariate analysis. This result seems to be in contrast with other observational studies which found a significant correlation with waiting time (2, 3).

This study suffers from some limitations. First of all, it took place in a single third level hospital trust;

this might limit results generalization, since larger hospitals tend to have higher proportion of LWBS (10). Secondly, data were mostly collected from administrative EMUR_PS dataset even if when available they were supplemented with clinical charts information. Differently from DAMA patients, it was possible to recognize only a few clinical information on LWBS patient; this impeded to perform sub-analysis including all collected variables with limited possibilities to plan specific interventional strategies as already underlined by other observational studies (10). Despite these limitation, the study was able to provide an overall view of risk factors associated to ED leaving in a universalistic healthcare system; results have shown the necessity to implement primary-ED integrated pathway care, to develop patient's awareness on appropriate use of Emergency Care, and finally to improve communication strategies for HCP and specific non-technical skills for ED team. In conclusion these interventions need to be oriented to frail sub-population such as foreign origin, paediatric and psychiatric patients according to our results to be at higher risk of uncompleted ED treatment.

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Rotavirus gastroenteritis hospitalization rates and correlation with rotavirus vaccination coverage in Sicily

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Summary. *Background and aim of the works:* Rotavirus (RV) is considered the main cause of gastroenteritis in children from 0 to 59 months and vaccination represents the only strategy to prevent hospitalizations due to RV. In 2013 Sicilian Region introduced universal RV vaccination for all newborns. The present study aims to estimate the reduction rotavirus gastroenteritis (RVGE) hospitalization rates among Sicilian children and their relations with vaccination coverages of the nine Sicilian Local Health Units (LHUs). *Methods:* Were analyzed hospital discharge records including a diagnosis of RVGE occurred from January 2009 to December 2017 in hospitalized children aged 0 to 59 months, residents in Sicily. Were reported data on complete RV vaccination cycles among Sicilian children under 12 months of age (vaccination coverage). *Results:* A 49.2% overall reduction of RVGE hospitalization rates was reported after RV vaccination introduction. A more considerable reduction of hospitalization rates was observed among children aged 0 to 11 months (-61.4%), followed by children aged 12-23 months (-51.2%) and 24-35 months (-48.8%). In all the nine Sicilian Local Health Units (LHUs), a reduction of RVGE hospitalization rates was observed after RV vaccine implementation. *Conclusions:* This study demonstrated the significant impact of RV vaccination on RVGE hospitalization rates in Sicily, especially among children aged 0 to 23 months. The reduction in RVGE hospitalization rates observed in the Sicilian LHUs after universal vaccination program implementation, were generally higher or consistent with average vaccination coverage reported from 2013 to 2017. (www.actabiomedica.it)

Key words: rotavirus, gastroenteritis, rotavirus vaccination, hospital discharge records, hospitalizations rate, local health units, vaccination coverage

Introduction

Worldwide, rotavirus (RV) is the leading cause of childhood gastroenteritis and nosocomial infection in Paediatric units among children under five years (1). In industrialized countries morbidity and health costs associated with RV infection are considerable, while in developing countries, rotavirus gastroenteritis (RVGE) represents an health emergency, with 600,000 children killed every year by dehydration (1).

In Europe, before the introduction of the vaccines against RV, this viral infection was responsible annually for about 3.6 million cases of gastroenteritis among children 0-59 months, including 87,000 hospital admissions and about 700,000 medical consultations (2).

The availability of vaccines has greatly modified the incidence and the economic burden of RV infections worldwide (3).

Anti-RV vaccination actually represents the most effective strategy for reducing RVGE among children

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and the introduction of RV vaccines in immunization schedule is strongly recommended by international health authorities (4, 5).

Since 2006, several countries adopted universal RV vaccination in their immunization schedules, reporting high vaccine effectiveness in reducing RVGE hospitalizations and outpatient visits (6-8).

In Sicily, the Regional Health Department introduced the universal rotavirus vaccination program into the immunization schedule in January 2013, as the first Region in Italy (9). Right after the vaccine implementation, a decrease in the number of hospital admissions for RVGE was observed in Sicily among children aged 0-59 months (10).

Universal RV vaccination demonstrated a substantial cost reduction for the Regional Health System, but also a decreasing trend in the mean age of hospitalized children and a smaller peak of RVGE hospital admissions observed in late winter and early spring (11).

The present work aimed to assess the impact of vaccination coverage achieved in Sicily, on RVGE hospitalization rates among 0-59 months children, after a five-years period of RV universal vaccination programme, and to evaluate the reduction according to different age-groups and Provinces.

Materials and Methods

Data collection

A retrospective observational study on Hospital Discharge Records (HDRs) of Sicily, the fourth most populous region in Italy with 5 millions inhabitants, including a cohort of 45,000-50,000 newborns per year, was conducted (12).

The Sicilian Region is divided into 9 Provinces (Agrigento, Caltanissetta, Catania, Enna, Messina, Palermo, Ragusa, Siracusa and Trapani). Each Province corresponds to a Local Health Units (LHUs), health organisations responsible for inpatient and outpatient medical care of all residents.

The Sicilian HDR database was established in 1994, including the complete data of patient hospitalized from both public and private regional hospital. Each HDR integrated demographic information (birthplace, residence, gender, and date of birth), admission and discharge dates, discharge status (categorized as "discharged/transferred" or "expired"), and up to six discharge diagnoses (one principal and five secondary diagnoses) coded according to International Classification of Disease, Ninth Revision, Clinical Modification (ICD-9-CM).

All HDRs included into the regional database with an ICD-9-CM diagnosis code of 008.61 as the first or other diagnosis position, corresponding to a diagnosis of "rotavirus gastroenteritis" occurred in children aged 0-59 months, from the 1st of January 2009 to the 31st of December 2017, were analysed.

Duplicate episodes of RVGE hospitalizations were considered unique if they occurred within 14 days between episodes, otherwise only the first episode was considered.

Statistical analysis

RVGE hospitalization rates observed in the pre-vaccination period (2009-2012) were compared with RVGE hospitalization rates of the post-vaccination period (2013-2017).

Vaccine coverages rates reported in the study correspond to the number of complete RV vaccination cycles per year on resident children younger than 12 months of age (birth cohort) and were obtained from the regional vaccination database, which is edited yearly according to the Italian Health Department recommendations.

The data of vaccination coverage reported for each Sicilian LHUs were intended as the average adherence data to RV vaccination in the first five years of vaccine implementation (from 1st January 2013 to 31st December 2017).

Quantitative variables (RVGE hospitalization rates, age class distribution) were evaluated during the pre (2009-2012) and post (2013-2017) vaccination periods and the corresponding percentage reductions were reported.

Hospitalization rates per 100,000 were calculated using the census population for children aged 0 to 59 months from 2009 to 2017 (12).

All statistical analyses were performed using the STATA v14.2 software package.

Results

RVGE hospitalization rates in Sicily, before (2009-2012) and after (2013-2017) the introduction of RV vaccination, are reported in Figure 1.

In particular, after the introduction of universal vaccination program, a decline in RVGE hospitalization rates among children aged 0 to 59 months was observed, decreasing from 394 per 100,000 in 2009-2012 to 200 per 100,000 in 2013-2017 (49.2% reduction overall).

In Figure 2, RVGE hospitalization rates documented in different age-groups, before and after RV vaccine introduction, were reported.

From 2013 to 2017, RVGE hospital admissions rates strongly decreased particularly among children aged 0-11 months (from 526 per 100,000 to 203 per 100,000; -61.4%).

A substantial reduction in RVGE hospitalizations rates was also observed among children between 11 and 23 months of age (from 657 to 321 per 100,000; -51.2%), followed by the age-groups 24-35 months (-49%), 36-47 months (-25.4%) and 48-59 months (-24%).

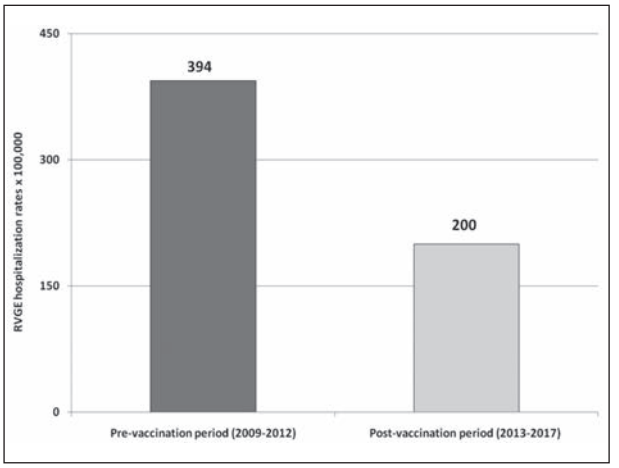

Figure 1. Average RVGE hospitalization rates (x 100,000) observed in Sicily among 0-59 months children before (2009-2012) and after (2013-2017) the introduction of RV vaccination

In Table 1, RVGE hospitalization rates observed in the 9 Sicilian LHUs before (from 2009 to 2012)

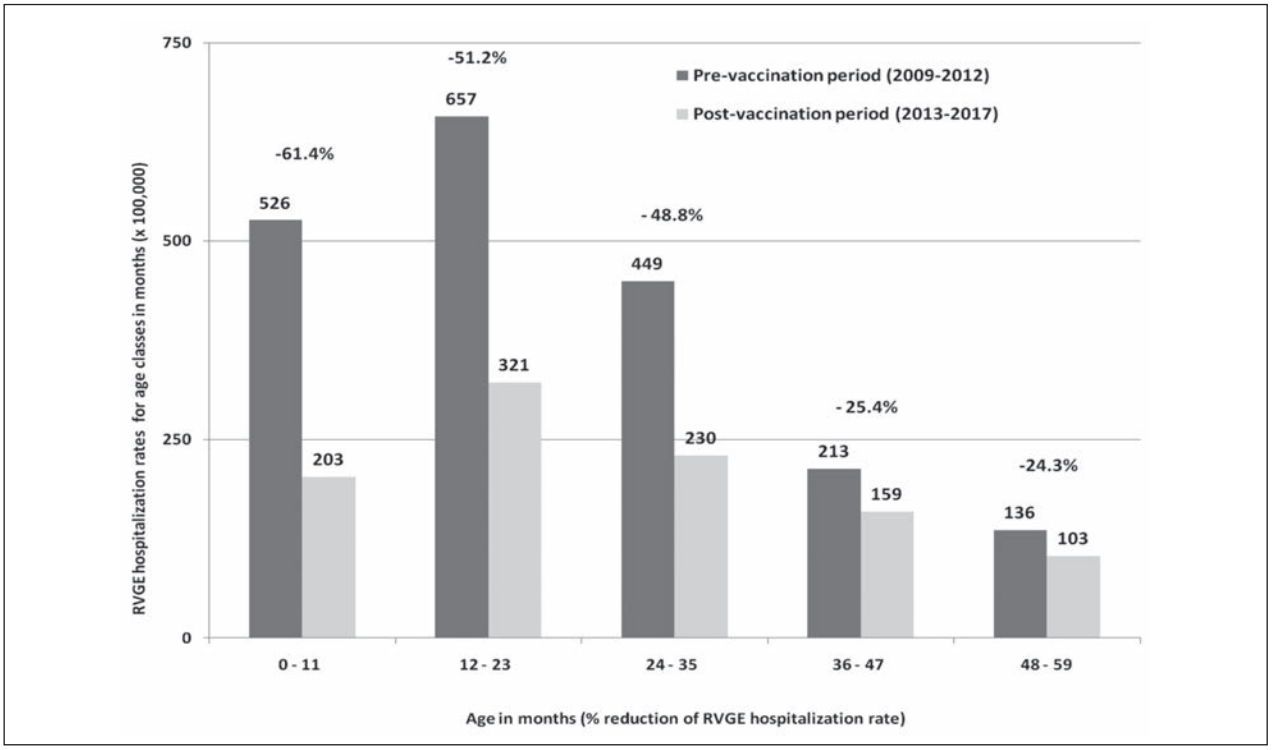

Figure 2. Average RVGE hospitalization rates (x 100,000) for age groups among 0-59 months aged children in Sicily, before (2009-2012) and after (2013-2017) the introduction of universal RV vaccination

Table 1. Average RVGE hospitalization rate observed in the nine Local Health Units (LHUs) of the Sicilian Region during pre (from 2009 to 2012) and post (from 2013 to 2017) vaccination period, and average RV vaccination coverage reported from 2013 to 2017

LHU	Prevaccination period (2009-2012) Average hospitalization rate	Postvaccination period (2013-2017) Average hospitalization rate	Difference (%)	Average vaccination coverage (2013-2017) (%)
Overall	394	200	-49.2	38.2
Agrigento	238	106	-55.5	48.1
Caltanissetta	239	194	-18.8	42.7
Catania	328	136	-59.5	30.6
Enna	461	226	-51	27.4
Messina	115	97	-15.7	19.1
Palermo	617	311	-46.6	51.5
Ragusa	451	243	-46.1	31.1
Siracusa	611	331	-45.8	34.1
Trapani	239	104	-56.5	58.6

and after (from 2013 to 2017) RV vaccination implementation and the average RV vaccination coverages, were reported.

Overall, the average RV immunization coverages from 2013 to 2017 among Sicilian children was 38.2% (range: 19.1%-58.6%; for Messina and Trapani, respectively).

A reduction in RVGE hospitalization rates in children aged between 0 and 59 months was observed in all Sicilian LHUs (range: 15.7%-59.5%; for Messina and Catania, respectively).

Discussion

Rotavirus is the main responsible of moderate/severe acute viral gastroenteritis in neonatal and paediatric age. These findings were reported in several studies that demonstrated a RV aetiology in over 50% of children hospitalized for diarrhoea (13, 14).

From 2003 to 2012, in Sicily, RV was responsible for at least 950 hospital admissions per year in children aged from 0 to 59 months, with an average hospitalization rate higher than 370 per 100,000 (15).

All European countries that introduced RV vaccination as part of the routine childhood immunization schedule, reported a significant reduction of RVGE burden in hospital wards, emergency rooms and outpatient admissions (16).

However, the majority of countries where a significant reduction of RVGE hospitalizations was observed, achieved coverage rates ranging from 60% to 85%, as early as the first year of vaccine implementation (17-19).

Conversely, even tough Sicily was the first Italian Region that introduced universal RV vaccination for all newborns in January 2013, average vaccination coverage was lower than 40% after 5 years of active and free offer (9, 20).

Moreover, among different Sicilian LHUs inequalities in the vaccination offer and uptake emerged. In particular, RV vaccination coverages in the Western Sicily Provinces (Trapani: 58.6%; Palermo: 51.5% and Agrigento: 48.1%) were considerable higher than in the Eastern Provinces such as Messina (19.1%), Catania (30.6%), Ragusa (31.1%) and Siracusa (34.1%).

Unfortunately, confidence of some Sicilian paediatricians and healthcare workers to RV vaccination was erroneously conditioned by the withdrawal of a previous version of RV vaccine administered until 1999, which was suspected to be responsible for a possible association with intussusception among vaccinated children (21). Assumption that was recently retabled in Sicily by some authors and promptly rejected by Sicilian Public Health Authorities (22).

Nevertheless, in all Regional LHUs, a substantial decrease of RVGE hospitalization rates in post-

vaccination period (2013-2017) (-49.2% overall) was observed.

The greater hospitalization rates reductions were found especially among children aged between 0-11 and 12-23 months (-61.4% and-51.2% respectively), that represented the age groups at higher risk for serious RVGE clinical presentations, often requiring hospitalization (23, 24).

RVGE hospital admission rates in the LHUs of Messina, Palermo, Trapani showed consistent reductions in post-vaccination period (2013-2017), with the correspondent average vaccination coverage rates.

In the LHUs of Agrigento, Ragusa and Siracusa, hospitalization reductions observed (between 10% and 20% higher than average vaccine coverage observed) could be attributable to the herd effect of RV vaccination, that protected not only vaccinated children from infections, but could also lead to an overall reduction of seasonal circulation of the pathogen (25,26).

On the other hand, the decrease in RVGE hospitalization rate observed in the LHUs of Catania and Enna (-59.5% and -51% respectively), much higher than vaccination coverage (30% and 27.4% respectively), and the small reduction of RVGE hospital admission of Caltanissetta (18.8%) compared with vaccination coverage (42.7%), could be influenced by annual changes in rotavirus circulation, often associated to different factors apart from vaccination (27).

Conclusions

In Sicily, RV universal vaccination implementation resulted in a considerable reduction in RVGE hospitalization rates during the period 2013-2017 (-49.2%).

The impact of vaccination in reducing the burden of hospitalizations for RVGE, especially among children aged 0-23 months and in all the 9 Sicilian LHUs were encouraging.

Currently, the most important challenge for Sicilian Public Health Authorities will be the improvement of both knowledge and attitudes of health care workers on RV vaccination, that actually did not recommend the immunization to newborns.

Only a continuous increase of vaccination coverages over next years could lead to further reduction of RVGE hospital admission rates, overcrowding of paediatric departments during RV epidemic seasons and RVGE economic burden on Regional Health System (11).

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GlaxoSmithKline Biologicals SA was given the opportunity to review a preliminary version of this manuscript for factual accuracy but the authors are solely responsible for final content and interpretation.
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Prevalence of the bullying phenomenon in a schools sample of Palermo, Sicily: a pre-post intervention observational study among teachers

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Summary. *Background and aim of the work:* Bullying involves a significant percentage of school-age children. According to the latest available surveillance data, in Sicily, the estimated prevalence among 11-15 years old children is 14%. This study aimed to estimate a prevalence of the bullying phenomenon, observed by teachers, in a sample of secondary schools of Palermo, Sicily. Moreover, after the conduction of preventive interventions among teachers, aimed to evaluate any modification in bullying prevalence. *Methods:* A cluster sampling selection according to socio-economic level of the school neighborhood was carried out. Two anonymous online questionnaires, pre and post-intervention, were administered to the 63 teaching staff, belonging to second and third year classes of ten secondary schools enrolled. Preventive interventions were conducted among teachers by experienced researchers. *Results:* Prevalence of bullying reported decreased significantly from 44.4% to 19.0% (p-value 0.001), comparing pre and post-intervention questionnaires. A reduction in the prevalence of verbal and physical bullying and a concomitant slight increase of indirect bullying were also observed. All the characteristics, reported by the teaching staff, for describing bullies, victims and observers of bullying have been categorized under three different content domains (affective-relational discomfort, socio-cultural context, and character/natural disposition). *Conclusions:* The present study estimated the prevalence and the characteristics of bullying phenomenon in a sample of secondary schools of Palermo, evaluating the reduction of bullying episodes among students, after a preventive interventions conducted among teaching staff. Data obtained confirmed the effectiveness of this approach and suggested an extension of the project at Regional Level.

Key words: bullying prevalence, secondary schools, teachers, socio-economic level

Introduction

Bullying is one of the most relevant social and health problem for school-age children and adolescents all over the world (1, 2).

In recent decades, the bullying phenomenon has gained increasing interest in public health, catalyzing many efforts in research, prevention and action frameworks (3-9).

Bullying can be defined as a systematic abuse of power that manifests itself with intimidation - forms of physical, verbal or psychological persecution - repeated over time, conceived and acted with the intention to cause fear, anguish or damage to the vic-

tim, perpetuated by a person or by a group of people, stronger within an unbalanced relationship with the victim (10).

Intentionality, duration over time and asymmetry in the relationship are the three main peculiarities of bullying. Moreover, victims of bullying are often selected for their condition of diversity and/or fragility.

In Italy, bullying involves a significant percentage of school-age children: 2 in 10 kids between 11-17 years reported to have been bullied two or more times in a month, with a higher prevalence among girls (11).

In Sicily, the first Italian region by territorial extension and the fourth by resident population, the estimated prevalence of children aged 11 to 15, who claims to have undergone at least one act of bullying in the last two months, was of 14% in 2011 (12).

According to literature, teachers demonstrate ignorance regarding some aspects, such as the lack of a clear motivation for the attacks, their repetition, and the fact that most of the time they happen far from the adults. Therefore, it is evident that teachers are aware of bullying, but incompletely, making it difficult to identify it in the classroom and differentiate it from other recurrent behavior in the school environment, such as jokes and lack of discipline (13-15).

The "Bullying In Sicilian Schools" (BIAS) study was designed with the aim to estimate the prevalence of the different form of bullying observed and perceived by teachers, at the beginning of the school year, in a representative sample of secondary schools of Palermo, the most populated city of Sicily, and at the end of the same school year, after the implementation of structured and targeted bullying prevention interventions addressed to teachers (16).

Material and methods

A pre-post intervention observational study involving 63 teaching staff members, belonging to second and third years classes of secondary schools of the city of Palermo, was conducted. Ten schools were enrolled in the study after a cluster sampling selection by neighborhood socio-economic index. To this purpose, schools were categorized in high (A), medium (B) and low (C) (16).

Two previously validated anonymous online questionnaires were administered pre and post intervention, respectively, to the teaching staff (16).

Operating procedures

In October 2017, during a dedicated meeting conducted by the BIAS's working group in collaboration with the Regional Bullying Observatory of Sicily (Italy), the project was presented to the bullying referent teachers of each school enrolled. The contents of the questionnaire and study timeline were illustrated in depth.

Afterwards, in November 2017, during the Class Council of each class recruited in the study, an online pre-intervention questionnaire was administered to the teachers' bullying referent, following a collegial consultation (16). Further, from January to May 2018, informative and formative interventions was dedicated to the enrolled teachers.

Finally, in June 2018, a post-intervention questionnaire was administered with the same proceeding of the pre-interventional one, with the aim to detect any changes in bullying prevalence.

Pre-intervention questionnaire

The pre-intervention questionnaire started with a preliminary question investigating whether bullying episodes, identified according to the World Health Organization definition, had occurred among students in the previous two months of school activities (September and October 2017) (10). If the answer was affirmative, the class council referent was in charge to complete the remaining part of the questionnaire.

The pre-intervention questionnaire was structured in 10 items, with multiple or open answers, aimed to investigate (16):

(1) the type of bullying mainly reported (verbal, physical or indirect);
(2) the places where bullying occurred;
(3) the number of students involved;
(4) bullying episodes reported by the victims or by other classmates to teachers, school chief or parents;
(5) the action taken to face or prevent bullying at school;

(6) the potential support provided by school staff to victims of bullying;
(7) the perceived need of specific interventions to prevent bullying;
(8) the type of intervention suggested by the teachers;
(9) the three main characteristics of bullies, victims or observers.

All the characteristics reported by the teaching staff to describe bullies, victims and observers of bullying have been categorized under three different content domains: affective-relational discomfort, socio-cultural context, and character/natural disposition. A qualitative analysis of such characteristics was then carried out.

Intervention

Five working groups (WGs) were defined to realize the intervention in the ten enrolled schools.

Each WG organized the intervention in two schools and was composed by at least three members of the BIAS project: a) a member of the Regional Scholastic Office, in charge of connecting the working group and the teachers; b) one or two Local Health Unit representatives with proven experience in the management of adolescent psychological problems and, particularly, bullying; c) one or two medical doctors from the University of Palermo, with an expertise in Public Health and Preventive Medicine.

The intervention was conceived to improve the teachers' ability to identify bullying episodes following the hypothesis to determine a positive cascade effect on the whole school community, starting from students' behavior. Thus, the targets of the intervention were all the 63 teachers of the classes enrolled from the sampled schools.

The intervention was implemented within four meetings lasting 3 hours each.

During the first meeting, data of the pre-intervention questionnaire were presented and discussed in a plenary session. Then, teachers were involved in conceiving and realizing effective activities oriented to increase awareness of bullying and in promoting preventive actions in the school context.

So, during the following 3 meetings, participatory approaches for planning (i.g. word café, role playing, goal oriented project planning) were implemented to structure an activity to be proposed by each WG.

As an expected outcome, during the intervention timeframe, part of the teachers voluntarily organized with their students initiatives, such as cooperative learning, peer education and role playing to address and prevent the bullying at school.

Post-intervention questionnaire

The post -intervention questionnaire included 10 multiple-choice or open-ended questions.

First of all, teachers were asked to indicate, if there had been any bullying in their class during the previous six months (January to May 2018). Again, questions about type of bullying, reported by the victims or other classmates were asked.

Modification in the frequency of bullying episodes were investigated as compared to pre-intervention period. Furthermore, an evaluation of the interventions implemented, with a specific concern on its methodology and the prevention strategies to be proposed for the next school years, was performed.

Finally, in July 2018, all the teachers and working group members plenary discussed and shared the initiatives carried out by each group, the results of the post-intervention questionnaire and the future perspectives of the study.

Data collection and analysis

The questionnaires were both self-administered throughout the use of a dedicated online form developed with the Google Forms platform. A McNemar test was performed to evaluate any prevalence modification between pre-and post-intervention.

All categorical variables were reported as absolute and relative frequencies (percentages). Chi-square test (with the Fisher's correction when appropriate) was used to compare categorical variables.

For the open-ended questions, a qualitative analysis was performed using a content analysis approach. The text strings were systematically read, coded and all the emerging contents were then categorized and classified by a medical doctor with expertise in adolescent mental health.

All the informatics supports (computers, servers, memories and portable disks, etc.) dedicated to the collection, conservation (even temporary) and the processing of data, have been provided with adequate security and protection mechanisms to prevent access to data by unauthorized persons.

The dataset, after being checked and cleaned of any errors or duplicates, was imported to EpiInfo ver.3.5.1 for statistical analysis.

The present study obtained the approval of the Palermo Ethical Committee 1, in the session of the 12th of July 2017 (protocol number: 07/2017) (16).

Results

Pre-post intervention questionnaires analysis

The prevalence of bullying episodes reported by teachers in the 63 classes decreased significantly from 44.4 % (n=28) of the pre intervention period to 19.0 % (n=12) of the post intervention period (p-value 0.001) (Table 1).

There was no substantial difference in bullying prevalence between second and third classes both in pre (45.5% vs 43.3%, respectively) and post-intervention (21.2% vs 16.7%, respectively) surveys (Table 1).

Furthermore, as shown in Table 1, a dissimilar significant prevalence (p-value <0.01) of bullying episodes was observed among schools by neighborhood socio-economic index. Specifically, a higher prevalence of bullying was observed in group B (66.6%), followed by C (36.8%) and A groups (25%).

Differently, after post-intervention questionnaire, the prevalence of reported bullying episodes was significantly higher in group C (36.8%), followed by group B (20.8%) and A (0%) (p-value <0.01).

As reported in Table 2, a reduction in the prevalence of verbal (n=20; 71.4% vs n=7; 58.3% respectively) and physical (n=4; 14.3% vs n=0; 0% respectively) bullying was observed comparing pre- and post-intervention data. Conversely, an increase of indirect bullying, passing from 14.3% (n=4) to 41.7% (n=5) was observed. More frequently the episodes of bullying were reported in classrooms (67.9%) followed by common spaces like hallways, bathrooms or school entrance (39.2%) and outside the school (32.1%) (table 2). In the majority of bullying phenomena observed before interventions, no more than five students were involved (82.1%). Both in pre- and post-intervention questionnaires the preferred reference person for the victims, to talk about bullying, resulted the teacher (53.1% and 50% respectively), followed by parents (28.6% and 33.3% respectively) and classmates (10.7% and 8.3%) (Table 2). Also a borderline statistically significant consistent decrease of the percentage of other students of the classrooms reporting bullying episodes involving classmates (from 46.4% to 16.7%) was observed (Table 2).

Table 1. Distribution of bullying episodes by school year attended and school neighborhood socio-economic index in pre- and post-intervention

n=63	Pre-intervention bullying, n (%)		p-value	Post-intervention bullying, n (%)		p-value
	Yes	No		Yes	No	
Reported bullying episodes in the last two (pre-intervention) and six (post-intervention) months						
	28 (44.4)	35 (55.6)		12 (19.0)	51 (81.0)	0.001
School year attended						
- *Second year (n=33)*	15 (45.5)	18 (54.5)	0.53	7 (21.2)	26 (78.8)	0.44
- *Third year (n=30)*	13 (43.3)	17 (56.7)		5 (16.7)	25 (83.3)	
Neighborhood socio-economic index						
- *High (A) (n=20)*	5 (25.0)	15 (75.0)	< 0.01	0 (0.0)	20 (100.0)	<0.01
- *Medium (B) (n=24)*	16 (66.7)	8 (33.3)		5 (20.8)	19 (79.2)	
- *Low (C) (n=19)*	7 (36.8)	12 (63.2)		7 (36.8)	12 (63.2)	

Table 2. Characteristics of the bullying phenomenon reported by the teaching staff of the n. 63 classes of the schools sampled in the City of Palermo reported in pre- and post-intervention questionnaires

	Bullying prevalence Pre-intervention n=28 n (%)	Bullying prevalence Post-intervention n=12 n (%)	p-value
Type of bullying occurred			
- *Verbal*	20 (71.4)	7 (58.3)	0.09
- *Physical*	4 (14.3)	0 (0.0)	
- *Indirect*	4 (14.3)	5 (41.7)	
Places were bullying phenomena occurred *(possible multiple responses)*			
- *Classrooms*	19 (67.9)	-	-
- *Common spaces (hallways, bathrooms, entrance)*	11 (39.2)	-	
- *Outside the school*	9 (32.1)	-	
Students involved in the bullying episodes			
- *At least five*	23 (82.1)	-	-
- *More than five*	5 (17.9)	-	
Victims of bullying talks with someone about episodes suffered *(possible multiple responses)*			
- *Yes, with teachers*	15 (53.6)	6 (50.0)	0.54
- *Yes, with classmates*	3 (10.7)	1 (8.3)	
- *Yes, with parents*	8 (28.6)	4 (33.3)	
- *No*	2 (7.1)	1 (8.3)	
Other students of the classrooms reported bullying episodes involving classmates			
- *Yes*	13 (46.4)	2 (16.7)	0.06
- *No*	15 (53.6)	10 (83.3)	
In counteracting bullying phenomena, who provided a support? *(possible multiple responses)*			
- *Colleagues*	28 (100.0)	-	-
- *School chief*	17 (60.7)	-	
- *Parents*	9 (32.1)	-	
Could a formative or educational intervention change the bullying attitude?			
- *Yes*	23 (82.1)	-	-
- *No/ I don't know*	5 (17.9)	-	
Suggested target for intervention on bullying prevention, (n=23)			
- *Teachers*	13 (56.6)	-	-
- *Selected students and parents*	5 (21.7)	-	
- *Whole classrooms*	5 (21.7)	-	
Bullying trend observed, (n=28)			
- *Increasing*	5 (17.8)		
- *Stable*	1 (3.6)		
- *Decreasing*	22 (78.6)		

Teachers received a strong support in counteracting bullying phenomena always by colleagues (100%), often by school chief (60.7%) and parents (32.1%). The majority of teaching staff enrolled required a formative or educative intervention for changing bullying attitudes (82.1%) and more than an half (56.5%) preferred an intervention among teachers (table 2).

As further shown in table 2, in the 78.6% of teaching staff reporting at least one episodes of bullying in the pre-intervention questionnaire (n=28), a decreasing trend of bullying in their classrooms after intervention was documented.

Finally, the 95.2% (n=60) of teaching staff considered helpful and useful the interventions carried out

and the 79.4% (n=50) of respondents implemented preventive intervention not only among collegues but also among students, throughout standardized and evidence-based methods, such as cooperative learning, peer education and role playing (data not shown in Tables).

Qualitative analysis

Table 3 shows the results of the qualitative analysis with the specific characteristics reported of bullies, victims of bullying episodes and observers, for each specific domain. In particular, within the area of "affective-relational discomfort" the characteristics categorized were for bullies (frustration, relational discomfort, roles disavowal, attention-seeking, warnings indifference, exclusion fear, emotional shortage), for victims (relational difficulties, lack of social skills, anxiety, inability to ask for help, fragility, social exclusion), and for observers (fear of marginalization, need to feel accepted, fear of social exclusion, fear to expose themselves, omertà/accomplice silence, need to identify with someone else). For the domain of "socio-cultural context" instead for bullies were reported: social discomfort, ignorance, lack of positive model, vulgarity, familiar discomfort, rules refusal, socio-economic and cultural disadvantage; for victims: socio-economic and cultural disadvantage, disability and isolation; and for observers: low or excessive involvement, passivity and poor solidarity. Lastly, within the domain of "character/natural disposition", bullies' characteristics reported were: prevarication, abuse, tyranny, arrogance, evil, aggressiveness, cockiness, immaturity, shallowness, self-doubt, low self-esteem, fragility; victims characteristics were: loneliness, fragility, subservience, insecurity, subjugation, weakness, shyness, low self-esteem, passivity; and for the observers: curiosity, superficiality, passivity, fear, cowardice, insecurity, indifference, lack of self-esteem, individualism, complicity.

Discussion

To the best of our knowledge, the BIAS study represents the first study conducted in Italy with the aims to estimate the prevalence of bullying among

Table 3. Qualitative analysis of the characteristics of bullies, victims and observer of bullying episodes reported by the teaching staff of the n. 63 classes participating to the pre-interventional study

Affective-relational discomfort	Socio-cultural context	Character/natural disposition
	Bullies	
Frustration, relational discomfort, roles disavowal, attention-seeking, disregards for warnings, exclusion fear, emotional shortage;	Social discomfort, ignorance lack of positive model, vulgarity, familiar discomfort, rules refusal, socio-economic and cultural disadvantage;	Prevarication, abuse, tyranny, arrogance, evil, aggressiveness, cockiness, immaturity, shallowness, self-doubt, low self-esteem, fragility;
	Victims of bullying episodes	
Relational difficulties, lack of social skills, anxiety, inability to ask for help, fragility, social exclusion;	Socio-economic and cultural disadvantage, disability, isolation;	Loneliness, fragility, subservience, insecurity, subjugation, weakness, shyness, low self-esteem, passivity;
	Observers of bullying episodes	
Fear of marginalization, need to feel accepted, fear of social exclusion, fear of expose yourself, omertà, need to identify with someone else;	Low or excessive involvement, passivity, poor solidarity;	Curiosity, superficiality passivity, fear cowardice, insecurity indifference, lack of self-esteem, individualism, complicity.

secondary school students by interviewing the teachers and to evaluate the potential reduction of bullying episodes after a preventive intervention.

We reported a 44% prevalence of the bullying phenomena before preventive interventions conducted.

This estimate is very high as compared to previous evidences available on the topic provided by the national survey "health-behavior in school-aged children" (HBSC), documenting a 14% prevalence of bullying episodes (12).

This difference could be explained by different aspects. As first, the HBSC survey, predominantly analyzing life-styles, attitudes and habits of Italian adolescents, posed only a generic question referring to potential episodes of bullying suffered at school (12). More in depth, an important difference in the definition used by the two questionaries should be noticed, since in HBSC the word "bullying" was used to detect the prevalence without a specific explanation, while in the BIAS study at the beginning of both pre and post intervention questionnaires, the universally and scientifically recognized definition of bullying was reported to help teachers in correct identification of bullying episodes (10). Furthermore, the BIAS study provided estimates by interviewing the teaching staff, while HBSC was addressed directly to student. All of the previous evidences taken together let us conclude that the HBSC study could have largely underestimated the bullying phenomenon in the school setting.

According to the survey results, about two thirds of the bullying episodes reported took place within the classrooms. This could be attributable to the specific perception of teachers that spend most of their time in classrooms. Contextually, about a third of bullying episodes took place in common areas and outside the school building, in accordance with literature data, indicating that places near or on the route, to and from school, are at high risk of bullying episodes (17 - 19).

Of interest, the majority of the interviewed teachers declared to have direct information from children victims of bullying. This data has to be taken into account because, as reported internationally, teachers represent the primary actors in creating and maintaining a positive classroom climate, as well as in promoting healthy interpersonal relations among their students and in the prevention of bullying episodes (20-22).

With regard to the effectiveness of the preventive interventions taken in place by the teachers, a significant decrease in bullying prevalence was highlighted through the post test questionnaire.

In particular, the episodes of bullying were zeroed in schools of higher socio-economic level and significantly decreased in those of average level. Only for the schools located in the most deprived areas of the city the prevalence has not changed. This trend is in line with the available literature, documenting the difficulty to prevent bullying phenomenon in disadvantaged context (23, 24).

Moreover, the qualitative analysis of the characteristics of bullies, victims and observers of bullying episodes reported by the teaching staff through the pre-intervention questionnaire highlighted three semantic domains: affective-relational discomfort, sociocultural context, and character/natural disposition.

In particular, teachers outlined a common ground for the sociocultural context within a disadvantage economic position for both bullies and victims, while for bullies emerged a specific role of the family in terms of "familiar discomfort, lack of positive model, vulgarity and rules refusal". Lastly, in line with the current literature disability was highlighted to play a role with regard to victims selection by bullies (25, 26).

Furthermore, according to the dimension explored by the character/natural disposition domain, it was possible to recognize the well-known bully profile, corresponding to the prevaricator features with a lack of confidence in him/herself (23). It is important to notice how "fragility" and "low self-esteem" were indicated for both bullies and victims, while features of "indifference", "passivity" and "individualism" emerged for observers. Attention should be also paid to "omertà/ accomplice silence" as one on the main characteristic attributed by the teachers to the observers, this probably reflecting the influence of the Sicilian cultural specific background within the genesis of phenomenon of bullying in this specific setting.

A controversial aspect of the methodology applied in our study could be the collegial answering approach to the questionnaires. In fact, although this could have overshadowed some perceptions of single

teachers, on the opposite, it has generated and has allowed at the same time the opportunity to improve and spread the dialogue within the teaching staff on the bullying issue.

An important limitation of our study is the involvement of schools from urban setting only, so introducing a potential selection bias that has to be prevented by extending the future investigation also to suburb and rural areas.

Last but not least, even if a comprehensive interpretation of the occurrence of the bullying phenomenon documented by the BIAS study will be possible only after the integration with the students' perception, we strongly believed that these findings could already have important implications for designing further intervention, strategies and programs for bullying prevention.

Conclusions

The BIAS study has allowed us to estimate the prevalence and the main characteristics of bullying phenomenon in a sample of secondary school of the Palermo city, together with the effectiveness of specific preventive interventions targeted to the teaching staff in reducing bullying episodes among students. Despite the discussed limitations, this preliminary findings could be considered as a first step of a wider project extended to students' perceptions on the topic. If the effectiveness of this approach in reduction and prevention of bullying phenomena will be confirmed also among students, the BIAS model should be applied on regional or national scale.

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