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EDITORIAL

PROBIOTICS, PREBIOTICS AND SYMBIOTICS IN INFLAMMATORY BOWEL DISEASES: STATE-OF-THE-ART AND NEW INSIGHTS

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Inflammatory bowel disease (IBD) consists of two distinct clinical forms, ulcerative colitis (UC) and Crohn's disease (CD), with unknown aetiology, which nevertheless are considered to share almost identical pathophysiological backgrounds. Up to date, a full coherent mechanistic explanation for IBD is still lacking, but people start to realize that the pathogenesis of IBD involves four fundamental components: the environment, gut microbiota, the immune system and the genome. As a consequence, IBD development might be due to an altered immune response and a disrupted mechanism of host tolerance to the non-pathogenic resident microbiota, leading to an elevated inflammatory response. Considering the available data arising from the scientific literature, here reviewed, in CD, a benefit of probiotics remains unproven; in UC, a benefit of probiotics remains unproven, even if *E. coli Nissle 1917* seems promising in maintaining remission and it could be considered an alternative in patients intolerant or resistant to 5-ASA preparations; in pouchitis, small controlled trials suggest a benefit from VSL#3 in the primary and secondary prevention of pouchitis; in IBD-associated conditions, a benefit of probiotics remains unproven. However, well-designed randomized control clinical trials are necessary to understand the undoubted role of these agents in the management of gut physiology in health and disease.

Inflammatory bowel disease (IBD) consists of two distinct clinical forms with unknown aetiology, ulcerative colitis (UC) and Crohn's disease (CD) which nevertheless share almost identical

Key words: Probiotics, prebiotics, synbiotics, inflammatory bowel disease

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pathophysiological backgrounds.

CD is predominantly associated with a type 1 helper-T-cell (Th1) and type 17 helper-T-cell (Th17) immune responses, characterized by increased production of interleukin IL-12, IL-23, IL-27, interferon-γ (IFN-γ) and tumour necrosis factor TNF-α. Diversely, UC seems to be associated with a type 2 helper-T cell (Th2) immune response, mainly leading to raised levels of IL-5 and transforming growth factor-β (TGF-β). The etiology of IBD is complex and multifactorial, where environmental, genetic and immunological components appear to play a role (1-3).

After the emergence of IBD about a century ago, numerous hypotheses were suggested as the possible mechanism, which included infection, toxicants, psychogenic disturbances, nutritional deficiencies, allergy to pollens or foods, abdominal trauma, impaired vascular or lymphatic circulation, lysozymes and other enzymes (4-7), or the excessive or deficient immune response due to reduced exposure to bacteria or helminthes (8-9)**.** Most of them were invalidated and forgotten. Up to date, a full coherent mechanistic explanation for IBD is still lacking, but people start to realize that the pathogenesis of IBD involves four fundamental components: the environment, gut microbiota, the immune system and the genome (10-12).

As a consequence, IBD development might be due to an altered immune response and a disrupted mechanism of host tolerance to the non-pathogenic resident microbiota, leading to an elevated inflammatory response. The strength of this hypothesis arises from many evidences, such as the fact that inflammation mainly occurs in the intestinal sites with the highest bacterial concentration (in UC), that antibiotic treatment often results in amelioration of disease symptoms (13), and that germ-free mice do not spontaneously initiate colitis (14).

We analyzed the international literature about the role of microbiota in IBD, using PubMed as primary source, focusing on the use of probiotics as complementary therapeutic approach in CD, UC and pouchitis. Aim of this review is to summarize the pathophysiological rationale for the use of probiotics in IBD, and to focus on clinical trial of such emerging therapeutic options.

Microbiota and etiopathogenesis of inflammatory bowel disease

The human normal flora, or microbiota, is extensive, both in its absolute quantitative mass and qualitative diversity. Conventional microbiological techniques fail to give a detailed inventory of the normal microbiota, but the development of recent high-throughput sequencing and molecular taxonomic methodologies have greatly increased our understanding of the population composition, dynamics, and ecology of the gut microbiota. The composition of intestinal flora is remarkably stable at different anatomic locations along the gut, but absolute numbers vary greatly, ranging from 10^{11} cells/g content in the ascending colon to 10^{7} to 10^{8} in the distal ileum and 10^{2} to 10^{3} in the proximal ileum and jejunum; anaerobes are several orders of magnitude more abundant than aerobes in the bacterial community, and a majority of the population (60-90%) comprises two divisions: the *Bacteroidetes and Firmicutes* (15).

The composition of the microbiota has been suggested to influence susceptibility to IBDs (16), which are mediated by both innate and adaptive arms of the host immune system. It is possible that distinct members of the commensal microbiota engage specific components of the immune system and, in doing so, they regulate intestinal immune homeostasis (15).

An important question is whether specific commensal microorganisms regulate the homeostasis of effector T cells in the lamina propria. For example, it has been reported that the gut commensal *Bacteroides fragilis* affects systemic Th1 responses through the action of the bacterial-derived polysaccharide A (PSA) (17). The lamina propria of the small intestine at steady state contains two populations of CD4 T cells, Th17 cells, and regulatory T cells (Treg); in particular, the former has been assumed to play a role in Crohn's disease and ulcerative colitis (18, 19**,** 20). Interestingly, Ivanov et al. (21) found that Th17 cells could be induced in the lamina propria of the small intestine in response to specific components of the commensal microbiota belonging to the *Cytophaga–Flavobacterium–Bacteroides* phylum, suggesting that the composition of the intestinal microbiota is likely to influence intestinal immunity, tolerance, and IBD susceptibility (22). More recently Ivanov et

al. (23) showed that Segmented Filamentous Bacteria (SFB) are potent inducers of Th17 cells in the lamina propria of the small intestine of mice. In particular, SFB colonization induced production of serum amyloid A (SAA) in the terminal ileum and SAA acted on dendritic cells in the lamina propria to promote Th17 cell differentiation. Also, the aforementioned CD4 Treg cells can be stimulated by commensal microbiota as evidenced by O'Mahony et al. (24), who showed that in mice the deliberate consumption of the commensal organism *Bifidobacterium infantis 35624* resulted in the induction of Treg cells, which protected the host from excessive inflammation during the course of infection caused by *Salmonella typhimurium*.

In particular, the reduction in the phlogistic response was achieved through the control of excessive pathogen mediated activation of nuclear factor kappa B, a transcription factor often involved in innate proinflammatory signalling in response to microbial exposure.

Natural killer (NK) cells play an important role in the innate immune system. It has been shown (25) that, in a germ-free mice, NKp46þ IL-22 producing cells were markedly reduced, suggesting that an environmental niche, operative in the gut, generated these unique effectors cells. Interestingly, more recently, Takayama et al. (26) conducted a clinical study that showed that NKp46þ cells were predominant in the intestinal mucosa of patients with Crohn's disease compared with controls or patients with ulcerative colitis. Upon interaction with intestinal inflammatory macrophages, these cells were also activated via IL-23 and produced IFNγ. Another interesting point concerning intestinal chronic diseases is the role of epithelial antimicrobial proteins as innate immune effectors; they probably play an important role in maintaining mutually beneficial host–microbe relationships by restricting contact between resident microbes and mucosal surfaces, and their deficiencies are associated with IBD. In particular, using a germ-free murine model, it has been shown (27) that resident gut bacteria drive intestinal epithelial expression of a C-type lectin that binds peptidoglycan and has direct antimicrobial activity; interestingly, the human counterpart of this protein (HIP/PAP) is usually overexpressed in intestinal mucosa of IBD patients (28) and it is also believed to be a biomarker of pancreatic ductal adenocarcinoma.

Commensal microbiota could also be implicated in stimulating immunoregulatory pathways through expression of specific heat shock proteins (HSPs). Molecular chaperones of the HSP family are evolutionarily conserved proteins that modulate a variety of intracellular functions including the folding of proteins, folding of multimeric proteins, the translocation of proteins across membranes, the degradation of proteins, and signal transduction. Apart from their chaperone activities, HSPs are involved in the regulation of innate immunity and mucosal immunity. Several reports have shown HSP reactive T cells to have an immunoregulatory phenotype, indicating that HSPs, particularly HSP60 and HSP70, constitute a group of autoantigens with the potential to trigger immunoregulatory pathways, which can suppress immune responses that occur in various human inflammatory diseases, such as rheumatoid arthritis (29, 30), type 1 diabetic mellitus (31, 32), atherosclerosis (33), and allergy (34). Intragastric administration or nasal immunization with HSPs was effective as preventive/therapeutic interventions of atherosclerosis in animal models, suggesting that mucosal immunization with HSPs is valuable for the regulation of chronic inflammation (35). Interestingly, it has been shown that the bacterial HSP60 GroEL, whose production is related to intestinal microflora, could generate CD4+CD25+ FoxP3+ T cells from naive T cells; owing to their ability to secrete GroEL, intestinal flora could be involved in intestinal homeostasis and UC pathogenesis (36). Luminal bacteria may also exert a protective role because some of their byproducts, such as small-chain fatty acids (SCFAs), are able to simulate intestinal expression of cytoprotective HSP25 and HSP72 (37-41).

Our group also reported his experience in this field. In a first work we investigated three heat shock protein/molecular chaperones: HSP10, HSP70, and HSP90. We found that the levels of these proteins are increased in UC patients at the time of diagnosis and decrease after therapy, supporting the notion that these proteins deserve attention in the study of the mechanisms that promote the development and maintenance of IBD, and as biomarkers of this disease (42). In another study we wanted to determine

Table I. *Controlled trials of probiotics for the induction and maintenance of remission in adults with Crohn's disease.*

Authors, year	Study design	Number of patients	Regimen	Rationale of the use of the probiotics employed	Duration (months)	Outcomes
Schultz et al., 2004	Randomized controlled trial	11	Lactobacillus GG versus placebo	- Inhibition of apoptosis of intestinal epithelial cells. - Decreased translocation of commensal bacteria via the mesenteric lymph nodes and liver.	6	No difference in obtaining remission
Malchow et al., 1997	Randomized controlled trial	28	E. coli Nissle 1917 versus placebo	- Downregulation of the expansion of newly recruited T cells into the mucosa - Intestinal inflammation regulation via TLR-2 and TLR-4 - Restoration of disrupted epithelial barrier in the colonic epithelial cell line T84	12	No difference in obtaining maintenance of remission
Guslandi et al., 2000	Randomized controlled trial	32	Saccharomyces boulargii 1g daily plus mesalamine versus mesalamine alone	- Limitation of infiltration of T-helper 1 cells into the mucosa NF-kB blocking and IL-8 downregulation	6	Probiotics plus mesalamine were superior in obtaining maintenance of remission, evaluated through Crohn's Disease Activity Index
Schultz et al., 2004	Randomized controlled trial	11	Lactobacillus GG versus placebo	- Inhibition of apoptosis of intestinal epithelial cells - Decreased translocation of commensal bacteria via the mesenteric lymph nodes and liver	6	No difference in obtaining maintenance of remission
Prantera et al., 2002	Randomized controlled trial	45	Lactobacillus GG versus placebo	- Inhibition of apoptosis of intestinal epithelial cells - Decreased translocation of commensal bacteria via the mesenteric lymph nodes and liver	12	No difference in preventing clinical post-surgical recurrence
Marteau et al., 2006	Randomized controlled trial	98	Lactobacillus johnsonii LA1 versus placebo	- Upregulation of intestinal MUC3 and MUC3 mRNA expression	6	No difference in preventing clinical post-surgical recurrence
Van Gossum et al., 2007	Randomized controlled trial	70	Lactobacillus johnsonii LA1 versus placebo	Upregulation of intestinal MUC3 and MUC3 mRNA expression	3	No difference in preventing endoscopic post-surgical recurrence

in colon mucosa of CD and ulcerative UC in relapse: a) the levels of the chaperonins HSP60 and HSP10; b) the quantity of inflammatory cells; and c) if the levels of chaperonins parallel those of inflammation cells. In this study, we found that HSP60 and HSP10 occurred in the cytoplasm of epithelial cells in CD and UC but not in negative controls.

HSP60 and HSP10 co-localised to epithelial

TableII. *Controlled trials of probiotics for the induction and maintenance of remission in adults with Ulcerative colitis.*

Authors, year	Study design	Number of patients	Regimen	Rationale of the use of the probiotics employed	Duration (months)	Outcomes
Rembacken et al., 1999	Randomized controlled trial	120	E. coli Nissle 1917 versus mesalamine	- Downregulation of the expansion of newly recruited T cells into the mucosa. - Intestinal inflammation regulation via TLR-2 and TLR-4. - Restoration of disrupted epithelial barrier in the colonic epithelial cell line T84.	3	No difference in induction rates
Tursi et al., 2004	Randomized controlled trial	90	VSL#3 plus balsalazide versus balsalazide alone or mesalamine alone	-Reduction of secretion of TNF-a and interferon-g . - Improvement of the colonic barrier function. - Inhibition of Salmonella Dublin invasion into T-84 cells. - Convertion of linoleic acid into conjugated linoleic acid. -Inhibition of TNF-a-induced IL-8 secretion, mitogen-activated protein kinase activation and NF-kB activation in HT-29 cells (CpG DNA). - Upregulation of mucin expression.	2	Better significant induction of remission in the probiotic group
Kato et al., 2004	Randomized controlled trial	20	Probiootic milk (Bifidobacterium breve, Bifidobacterium bifidum and Lactobacillus acidophilus 1) versus placebo	- Increase in IL-10 secreted by mesenteric lymph nodes (Bifidobacterium-fermented milk) - Reduction of MPO activity, tissue contents of immunoglobulin, TNF-a. - Upregulation of intestinal MUC3 and MUC3 mRNA expression.	3	Significantly better change in clinical activity index, histological score in the probiotic group, in evaluating the induction of remission
Furrie et al., 2005	Randomized controlled trial	18	Symbiotic (Bifidobacterium longum and fructooligosaccharide/ inulin mix) plus Standard treatment versus Standard treatment alone	- Upregulation of IL-10 expression in dendritic cells. - Inhibition of disorderd T-cell activation.	1	Improved sigmoidoscopy scores and cytokine profiles in the probiotic group, in evaluating the induction of remission
Kruis et al., 1997	Randomized controlled trial	120	E. coli Nissle 1917 versus mesalamine	Downregulation of the expansion of newly recruited T cells into the mucosa. - Intestinal inflammation regulation via TLR-2 and TLR-4. - Restoration of disrupted epithelial barrier in the colonic epithelial cell line T84	3	No statistically significant difference in the relapse rate in evaluating the maintenance of remission
Ishikawa et al., 2003	Randomized controlled trial	21	probiotic milk (Bifidobacterium Breve, Bifidobacterium Bifidum and Lactobacillus acidophilus) with Standard tretment versus Standard treatment alone	- Increase in IL-10 secreted by mesenteric lymph nodes (Bifidobacterium-fermented milk). - Reduction of MPO activity, tissue contents of immunoglobulin, TNF-a - Upregulation of intestinal MUC3 and MUC3 mRNA expression	12	Fewer relapses in probiotic group, ikn evaluating the maintenance of remission
Kruis et al., 2004	Randomized equivalence trial	312	E. coli Nissle 1917 versus mesalamine	- Downregulation of the expansion of newly recruited T cells into the mucosa. - Intestinal inflammation regulation via TLR-2 and TLR-4. - Restoration of disrupted epithelial barrier in the colonic epithelial cell line T84	12	Equivalence in the relapse rate, in evaluating the maintenance of remission
Zocco et al., 2006	Randomized controlled trial	186	Lactobacillus GG versus mesalamine versus both	- Inhibition of apoptosis of intestinal epithelial cells. - Decreased translocation of commensal bacteria via the mesenteric lymph nodes and liver.	12	Lactobacillus GG is showed to prolonge the time of relapse

Table III. *Controlled trials of probiotics for the induction and maintenance of remission in adults with pouchitis.*

Author, year	Study design	Number of patients	Regimen	Rationale of the use of the probiotics employed	Duration (months)	Outcomes
Gionchetti et al., 2003	Randomized controlled trial	40	VSL#3 versus placebo	-Reduction of secretion of TNF-a and interferon-γ. - Improvement of the colonic barrier function. -Inhibition of Salmonella Dublin invasion into T-84 cells. -Convertion of linoleic acid into conjugated linoleic acid. -Inhibition of TNF-a-induced IL-8 secretion, mitogen-activated protein kinase activation and NF-kB activation in HT-29 cells (CpG DNA). - Upregulation of mucin expression.	12	Significant reduction in the onset of acute pouchitis with probiotic group in evaluating the prophylaxis
Gionchetti et al., 2000	Randomized controlled trial	40	VSL#3 versus placebo	- Reduction of secretion of TNF-a and interferon-γ. - Improvement of the colonic barrier function. - Inhibition of Salmonella Dublin invasion into T-84 cells. - Convertion of linoleic acid into conjugated linoleic acid. - Inhibition of TNF-a-induced IL-8 secretion, mitogen-activated protein kinase activation and NF-kB activation in HT-29 cells (CpG DNA). - Upregulation of mucin expression.	9	Significant decrease in relapse in the probiotic group in evaluating the maintenance of remission
Kuisma et al., 2003	Randomized controlled trial	20	Lactobacillus GG versus placebo	- Inhibition of apoptosis of intestinal epithelial cells. - Decreased translocation of commensal bacteria via the mesenteric lymph nodes and liver.	3	No difference in disease activity in evaluating the maintenance of remission
Mimura et al., 2004	Randomized controlled trial	36	VSL#3 versus placebo	-Reduction of secretion of TNF-a and interferon-γ. - Improvement of the colonic barrier function. - Inhibition of Salmonella Dublin invasion into T-84 cells. - Convertion of linoleic acid into conjugated linoleic acid. - Inhibition of TNF-a-induced IL-8 secretion,.mitogen-activated protein kinase activation and NF-kB activation in HT-29 cells (CpG DNA). - Upregulation of mucin expression.	12	Significantly decreased relapse in the probiotic group in evaluating the maintenance of remission

cells of mucosal glands but not always in connective tissue cells of lamina propria, where only HSP60 or, less often, HSP10 was found; cells typical of inflammation were significantly more abundant in CD and UC than in negative controls. Since chaperonins are key factors in the activation of the immune system leading to inflammation, we propose that they play a central role in the pathogenesis of the two diseases, which, consequently, ought to be studied as chaperonopathies (43). Furthermore, it is intriguing the recent discovery of genetic polymorphisms (involving pattern recognition receptor genes) related to serum levels of anti-microbial antibodies in CD patients but not in negative controls (44); on the basis of this finding it could be postulated an abnormal immunological response to alteration of microbiota in patients carrying these polymorphisms.

Probiotics

Probiotics are microorganisms that have beneficial properties for the host (45). Several mechanisms of action of probiotics relative to prevention and treatment of IBD have been reported, such as antimicrobial activity and suppression of bacterial growth, immunomodulation and initiation of an immune response, improvement of intestinal barrier

function, suppression of human T-cell proliferation (46-50), and modulation of pain perception (51-55). Probiotics have also been found to induce their effect by means of their DNA, as shown by experiments using probiotic DNA (56-58) and subcutaneous administration of probiotic DNA (59). Derived originally from cultured food, especially dairy products, this group includes *Lactobacillus species*, *Bifidobacterium species*, *E. coli Nissle 1917* (a nonpathogenic *E. coli* strain), *Saccharomyces boulardii*, *Clostridium butyricum*, *VSL#3* and *Lactococcus lactis* genetically engineered to secrete IL-10.

Probiotics in Crohn's Disease

The literature on the induction and maintenance of remission in CD is heterogeneous and difficult to interpret. The reasons for such heterogeneity are several, as the different probiotics (and doses) used, the differences in study duration, the features of the included patients, and the measured endpoints.

In the setting of inducing remission, in a pivotal study performed by Schultz and coworkers, with only 11 patients, probiotics provided no additional benefit to steroids and antibiotics in inducing remission (60). Subsequently, Fujimori and coworkers, in an open-label study with 10 patients who were refractory to standard therapies (prednisolone and aminosalicylates), tried a combination of probiotics (*Bifidobacterium breve, Bifidobacterium longum,* and *Lactobacillus casei*) and a prebiotic (psyllium) simultaneously. A complete response was found in 6 of 10 patients without any adverse events (61).

In the paediatric setting, Gupta and coworkers used, in an open-label trial, *Lactobacillus rhamnosus* GG (62). In this study, 3 of the 4 children were reported to have improved Pediatric Crohn's Disease Index (PCDAI) scores or serial determinations over the 6 months of the trial. Specifics with regards to ESR or CRP are not reported even if the ESR is a component of the PCDAI (63).

The placebo-controlled trial, using *Lactobacillus rhamnosus* GG was the sole study included in a Cochrane review, performed by Butterworth and coworkers, concerning the efficacy of probiotic supplementation for the induction of remission in CD that met the inclusion criteria of being a randomized controlled trials of participants with CD whose disease was active at the time of entry into the study (64). In the study 11 patients were enrolled; four of 5 patients in the probiotic group achieved remission compared to 5 of 6 in the placebo group (OR 0.80; 95% CI 0.04 to 17.20). Thus, this one small study did not show that probiotics had any effect in treating active CD (64). More controlled studies have been performed on the maintenance of remission in adults with CD, but in general these studies fail to show any benefit of probiotic administration (55).

Initial randomized trials in CD were reported with probiotics used as sole maintenance therapy following corticosteroid therapy (65) or in combination with lower does of 5-aminosalicylate therapy compared to controls for maintenance therapy in those already in remission (66). Subsequent trials have focused on *Lactobacillus rhamnosus* strain GG for maintenance therapy following induction of remission with corticosteroids (60) and maintenance of remission with probiotic used as additional maintenance therapy (67).

In the largest maintenance trial to date, Bousvaros and coworkers (68) also reported no difference in the proportion of those developing relapse on *Lactobacillus rhamnosus* strain GG 2 × 10 (10) CFU/day (31%; 12 of 39) or placebo (17%; 6 of 36, $p = 0.18$) (60).

Data are also more robust on the prevention of relapse following surgical intervention, but again probiotics fail to prevent endoscopic and clinical recurrence.

The meta-analysis performed by Doherty and coworkers (69) of the effects of probiotics as a class suggested that their effect was not different from placebo. The relative risk of clinical recurrence with any probiotic relative to placebo ($n = 213$) was 1.41 (95% CI 0.59 to 3.36), any endoscopic recurrence ($n = 333$) was 0.98 (95% CI 0.74 to 1.29) and severe endoscopic recurrence ($n = 213$) was 0.96 (95% CI 0.58 to 1.59) (64). Table I shows the outcomes of controlled trials of probiotics for the induction and maintenance of remission in adult with CD.

Probiotics in Ulcerative Colitis

Although various probiotic species have shown promising in the treatment of UC, given the small number of patients in these studies and the risk associated with probiotics, two systematic reviews

have concluded that there is insufficient evidence to support the use of probiotics for the induction or maintenance of remission in ulcerative colitis (70, 71, 102).

In fact, several trials have been published examining probiotics in the induction and remission of UC, however, only few of these are randomized controlled trials (RCTs). Most are with different probiotic formulations and overall have been performed in a relatively small number of patients. For induction of remission, the first and largest controlled trial to date performed by Remnacken and coworkers showed no additional efficacy of *Escherichia coli Nissle 1917* than steroids, mesalamine, and antibiotics (63).

Three additional trials, all small in number of patients, and of short duration of therapy and with variable standard of care, showed improvement in various measures of disease activity and even cytokine profiles (72-74, 55), whereas the rectal administration of *Escherichia Coli 1917 Nissle* enema has equivocal results (75). It was showed that the combination of VSL#3™ plus balsalazide was slightly more effective than balsalazide or mesalamine alone, in a randomized trial of patients with acute mild-to-moderate UC (75). Similar results were found in the paediatric setting by Miele and coworkers (76).

Further evidences show that VSL#3™ could induce remission and reduce disease activity in patients with mild-to-moderate active ulcerative colitis (77-80). A randomized trial of 77 patients, performed by Sood and coworkers, proved that VSL#3™ was more effective than placebo in improving the ulcerative colitis disease activity index (UCDAI) by 50 percent at week 6 (33% versus 10%) and inducing remission at week 12 (43% versus 16%) (77). Another randomized trial by Tursi and coworkers examined 144 patients who were receiving a 5-ASA, azathioprine, and/or methotrexate (80). This trial also showed that patients receiving VSL#3™were more likely to have at least a 50% decrease in UCDAI at week 8 compared with patients receiving placebo (63% versus 41 %), with a similar increase in remission rate in the VSL#3™group (48% versus 32%), even if histologic scores were not significantly improved with VSL#3™ therapy.

With regard to maintenance of UC remission, probiotics have been tested in a larger number of patients. One trial by Kruis and colleagues tested *Escherichia coli Nissle 1917* and found no difference in relapse rates in patients on a probiotic versus mesalamine (81). A trial by Zocco and colleagues also found no difference in relapse rates at 6 or 12 months when comparing *Lactobacillus GG* with mesalamine with a combination of the two (82). Those patients who took the probiotic did appear to have a longer time to relapse (55).

However, a small double-blind placebo controlled trial showed no significant difference in the rates of maintenance of remission in patients with left sided UC randomized to 52 weeks of *Lactobacillus basilicus La-5* and *Bifidobacterium animalis subsp. Lactis Bb-12* or placebo (relapse rate 75% versus 92%) (65).

All of these studies support the idea that probiotics may be as effective as mesalamine in maintaining remission, almost in the short-term trial. Table I shows the outcomes of controlled trials of probiotics for the induction and maintenance of remission in adult with UC.

Probiotics in pouchitis

With regard to the surgical treatment of UC and familial adenomatous polyposis, proctocolectomy with ileal pouch-anal anastomosis (IPAA) is the favored alternative to proctocolectomy with permanent ileostomy, since it preserves intestinal continuity and sphincter function and removes almost all of colorectal mucosa. After proctocolectomy with IPAA, pouchitis or acute and chronic inflammation of the ileal reservoir is the most frequent long-term complication of this operation, occurring in up to 20% of patients at 1 year. Studies of the microflora in the pouch have revealed deficiency of streptococcal species (83, 55).

Most patients will develop this problem in the short term (within the first year) and antibiotics may be considered an effective form of therapy in many (84, 85) but for those that do not respond other forms of therapy are required (85). For some, antibiotics improve the pouchitis but there is a relapsing course of the pouchitis following the discontinuation of antibiotics. As antibiotics can provide relief for most with pouchitis, a basic assumption has been

the importance of the microbiota of the pouch in the development and chronicity of pouchitis. Thus, alteration of the microbiota by addition of probiotics was considered (65). In fact, the strongest evidence for the use of probiotics in IBD is in prevention and treatment of pouchitis (86-89).

Trials about the treatment of mild/moderate pouchitis are few, with small numbers of adult patients. Kuisma and coworkers (87) recruited 20 patients (10 intervention arm) for a trial of *Lactobacillus rhamnosus GG* 2×10^{10} CFU/day for 3 months. Patients with chronic, active pouchitis were excluded. The Pouchitis Disease Activity Index (90) was utilized for evaluation of clinical effect. Prior to study entry, the mean PDAI was in the mild range (8.0±0.8) and there was no difference following the intervention period with clinical response (defined as a PDAI score reduction of ≥3) occurring in 1/10 (10%) patients in the probiotic group and 0/10 (0%) patients in the placebo group (10% *vs* 0%, *P*=0.32).

In an open-label trial of 51 UC patients post ileal pouch-anal anastomosis, performed by Laake and coworkers, and using a fermented milk product with a blend of probiotic strains (*Lactobacillus acidophilus* strain La5 + *Bifidobacterium lactis* strain Bb12) containing 5×10^{10} CFU/day (91) however, there was a reported improvement in endoscopic evaluation. In another open label trial by Gionchetti and coworkers, twenty-three consecutive patients with mild pouchitis as defined using Pouchitis Disease Activity Index (scores 7-12) were treated with 3.6×1012 CFU/day of VSL#3™ for 4 weeks (92). Sixteen of 23 patients (69%) with mild pouchitis were in remission after treatment and the median total Pouchitis Disease Activity Index scores reported before therapy improved following therapy (10 *vs* 4, *P*<0.01). Thus, there is limited evidence for a role of probiotics as monotherapy for mild to moderate pouchitis at the present time.

Two trials have studied whether there is an advantage to initiate probiotics immediately following ileal pouch-anal anastomosis to evaluate the eventual delay in onset of development of pouchitis. Gionchetti and coworkers performed a placebo-controlled trial (89) where, at the end of one year, 2 of 20 (10%) of the patients in the intervention arm had developed colitis as determined compared to 8 of 20 (40%, no episodes 80% *vs* 60%, *P*=0.03) of the control arm participants using the PDAI with endoscopy. The Peto odds ratio for prevention of pouchitis by VSL#3™ compared with placebo was 4.76, 95% CI 1.16 to 19.56 (93).

The other randomized trial or probiotics also studied VSL#3™ in an open-label design performed by Pronio and coworkers, that compared the probiotic to no treatment over a 12 month period (94), where none of the 16 patients in the group administered probiotic compared to one of 12 (8.3%, no pouchitis 100% *vs* 92%, *p* =0.24) developed pouchitis.

Small controlled trials have also suggested that at least one probiotic preparation (VSL#3™) may be effective in prevention of recurrent pouchitis after antibiotic induction of remission.

The first randomized trial addressed to evaluate this outcome, performed in the year 2000 by Gionchetti and coworkers, included 40 patients with a history of chronic, relapsing pouchitis who were placed into clinical and endoscopic remission with broad spectrum antibiotics (88). Patients were randomly assigned to VSL#3™ 6 g/day or placebo. After nine months of daily treatment, significantly fewer patients in the probiotic group had experienced a relapse (15% *vs* 100%). Within 3 months of stopping treatment, all patients in the probiotic group had relapsed. Interestingly, fecal *Lactobacillus* and *Bifidobacteria* concentrations returned to pretreatment levels within one month after therapy withdrawal, indicating that permanent colonization with the probiotic species did not occur.

A similar result was noted in another European trial of VSL#3™ that also evaluating the prevention of recurrence of pouchitis in relapsing or chronic pouchitis patients (86). Remission of the pouchitis was induced in these participants by administering 4 weeks of a combination of antibiotics (metronidazole + ciprofloxacin) that was followed by either VSL#3™ or a placebo. In the treatment group remission was maintained in 17 of 20 (85%) but only 1 of 16 (6%, *p*<0.0001) on placebo. The pooled Peto odds ratio for these two studies for the combined rate of maintenance of remission with probiotic bacteria compared to placebo (97% *versus* 3%, *P*<0.0001) was 25.39 (95% CI 10.37 to 62.17). The number needed to treat with oral probiotic therapy to prevent one additional relapse was 2 (92).

A third study included 40 consecutive patients

who underwent IPAA for ulcerative colitis (89). Patients were randomly assigned to receive VSL#3™ 3 g/day or placebo immediately after ileostomy closure for one year. Patients receiving the probiotic had significantly fewer episodes of pouchitis (10% versus 40%); furthermore, probiotic treatment was also associated with significant improvement in quality of life, compared to placebo.

In contrast, an open label trial by Shen and colleagues (95) reported lesser responses. In their trial, 31 subjects were prescribed a 2-week treatment of a single antibiotic (ciprofloxacin) followed by VSL#3™. Also in contrast to the other studies, the VSL#3™ was bought by patients rather than be supplied through the study. Probiotic therapy was stopped by 9 of 31 (29%) seven weeks into therapy and 25 of 31 (81%) by 8 months had discontinued the probiotic because of failure to prevent pouchitis (n=23) or side effects of the probiotic administration (n=2). Only 6 of 31 (19%) did not develop clinical evidence of pouchitis by the end of the 8-month trial period. Even among these 6 subjects endoscopy revealed some level of pouch inflammation. In this trial (95), there was a single antibiotic administered and endoscopy was not performed prior to probiotic administration to ensure pouch inflammation had completely resolved.

A recent clinical practice guideline on management of pouchitis (96) has suggested that for those patients with prompt recurrence of pouchitis following antibiotic usage or having multiple recurrences of pouchitis despite antibiotics either VSL#3™ or chronic use of antibiotics but does not suggest probiotics for acute treatment of pouchitis. Table III shows the outcomes of controlled trials of probiotics for the induction and maintenance of remission in adult with pouchitis.

3.4 Probiotics in extraintestinal manifestations of IBD

3.4.1 Arthralgia: Karimi and coworkers performed an open label trial where 16 patients with either CD or UC completed a 3-month trial of ingesting 9×10^{11} CFU/day of VSL#3™ to assess whether there was a clinical improvement in arthralgia (97). Participants had quiescent IBD at entry and no clinical or laboratory evidence of arthritis, were not taking non-steroidal anti-inflammatory medications and other medications were unchanged. An improvement in peripheral but not axial arthralgia was reported using an articular index score (65).

3.4.2 Spondylarthropathy: In an interesting internet-based randomized control trial (98) of probiotic in patients with spondylarthropathy (7% of patients were IBD patients), Brophy and coworkers aimed to determine whether an internet-based trial of a complementary and alternative medicine could fulfill the revised CONSORT (Consolidated Standards of Reporting Trials) statement quality checklist for reporting of RCTs. However a secondary aim was to study the effect of probiotics on improving well-being. Well-being was measured by self-assessment using a visual analogue scale and 96 of 147 (65%) of people randomized to receive a blend probiotic completed a 3-month trial. No statistically or clinically significant difference between placebo and probiotic groups in terms of global well-being was found in this study (98).

3.4.3 Sclerosing Cholangitis: Vleggaar and coworkers randomized fourteen patients with concurrent IBD to treatment with a blend probiotic (*Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus salivarius*, *Lactobacillus lactis*, *Bifidobacterium bifidum and Bifidobacterium lactis*; total daily dose of 1010 CFU/day) or placebo during 3 months in a double-blind crossover design that included a 1-month washout period (99). The subjects remained on their ursodeoxycholic acid. The results of this study showed no evidence of a beneficial effect of the probiotics on PSC-related symptoms, serum liver biochemistry or liver function.

CONCLUSIONS AND PERSPECTIVES

Many concerns remain about the use of probiotics, such as the optimal number of colony forming units delivered into the gut, the survival of administered probiotics as they transit into the gut, the best method of delivery (e.g. yogurt versus milk), the difference in the action of probiotics related to the age of the patients, the use of combination or single probiotic preparation, and the optimal duration of the therapy. Other questions and concerns have been raised, however, about the safety of probiotic administration in the setting of a severe illness: for example, worsening of Crohn's disease (CD) in

patients taking some probiotic formulations (100) or exacerbation of indomethacin- induced enteropathy in animal models by *Lactobacillus GG* had been observed (101). As rare as these complications appear to be, probiotic safety profile needs to be specifically studied, particularly in hospitalized patients.

In conclusion, considering the available data arising from the scientific literature:

– In IBD, probiotics could have a promising therapeutic role, associated to conventional drugs.

– in UC, a benefit of probiotics remains unproven, even if *Escherichia coli Nissle 1917* shows promising in maintaining remission and could be considered an alternative in patients intolerant or resistant to 5-ASA preparations;

– in pouchitis, small controlled trials suggest a benefit from VSL#3 in the primary and secondary prevention of pouchitis;

Well-designed randomized control clinical trials are necessary in order to understand the undoubted role of these agents in the management of gut physiology in health and disease.

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EDITORIAL

ANTI-INFECTIVE PROPHYLAXIS FOR PRIMARY IMMUNODEFICIENCIES: WHAT IS DONE IN ITALIAN PRIMARY IMMUNODEFICIENCY NETWORK CENTERS (IPINet) AND REVIEW OF THE LITERATURE

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Primary immunodeficiencies (PIDs) are rare diseases characterized by an increased susceptibility to infections. Early diagnosis and appropriate treatment are critical for reducing morbidity and mortality. Based on available data, the efficacy of antibiotic administration for the prophylaxis of infections remains uncertain, and recommendations supporting this practice are poor. The use of antimicrobial prophylaxis is mainly based on single institution-specific experience without controlled measurements of patient safety and quality health outcomes. To address this issue an Italian Network on Primary Immunodeficiencies (IPINet) has been set up in 1999 within the Italian Association of Pediatric Hematology and Oncology (AIEOP) to increase the awareness of these disorders among physicians. Further, diagnostic and treatment guideline recommendations have been established to standardize the best clinical assistance to all patients, including antibiotic prophylaxis, and for a national epidemiologic monitoring of PIDs. The aim of this review is not only to give a scientific update on the use of antimicrobial prophylaxis in selected congenital immunological disorders but also to draw a picture of this practice in the context of

Key words: antimicrobial prophylaxis, infections, primary immunodeficiencies, IPINet-AIEOP

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the Italian Primary Immunodeficiency Network (IPINet). Controlled multicenter studies are necessary to establish if, when and how you should start an efficacious antimicrobial prophylaxis.

Primary immunodeficiencies are rare disorders of the innate and/or adaptive immune system that cause increased susceptibility to infections with increased morbidity and mortality. Antimicrobial prophylaxis is often used in clinical practice to reduce infection rates in several conditions, including the immunocompromised patient. However, the most appropriate antimicrobial agent or combination of drugs, dosage regimen, duration and predictive factors for its use must be defined.

There is a great variability among different pediatric immunologic centers in their prevention strategies with prophylaxis. This heterogeneity is not only due to the specific pathogens associated with each distinct immunodeficiency, but also to the age, the clinical condition of the child, the presence of predisposing factors and costs.

Here we give an update on the use of anti-infective prophylaxis in select primary immunodeficiencies. We focus on those PIDs where data from IPINet registries are available such as a) antibody defects, including X-linked Agammaglobulinemia (XLA), Common Variable Immunodeficiency (CVID), Selective IgA Deficiency (sIgAD) and Transient Hypogammaglobulinemia of Infancy (THI); b) immunodeficiency syndromes, i.e Di George Syndrome; c) congenital defects of phagocytes, i.e Chronic Granulomatous Disease (CGD). Other defects, such as Ataxia-Telangiectasia (AT), Wiskott-Aldrich Syndrome (WAS) and Immunodysregulation, Polyendocrinopathy, Enteropathy, X-linked Syndrome (IPEX), are mentioned but more extensive data are needed because of the rarity and limited size of these patients.

HUMORAL IMMUNODEFICIENCIES

X-linked Agammaglobulinemia; Common Variable Immunodeficiency; Selective IgA Deficiency; Transient Hypogammaglobulinemia of Infancy

The increased susceptibility to infections is a typical feature of all primary immunodeficiencies. Most PIDs, whatever is their origin, have a humoral impairment as an expression of the altered communication among the different components of the complex immune system machinery. Predominantly antibody deficiencies represent about 50-60% of all immunodeficiencies and are all characterized by a marked reduction or absence of immunoglobulins due to a block in B-cell development, differentiation and/or function. Their clinical and immunological phenotypes may be variable although they are mostly characterized by recurrent pyogenic infections (*Haemophilus influenzae, Streptococcus pneumoniae, Moraxella catarrhalis* and *Staphylococcus aureus*) that typically affect the respiratory tract, skin or other organs. *Giardia lamblia* may be responsible for protracted diarrhea and *Helicobacter pylori* colonization may cause gastric lesions that need eradicating treatments. Viral infections are uncommon although enteroviral meningoencephalitis and persistent Parvovirus infection may be a presenting feature of humoral immune deficiency. Opportunistic infections caused by *Pneumocystis jirovecii*, *Cryptosporidium* and fungi have been rarely reported (1).

X-linked Agammaglobulinemia (XLA) is the prototype of antibody deficiencies. Molecular epidemiological analysis on the genes responsible for the XLA phenotype has allowed the identification of distinct mutations in Bruton's tyrosine kinase (BTK), a member of Tec Family, as responsible for XLA disease in the majority of patients, including the Italian cohorts (2, 3). Genetic defects involving components of the pre-B-cell receptor (pre-BCR), such as μ heavy chain, surrogate light chain, BLNK, p85alpha, Igα and Igβ have been found in autosomal recessive agammaglobulinemia. Peripheral B cells of these patients are usually 1–2% of normal and they are unable to make functional antibody responses; consequently, serum immunoglobulins are profoundly decreased. The normal number of pro-B cells and the marked reduction of pre-B cells suggest impaired cellular proliferation or increased cell death during early B-cell development (1).

Common Variable Immunodeficiency (CVID) is the most common symptomatic antibody deficiency and, although mainly reported in adults, it may present from childhood. This disorder includes heterogeneous conditions with altered terminal B-cell differentiation and maturation, leading to dysfunctional antibody

production. So far, most cases of CVIDs remain genetically undefined and the diagnosis is made in presence of a marked decrease in at least 2 isotypes of immunoglobulins, poor response to vaccines and exclusion of other causes of hypogammaglobulinemia. Indeed, disturbed germinal center reaction, immune dysregulation and clinical immunodeficiency are variably expressed. However, in 10-15% of CVID patients, a molecular defect has been recently identified and the definition of Inducible T-cell costimulator gene (ICOS) deficiency, BAFF-R deficiency, CD19 deficiency, CD20 deficiency, CD21 deficiency, CD81 deficiency, LRBA deficiency seems more appropriate than a generic definition of CVID. Mutations of the Tumor Necrosis Factor Receptor Soluble Factor 13B (TNFRSF13B) gene, which encodes the transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI), have been associated to the CVID phenotype. Clinical variability of patients and family members carrying the C104R mutations ranging from hypogammaglobulinemia to autoimmune disorders have been reported (4).

Humoral defects are usually treated with intravenous (i.v.) (400 mg/kg every 3-4 weeks) or subcutaneous (s.c.) (100 mg/kg/week) replacement immunoglobulin therapy (5, 6). However, these doses need to be tailored according to patient's compliance and tolerance but also to the possible development of a chronic lung disease which might need higher doses (7).

Chronic lung disease (CLD) is a well-recognized complication of primary antibody deficiency and one of the primary causes of morbidity and mortality both in CVID and XLA patients. In our Italian cohorts a high prevalence of patients with CLD was present at time of diagnosis in XLA (38%) and CVID (34%), with an increased percentage during follow-up either in adult and younger age groups (3, 8). Duration of follow-up has been shown as the only significant variable on CLD development (8). Of interest, immunoglobulin replacement treatment did not control the increased number of XLA and CVID patients with CLD and chronic sinusitis. Several studies, including the one by IPINet, report that the severity of CLD is related to a delay of diagnosis, suggesting that it may develop as a consequence of chronic pulmonary infections and lack of an appropriate aggressive therapy (3). Also, the progression of chronic pulmonary disease remains a significant problem for these patients even after starting immunoglobulin replacement therapy.

Bronchiectasis remains a disease with poor international guidelines for its management, airway clearance techniques and antibiotic therapy. Recently, a primary care summary of the British Thoracic Society has been published on the management of non-cystic fibrosis bronchiectasis to promote a higher level of knowledge and care (9). An existing Cochrane Review showed that continued cycles of antibiotic therapy are effective in reducing the volume and purulence of sputum and the ongoing airways inflammation (10).

IPINet records show that, whereas the use of antibiotic prophylaxis is heterogeneous in CVID patients, it increases up to 50% in XLA patients with chronic lung disease (Table I, revised data from IPINET-AIEOP database). Since its introduction, IPINET recommendations have been continuously evaluated and revised according to biannual meetings among participating centers (http://www.aieop.org/?q=node/367). Since it has been suggested that though IgG serum levels >800 mg/dl may lower the incidence of bacterial infections (3, 8, 9) we have also increased the monthly i.v. and s.c. immunoglobulin dosage in approximately 15% of patients (8). However, the IgG levels did not correlate with the development of chronic lung disease and the potential benefit of a more aggressive treatment, including antibiotic prophylaxis and continuous cycles of respiratory rehabilitation have been raised. Accordingly, in XLA and CVID Italian patients continuous antibiotic prophylaxis is currently recommended with a program of respiratory physiotherapy, as prescribed by the physiatrist, in patients presenting more than one episode of bronchopneumonia a year (recurrent BPN) or in those with chronic bronchopneumopathy or chronic bronchitis. In addition, a patient education program is adopted to make the patient aware of his disease and related complications and to learn how to manage exacerbations. Further, HRCT has been proven very useful in delineating the extent of lung damage. The correlation between Bhalla score, clinical findings and the favourable outcome of the disease have suggested that in most IPINet patients chest HRCT should not be repeated annually (11,

12).

As reported by Freeman and Holland, many clinicians elsewhere associate antimicrobial prophylaxis with lung physiotherapy and immunoglobulin replacement therapy in patients with chronic sinusitis and bronchiectasis. However, some of them start antibiotic prophylaxis when recurrent infections exceed three per year during intravenous immunoglobulin (IVIG) therapy or in the presence of severe infections; others administer prophylaxis to patients with severe antibody deficiencies on IVIG; whereas a third group of specialized centers treat only acute infections (13). In XLA-patients no data are available about the efficacy of antimicrobial prophylaxis due to the different standards in medical practice in various countries. Also, the risk of bacterial resistance makes this practice often poorly widespread. The prolonged administration of a single drug (i.e. cotrimoxazole or amoxicillin) is favoured instead of a cyclic use of antibiotics because of a lower risk of resistance (13).

Therefore, in addition to the practice of higher serum IgG levels (> 600-800 mg/dl) obtained by replacement therapy, few specialized centers adopt antibiotic prophylaxis according to the different type of bacteria isolated from sputum, although it is still unclear when and in what patients this therapy should be performed (14). It has recently been described that azithromycin may improve lung function and reduce pulmonary exacerbations not only due to its antimicrobial effect on gram-positive bacteria and atypical pathogens, but also due to its anti-inflammatory properties. In a recent study investigating the effect of antibiotic therapy with erythromycin (250 mg once daily), the authors reported 50% fewer exacerbations than in the previous 12 months and a decrease of days of antibiotic administration per year after 12 months' treatment (15). Indeed, macrolides prevent the development of the biofilm produced by bacteria that colonize the respiratory epithelium (16).

Selective IgA Deficiency is the most common immunological disease with an incidence of 1:400-600 in the general population. The presence of low IgA values (<7 mg/dL) in a child older than four years with normal values of other immunoglobulin isotypes represents the main diagnostic criteria. Patients are usually asymptomatic and the diagnosis is often accidental, however recurrent respiratory and gastrointestinal infections, allergies, autoimmune conditions and malignancies may occur (17). Respiratory infections are mostly caused by bacteria, such as *Haemophilus influenzae* and *Streptococcus pneumoniae*, however Giardiasis is also frequent. Higher incidence of severe infections and complications is observed in patients with associated antibody deficiency such as IgG subclass deficiency. Since IgA deficiency is usually asymptomatic, treatment is often unnecessary. Antibiotic therapy is usually performed during infectious episodes but in the presence of recurrent infections continuous or intermittent antibiotic prophylaxis may be attempted (17).

The IPINet recommends that in patients with IgA deficiency and recurrent/severe infections respiratory physiokinesitherapy and prompt antibiotic treatment should be considered; continuous antibiotic prophylaxis may be indicated in severe cases. In Table I use of antibiotic prophylaxis at time of diagnosis is reported; the recent introduction of the IPINet protocol for selective IgA deficiency does not allow extensive follow-up data yet.

Transient Hypogammaglobulinemia of Infancy (THI) is an heterogeneous disorder characterized by reduced serum IgG values (below 2 SD of normal values for age) in early childhood. THI can be only suspected after the exclusion of other causes of hypogammaglobulinemia (drugs, genetic disorders, chromosomal abnormalities, infectious diseases, cancer, systemic diseases) and the definitive diagnosis can only be made a posteriori with the normalization of IgG levels which usually occurs by 24 months of life. Patients suffer from recurrent infections, allergies and autoimmune diseases (18, 19). Actually, no data are available to give specific guidelines, however a supportive therapy and appropriate antibiotics are sufficient during acute infectious episodes for symptomatic patients. In Table I data on the use of antibiotic prophylaxis in THI patients, recorded in the context of IPINet, are reported.

In patients with humoral immunodeficiencies inactivated or acellular vaccines can be administered with no risk in the early months of life when the diagnosis has not been ascertained yet, however the type and severity of their defect may affect their effectiveness (18) (Table II). Children and adults with

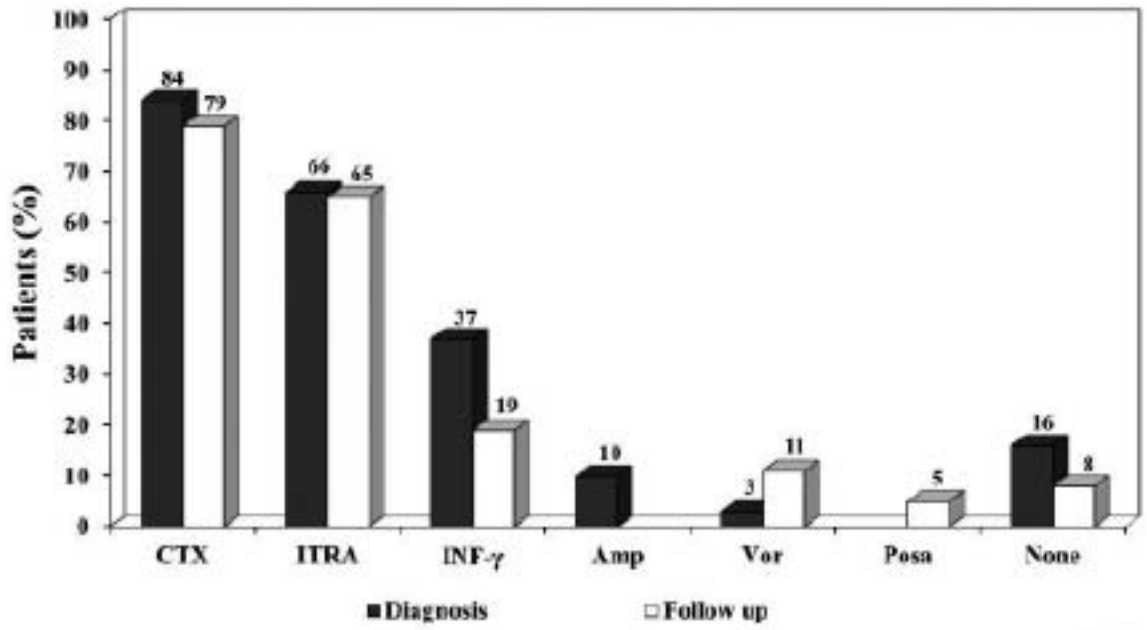

Fig. 1. *Anti-infective prophylaxis in CGD patient at diagnosis and follow-up. IPINet-AIEOP data. (Update Dec 2012).* ***CTX****: Cotrimoxazole;* ***ITRA****: Itraconazole;* ***Amp****: Amphotericin B;* ***Vor****: Voriconazole;* ***Posa****: Posaconazole.*

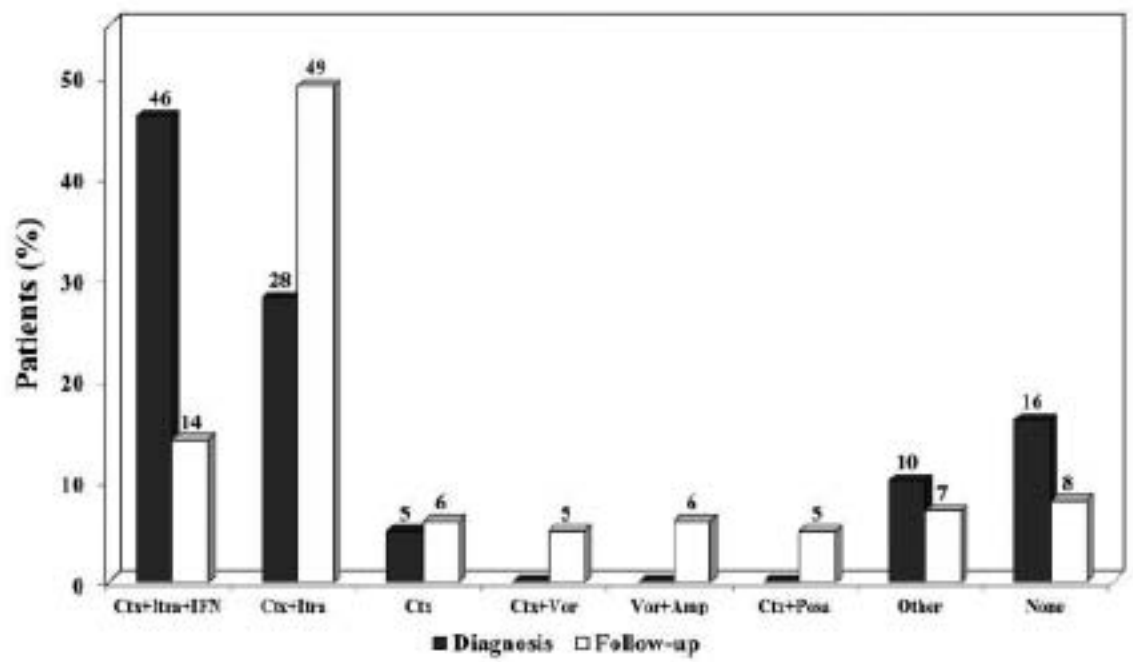

Fig. 2. *Analysis of prophylactic regimens performed at the time of diagnosis and follow-up in CGD patients according to IPINet-AIEOP Registry data (Update Dec 2012).* ***Ctx****: Cotrimoxazole;* ***Itra****: Itraconazole;* ***Vor****: Voriconazole;* ***Amp****: Amphotericin B;* ***Posa****: Posaconazole.*

bronchiectasis should receive an annual influenza vaccination and pneumococcal vaccination 5-yearly after completion of their primary series. Not only vaccination represents a critical preventive strategy in the control of infectious diseases in the healthy and PID patient (see Table II for recommended vaccines in selected PIDs) but also a major diagnostic tool in the clinical evaluation for immune defects. Measurement of functional antibody responses may provide a useful criteria to assess those patients requiring immunoglobulin replacement therapy. Diagnostic vaccination is better evaluated with those antigens where tests to measure antibody response and interpretation are available. Indeed, the use of pneumococcal polysaccharide vaccine is warranted to ascertain response to polysaccharide antigens whereas tetanus toxoid to investigate response to protein antigens. Live viral vaccines are contraindicated in selected immune defects (18, 19) as reported in Table II.

IMMUNODEFICIENCY SYNDROMES

Di George Syndrome

Several genetic disorders are associated with immunodeficiency that contributes to worsen the prognosis.

Di George Syndrome (DGS) is commonly associated with chromosome 22q11 deletion and less frequently with chromosome 10p13 deletion. The majority of patients have an impaired development of the thymus and parathyroid glands, congenital heart defects, palatal anomalies, hypocalcemia, facial dysmorphisms, feeding difficulties and psychiatric disorders. According to the severity of the immunological defect, patients can be divided into two groups: patients with partial immunological defect (pDGS) and patients with complete immunological defect (cDGS). cDGS form is rare (0.5-1.5%) and presents with a picture of severe combined immunodeficiency, characterized by marked lymphopenia or no T-cell responsiveness; humoral defects are typical. The immunological defect in pDGS is generally modest. Indeed, patients show a continuum of immunological defects between these two extremities. TCR rearrangement excision circles (TRECs) levels may be useful to monitor thymic function in this syndrome (20). Antibody concentration as well as the specific antibody response after vaccination may be normal or reduced (20). Children with an impaired response to polysaccharide antigens usually present recurrent pulmonary infections. Since for the majority of DGS cases immunodeficiency is not severe and may improve with age, prophylactic antibiotics may be unnecessary in these cases. Conversely, prophylaxis against *Pneumocystis jiroveci* is recommended in patients with a significant defect of $CD4^+$ cells as reported for children with HIV infection: it should be started when $CD4^+$ cells are $<750/mm^3$ in children <12 months, $<500/mm^3$ in children aged between 1-5 years and $<200/mm^3$ in children over 5 years of age. Prophylaxis should be discontinued once $CD4^+$ cell counts reach normal values. The clinical and immunological status of Italian patients is quite heterogeneous and, as reported in Table I from IPINeT registry, approximately 15% (22/147) of patients with DGS suffer a severe T-cell lymphopenia and use antibiotic prophylaxis (21). According to IPINet recommendations children with DGS may have an enhanced susceptibility to infections which is partly due to the immune defect and partly to the facial anomalies favoring recurrent upper airway infections. Prompt aggressive antibiotic treatment is recommended in all cases to control acute infectious episodes. Antibiotic prophylaxis can be administered in the case of recurrent infections and the duration must be determined in individual cases by the attending physician.

Administration of vaccines with purified antigens (against *Tetanus, Diphtheria, Pertussis, Hepatitis, Hemophilus influenzae, Influenza, Pneumococcus, Meningococcus*) are recommended and immunization carries no risk (Table II). However, the extent of vaccine antibody response depends on the severity of the immunological defect (21).

CONGENITAL DEFECTS OF PHAGOCYTES

Chronic Granulomatous Disease

Neutrophils are the first line of defense against bacterial and fungal infections. A numerical and functional defect of these cells leads to a marked susceptibility to infections that are prone to become chronic and poorly responsive to antibiotics. Infections are localized in the skin, mucous

Table I. *Use of antibiotic prophylaxis in XLA, CVID, THI and DEL22 patients: results from IPINet-AIEOP Registry (as at Dec 2012).*

Primary Immunodeficiency	Total patients	Number (%) of patients on prophylaxis	Antibiotic prophylaxis (isolated or combined)*
XLA	145	41 (28,3%)	***24 patients with chronic lung disease:*** 3- cyclic use of **Cefaclor – Cotrimoxazole – Clarithromycin** 3- **Cotrimoxazole** 3- **Clarithromycin** 2- cyclic use of **Clarithromycin- Cotrimoxazole** 2- **Ciprofloxacin** 2-**Amoxicillin/Clavulanic Acid** 1-cyclic use of **Cotrimoxazole– Clarithromycin – Amoxicillin/Clavulanic Acid** 1- cyclic use of **Cotrimoxazole–Amoxicillin** 1- cyclic use of **Cefixime - Ciprofloxacin** 1- cyclic use of **Amoxicillin/Clavulanic Acid – Levofloxacin** 1- cyclic use of **Amoxicillin/Clavulanic Acid – Pentamidine** 1- **Cefaclor** 1- **Amoxicillin** 1- **Azithromycin** 1- **Levofloxacin** ***14 patients without chronic lung disease:*** 2- cyclic use of**Amoxicillin-Cefaclor-Cotrimoxazole** 2- cyclic use of**Clarithromycin – Amoxicillin/Clavulanic Acid** 2- **Amoxicillin/Clavulanic Acid** 2- **Azithromycin** 1-cyclic use of **Amoxicillin-Cefaclor-Clarithromycin** 1- cyclic use of **Amoxicillin-Cotrimoxazole-Amoxicillin/Clavulanic Acid** 1- cyclic use of **Cefuroxime Axetil- Amoxicillin/Clavulanic Acid** 1- **Cotrimoxazole** 1- **Ciprofloxacin** 1- **Levofloxacin** N.B. chronic lung disease not specified in 3 patients treated with Amoxicillin/Clavulanic Acid
CVID	578	49 (8,5%)	12- **Azithromycin** 10- **Cotrimoxazole** 5- **Clarithromycin** 4- **Amoxicillin/Clavulanic Acid** 3- **Ciprofloxacin** 2- **Amoxicillin** 2-**Diaminocillin** 2- **Erythromycin** 2- **Levofloxacin** 1- cyclic use of **Cefaclor – Cotrimoxazole. Erythromycin** 1- cyclic use of **Cefaclor – Erythromycin- Cotrimoxazole** 1- cyclic use of **Amoxicillin/Clavulanic Acid - Ciprofloxacin** 1- cyclic use of **Amoxicillin - Cotrimoxazole** 1- **Cefaclor** 1- **Clindamycin** Not specified in 1 patient
THI	86	12 (10,8%)	4- **Amoxicillin** 3- **Cefaclor** 2- **Cotrimoxazole - Trimethoprim** 1-**Amoxicillin/Clavulanic Acid** Not specified in 2 patients
DEL22	284	22 (7,7%)	10- **Cotrimoxazole** 6- **Amoxicillin/Clavulanic Acid** 2- **Azithromycin** 1- cyclic use of **Cefaclor – Amoxicillin/Clavulanic Acid** 1-**Diaminocillin** 1- **Cotrimoxazole-Fluconazole** 1-**Amoxicillin**

** the number of patients exceeds the total because some patients had a combined prophylaxis*

Table II. *Vaccinations in selected primary immunodeficiencies.*

	Specific immunodeficiency	Recommended vaccines	Contraindicated vaccines
B-lymphocyte defects	Severe antibody deficiencies (e.g.XLA, CVID)	Pneumococcus Influenza (TIV) Consider measles and varicella vaccinations.	LAIV BCG Salmonella
	Less severe antibody deficiencies (selective IgA deficiency, THI)	Pneumococcus Influenza (TIV)	BCG Salmonella
T- lymphocyte defects	Complete defects (e.g. SCID, complete DiGeorge Syndrome)	Pneumococcus Influenza (TIV)	All live vaccines * °
	Partial defects (e.g. Wiskott-Aldrich Syndrome, Ataxia-telangiectasia, IPEX Syndrome)	Pneumococcus Meningococcus Haemophilus Influenza (TIV)	All live vaccines * °
Phagocyte defects	CGD	Pneumococcus Influenza (TIV) Meningococcus	Live bacterial vaccines °

** Live viral vaccines: measles-mumps-rubella, live attenuated influenza vaccine (LAIV), varicella;*
° Live bacterial vaccines: BCG, Salmonella typhi.

membranes and lymph nodes, that are the first barriers against microbial invasion; from these sites they can spread to the rest of the body (22).

Chronic Granulomatous Disease (CGD) is a rare inherited disorder of the phagocytes of the innate immune system that is caused by defects in NADPH oxidase enzyme complex. The disease is due to a mutation in any of the genes encoding NADPH oxidase subunits. The mutations in X-linked CYBB gene, encoding the gp91 phox, account for approximately two thirds of CGD cases. Mutations of NCF1 gene, encoding the p47, p22 and p40 phox subunit, are mostly responsible for the autosomal recessive forms. The defective NADPH oxidase leads to an impaired anti-microbial response, particularly to intracellular microorganisms. Fungi, i.e *Aspergillus* species; yeasts, i.e. *Candida* species; and several bacteria, such as the most prominent *Staphylococcus aureus*; Gram-negative bacteria, i.e. *Escherichia coli*, non-typhoid *Salmonella* species, *Klebsiella pneumonia*, *Serratia marcescens* and *Burkholderia cepacia*; and more rare species (*Mycobacteria*, *Actinomyces israelii* and *Peptostreptococcus*) may be isolated (23).

Patients with CGD suffer from recurrent life-threatening bacterial and fungal infections with formation of abscesses and from abnormally autoinflammatory responses leading to granuloma formations in multiple organs. In our IPINet cohorts the majority of patients were symptomatic before the age of 1 year and lymphadenitis was the most common clinical feature. Pneumonia, skin and oro-gastrointestinal infections were other causes of morbidity (23). After the diagnosis of CGD is made, there is a general consensus on the use of long term oral prophylaxis with antibacterial and antifungal drugs to reduce the rate of infections. Although a further reduction of the infection rate has been described with IFN-γ (50 mg/m^2 s.c. with a body surface >0.5 m^2; 1.5 mg/kg/dose s.c. with a body surface <0.5 m^2; for 3 days/week), the efficacy of its use for prophylaxis has been much debated. Our first extensive survey of CGD in Italy in the context of Italian Primary Immunodeficiency Network- Italian Association of Pediatric Hematology and Oncology studies (IPINet-AIEOP) has not provided evidence of efficacy (24). Different standard medical procedures adopted in various countries and/or composition of

patient cohorts with changing clinical expression of the disorder over time might be some of the factors causing conflicting observations. The most common prophylactic schedule commences with trimethoprim/sulfamethoxazole (co-trimoxazole, CTX), at a dosage of 6-8 mg/kg/die of trimethoprim orally in two doses to a maximum of 160 mg daily. The development of antimicrobial resistance is a rare event. In the case of patients with allergy or intolerance to CTX, desensitization could be performed (13). Alternatively the dicloxacillin (25-50 mg/kg/die orally in 4 doses) could be used. The CTX is contraindicated in patients with G6PD deficiency. Second and third generation of cephalosporins can prevent most infections caused by gram-negative bacteria and methicillin-sensitive *Staphylococcus aureus*. Antifungal prophylaxis is adopted with itraconazole (in children: 10 mg/kg/die orally in two doses, up to a maximum of 200 mg/day <12years and 400 mg/day >12 years), also active against *Aspergillus spp* (25). It is preferable to use the syrup than the capsules for bioavailability reasons. Recently, the use of voriconazole and posaconazole looks promising for their spectrum of antifungal coverage and absorption (13). Last IPINet follow-up data from CGD patients show an higher adherence to international recommendations with a more popular use of cotrimoxazole and itraconazole as prophylactic regimens (Fig. 1, unpublished data, courtesy of Dr. B. Martire). Moreover, the same registry reported a reduction in the use of IFN-γ from 37% to 19% (Fig.1) and the introduction of voriconazole or posaconazole in combination with cotrimoxazole prophylaxis (Fig.2). CGD is not a contraindication to regular vaccination schedule except for live bacterial vaccines. Flu vaccine, anti-pneumococcal and anti-meningococcal vaccines are yearly recommended as for other PIDs (Table II) (26).

OTHER DEFECTS

Ataxia-Telangiectasia; Wiskott-Aldrich syndrome; IPEX syndrome

Ataxia-Telangiectasia (AT) is an autosomal recessive disease that usually presents with progressive cerebellar ataxia in early life. It is due to a defect of the ataxia–telangiectasia mutated (ATM) protein kinase which has a crucial role in the coordination of the cellular response to DNA damage. Recently, ATM has been also identified as a redox sensor that controls the levels of reactive oxygen species; the increased oxidative stress in cells due to ATM deficiency is likely to contribute to neurodegeneration, metabolic dysregulation and oncogenesis (27).

AT is characterized by cerebellar degeneration, progressive ataxia, oculo-cutaneous telangiectasia, primary immunodeficiency, type 2 diabetes and an increased incidence of lymphoid tumorigenesis. The immune deficiency is expressed with progressive decrease of T-lymphocyte numbers and function and hypogammaglobulinemia (28). In addition to the immunodeficiency, mechanical problems (pneumonia ab ingestis caused by impaired swallowing; diminished chest expansion and difficulty in walking due to neurological problems) account for the increased susceptibility to infections. The most commonly isolated bacteria are *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Pseudomonas aeruginosa*. The IPINet protocol for AT recommends prophylaxis against *Pneumocystis jiroveci,* in case of a significant CD4+ defect, as in children with HIV infection, with CTX (6 mg /kg/day of trimethoprim orally in one or two doses) or azithromycin (500 mg/day for 6 days, 250 mg/day for 6 days, then 250 mg Monday/Wednesday/Friday every week) (29). However, *Pneumocystis jirovecii* infection is extremely rare and controlled studies are needed. Immunoglobulins are only used in those patients with hypogammaglobulinemia or specific antibody deficiency when antibiotic prophylaxis does not prevent recurrent respiratory infections (29). The use of active immunoprophylaxis with pneumococcal and meningococcal conjugates, and anti-Haemophilus influenzae vaccines is recommended (Table II) (29).

The Wiskott–Aldrich Syndrome (WAS) is a X-linked primary immunodeficiency disorder characterized by the triad of thrombocytopenia, eczema and progressive immunodeficiency. However, only one third of the patients show the triad during their infancy. Older WAS subjects have an increased risk for autoimmune disorders and lymphoid malignancies. Clinical manifestations and severity of the disease depend on the type of mutations in WASP gene. X-linked Thrombocytopenia (XLT) represents a milder variant of WAS. The

identification of WASP mutations in XLT confirms that XLT and WAS are allelic diseases. WASP is a member of a family of proteins involved in signaling and cytoskeletal organization whose defect affects immune cell effector function and immunological synapse formation. A scoring system (0 to 5) has been proposed by Zhu et al. to help in the diagnosis, treatment and prognosis of these disorders (30).

WAS patients have an increased susceptibility to bacterial infections, but also to viral and fungal infections (31). There is a particular risk of infections caused by encapsulated bacteria because of the reduced ability to produce antibodies of the IgG2 subclass against polysaccharide antigens. *Herpes simplex* type 1 and 2 may cause recurrent infections, severe and/or disseminated, but warts and *Molluscum contagiosum*, both difficult to treat, are also common. Fungal infections and opportunistic infections, especially pneumonia caused by *Pneumocystis jiroveci*, have been reported with considerable frequency (32). The clinical management of these patients is complex and variable. Some specialized Centers use IgG replacement to reduce the risk of infections. Antimicrobial prophylaxis with CTX is commonly used to reduce or eliminate the risk of interstitial pneumonia caused by *Pneumocystis jiroveci* (cotrimoxazole: 6 mg/kg/day of trimethoprim orally in one or two doses). There is no general consensus on antiviral prophylaxis; however IPINet protocol recommends prophylaxis with Acyclovir (5 mg/kg/dose orally every 8 hours) for those patients with a history of at least one episode of severe HSV infection. Some patients survive into adulthood, but most of them die around 10 years of life due to bleeding, tumors, infections and complications of transplantation, which still represents the ultimate therapeutic option (32, 33). Active immunizations against *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Neisseria meningitidis* and Flu are recommended in both splenectomized and not splenectomized patients (Table II).

The IPEX Syndrome is a rare X-linked genetic disorder caused by FOXP3 mutation, a transcriptional regulator with a key role in the development of CD4 regulatory T (Treg) cells. Treg deficiency or dysfunction alters immune homeostasis and self-tolerance leading to severe, multi-organ, autoimmune phenomena including enteropathy, chronic dermatitis, endocrinopathy and other organ-specific diseases including anaemia, thrombocytopenia, neutropenia, hypothyroidism, thyroiditis, nephritis and hepatitis (34). Many of these patients have a history of serious infections such as sepsis, meningitis, pneumonia and osteomyelitis caused by enterococci, staphylococci, Candida and CMV pathogens (35). According to IPINet protocol, antimicrobial prophylaxis must be tailored for each patient in consideration of potential skin and intestinal infections, and sepsis secondary to central venous catheter insertion which is required for parenteral nutrition and i.v. drug administration. Needless to say, these patients have such a debilitating status that an infectious episode may be fatal. In Table II are reported recommended vaccinations for this disorder.

CONCLUSIONS

The existing literature is limited in the field of antibiotic prophylaxis in PIDs and what is known is mainly based on single institution-specific experience without controlled measurements of patient safety and quality health outcomes. There is an urgent need to standardize antibiotic prophylaxis in distinct PIDs according to evidence-based practices. Implementation of a networking among researchers in the field of PIDs is designed to enable clinicians to translate knowledge in high-quality health-care. In this context the IPINet model represents an useful platform to promote further studies and find consensual strategies for an optimal clinical management of PID patients, including antibiotic prophylaxis, with a strong impact on public health.

The growth in size and importance of National and International Clinical Networks, combined with their integration and adequate interest of pharmaceutical companies, will allow these studies to be successfully performed.

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EDITORIAL

THE ROLE OF OXYTOCIN IN PLASTICITY, MEMORY AND ATTACHMENT DYNAMICS

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The peptide hormones oxytocin (OT) and arginine vasopressin (AVP) have been implicated in the regulation of mammalian social behavior. There is considerable evidence implicating both oxytocin and vasopressin in social recognition and social memory. This review explores their role in attachment dynamics. Oxytocin is one element in a complex network of interactions observed in natural phenomena ranging from molecular biology, etology, social behavior and human psychology.

The cognitive neuroscience of attachment dynamics is a complex and multidisciplinary field (1-2). Following Bowlby's observations (3) on attachment behavior, the mother-infant bond has been studied as a strong coupling organization mainly regulated by its intra-systemic emotional signals. In a series of studies on rodents and primates, Myron Hofer and other developmental biologists investigated several hidden regulatory mechanisms (4). Hidden physiological factors act on different sensory channels: nutritional, olfactory, tactile, thermal, visual and vestibular. For example, the body temperature of infant rats is largely determined by the body temperature of the mother. Body temperature has been proven to regulate levels of brain peptides, nucleic acids and neuro-amines.

In humans, these regulatory mechanisms are presumed to continue in adult life, and their functioning becomes more intermingled with other emotional, cognitive and social factors. This review focuses on the role of oxytocin in the evolution of psycho-social attachment bonds. Works by Margaret Mahler (5) on the vicissitudes of human symbiosis and contributions of Bowlby (3), Winnicott (6), and Orsucci (2-7) can be a general psychological framework for these findings based on multidisciplinary research.

Harlow (8) described different behavioral processes involved in the formation of parent–infant, filial, and pair (couple) bonds. Each of these bonds is based on multi-sensory processing (predominantly olfactory in rodents and visual in primates) and complex motor responses (for example, proximity seeking, nurturing responses, and defensive behaviors).

In animals, attachment can be studied as proximity seeking, social preference or separation response. In humans, the ultimate form of attachment is what is experienced as love, in its different forms.

Recent studies have begun to show the neural mechanisms for social recognition, nurturing behavior, and social preferences. Studies on the neural basis of attachment are based on three main features.

Key words: oxytocin, vasopressin, plasticity, attachment, complexity, autism

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DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

- First, observing a clear onset of specific behavior in order to identify factors that initiate, inhibit, stabilize or discontinue attachment bonds.
- Second, attachment behavior must be, by definition, selective and enduring. Selectivity distinguishes attachment from generalized social interaction. Duration distinguishes a bond from a transient preference.
- Finally, opportunity and capacity to measure and manipulate these behaviors.

Studies on social affiliation and attachment have spanned from gene targeting in *Caenorhabditis elegans* worms to psychodynamic approaches in humans.

Imprinting

The study of neural mechanisms that underlie infant attachment has progressed furthest in species with early offspring. Within a discrete developmental window, the newly hatched chick shows visual imprinting, an enduring selectivity for following the mother (or a mother-like object) that can be quantified with great accuracy.

Imprinting in the chick is not a single process but consists of at least three largely independent processes.

- First, there is the approach response, which is associated with increased arousal and inhibition of avoidance.
- This is followed by the acquisition or learning stage when chicks form a long-term memory for the imprinted stimulus, a stimulus that is partially pre-specified.
- Finally, marking the end of the sensitive period for learning, there is a reversal of the approach–avoidance bias as chicks begin to avoid new objects while continuing to follow the familiar imprinted object.

There is a region within the intermediate medial part of the hyperstriatum ventrale of the chick brain critical for acquisition and early consolidation of the memory of an imprinted visual stimulus. A related region, the mediorostral neostriatum, responds selectively to imprinted auditory stimuli. In the chick brain the learning phase of imprinting involves enhancement of pre-synaptic release of new amino acids, as well as changes in post-synaptic mechanisms.

Studies on infant attachment in mammals have not yet identified a specific neural circuit or a predominant neurochemical system modulating ultra-structural changes that are necessary for imprinting; however recent studies with chicks, rats, sheep, voles and humans have now begun to reveal some important candidates for the neurobiology of social attachment.

Oxytocin and mother-infant attachment

Of the roughly 100 neuropeptides described in the mammalian brain, most are synthesized and released from the hypothalamus, often with peripheral effects as endocrine hormones.

Neuropeptides usually interact with G-protein coupled receptors through which they act as slow neurotransmitters or neuromodulators. Nonapeptides are one of the oldest families of neuropeptides: each with nine amino acids and a genetic structure that includes a large precursor protein known as neurophysin.

Across these diverse species, three aspects appear to be conserved (9):

(a) nonapeptides are usually expressed selectively in brain and gonads;

(b) nonapeptides and their receptors are influenced by gonadal steroids, seasonality, and gender; and

(c) nonapeptides are important for social behavior, often in a highly species-typical fashion.

The peptide hormones oxytocin (OT) and arginine vasopressin (AVP) have been implicated in the regulation of mammalian social behavior. OT and AVP are highly conserved across species in terms of structure and function (10).

Both are composed of nine amino acids (sharing seven in common) and have peripheral and central effects. AVP modulates a number of behaviors exhibited only by males (11) while OT is more closely involved in female behavior.

Peripherally, OT regulates uterine contractions during labour and milk ejection during lactation. It is synthesised in magnocellular neurons of the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus which project to the posterior pituitary where OT is released into peripheral circulation. Peripheral effects are typically measured by plasma radioimmunoassay. Centrally, OT acts as a neuromodulator. It is synthesised in the parvocellular

neurons of the hypothalamic PVN which projects to limbic sites (hippocampus, amygdala, striatum, hypothalamus, nucleus accumbens) and to mid- and hind-brain nuclei. Central OT is typically measured in cerebrospinal fluid. Central OT release and neuronal activity can be elicited by sexual and reproductive stimuli (copulation, genital and breast stimulation, birth, olfactory stimuli, suckling) and non-sexual stimuli including grooming, light massage and exposure to offspring. Stimulus-induced and peripherally or intra-cranially injected OT facilitates milk ejection, uterine contractions, parturition, lordosis, copulation, ejaculation, maternal behaviors, partner and offspring preference and social contact (12).

Oxytocin is of special relevance to females because OT and OT receptors are regulated by estrogen (13). Estrogen receptor β is expressed in hypothalamic neurons that synthesise OT and estrogen receptor α is needed for the synthesis of OT receptors in the amygdala (14). Gonadal steroids play a facilitating role in modulating central and peripheral OT synthesis and OT receptor proliferation, density and affinity.

In mammals, maternal behavior presents many differences from species to species, ranging from species that spend only few minutes each day in contact with their young to species showing maternal care all across the life cycle. Nevertheless, there are some species, such as rats, whose maternal care is restricted to the post-partum phase. These species provide an optimal and restricted setting for the study of neural mechanisms of mother-infant attachment. For example, neuropeptides involved

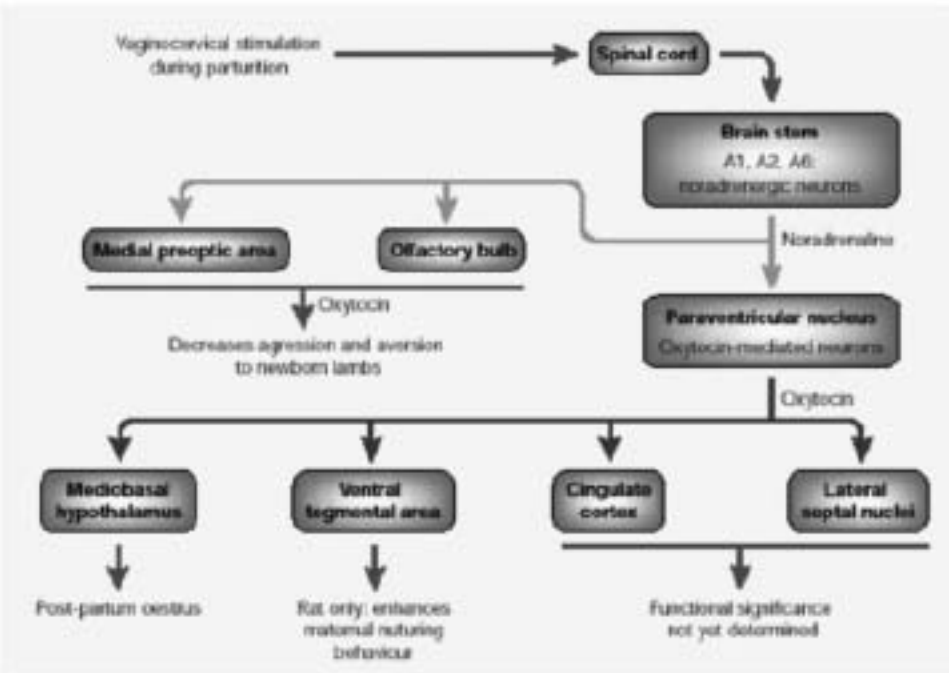

Fig. 1. *Oxytocin and maternal behaviour in the sheep brain. Within 2 hours of parturition the ewe develops a selective, permanent bond to her lamb. One neurobiological model for this process posits that afferent stimulation through the spinal cord from vaginocervical dilation during parturition increases the activity of noradrenaline cells in the brainstem (A1, A2 and A6), which project to the paraventricular nucleus (PVN) in the hypothalamus, as well as to the olfactory bulb. Stimulation of oxytocin cells in the PVN facilitates maternal behavior through coordinated effects on several regions in which oxytocin increases GABA (-aminobutyric acid) and noradrenaline release. Oxytocin in the olfactory bulb and medial preoptic area reduces aggressive or aversive responses to newborn lambs. Oxytocin in the mediobasal hypothalamus inhibits post-partum oestrus. On the basis of data from rats, oxytocin in the ventral tegmental area might facilitate the onset of maternal nurturing behaviors, although this has yet to be shown in the sheep brain. Projections to the lateral septum and cingulate cortex have not yet been studied for their functional significance. (Figure modified from Kendrick, 1997, with permission from Elsevier Science, and includes release data based on microdialysis studies and behavior data based on retrodialysis of oxytocin into regions noted.)*

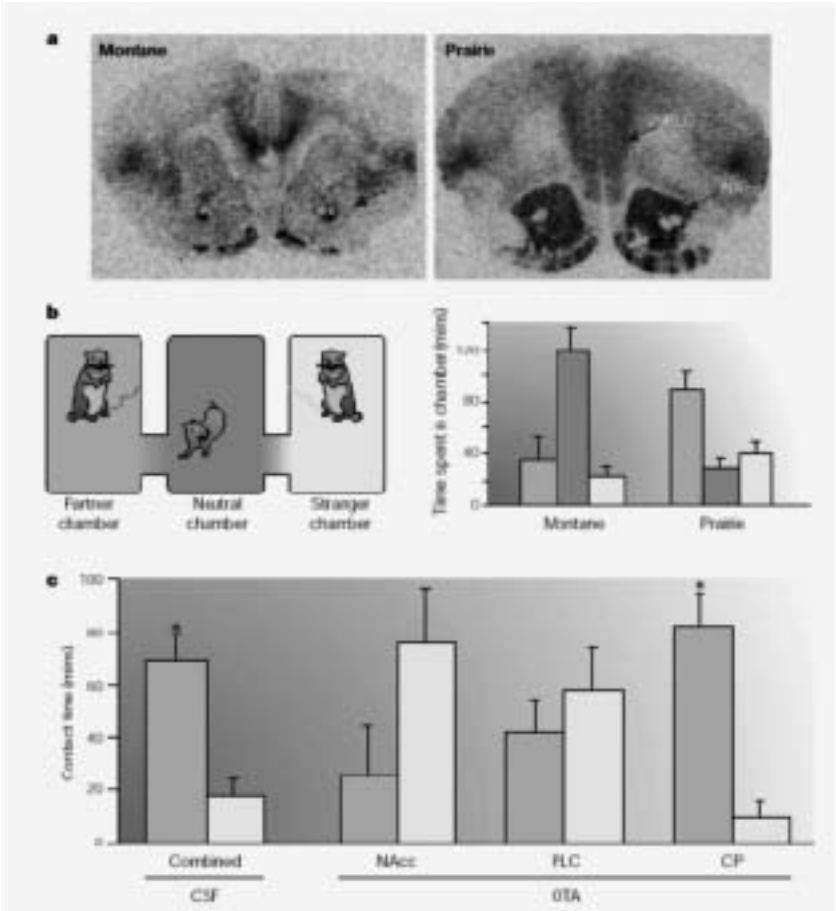

Fig. 2. *Oxytocin and social attachment in the monogamous prairie vole female.* ***a****: Prairie and montane voles have different distributions of oxytocin receptors in the brain, particularly in the nucleus accumbens (NAcc) and the prelimbic cortex (PLC; arrow).* ***b****: In the laboratory, pair bonding is assayed using a partner-preference test. After mating or cohabitation with a 'partner,' the experimental vole is place in a three-chambered arena in which the partner is tethered in one chamber and an equivalent non-familiar vole or 'stranger' is tethered in another chamber. The experimental animal has free access to both chambers. After mating, female prairie voles spend more time in the partner's chamber than in the neutral or stranger's chamber, indicating a partner preference. By contrast, mated montane voles show no preference for either the partner or the stranger, indicating the absence of a mating-induced social attachment.* ***c****: Partner preference formation is blocked in the mating female prairie vole by infusions of oxytocin receptor antagonist (OTA) into the NAcc as well as the PLC, whereas cerebrospinal fluid (CSF) into either area or OTA infused into the caudate putamen (CP) had no effect on partner preference formation. OTA had no effect on mating. (Panel c modified from Sharer, 2000).*

in lactation have been considered as central neuroendocrine mediators of rat maternal care.

The facilitation of maternal behavior by oxytocin might be mediated either through the ventral tegmental area, the medial preoptic area, or from within the olfactory bulb, as injections of a selective oxytocin antagonist into each of these sites can block the onset of maternal care.

In many mammals, such as rats, voles and sheep the onset of maternal behavior involves overcoming a natural avoidance of neonate odors. Differing from rats, sheep maternal care is highly selective and probably follows more rigorous models of attachment, considering that the post-partum ewe rejects any lamb that is not her own. This can be only partially explained by experimental studies showing how oxytocin mRNA and oxytocin mRNA receptors are increased regionally in the sheep post-

partum brain. Recent investigations have underlined that the form of permanent imprinting that occurs in sheep involves a reorganization of the olfactory bulb. In fact, there is an increase of mitral cells in the bulb that respond preferentially to lamb odors, particularly cells specifically responding to the ewe's own lamb. The process of olfactory learning can be strictly linked to the motivation for maternal care.

An interesting study by Strathearn, Fonagy, Amico and Montague (15) on how adult attachment predicts maternal brain and oxytocin response to infant cues is bridging the gap between infant and adult domains. They examined first-time new mothers to test whether differences in attachment, based on the Adult Attachment Interview, were related to brain reward and peripheral oxytocin response to infant cues. On viewing their own infant's smiling and crying faces during functional MRI scanning, mothers with secure attachment showed greater activation of brain reward regions, including the ventral striatum, and the oxytocin-associated hypothalamus/pituitary region. Peripheral oxytocin response to infant contact at 7 months was also significantly higher in secure mothers, and was positively correlated with brain activation in both regions. Insecure/dismissing mothers showed greater insular activation in to their own infant's sad faces. These results suggest that individual differences in maternal attachment may be linked with development of the dopaminergic and oxytocinergic neuroendocrine systems.

Oxytocin and adult-adult attachment

Studies on voles provide an animal model to study how changes in gene structure could modify the expression of regional receptors, and affects the functional response to endogenous or exogenous ligands. There is considerable evidence that implicates both oxytocin and vasopressin in social recognition or social memory.

Voles are rodents found in diverse habitats in North America. For social neuroscience, voles offer good models for comparative studies because closely related species exhibit marked contrasts in social organization and social behavior (16). Prairie voles (Microtus ochrogaster) and pine voles (Microtus pinetorum) are monogamous species living in burrows. Male prairie voles show a striking change in behavior following mating, including an enduring selective preference for their mate (increased affiliation), increased aggression towards other males (mate guarding), and increased paternal care (16).

These changes are not seen in mountain or meadow voles following mating, suggesting that these mating induced changes reflect pair bonding and are fundamental to monogamous social organization in prairie voles. As AVP and OT are released with mating (17), does either peptide have a role in the prairie vole's pair bond formation? AVP, given centrally to prairie vole males who have not mated, induces each of these pair bonding behaviors.

Why does the same peptide have such different effects in two closely related species? The neuroanatomical expression maps for the V1a receptor and the OT receptor are markedly different across vole species. In prairie voles, which pair bond follows mating, V1a receptors are highly concentrated in the ventral pallidum and OT receptors are expressed most heavily in the nucleus accumbens, both regions associated with reward and reinforcement. In montane voles or meadow voles, V1a and OT receptors are more heavily expressed in the lateral septum and amygdala. One model (Figure 2) would suggest that the neuropeptides are released in both species with mating but that the neurobehavioral consequences of mating are different because the neuropeptides are activating different pathways: in pair bonding species mating is reinforcing and leads to attachment. In non-pair bonding species, mating has no enduring effects (18).

Why the gender difference in response? There are no striking gender differences in receptor expression, yet the path to pair bonding appears preferentially to use AVP in males and OT in females, just as found for effects on many other socio-sexual behaviors in other species (19).

What are the downstream effects of AVP binding to its receptor in the ventral pallidum or OT binding to the nucleus accumbens? Wang and his colleagues have demonstrated the importance of an interaction between OT and dopamine, specifically the dopamine D2 receptor, in the nucleus accumbens for pair bond formation in female prairie voles (20-21-22). In the nucleus accumbens shell, the cellular output appears to involve cAMP (cyclic adenosine monophosphate)

signaling: decreased cAMP signaling facilitates pair bond formation and increased cAMP signaling or activation of PKA (protein kinase A) inhibits pair bond formation (23). The cellular nature of the enduring pair bond – whether epigenetic or some form of cellular plasticity - is not yet known, although an intriguing body of work from Wang's lab points to a role for dopamine D1 receptors in the maintenance of pair bonding (24).

Finally, perhaps the most fundamental question is how such closely related species could have evolved such different neuroanatomical receptor expression maps? In fact, the expression of V1a receptors and OT receptors are strikingly different across mammalian species, in contrast to most neuropeptide receptors which have conserved patterns of expression. This pattern may be more than coincidence that other monogamous species, such as marmosets and California mice (25) (Peromyscus californicus), resemble prairie voles in that they also have OT or V1a receptors in nucleus accumbens or ventral pallidum, areas respectively and these are associated with reinforcement and reward (25-26).

In summary, here we have evidence bridging from gene variation to cellular expression to neural network to affiliative behavior. The results are parallel in many ways to the C. elegans research on social feeding, which also involved a neuropeptide receptor and a genetic variant (27). There are three critical principles from the vole story that may be relevant to a molecular basis for social neuroscience. First, comparative studies have proven important: species differences reveal candidates for individual differences within a species.

Second, differences in neuropeptide receptor genes, thus far, appear more informative than differences in the genes for neuropeptides. Importantly, there are no evident differences in the levels or distribution of OT or AVP cells in different vole species. Third, genetic sequence differences, especially in promoter regions, can alter patterns of receptor expression and the geography of receptors in the brain appears to be, thus far, the best correlate of social organization. It follows from each of these principles that (a) one must be cautious about generalizing from one species to another, (b) measuring neuropeptide levels may not be as informative as mapping receptor expression patterns, and (c) the effects of administering neuropeptide agonists or antagonists will depend on receptor expression. Can the principles gleaned from these studies in nematodes and voles inform human social neuroscience?

What are the effects of OT and AVP on human social behavior? The absence of non-peptide agonists that readily cross the blood brain barrier has required investigators to administer the peptides intranasally to explore effects on behavior and cognition. Recognizing that negative studies may be less likely to be reported, the available published literature converges around the notion that OT increases trust, empathy, eye contact, face memory, and generosity (28-29-30). In line with animal research showing that OT reduces anxiety (31), there is also evidence that intranasal OT facilitates response to exposure therapy in people with social anxiety disorder(32). In an important extension of this work to neuroimaging, Kirsh and colleagues reported that OT reduces the amygdale activation following threatening stimuli (33). While not conclusive, the result suggest that the OT effect on amygdala activation could be more evident in the response to social threats (faces) versus non-social threats (scenes) (33). In fact, OT appears to reduce the amygdala response to emotional expression irrespective of valence (34).

The vole research on affiliation, the mouse studies of social cognition, and the human research suggesting pro-social effects of OT and AVP all lead to the question: are these peptides involved in autism? Autism is a developmental disorder with onset by age three of social deficits, absent or abnormal communication, and a tendency to repetitive or stereotyped behaviors. There have been three lines of evidence exploring the link between OT, AVP, and autism: genetics, plasma levels, and peptide treatment studies (35).

Oxytocin knockout mice, capable of full maternal behavior, shows a profound social amnesia without other evident cognitive deficits. In contrast to the sheep studies of olfactory memory, in these knockout mice a social stimulus elicits normal amounts of Fos activation in the olfactory bulb, but fails to activate the medial amygdala and its projection sites, the bed nucleus of the stria terminalis and the medial preoptic area. In the mouse brain, the medial nucleus of the amygdala is enriched with oxytocin receptors and in

the knockout mouse, injections of oxytocin into this region (but not into the olfactory bulb) restores social recognition.

Therefore, it remains possible that the absence of partner preferences in prairie voles treated with oxytocin and vasopressin antagonists results from an inability to recognize the partner, not from an absence of pair bonding. Wang et al. (27) have shown that D2 dopamine receptor blockade given just before a preference test does not interfere with recognition of the partner but, given just after mating, a D2 dopamine antagonist inhibits consolidation of the memory for the mate. Analogous studies have yet to be done with oxytocin or arginine vasopressin in prairie voles. Oxytocin has emerged as one candidate from studies in rats, sheep and monogamous voles, but we do not understand fully how this neuropeptide, released during nursing and copulation, fits into a systems model for motivation. Oxytocin is, at most, one element in a cascade. Endogenous opioids have been shown to influence affiliative and maternal behaviors in sheep and primates, possibly by stimulating oxytocin release or possibly by an independent effect on reinforcement.

Complexity

From this neurodynamicpoint of view, mammals are "embodied in the entirety of the brain-body dynamics", in a comprehensive biophysical identity (7). We find this definition more grounded and explicit, but in the same line of the approach suggested, with different nuances, as embodiment (36), proto-self (37), synaptic self (38).

Many biological systems consisting of interacting agents, ranging from bio-molecular complexes to social populations, are normally found in configurations where the ensemble is segregated into groups with specific functions (2-39-40).

While the evolution of individual elements is highly correlated inside each group, the collective dynamics of different groups is much more independent. Usually, clustering is a dynamic process, where groups may preserve their identity in spite of the fact that single elements are continuously migrating between them. The individual motion towards or away from clusters may also be controlled by the internal state of each element, which favors or inhibits grouping with other elements. This is observed in natural phenomena ranging from complex chemical reactions, where biomolecules react with each other only when they have reached appropriate internal configurations, to social systems, where the appearance of organizational structures requires compatibility between the individual changing states of the involved agents.

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EDITORIAL

LUTEOLIN INHIBITS MAST CELL-MEDIATED ALLERGIC INFLAMMATION

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Mast cells are ubiquitous in the body and multifunctional immune cells; they are known to be primary responders in allergic reactions, orchestrating strong responses to minute amounts of allergens. Mature mast cells perform important beneficial roles in host defense, both in IgE-dependent immune responses to certain parasites and in natural immunity to bacterial infection. In IgE-associated biological responses, the crosslinking of FcεRI-bound IgE with multivalent antigens initiate the activation of mast cells by promoting aggregation of FceRI. This cross-linking receptor-bound IgE by multivalent Ag initiates a cascade of intracellular reactions leading to mediator release such as proinflammatory mediators, chemokines and cytokines. Luteolin belongs to a flavone group of compounds called flavonoids, it has anti-oxidant properties, inhibits some cancer cell proliferation and exerts a regulatory effect on mast cell-mediated inflammatory diseases and allergy. Here, we report the impact of luteolin on mast cell activation.

Traditional medicine is becoming an increasingly attractive approach for the treatment of various diseases and natural compounds have been extensively studied. Cell cultures and animal tumor models demonstrate that a large number of natural compounds from the diet could lower cancer risk and some of them could sensitize tumor cells in anti-cancer therapies. Flavonoids are polyphenolic

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compounds that are ubiquitous and appear to play an important role in inflammation, allergy, cancer and impacting blood and microvascular endothelial cells (1). They are comprised of a large group of low molecular weight polyphenolic secondary plant metabolites and are found in fruits, vegetables, nuts, seeds, stems, flowers, roots, bark, tea, wine and coffee and are thus common substances in our daily diet. More than 8,000 different flavonoids have been isolated and are divided in eight groups: flavans, flavanones, isoflavanones, flavones, isoflavones, anthocyanidins, chalcones and flavonolignans. They may have beneficial health effects because of their antioxidant properties and their inhibitory role in various tumors. It is generally accepted that a diet rich in plant-derived foods contained flavonoids may play an important role in the decreased risk of chronic diseases and that the cancer chemopreventive effects of green tea are mediated by its abundant polyphenol, epigallocatechin gallate (2-3). Flavonoids play an important role in defending plant cells against microorganisms, insects, and UV irradiation, and they are also beneficial to human health (4). Flavonoids are antimicrobial agents, antioxidants, estrogenic regulators, and posses anti-cancer activity interfering with regulation of cell cycle (at the G1/S or G2/M), apoptosis, invasion, metastasis, and angiogenesis, through the inhibition of kinases (5). Flavonoids have also been shown to suppress cysteinyl leukotriene synthesis, an arachidonic cascade compound, through an inhibition of phospholipase A_2 and/or 5-lipoxygenase (6). However, the biological activities of these natural compounds are generally not potent enough and higher concentrations would be required to achieve the expected biological effects and the results of some studies do not support the hypothesis that intake of flavonoids is protective against cancer risk (7).

Luteolin, 3′,4′,5,7-tetrahydroxyflavone (Fig. 1), belongs to the flavone group of compounds called flavonoids, has a C6-C3-C6 structure and possesses two benzene rings (A, B), a third, oxygen-containing (C) ring, and a 2−3 carbon double bond. Luteolin is heat stable and losses due to cooking are relatively low (8). The biological activity of luteolin formula is due to the hydroxyl moieties and 2−3 double bond. Luteolin, like most flavonoids, are regarded as antioxidants and may exert their effects by protecting or enhancing endogenous antioxidants such as glutathione-S-transferase and reductase, superoxide dismutase and catalase (9).

Luteolin has the capacity to arrest the cell cycle during the G1 phase on several cancer cell proliferation *in vivo* and *in vitro* and it is a strong activator of apoptosis with a non clear mechanisms (5). Data from previous studies suggest luteolin is a promising agent for anticancer therapy. In several carcinomas, luteolin enhances the expression of Fas through triggering the degradation of STAT3 (10). The intake of luteolin is inversely associated with subsequent coronary heart disease in some but not all prospective epidemiological studies (11). Modulation of ROS levels, reduction of NF-kappaB and AP-1 activity, stabilization of p53, and inhibition of PI3K, STAT3, IGF1R and HER2 may represent possible mechanisms involved in the biological activities of luteolin. In addition, it has been also reported that luteolin exerted a regulatory effect on mast cell-mediated inflammatory diseases, such as allergy disease. (12).

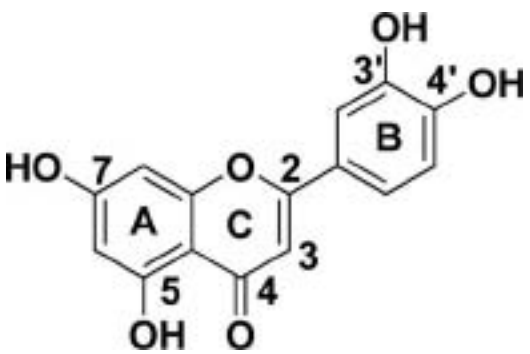

Fig. 1. *Lutelin structure.*

MAST CELLS

Mast cells are ubiquitous in the body, including skin, intestine, and lungs, and brain and express high affinity IgE receptor (FcεRI) playing an important role in allergic inflammation through releasing chemical mediators (such as histamine and serotonin), cytokines, chemokines, PGD2 and cysteinyl leukotrienes (13). Mast cells in the presence of IL-6 and transforming growth factor β (TGFβ) are necessary for the production of Th17 cells (14).

Reduced consumption of vegetables containing

antioxidants increase omega-6 PUFA (precursors for leukotriene C4 promoting inflammation) intake and reduced omega-3 PUFA (decreases LTC4), compounds implicated in atopic diseases and allergic inflammation. Flavonoids inhibit leukotriene synthesis though the inhibition of 5-lipoxygenase and phospholipase (15-16). Changes in environmental factors may contribute to the increase in the prevalence of allergic diseases and dietary change might contribute to the onset of allergic diseases. Flavonoids have been recognized to exert antioxidant, anti-bacterial and anti-viral activity, and possess anti-inflammatory, anti-angionic, analgesic, hepatoprotective, cytostatic, apoptotic, estrogenic or anti-estrogenic properties as well as anti-allergic effects (17). Luteolin regulates the production of TNF-α, IL-6, IL-8 and GM-CSF and it has been reported that luteolin inhibits not only the release of histamine, leukotrienes and prostaglandin D_2 but also the secretion of granulocyte macrophage-colony stimulating factor, IL-6 and TNF by human cultured mast cells in response to cross-linkage of FcεRI (18). Luteolin, also strongly inhibits both IL-4 and IL-13 synthesis (with a consequence of B cell inhibition) by allergen or anti-IgE antibody-stimulated mast cells and is considered as potential natural IgE inhibitors and antiallergic substance (19).

Mast cells promote cancer growth by stimulation of neoangiogenesis, tissue remodeling and by modulation of the host immune response (19). Angiogenesis represents an essential step of disease progression in several malignancies and is integral to tumor development, providing nutrients necessary for tumor growth. In addition, angiogenesis is a necessary step for metastasis of solid tumors to distal sites in the body and VEGF (vascular endothelial growth factor), a tumor cell's secret angiogenic factors, is a well-characterized regulator of physiological and pathological angiogenesis. It has been reported that luteolin is a potent angiogenesis inhibitor capable to inhibit tumor growth by inhibiting VEGF and hyaluronidase (20).

In addition, luteolin inhibits lipoxygenase, cyclooxygenase, and ascorbic acid-stimulated malonaldehyde formation in lipids and the generation of certain cytokines such as TNFα and IL-6 that can stimulate cancer cell migration and metastasis and signal transduction pathways (21). The ability of luteolin to inhibit xanthine oxidase activity and angiogenesis, to induce apoptosis, to prevent carcinogenesis in animal models, to reduce tumor growth in vivo and to sensitize tumor cells to the cytotoxic effects of some anticancer drugs, suggests that this flavonoid has cancer chemopreventive and chemotherapeutic potential (22). Moreover, luteolin induces apoptotic cell death (probably via inhibition of preteasome activity) and may be a putative anticancer therapeutic agent (23). Furthermore, lipopolysaccharide (LPS) a component of Gram-negative bacteria, capable of inducing inflammation through the activation of inflammatory cytokines and free radicals, is inhibited by luteolin. Inhibition of NF-κB may reduces the expression of inflammatory genes; therefore, luteolin results an anti-inflammatory compound probably by inhibiting two major pathways NF-kB and MAPK, an effect shown in macrophages (24). The regulation of the NF-κB signal pathway by luteolin is a potentially attractive and characteristic probe for studying mast cell-mediated inflammatory and allergic diseases.

In conclusion, these studies suggest that the anti-inflammatory effect of luteolin may be through potent inhibition of mast cell activation (25). Mast cell activation amplify inflammation which is inhibited by luteolin; therefore, targeting mast cells may have potential therapeutic effects in preventing or alleviating the development and progression of allergic inflammation. However, luteolin which has strong antioxidant activities is completely ineffective in suppressing some stimulus on certain cells, and probably its inhibitory action in different cells is not directly associated with its antioxidant properties.

However, more preclinical work is needed for establishing the efficacy and safety of luteolin alone or in combination with other therapeutics before conducting clinical trials. Therefore, further studies are necessary, especially focusing on molecular targets, mechanism-based animal and clinical studies to address the safety issues of luteolin with doses effective for cancer prevention and therapy in humans. These successful examples of inflammatory inhibition by luteolin will encourage researchers to synthesize, screen and discover more and better natural compound analogs that will eventually benefit cancer patients, inflammatory diseases and allergic disorders in the clinic.

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CYTOLOGICAL CHARACTERISTICS OF LUNG WASHINGS FROM CHILDREN IN DEPLETED URANIUM STROKED REGION

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The study was based on theoretical interpretation of authentic findings of Lupus Erythematosus Cells (LEC) in bronchoalveolar lavage (BAL) samples of children who underwent flexible bronchoscopy for clinical symptoms and radiological changes consistent with persistent pulmonary infiltrates during the first year after the bombing of Serbia in 1999. Differential cell counts were compared and statistical significance of differences for estimated cell population percentages calculated in groups of LEC positive (LEC+) and LEC negative (LEC-) BAL specimens. Significant increase of percentages of neutrophils and eosinophils and decreased percentages of macrophages were found in the group of LEC+ in comparison with LEC- BAL specimens ($p<0.05$, $p<0.001$, $p<0.001$, respectively). Presence of decreased percentages of cells of monocyte-macrophage lineage with consequent expansion of white blood cells in BAL, argue for understanding the nature of LEC+ alveolitis as a possible nonspecific finding of radiation-induced biological response of pulmonary tissue. LEC phenomenon may be understood as an early radio adaptive tissue response. Depleted uranium (DU) radiotoxic effect with concomitant alpha particles radiation, has been associated with unpredictable and everlasting biological effects. The emission of radiation in the course of several decades due to corrosion of scattered remnants of DU armaments, which has been potentiated by the repeated bombing of the regions within range of the transfer of radioactive particles by air, strikes a broad territory and numerous populations, and unavoidably leads to *in vivo* Petkau effect. Except the war, peacetime nuclear disasters in various parts of the world, such as Fukushima, Chernobyl and others, contribute to this effect too. In this way, the Petkau effect is a challenge for science to declare the future health strategy with the main goal focused on minimizing the early, as well as delayed *in vivo* effects of depleted uranium.

The first evidence of the Lupus Erythematosus Cell phenomenon (LEC) in bronchoalveolar lavages (BAL) was published during 1996 (1). Recently, LEC phenomenon in pediatric lung washings was discussed in relation to a possible influence of depleted uranium (DU) particles exposure. After a ten-year (1992-2002) evaluation of the results of differential cell counts of pediatric bronchoalveolar lavages, increase in percentages of LEC in BAL specimens was detected, particularly in the period July-December 1999 (2).

Having in mind the repeated bombing of the region within range of air transferable radioactive particles, special attention was focused on the geographical origin of patients included in the present study. Fig. 1 was based on reported data related to the detection of radiation in the remote regions of Europe, which coincided with operations in the Persian Gulf,

Key words: Radiation-induced alveolitis, The Petkau effect, bronchoalveolar lavage

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Bosnia and Herzegovina and Serbia. According to the author's civilian access to the data and theoretical interpretation of observation given by Moret (3) it was possible to estimate approximately 2400 miles radius around the Persian Gulf, as well as around of Bosnia and Herzegovina and Serbia, with the putative expansion of air pollution containing DU particles, but not taking into account any geographic or meteorological peculiarities of potentially exposed area.

Although the Petkau phenomenon was described in the literature as in vitro phenomenon (4), repeated bombing of relatively close areas in Persian Gulf and in Balkans, with subsequent emission of ionizing radiation and prolonged release of alpha-particles from corroded armaments from DU radioactive decay, provides an opportunity for the estimation of *in vivo* Petkau phenomenon and its effects.

Repeated low-slow radiation doses could induce remarkable harmful effects in the living tissues, which is represented by anticipated Petkau effect with, in time, superimposing increasing trend, opposing to the simultaneous decreasing trend of radiation (Fig. 2). There are many data related to the quantities of DU that was used in military campaigns since 1990 (5).

Alpha particles originating from DU cause ionization whose main effect is bystander effect in the living tissue. The bystander phenomenon is predominant at low tissue doses; at higher doses, cell death is more probable than the stimulation of radioadaptive tissue response (6). LEC phenomenon in BAL was recently discussed as a radioprotective/ radioadaptive tissue response (2). It is a question when tissue protective effects get their pathogenic feature?

The aim of study was to analyze and compare the cytological peculiarities of LEC+ vs. LEC-alveolitis in pediatric patients without proven autoimmune disease at the time of bronchological examination, who originated from the region bombed by DU projectiles.

MATERIALS AND METHODS

Bronchoalveolar lavage specimens were obtained from 20 unrelated children who underwent flexible bronchoscopy for clinical symptoms and radiological changes consistent with persistent pulmonary infiltrates at The Department of Pulmonology, Mother and Child Institute in Belgrade. They showed no clinical and/or laboratory signs of systemic Lupus Erythematosus or any other connective tissue disease. BAL differential cell counting was performed in the Laboratory for Microanalytic Diagnostics, Clinical Center of Serbia,

Fig. 1. *Schematic presentation of the potentially exposed area after bombing of targets in the Persian Gulf and the Balkans with depleted uranium projectiles. The thick black circle outlines approximately 2400 miles radius around the Persian Gulf; thin black circle schematically represents approximately 2400 miles around Bosnia and Herzegovina and dashed black circle schematically represents approximately 2400 miles around Serbia.*

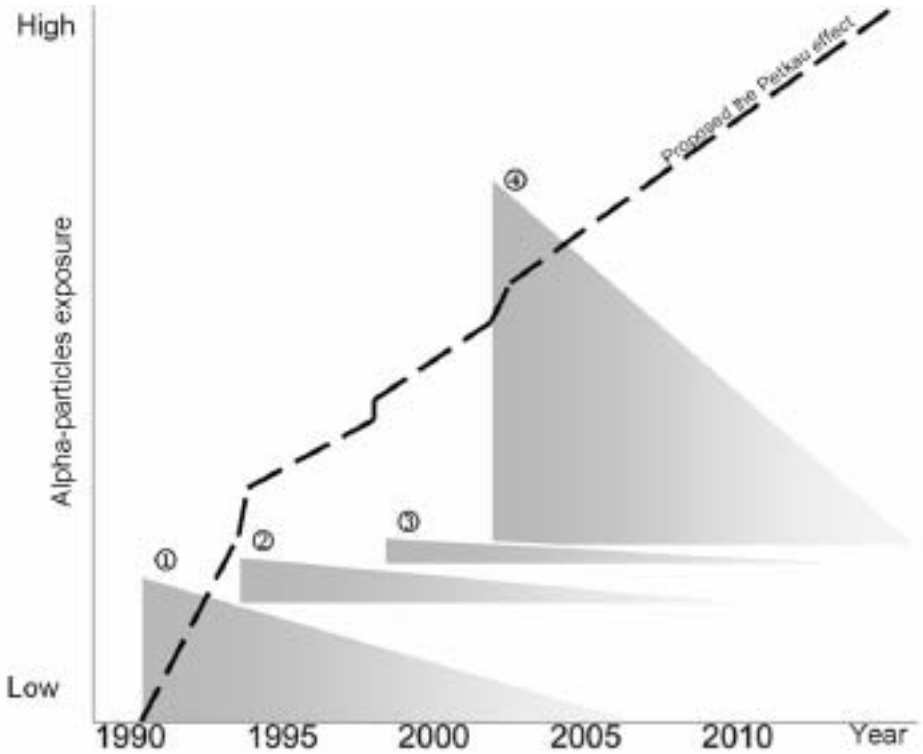

Fig. 2. *Presentation of the proposed in vivo Petkau effect in relation to repeated exposures to depleted uranium. The dark gray to pale grey contours represent proposed exposures to alpha particles that originated from depleted uranium missiles in military campaigns:* ***1)*** *First Gulf War (1990-1991);* ***2)*** *bombing of Bosnia and Herzegovina (1994-1995);* ***3)*** *bombing of Serbia (1999);* ***4)*** *second Gulf War (2003-2011); different size of the shaded areas symbolizes the difference in quantities of depleted uranium armaments used during military campaigns. The black dashed line symbolizes proposed the in vivo Petkau effect.*

Institute of Nuclear Medicine through the period 1999-2000 after the bombing of Serbia (March-June 1999). The samples were spun down freshly and pellet was adjusted with Hemaccel solution and cytocentrifuged, then air-dried and stained with May-Grünwald-Giemsa for differential cell counting. Slides were analyzed on an OPTON Axioplan light microscope for at least 500 cells.

From all cell populations and subpopulations within the total differential counts, the study evaluated the yield of neutrophils, eosinophils, lymphocytes, alveolar macrophages (expressed as the sum of alveolar macrophages with clear cytoplasm, alveolar macrophages with ingested particles, alveolar macrophages with mitotic figures, alveolar macrophages vacuolated, poly-segmented alveolar macrophages, giant cells and foamy alveolar macrophages), mast cells, LEC and other cells (expressed as the sum of plasmocytes, epithelial cells (the sum of ciliaed and squamous epithelial cells) and monocytes). It was possible to recognize monocytes in the BAL specimen by observing the contours of nuclei or the cell size in comparison with same peculiarities of alveolar macrophages, in the same BAL specimen, but having in mind that very precise distinguishing between BAL monocytes and alveolar macrophages could not be performed with the light microscopy only.

All patients included in the current study were from Serbia and Montenegro seaside and Bosnia and Herzegovina, territories of former Yugoslavia (Fig. 3) and they were classified in two groups dependent on presence of LEC in their BAL specimens: group 1 (patients with LEC- BAL specimens) and group 2 (patients with LEC+ BAL specimens).

Statistical analysis included calculation and comparative analysis of differential cell counts of LEC positive vs. LEC negative BAL specimens. Relative percentages of the LEC, as well as other cell populations

Fig. 3. *Schematic representation of territorial origin of patients who underwent bronchoscopy examination after the bombing of Serbia 1999. Distribution of patients from the territory of Former Yugoslavia: ○: Lupus Erythematosus Cell-negative bronchoalveolar lavage specimens; ● Lupus Erythematosus Cell-positive bronchoalveolar lavage specimens.*

in BAL specimens were calculated and comparison of results was done by two sample unequal variance 2-tailed *t*-test for groups investigated. Correlation analysis was made using the Vassar Stats utilities: Website for Statistical Computation, http://www.vassarstats.net/corr_big.html.

RESULTS

Numerous BAL cell populations and subpopulations were taken into account for calculation of differential cell counts for each patient. Results of differential cell counting of lung washings of patients from group 1 and group 2, with respect of neutrophils, eosinophils, mast cells, alveolar macrophages, LEC and other cells (related to the sum of epithelial cells, plasmocytes and monocytes in the BAL specimens) were presented in Table I.

Statistical significance of differences for mean percentages of different cell populations in BAL was presented in Table II.

There is significant increase of percentages of neutrophils and eosinophils and decreased percentages of macrophages in LEC-positive BAL specimens in comparison with LEC-negative ones ($p<0.05$, $p<0.001$, $p<0.001$, respectively).

The results of correlation analysis of BAL cytological parameters in group 1 and group 2 were presented in Table III.

Statistically significant negative correlation was found between BAL eosinophils and alveolar macrophages in LEC+ BAL specimens. LEC- BAL specimens showed statistically significant negative correlations between neutrophils and lymphocytes.

DISCUSSION

The conventional understanding of nature and pathogenesis of LEC appearance in the body fluids and tissues has been in relation with coexistent autoimmune diseases, particularly Systemic Lupus Erythematosus (7, 8). The author's observations since 1996 positioned the presence of LEC in BAL as a nonspecific, in pneumopathies that occurred

Table I. *Cytological parameters of LEC- and LEC+ BAL specimens.*

Cell populations in BAL differential cell count (%)	N	E	Ly	AM	Mast	LEC	Other BAL cells*
Group 1							
1	0.60	3.60	10.40	73.70	0.60	0.00	11.10
2	29.00	8.00	5.70	52.70	0.20	0.00	4.40
3	14.90	16.30	11.20	55.70	0.40	0.00	1.50
4	1.00	17.70	19.60	46.10	1.00	0.00	14.60
5	3.80	8.00	23.40	36.90	0.60	0.00	27.30
6	6.10	7.80	10.10	67.10	0.20	0.00	8.70
7	3.50	6.40	17.20	63.70	0.00	0.00	9.20
8	1.60	1.30	29.50	58.90	0.60	0.00	8.10
9	4.50	9.30	22.50	51.00	0.30	0.00	12.40
10	5.90	2.30	2.80	87.50	0.90	0.00	0.60
Group 2							
1	21.90	25.20	16.62	21.05	1.38	0.69	13.16
2	42.55	21.70	8.37	22.26	0.56	0.40	4.16
3	14.86	41.89	19.22	15.31	0.50	4.05	4.17
4	14.30	22.45	18.20	27.25	0.15	8.04	9.61
5	7.50	38.31	17.75	11.17	0.90	4.67	19.70
6	5.70	20.96	18.4	42.6	0.60	1.50	10.24
7	32.65	40.60	4.84	14.17	0.40	3.92	3.42
8	15.20	19.30	17.80	41.40	0.60	0.20	5.50
9	51.96	23.00	3.80	12.40	1.20	1.20	6.44
10	7.60	13.40	8.50	68.10	0.00	0.60	1.80

*BAL: bronchoalveolar lavage; LEC+: - Lupus Erythematosus Cell-positive samples; LEC-: Lupus Erythematosus Cell-negative samples; N: neutrophils; E: eosinophils; Ly: lymphocytes; AM: alveolar macrophages; Mast: mast cells; LEC: Lupus Erythematosus Cells. *Other BAL cells: the sum of plasmocytes + monocytes + epithelial cells.*

Table II. *Statistical parameters of differential cell counts in Lupus Erythematosus cell-positive and negative BAL samples.*

	LEC- Mean & SD	LEC+ Mean & SD	Significance of differences
N	7.09 ± 2.76	21.42 ± 15.89	$p<0.05$
E	8.07 ± 5.42	26.68 ± 9.90	$p<0.001$
Ly	15.24 ± 8.55	13.35 ± 6.19	No significant
AM	59.33 ± 14.47	27.57 ± 18.13	$p<0.001$
Mast	0.48 ± 0.32	0.63 ± 0.43	No significant
LEC	-	2.52 ± 1.70	-

BAL: bronchoalveolar lavage; LEC-: Lupus Erythematosus cell negative BAL specimens; LEC+: Lupus Erythematosus cell positive BAL specimens; N: neutrophils; E: eosinophils; Ly: lymphocytes; AM: alveolar macrophages; Mast: mast cells; LEC: Lupus Erythematosus Cells.

Table III. *Correlation analysis of bronchoalveolar lavage cyological parameters in Lupus Erythematosus Cell positive and Lupus Erythematosus Cell negative specimens.*

Correlations	LEC-	LEC+
N/Ly	r=-0.75, p<0.05	No significant
E/AM	No significant	r=-0.75, p<0.05
Ly/AM	No significant	No significant
AM/Mast	No significant	No significant

BAL: bronchoalveolar lavage; LEC-: Lupus Erythematosus cell negative BAL specimens; LEC+: Lupus Erythematosus cell positive BAL specimens; N: neutrophils; E: eosinophils; Ly: lymphocytes; AM: alveolar macrophages; Mast: mast cells; r: correlation coefficient.

coincidentally with the radiation exposure in a territory that was stroked by missiles with DU. One of recent papers pointed out that the presence of LEC in BAL could be manifestation of early radioadaptive/ radioprotective tissue response to exposure to low doses of radiation, particularly alpha particles that were trapped in the airways (2). All the patients whose BAL samples were analyzed in this study, originated from geographically close territories that were repetitively stroked by DU armaments and all of them have the history of the respiratory diseases in the first year after the bombing of Serbia.

The study compared characteristics of BAL differential cell counts performed in 10 patients with LEC+ and 10 patients with LEC- findings. In LEC+ BAL specimens predominates neutrophilic/ eosinophilic alveolitis with marked decrease of AM (Table 2). It was published earlier that radiation affects the blood cells, thus leading to a dominant decrease in the number of monocytes (9). Having in mind that AM are tissue counterparts of circulating monocytes, results support an idea that LEC+ BAL specimens may reflect radiation induced changes.

The neutrophils are not only major effectors of acute inflammation, but they can also contribute to chronic inflammatory conditions and adaptive immune responses (10). With a deeper look into the mechanism of pathogenesis of LEC+ alveolitis, based on the results in Tables II and III, it is very likely that a massive migration of neutrophils from the circulation to the pulmonary tissue takes place at the expense of lymphocytes, which also, but to a lesser extent migrate from the bloodstream to the pulmonary tissue. Defective apoptotic clearance, due to impaired phagocytosis by macrophages, may induce overexpressed phagocytosis by other cells of white lineage with concomitant LEC appearance (2, 11). Negative correlation between BAL eosinophils and lymphocytes in LEC- BAL specimens was absent in LEC+ BAL specimens (Table III). This finding reflects the expected reciprocity in migratory potential of different cell populations from the circulation into the tissue (namely monocytes that mature into the AM and eosinophiles). Slight increase of mast cells (Table II) may reflect aggressive pathogenic properties of LEC+ alveolitis, probably regulated by cytokines and deranged intercellular interactions.

The changed migratory capacity of cells from the bloodstream to the pulmonary tissue, as well as the nature of cell-to-cell interactions may be the cause of key differences between LEC+ and LEC- alveolitis. Very possibly, recruitment of different cell populations from circulation into the tissue is stimulated by the same harmful agent, but cell-to-cell communication may be changed in conditions of simmering bronchopulmonary pathogenic process. At the same time, the tissue opposes long-lasting inflammation as well as diminished apoptotic clearance by alveolar macrophages, inducing adaptive mechanisms including the LEC phenotype expression.

There is a reciprocal relationship between low radiation doses and their harmful effects on the cell (Fig. 2). Biological effects are extensive and unpredictable, because of complex regulation, dependent on exposition, as well as genetic predisposition, health, age and other individual factors.

After the bombing with DU projectiles widespread air pollution due to the release of radioactive particles, both in the explosion, and in later years due to corrosion of missiles, can be expected (Fig. 1, 2). In randomly arrived patients, there are findings of both, LEC+ and LEC- alveolitis and different diagnoses on admission, although the patients came from a relatively limited region within a radius of about 300 miles (Fig. 3, Table I). Due to long pulmonary retention of 1,470 days what is expected in the case of inhalation of uranium oxides (12) a wide range of clinical manifestations can occur, depending on the individual predispositions of the exposed persons.

Disclosure the incidence of diseases in the regions affected by DU radiation, and results of radiation monitoring from the period before and after the first military use of DU in the Persian Gulf in 1991 (13, 14) would allow a valuable comparative study whose data may be of great importance in defining future health strategies.

In conclusion, results of this study support the contention that under the conditions of exposure to radiation originating from DU, the fate of the individual patient depends not only on environmental impacts, but also on individual differences - genetic predisposition, general health status, etc. For this reason, the future strategy for the control of both, the early and deferred health effects of DU has to be based on an individualized approach to each patient, in diagnostics, as well as in preventive and treatment procedures.

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COMPARISON OF CHANGES IN THE PERCENTAGES OF $CD8^+CD28^-TCR\alpha\beta^+$ T CELL SUBPOPULATIONS IN ALLERGIC ASTHMA SUBJECTS *vs* CONTROLS BEFORE AND AFTER ANTI-CD3/ANTI-CD28/IL-2 STIMULATION *IN VITRO*

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Asthma is a chronic inflammatory disease characterized by the migration of activated T cells into the bronchial mucosa. TGF-β and IL-10 have proved to regulate airway hyper-responsiveness and leukocytes recruitment to the airways of ovalbumin (OVA) sensitized mice. We examined relative changes in $CD8^+$ T cell subpopulations between fifty allergic asthma subjects and twenty five aged-matched healthy adults before and after anti-CD3/CD28 and IL-2 stimulation in the presence of IL-10 or TGF-β, focusing on CD62L and FoxP3 expressing $TCR\alpha\beta^+$ cells. Severe asthma group had a significantly higher percentage of $CD8^+CD28^-$ and $CD8^+CD28^-TCR\alpha\beta^+CD62L^{high}FoxP3^{bright}$ T cells than other groups after enrichment. Compared to the baseline, co-stimulation with either IL-10 or TGF-β increased the percentage of $CD8^+CD28^-$ but decrease the percentage of $CD8^+CD28^+$ T cells within anti-CD3/anti-CD28/IL-2 activated $CD8^+$ T cells in all groups. Co-stimulation with anti-CD3/anti-CD28/IL-2 in presence of either IL-10 or TGF-β decreased the frequencies of $CD8^+CD28^-TCR\alpha\beta^+CD62L^{high}FoxP3^{bright}$ T cells in severe asthma subgroup but increased this parameter in other groups. We suggest that altered high level expression of CD62L and FoxP3 on $CD8^+CD28^-TCR\alpha\beta^+$ T cell is relevant to allergic asthma. These data have implications for further characterization of $CD8^+CD28^-TCR\alpha\beta^+$ T cell subsets, with special emphasis on their implication in healthy or allergic immune response.

Allergic asthma is a chronic inflammatory disorder of the lung (1) initiated and maintained by the migration of allergen-specific T cells from the blood into the lung parenchyma. In recent years, several subsets of $CD8^+$ T cells have been defined based on their differential expression of the T-cell costimulatory marker CD28 and the transcription factor FoxP3. Studies from several research groups including ours demonstrated the contribution of $CD8^+CD28^-$ T cells to the pathogenesis of asthma (2). Moreover, $CD8^+Foxp3^+$ T cells have been shown to inhibit allergen-induced Th_2 immune response (3). $CD8^+$ T cells can also be distinguished based on their surface expression of the lymph node homing molecules L-selectin (CD62L) into $CD62L^{high}$ "central" (T_{CM}) and $CD62L^{low}$ "effector" (T_{EM}) memory subsets (4). Human leukocytes express CD62L, the human homologue of the murine lymphocyte homing receptor, mel-14. This molecule mediates various aspects of immune system including cell communication, adhesion, and migration from the circulation into the lung. Altered

Key words: IL-10, TGF-β, FoxP3, anti-CD3/CD28, $CD8^+TCR\alpha\beta^+$ T cells, allergic asthma

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expression of this molecule correlates with impaired recruitment of leukocytes into the inflamed lung (5). Human $CD62L^+$ T cells are proved to influence the production of IgG and IgA-producing cells by B cells (6). These findings lend strong support to the idea that CD62L plays a primordial role in allergic mediated pulmonary inflammation. Previous studies have shown that thymic derived conventional T cells, which co express T cell receptor (TCR) αβ and either CD4 or CD8 molecules migrate to the periphery, whereas their unconventional counterparts which are characterized by the expression of either TCRαβ or TCRγδ with or without CD4 or CD8 populate non-lymphoid organs (7). Convincing reports indicate that $CD8^+CD28^-TCR\alpha\beta^+$ T cells represent the most commonly encountered circulating T cells subset in the peripheral blood of adult subjects (8). However, the distribution of circulatory $CD8^+CD28^-TCR\alpha\beta^+$ T cells expressing CD62L and FoxP3 has not been well examined. Immunomodulatory drugs modulate allergic immune responses through inhibition of the interleukin (IL)-2-dependent proliferation of human peripheral blood mononuclear T cell clones. Interestingly, co stimulation of T cells with the combination of anti-CD3/CD28 and IL-2 resulted in an increased expansion rate compared with cultures stimulated with IL-2 alone (9). Furthermore, in view of previous findings that TGF-β and IL-10 regulate antigen-induced airway hyper-responsiveness and the recruitment of leukocytes to the airways of ovalbumin (OVA) sensitized mice (10), we hypothesized that these cytokines may influence activated T cells adhesion to the lung vascular endothelium. To that end, we analyzed the distribution of different $CD8^+$ T cell subsets before and after short term culture with anti-CD3/anti-CD28/IL-2 in the presence of IL-10 or TGF-β. We showed differences between allergic asthma and healthy subjects in regards to the relative percentages of enriched or anti-CD3/CD28 and IL-2 activated $CD8^+CD28^-$ T cell subpopulations. We present evidence that IL-10 and TGF-β regulate the expression of TCRαβ, CD28, CD62L and FoxP3 on untouched or anti-CD3/anti-CD28/IL-2 treated $CD8^+$ T cells from healthy and allergic asthma subjects. These observations have implications for further characterization of the $CD8^+CD28^-TCR\alpha\beta^+$ T cell subsets and with special emphasis on their role in allergic asthma.

MATERIALS AND METHODS

This study was approved by the Ethics Committee of the Medical University of Lodz, Poland. All participants were non-smokers and provided written informed consent in accordance with the Declaration of Helsinki before sample collection and assessment.

A total of twenty five severe allergic asthmatics (SA), twenty five mild to moderate asthmatics (MA), and twenty five healthy controls (NC) were studied. The participants underwent medical examination; spirometry and skin prick tests. The diagnosis of asthma was based on history of periodic wheezing, reversible airway obstruction as described in GINA guidelines. The severity distribution was based on the European Network for Understanding Mechanisms of Severe Asthma (ENFUMOSA) classification. Patients were clinically stable and had no recent history of an upper respiratory tract infection.

Blood collection

Anti-coagulated peripheral blood was drawn within one hour prior to skin prick test (SPT) to avoid the probable influence of this procedure on our results. The samples were kept on ice to prevent adhesion molecules receptor modulation.

Peripheral blood mononuclear cells (PBMC) isolation

Peripheral blood mononuclear cells (PBMC) were isolated using density gradient centrifugation (Histopaque 1077, density 1.073 kg/L). PBMCs purity was at least 97% as assessed by hematologic analyzer.

$CD8^+$ T cell enrichment by magnetic-activated cell sorting (MACS)

$CD8^+$ T cells were purified from PBMCs by negative selection using a Naive CD8 T Cell Enrichment Set (BD Biosciences, California). The cells were initially incubated with $CD8^+$ T Cell Enrichment Cocktail (5 µl per $1x10^6$ cells) for 15 min at 4°C in the dark. After washing with a 10X excess volume of 1X BD IMag™ buffer, cells were centrifuged at 300×g for 10 min, and the supernatant was decanted. The cells were incubated with BD IMag™ Streptavidin particles for 30 min at 4°C. The labeled cells were enriched using a BD IMagnet™. The purity of $CD8^+$ T cells was always higher than 97% as determined by flow cytometry.

$CD8^+$ T cells culture conditions

For *in vitro* stimulation, freshly isolated $CD8^+$ T cells were washed twice with RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) and suspended (1.0×10^6 cells/100 µl) in the same medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA), amino

acid supplements (2 mM glutamine), and antibiotics (100 U/mL penicillin; 100 mg/mL streptomycin) (Sigma). Cells were stimulated with anti-CD3 (10 µg/ml), anti-CD28 (1 µg/ml), and recombinant IL-2 (20 U/mL) in the presence of 10 ng/ml of IL-10 or TGF-β. Cultures were incubated for 3 days at 37°C under 5% CO_2 and 95% air atmosphere.

Immunofluorescence

Freshly isolated and cultured $CD8^+$ T cells were stained according to standard procedures. Cells in suspension ($1x10^6$ cells/100 µl) were incubated with 10 µL of the following conjugated monoclonal antibodies against CD3 [fluorescein isothiocyanate (FITC)] (clone UCHT1, IgG1, κ), CD8 [American Cyanamid (Am Cyan)] (Clone SK1, IgG_1, κ), CD28 [peridinin chlorophyll protein- Cy5 (PerCP-Cy5)] (Clone CD28.2, IgG_1, κ), CD62L [phycoerythrin cyanine-5 (PE-Cy™ 5)] (Clone DREG-56, IgG_1, κ), and TCRα/β (Pacific blue) (Clone T10B9.1A-31, IgG_1, κ) for 30 min in the dark. Cells were re-suspended in wash buffer and then incubated with 2 ml of 1x Human FoxP3 Buffer A for 10 min in the dark. Thereafter, cells were washed twice with 2 ml of BD Pharmingen Stain Buffer (FBS) and centrifuged. The aliquots of labeled $CD8^+$ T cells suspension was washed with 2ml of BD Pharmingen Stain Buffer (FBS) and incubated with anti-anti-Human FoxP3 (allophycocyanin (APC)) (clone 259D/C7, IgG_1, κ) for 30 min in the dark. All steps were performed at 4°C. The labeled cells were washed with cold phosphate-buffered saline (PBS), and subsequently resuspended in 500 µl of staining buffer prior to analysis.

Flow cytometry analysis

Samples were run on a bench top FACS CANTO II cytometer (BD Biosciences, USA) according to the manufacturer's instructions. Data were analyzed with FACS Diva software (Becton Dickinson). Gating of CD8, CD28, TCRαβ, CD62L surface markers and intracellular FoxP3 (Fig. 2) were determined using Fluorescence Minus One (FMO). This technique consists of selecting controls containing all markers except the one of interest. A total of 10.000 cells were detected at each time for each sample. Lymphocytes were gated by forward and side scatter characteristics (Fig. 1). Gates were standardized within individual samples to ensure a fully comparative data. Results are presented as median percentages of viable $CD8^+$ T cell within the specific gated lymphocyte compartment.

Statistical analysis

All statistical analyses were performed using an unpaired, two-tailed Mann-Whitney test and Stat Soft's software (z o.o., Krakow, Poland). $P<0.05$ was considered statistically significant.

RESULTS

Comparison of patient demographic data

Fifty patients with allergic asthma, including 25 males and 25 females, were enrolled in this project. Twenty-five healthy volunteers served as controls.

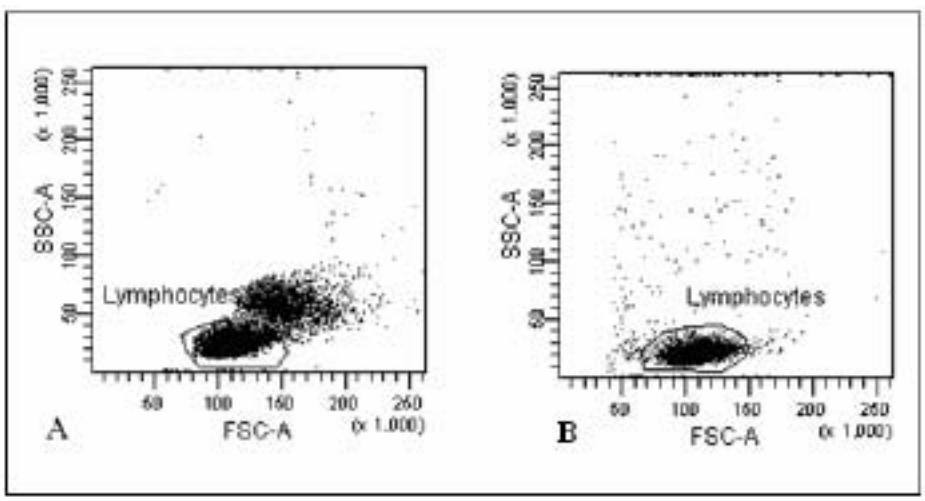

Fig. 1. *Representative dot plots for lymphocytes. Cells were isolated from peripheral blood prior to staining. Lymphocytes were gated based on forward scatter/side scatter. Dot plots representing the lymphocyte gate after isolation (**A**) and magnetic bead purification (**B**) are shown.*

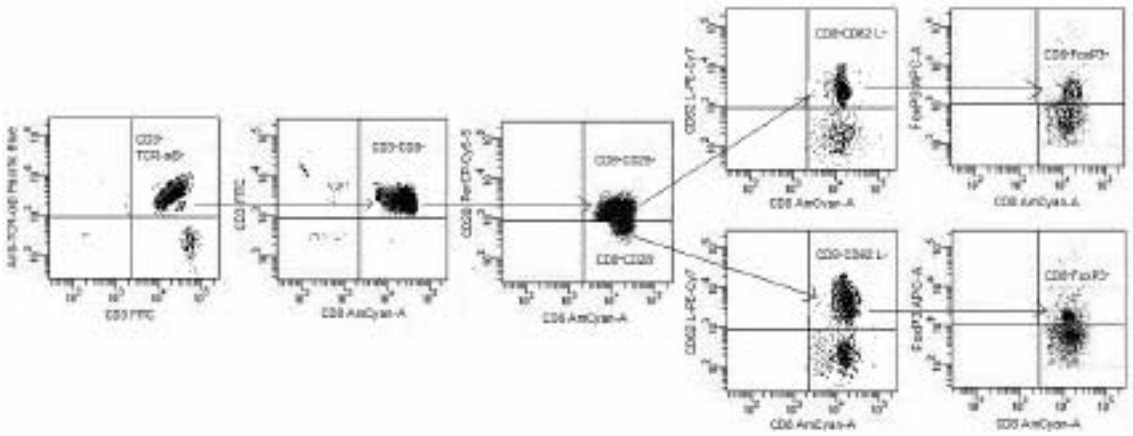

Fig. 2. *Flow cytometric analysis of CD8⁺ T cells co expression CD28, CD62L, TCRαβ, and FoxP3 molecules. Untreated and treated CD8⁺ T cells from each group were separately stained with anti- TCRαβ, anti-CD3, anti-CD8, anti-CD28 and anti-CD62L and anti-FoxP3 monoclonal antibodies. CD3⁺CD8⁺T population were evaluated for the expression of selected markers by gating : CD3⁺ lymphocytes from TCRαβ⁺ cells population; CD8⁺ lymphocytes from CD3⁺ cells; CD28⁺ lymphocytes from CD8⁺ cells; CD62L⁺ lymphocytes from CD28⁺ or CD28⁻ cells; FoxP3⁺ lymphocytes from CD62L⁺ cells. Quadrants show staining of populations gated according to fluorescence-minus-one (FMO) control technique.*

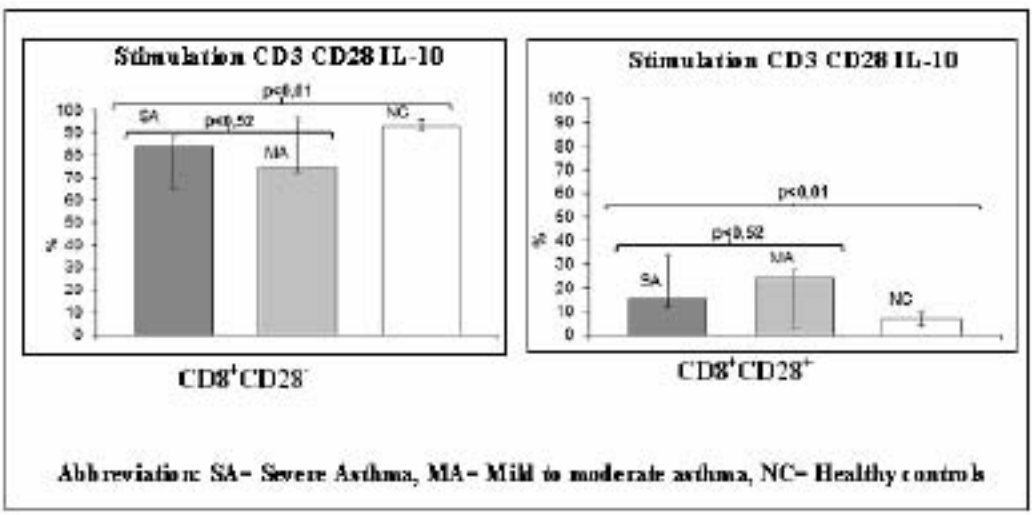

Fig. 3. *Comparative analyses of the percentages of enriched CD8⁺CD28⁻ and CD8⁺CD28⁺ T cells using the Wilcoxon Mann-Whitney test. Untouched CD8⁺ T cells were magnetically purified by negative selection from peripheral blood mononuclear cells (PBMC) obtained from buffy coats fraction of peripheral blood according manufacturers' instructions. After staining with antibodies, the surface expression of CD3, CD8, and CD28 was assessed by flow cytometry. Shown are the relative percentages of CD8⁺CD28⁻ and CD8⁺CD28⁺ T cells. The bar graph shows the median ± Interquartile (IQ) of 4 independent experiments. P values of 0.05 or less were considered statistically significant.*

The severe asthma group comprised 12 men and 13 women whereas the mild to moderate asthma group included 13 men and 12 women. The control group comprised 13 men and 12 women. The mean year of: severe asthma subjects was 48±14, mild to moderate asthma subjects 42±13, and controls 48.5±14.5 patients.

Comparison of patients' clinical information

Allergic asthma subjects had common asthma symptoms and were skin prick test-positive (>3 mm wheal) for at least 1 of 9 common aeroallergens

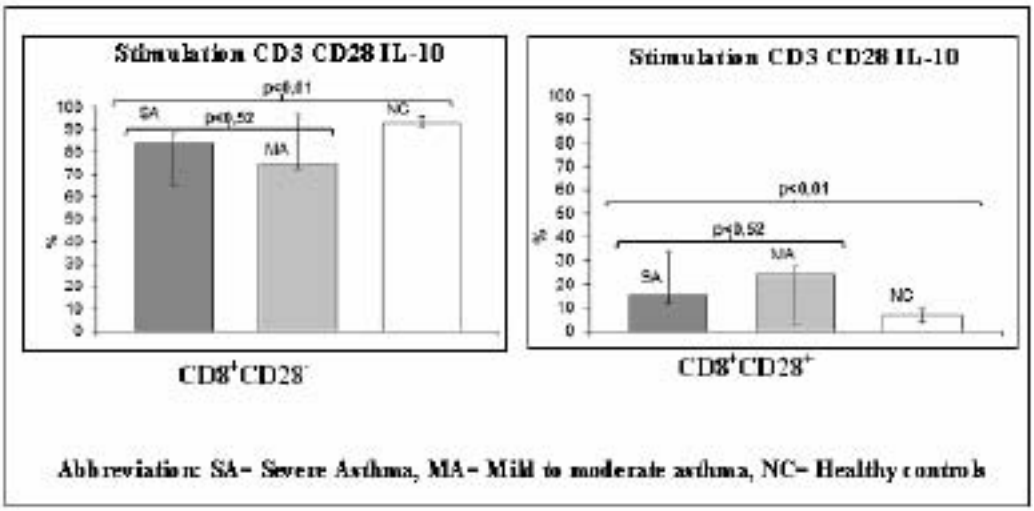

Fig. 4. *Comparison of the percentages of in vitro activated $CD8^+CD28^-$ and $CD8^+CD28^+$ T cells cultures using the Mann-Whitney test. Anti-CD3/anti-CD28/IL-2-activated $CD8^+$ T cells were cultured in the presence of IL-10. After 3 days in culture, the surface expression of CD3, CD8, and CD28 was assessed by flow cytometry. Shown are the relative frequencies of $CD8^+CD28^-$ and $CD8^+CD28^+$ T cells. The bar graph indicates the median ± Interquartile (IQ). P values of 0.05 or less were considered statistically significant.*

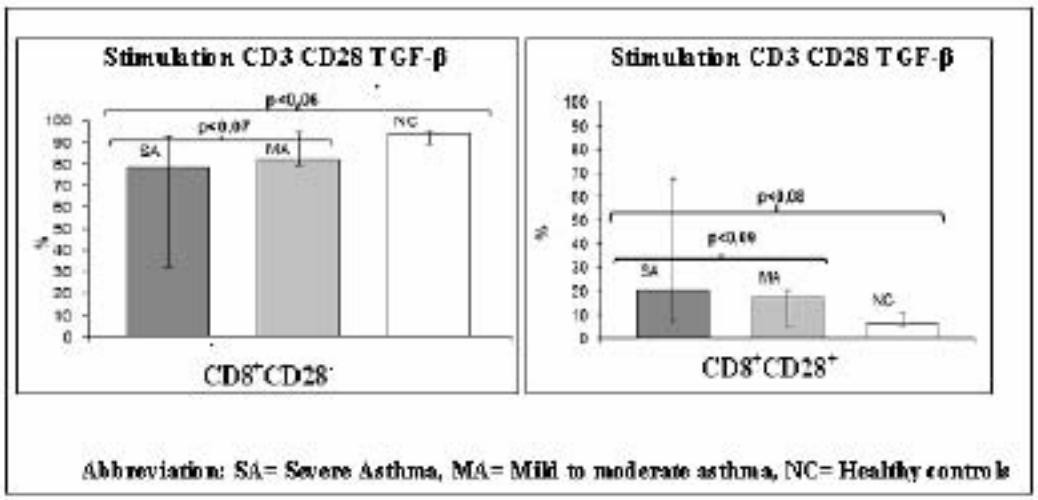

Fig. 5. *Comparison of the percentages of in vitro activated $CD8^+CD28^-$ and $CD8^+CD28^+$ T cells cultures using the Mann-Whitney test. Anti-CD3/anti-CD28/IL-2-activated $CD8^+$ T cells were cultured in the presence of TGF-β. After 3 days in culture, the surface expression of CD3, CD8, and CD28 was assessed by flow cytometry. Shown are the relative percentages of $CD8^+CD28^-$ and $CD8^+CD28^+$ T cells. The bar graph indicates the median ± Interquartile (IQ). P values of 0.05 or less were considered statistically significant.*

extracts. Airway reversibility was defined as an increase of at least 12% or 200 ml in forced expiratory volume in 1s (FEV_1) 30 min after inhalation of two puffs of salbutamol (2x100 μg). Airflow obstruction was determined whenever FEV_1 and FVC were beneath 80% of the predicted values and FEV_1/FVC ratio was below 75% of the predicted values. Severe asthma subjects had longer duration of disease 16±8.7 than those with mild to moderate asthma 10±6.3. The percentage predicted FEV_1 of severe asthma subjects was 67±15.98, mild to moderate asthma 87.71±16.12, and controls 96.6±4. Patients

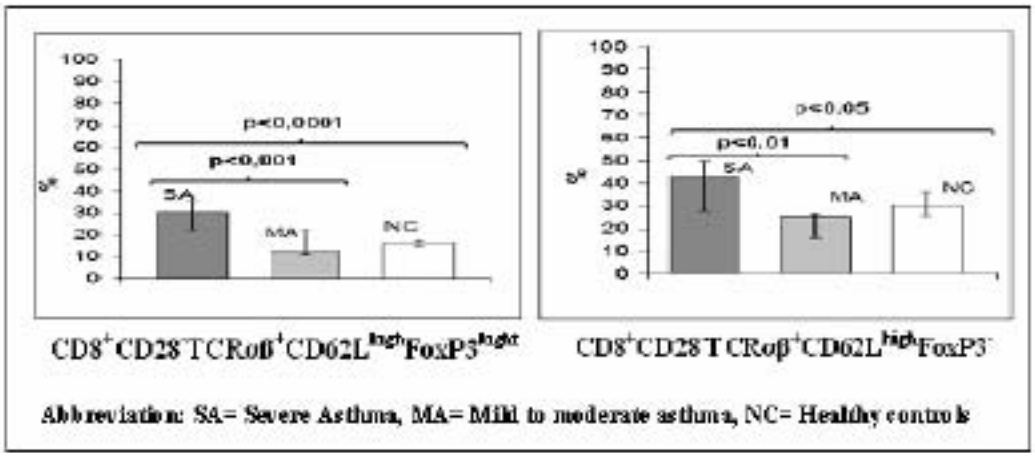

Fig. 6. *Phenotype of enriched $CD8^+CD28^-TCR\alpha\beta^+CD62L^{high}FoxP3^{bight}$ T cells and $CD8^+CD28^-TCR\alpha\beta^+CD62L^{high}FoxP3^-$ T cells. Untouched $CD8^+$ T cells were purified by negative selection from PBMC obtained from peripheral blood according manufacturers' instructions. After staining with antibodies against specific antibodies, the surface expression of TCRαβ, CD3, CD8, CD28, CD62L, and the intracellular transcription factor FoxP3 by enriched $CD8^+$ T cells was assessed by flow cytometry. Shown are the relative percentages of $CD8^+CD28^-TCR\alpha\beta^+CD62L^{high}FoxP3^{bright}$ T cells as well as $CD8^+CD28^-TCR\alpha\beta^+CD62L^{high}FoxP3^-$ T cells. The bar graph shows the median ± Interquartile (IQ). P values of 0.05 or less were considered statistically significant.*

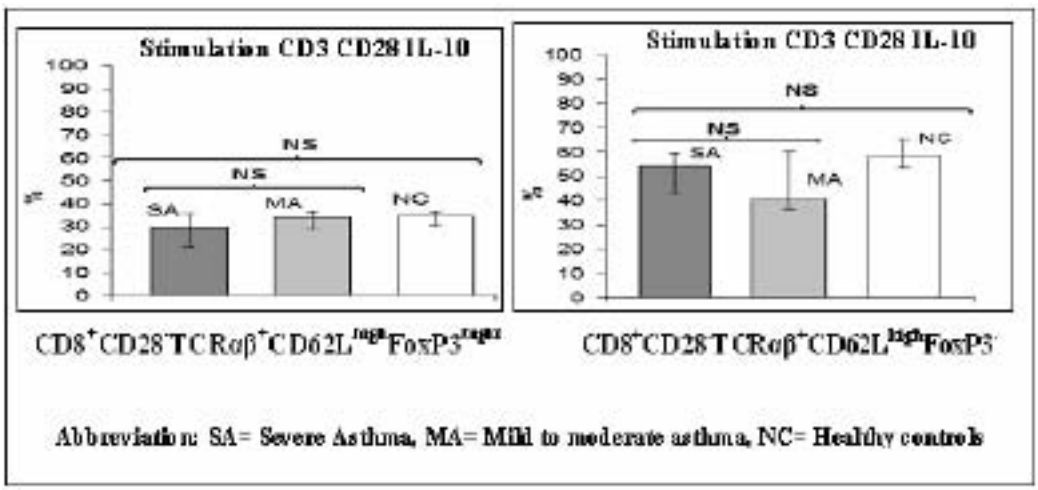

Fig. 7. *Comparison of the effects of anti-CD3/anti-CD28/IL-2 and IL-10 co-stimulation on the proportion of $CD8^+CD28^-CD62L^{high}FoxP3^{bright}$ and $CD8^+CD28^-CD62L^{high}FoxP3^-$ T cells using the Mann-Whitney test. After 3 days in culture, the surface expression of TCRαβ, CD3, CD8, CD28, CD62L, and the intracellular transcription factor FoxP3 by $CD8^+$ T cells was assessed by flow cytometry. The $CD8^+CD28^-TCR\alpha\beta^+CD62L^{high}FoxP3^{bright}$ T cells and $CD8^+CD28^-TCR\alpha\beta^+CD62L^{high}FoxP3^-$ T cells frequencies are shown. The bar graph shows the median ± Interquartile (IQ). P values of 0.05 or less were considered statistically significant.*

with severe asthma have taken either high dose of inhaled glucocorticosteroids (ICS) (800-1600µg), long-acting ß-agonists (LABA) for at least a year. Patients with mild to moderate asthma received daily inhaled steroids (200-800µg) in combination with symptomatic therapy. All healthy controls had negative-skin prick test to all tested allergens, normal chest X-Ray and baseline FEV_1.

Severe asthma group had a significantly higher percentage of $CD8^+CD28^-$ but lower percentage of $CD8^+CD28^+$ T cells in peripheral blood than others

Patients with severe allergic asthma (73.2±65.6%) had significantly higher relative percentage of

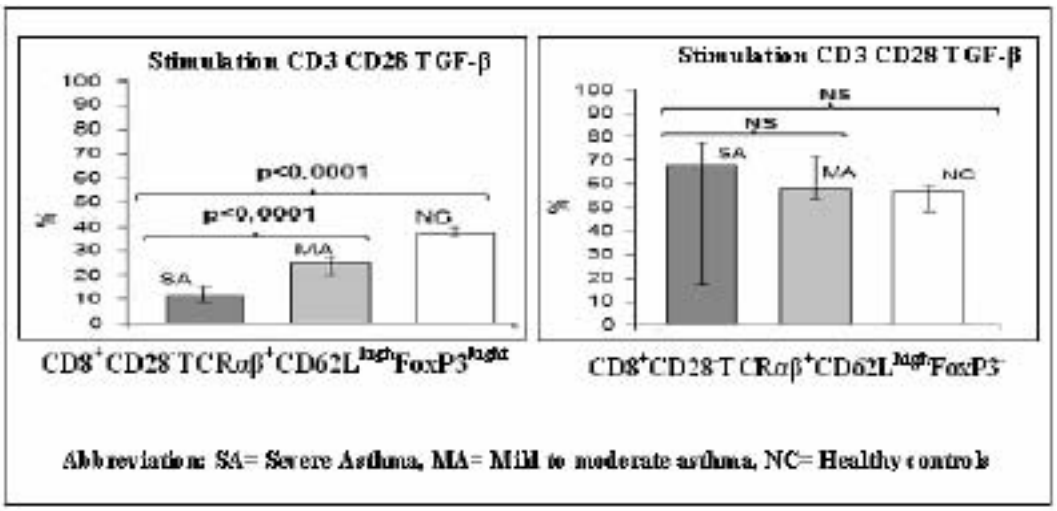

Fig. 8. *Comparison of the effects of anti-CD3/anti-CD28/IL-2 and TGF-β co-stimulation on the proportion of $CD8^+CD28^-CD62L^{high}FoxP3^{bright}$ and $CD8^+CD28^-CD62L^{high}FoxP3^-$ T cells using the Mann-Whitney test. After 3 days in culture, the surface expression of TCRαβ, CD3, CD8, CD28, CD62L, and the intracellular transcription factor FoxP3 by $CD8^+$ T cells was assessed by flow cytometry. The $CD8^+CD28^-TCR\alpha\beta^+CD62L^{high}FoxP3^{high}$ T cells and $CD8^+CD28\ TCR\alpha\beta^+CD62L^{high}FoxP3^-$ T cells frequencies are shown. The bar graph shows the median ± Interquartile (IQ). P values of 0.05 or less were considered statistically significant.*

$CD8^+CD28^-$ T cells within the isolated $CD8^+$ T cells than those with mild to moderate allergic asthma (37.5±37.35%) ($p<0.001$) and controls (46.3±47.6%) ($p<0.05$). In contrast, the percentage of circulating $CD8^+CD28^+$ T cells within the freshly isolated $CD8^+$ T cells was significantly lower in the severe asthma group (26.8±34.35%) than in the mild to moderate phenotype (61.7±62.75) ($p<0.01$) and controls (54.2±52.4) groups ($p<0.02$) (Fig. 3).

Co-stimulation with either IL-10 or TGF-β caused an increase in the percentage of $CD8^+CD28^-$ but a decrease in percentage of $CD8^+CD28^+$ T cells within anti-CD3/CD28 and IL-2 activated $CD8^+$ T cells from all groups

Findings from immunotherapy trials suggest that anti-CD28 (αCD28) can enhance the stimulatory effects of αCD3 on T cells due to its capacity to mimic the B7.1 or B7.2 ligand (11). IL-2, which is an autocrine cytokine play a crucial role in the activation, growth, proliferation of antigen-specific T-cell clones. It has been demonstrated that anti-CD3/CD28 and IL-2 regulate T-cell proliferation in different ways (12). Some reports suggest that the stimulatory effects of αCD3/CD28 on $CD8^+$ T cells are IL-2 independent (13) whereas other studies have proven otherwise. Because IL-10 and TGF-β direct T-cell differentiation, we asked whether they play a role in the pathogenesis of AA, and whether they have some effects on TCRαβ, CD28, CD62L and FoxP3 expression on αCD3/αCD28/IL-2 treated $CD8^+$ T cells. In relation to the baseline, co-stimulation with αCD3/αCD28/IL-2 and IL-10 resulted in a 1.14 (73.2±65.6% to 84.1±77%) fold upgrade in the percentage of $CD8^+CD28^-$ T cells in the SA subgroup compared with 1.99-fold (37.5±37.35% to 74.6±84.65%) in the MA subgroup and 2-fold (46.3±47.6% to (92.9±93.3%) increase in the controls group (Fig. 4). In contrast, co-stimulation with αCD3/αCD28/IL-2 and TGF-β resulted in a 1.77-fold (26.8±34.35% to 15.1±22.9%) decrease in the relative percentage of $CD8^+CD28^+$ T cells in the SA subgroup compared with 2.56-fold (61.7±62.75 to 24.1±15.35%) in the MA subgroup and 7.74 fold (54.2±52.4) to (7±6.65 %) in the NC group (Fig. 5).

Severe allergic asthma group had increased $CD8^+CD28^-TCR\alpha\beta^+CD62L^{high}FoxP3^{bright}$ T-cell frequencies than other groups after enrichment

Continuing OVA aerosol challenge has proven to increase the number of $TCR\alpha\beta^+$ cells (14). Here, we identify and characterize $CD8^+CD28^-$

TCRαβ^{+}CD62L^{high}FoxP3^{bright}T cells in peripheral blood of healthy and allergic asthma subjects before stimulation (baseline). The severe asthma subgroup had significantly higher relative proportion of CD8^{+}CD28^{-}TCRαβ^{+}CD62L^{high}FoxP3^{bright} T cells (30.3±28.85) than subjects with the mild to moderate asthma (12.8±16.55) ($p<0.001$) and controls (16.2±16.45) ($p<0.0001$), $p<0.05$ (Fig. 6).

Compared to the baseline, αCD3/αCD28/IL-2 and IL-10 or TGF-β stimulation decreased the frequencies of CD8^{+}CD28^{-}TCRαβ^{+}CD62L^{high}FoxP3^{bright} T cells in severe asthma subgroup

Compared to the baseline, stimulation with αCD3/αCD28/IL-2 and IL-10 slightly decreased the percentage of CD8^{+}CD28^{-}TCRαβ^{+}CD62L^{high}FoxP3^{bright} T cells in severe asthma group (from 30.3±28.85 to 29.8±28.6) (Fig. 7). Similarly, co-stimulation with αCD3/αCD28/IL-2 and TGF-β caused 2.75-fold (from 30.3±28.85 to 11.0±11.75) decrease in this parameter in this subgroup (Fig. 8).

Compared to the baseline, co-stimulation with αCD3/αCD28/IL-2 and IL-10 or TGF-β increased the frequencies of CD8^{+}CD28^{-}TCRαβ^{+}CD62L^{high}FoxP3^{bright} T cells in mild to moderate asthma and controls groups

Co-stimulation with αCD3/αCD28/IL-2 and IL-10 resulted in 2.66-fold increase (12.8±16.55 to 34.1±32.55) in CD8^{+}CD28^{-}TCRαβ^{+}CD62L^{high}FoxP3^{bright} T cell frequency in the mild to moderate asthma group and 2.12-fold increase (from 16.2±16.45 to 34.5±33.65) in controls group (Fig. 7). Similarly, co-stimulation with αCD3/αCD28/IL-2 and TGF-β led to 1.90-fold (from 12.8 ±16.55 to 24.4 ±23.5) increase in this parameter in the mild to moderate asthma and 2.29-fold (from 16.2±16.45 to 37.2±37.85) increase in controls groups (Fig. 8).

DISCUSSION

Previous studies examining human T cells have reported that altered numbers of intraepithelial CD8^{+}TCRαβ^{+} T cells correlate with perpetuated inflammation (15). Moreover, an altered ditribution of CD8^{+}TCRαβ^{+} T cells has been observed in bronchial alveolar lavage (BAL) of allergic animal models (16). A growing body of evidence suggests an association between the expression levels of L-selectin, airway hyper responsiveness, and chronic allergic asthma (17). Increase percentages of circulating Foxp3^{+}CD62L^{+} T cells are seen in graft-versus-host disease (GvHD) (18). Initial results from our research group established a link between frequencies of human circulatory CD8^{+}CD25^{+} T cells expressing high level of FoxP3 and the development of allergic asthma (19). In light of these evidences, the present study shows an altered expression of CD8^{+}CD28^{-}TCRαβ^{+} T cells subset expressing high level of CD62L and FoxP3 in allergic asthma subjects, indicating a role for this subset in the course and clinical outcome of allergic airway inflammation. This observation led us hypothesize that the migration of circulating FoxP3 expressing CD8^{+}CD28^{-}TCRα/β^{+}CD62L^{high} T cells to the airways is impaired in allergic asthma subjects as evidenced by their altered expression of adhesion molecule CD62L. Our observation is consistent with previous studies (20) demonstrating an increase expression of FoxP3^{+} T cells to offset the aggravated atopy and airway inflammation. However, it detracts from other reports which found no significant differences between the levels of L-selectin expressing-T cells from patients with asthma and healthy subjects (21). Previous studies have established a correlation between the proportion of FoxP3 and CD62L (22) expressing T cells and the levels of serum Immunoglobulin E (IgE). Due to the fact that the levels of serum IgE determine clinical expression and severity of allergic asthma (23), one would expect a higher expression of CD62L in allergic asthma subjects compared to controls. Surprisingly, the percentage of CD8^{+}CD28^{-}TCRα/βCD62L^{high}FoxP3^{bright} T cells was increased in severe asthma subgroup whereas this parameter was decreased in mild to moderate asthma subgroup. There are several possible explanations for the discrepancies between the severe and mild to moderate asthma in relation to controls. T helper 2 (Th_2) cells and related cytokines such as IL-4 and IL-5 have long been presumed to play an exclusive role in asthma and allergy. In this perspective, Th_1 cells were thought to down modulate the effects of Th_2 cells, thus their protective role in asthma. Recently,

the pro-inflammatory properties of interferon-γ (IFN-γ), a cytokine secreted by Th_1 has begun to emerge. IFN-γ and IL-4 are proven to cross regulate each other. The former is thought to suppress Th_2 cells whereas the latter appears to inhibit Th_1 subset. It is becoming clear that IFN-γ secreting-Th_1 cell can predispose individuals to developing severe asthma. Interestingly, IFN- γ has been shown to increase CD62L expression on circulating leukocytes (24). Therefore, differences in cytokines profiles between severe and mild to moderate asthma subjects offer a possible explanation for our findings. Some reports indicate that IL-4 mediates airway inflammation by inhibiting IFN-γ (25), whereas others provide reliable evidences that IFN-γ inhibits IL-4 effects and up-regulate Foxp3 and CD62L (26). Thus, it could conceivably be hypothesized that, in severe allergic asthma subjects, IFN-γ suppresses IL-4, the dominant Th_2 cytokine to enhance Foxp3 and CD62L expression. Furthermore, Q576R polymorphism in the gene encoding the IL-4 receptor has been identified in patients with severe asthma (27). The difference between the allergic asthma groups may be partially explained by the IL-4 receptor polymorphism in severe asthma subjects. It has also been demonstrated that glucocorticoids have some effects on intracellular L-selectin mRNA (28) and FOXP3 expressions. We consider difference in glucocorticosteroids dose intake between asthma subgroups as another probable explanation for some discrepancies between expected theoretical and experimental data. Overall, considering the importance of homing adhesion molecule CD62L in T cell migration in the lungs, we speculate that any alteration of its expression can impede the infiltration of $CD8^+CD28^-TCR\alpha/\beta CD62L^{high}FoxP3^{bright}$ T cells into the airways, perpetuating inflammation. It has been documented that costimulation of T cells with αCD3/CD28 and IL-2 greatly increases the proportion of $FoxP3^+$ T cells (29). Our findings lend support to this idea by showing an increase frequency of $CD8^+CD28^-TCR\alpha/\beta CD62L^{high}FoxP3^{bright}$ T cells in mild to moderate asthma and controls groups. Surprisingly, culture under similar condition led to a decrease in the relative percentage of this subset in severe allergic asthma subgroup. It has been shown that TCR-stimulated T cells from allergic asthma subjects are resistant to certain inhibitory effects of TGF-β and IL-10 as compared with healthy controls (30). We hypothesized that the differential response of $CD8^+CD28^-TCR\alpha\beta^+CD62L^{high}FoxP3^{bright}$ T cells from allergic asthma subjects to the effects of TGF-β and IL-10 may provide another explanations for our findings. Albeit suggestive, our hypotheses need to be confirmed by larger and unbiased studies. In summary, we established a link between altered expression of high level of CD62L and FoxP3 on $CD8^+CD28^-TCR\alpha\beta^+$ T cell and allergic asthma. These data have important implications for further characterization of $CD8^+CD28^-TCR\alpha\beta^+$ T cells subsets and with special attention on their role in healthy or allergic immune response.

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INCREASED URIC ACID LEVELS IN BIPOLAR DISORDER: IS IT TRAIT OR STATE?

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The aim of the present study was to compare uric acid levels between disease and remisson episodes of bipolar disorder (BD) and healthy individuals and to investigate whether uric acid levels were related with clinical properties and the course. Uric acid levels were compared in 43 BD patients with manic episode, 20 BD patients with depressive episode, 41 BD patients in remission and 43 healthy individuals. Informations regarding disorder was recorded using SKIP-TURK, the severity of episode was measured with Young Mania Rating Scale (YMRS) and Hamilton Depression Rating Scale (HDRS). Uric acid levels were found higher in manic, depressive and euthymic bipolar patients than those in healthy individuals. In cases in remission period, a moderate relation was present between uric acid levels and the age of onset. A moderate relation was found in manic episode between first and second week YMRS scores and uric acid levels, and a strong relation was found in depressive episode between first and second week HDRS scores and uric acid levels. While decrease in uric acid levels in manic episode was found to be associated with the decrease in YMRS scores, no such relation was shown in depressive episode. Our findings stress the impairment in purinergic functions in all episodes of BD. This impairment seems to be associated with clinical properties of BD.

Purinergic system plays role in the regulation of mood, motor activity, cognitive function, sleep and behavior (1). Uric acid is the end product of purin metabolism and is produced by xantine dehidrogenase. Increased levels of uric acid mean accelerated purinergic transformation and decreased adenosinergic transmission (2). Adenosinergic receptors limit cellular excitability by inhibiting neurotransmitter release in central nervous system (CNS).

Increase in uric acid levels changes the control on cellular excitability by decreasing adenosinergic transmission (3).

Increasing evidence suggest that impairment in the function of purinergic system plays part in the pathophysiology and treatment of Bipolar Disorder (BD) (4, 5). Increased uric acid levels have been shown both in chronic BD, and in first attack manic patients (6, 7). In a randomised controlled study, Machado-Vieira et al. (2008) demonstrated that the addition of allopurinole, which is an inhibitor of xantine dehidrogenase, to lithium is quite effective in the treatment of acute mania (6). Machado-Vieira (2012) went one step further and suggested the use of uric acid levels as a screening test in mania (8).

The aim of the present study was to compare the uric acid levels between episodes and remission periods of BD and healthy individuals and to investigate whether uric acid levels are related with clinical properties and the course of the disorder.

MATERIALS AND METHODS

Sample

With this aim, between January 2011 and January

Key words: Bipolar disorder, mania, depression, remission, uric acid

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DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

2012, 50 patients in manic episode and 50 patients in depressive episode, all of whom were diagnosed with BD according to DSM-IV and admitted to our clinic from emergency service or our outpatients clinic were included consecutively in the present study. Their written informed consents were obtained by themselves or their relatives. For patients with active episode, not using any medications was required. For this purpose 7 manic and 15 depressive patients were excluded.

Fifty patients in remission period at least for 4 weeks who were diagnosed with BD according to DSM-IV, admitted to our outpatients clinics and whose consent form was signed by themselves were included. Five patients who were taking antipsychotics as maintenence treatment were excluded.

Control group comprised 43 healthy individuals who were at similar mean age with the other three groups and without any previous history of psychiatric diagnosis or treatment and current psychiatric complaints.

Subjects with gout, chronic inflammatory disease, hypertension, renal disease, hypertriglyceridemia as associated with hyperuricemia and other Axis I disorders and/or severe or unstable medical illnesses were excluded. Hence, 1 patient with depressive episodes and 4 patients in remission were excluded. All subjects were medically healthy, as determined by physical and neurological examination and laboratory tests. All subjects were hindered for a diet rich in proteins at last one week. 14 patients with depressive episodes and 7 healthy controls were excluded as they refused to comply with this diet. As a result, data of 43 patients with manic episodes, 20 patients with depressive episodes, 41 patients in remission and 43 healthy controls were included in statistical analysis.

Assessment tools

SCID-I (Structured Clinical Interview for DSM-Axis I Disorders-SCID-I):Turkish version of structured clinical interview for DSM-IV axis I disorders (9).

SCID-NP (Structured Clinical Interview for DSM-Nonpatient form-SCID-NP): Turkish version of clinical interview form for those who are not patients DSM-IIIR (10).

Hamilton depression rating Scale (HDRS): It was developed to measure the level of depression and changes in its severity (11). Its reliability and validity study in Turkish was carried out by Akdemir et al. (12).

Young mania rating scale (YMRS): It is used to measure the severity of manic symptoms before treatment in manic cases and to confirm the state of remission in recovery period. This scale filled by the interviewer was developed by Young et al. (13) and its validity and reliability study in Turkish was carried out by Karadağ et al. (14).

Process

Approval for the study was obtained from Erenköy Psychiatry Hospital Training and Scientific Investigations committee. The cost of the measurement of plasma uric acid levels was met from the Investigation Budget Fund of our hospital.

Information regarding disease was recorded by using SKIP-TURK. All cases were evaluated by YMRS and HDRS when they were admitted to clinic (for manic and depressive episodes) and at 1st and 2nd weeks of treatment. Also plasma uric acid levels of the patients were measured at same evaluation points . In patients in remission period and in healthy individuals, uric acid levels were determined by a single measurement. In cases diagnosed with BD, the criteria for remisson was set at HDRS<8 for depression and YMRS<5 for mania. Plasma uric acid levels were recorded in mg/dl after being rotated in centrifuge with 3000 rotations and kept at, -80°C.

Statistical analysis

Group comparisons of demografic variables used ANOVAs for continuous measures and Fisher's Exact Test for categorical variables. Analysis of variance was used to compare uric acid levels between patients and healthy controls. Gender and age were added as covariates to determine whether those factors would alter the results. Levene's test was used to examine homogenity of variance, Shapiro-Wilk's test was used to examine normality. The comparison of the repetaed measurments in the same cases was made by variance analysis.

For correlation analysis, Pearson correlation test was used. Results were considered significant at $p<0.05$, two tailed. Bonferroni corrections were applied separately for the group comparisons and correlations and for whole group and individual group correlations.

RESULTS

Gender distribution and average age was similar between groups (Table I). There wasn't any difference between age at onset and manic and depressive episode frequency between patient groups.

Uric acid levels in manic, depressive and euthymic bipolar patients were found to be higher than those in healthy individuals ($F=4.035$, $p=0.043$), (Table II). In cases in remission period, a moderate relation was present between uric acid levels and the age of onset ($r=0.42$).

A moderate relation was found in manic episode

Table I. *Demographical and clinical properties of groups.*

	Mania n= 43	Depression n= 20	Remission n= 41	HC n= 43	Analysis x^2/F p
Gender (F/M)	19/24	11/9	20/21	22/21	2.641 0.235
Age (Mean±SD)	36.4±8.6	38.1±11.5	37.5±10.9	38.4±12.6	1.122 0.457
Age of onset (Mean±SD)	26.4±10.7	28.3±8.6	28.7±11.6	-	1.008 0.486
Frequency of manic episote (Number of episode/Duration of illness – year-), (Mean±SD)	0.28	0.24	0.26		0.843 0.651
Frequency of depressive episote (Number of episode/Duration of illness – year-), (Mean±SD)	0.31	0.34	0.29	-	0.754 0.688

Gender distribution and average age was similar between groups. There wasn't any difference between age at onset and manic and depressive episode frequency between patient groups.

Table II. *Uric acid levels in patients and healthy controls.*

Mania, n= 43	Depression , n= 20	Remission, n= 41	HC, n= 43
5.7±1.5	5.3±1.4	5.4±1.3	4.8±0.8

Uric acid levels in manic, depressive and euthymic bipolar patients were found to be higher than those in healthy individuals.

Table III. *Uric acid levels(UA) and Young Mania Rating Scale(YMRS) scores in manic episode.*

	Baseline	1.week	2.week
UA (mean±SD)	5.7±1.5	5.5±1.3	5.4±1.3
YMRS (Mean±SD)	30.2±4.4	18.4±7.5	10.2±3.6

Decrease in uric acid levels in manic episode was found to be associated with the decrease in YMRS scores.

between first and second week YMRS scores and uric acid levels, whereas a strong relation was found in depressive episode between first and second week HDRS scores and uric acid levels (respectively, r=0.41, 0.39, 0.51 and 0.52), (Fig. 1-4). While decrease in uric acid levels in manic episode was found to be associated with the decrease in YMRS scores (r=0.27, p=0.034), no such association was shown in depressive episode

Table IV. *Uric acid levels(UA) and Hamilton Depression Rating Scale(HDRS) scores in depressive episode.*

	Baseline	1.week	2.week
UA (mean±SD)	5.3±1.4	5.3±1.3	5.3±1.1
HDRS (mean±SD)	24.2±4.4	17.4±4.2	12.2±1.7

No association was found between uric acid levels in depressive episode and decrease in HDRS scores.

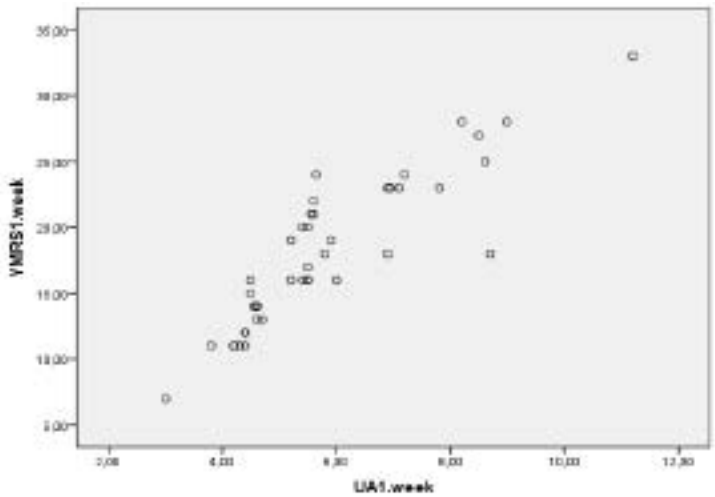

Fig. 1. *Correlation between UA and YMRS in first week (r=0.41, p=0.037).*

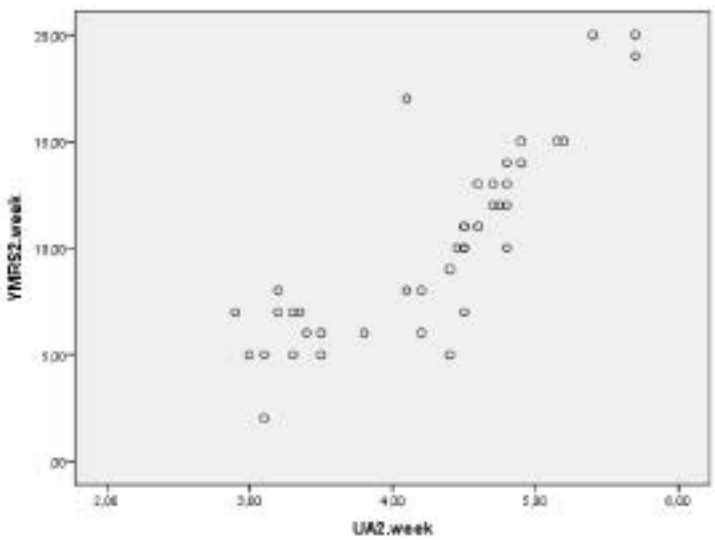

Fig. 2. *Correlation between UA and YMRS in second week (r=0.39, p=0.038).*

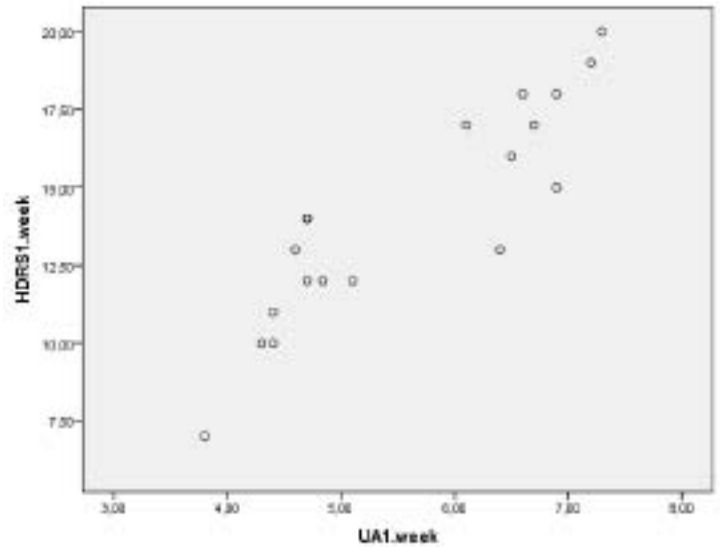

Fig. 3. *Correlation between UA and HDRS in first week (r=0.51, p=0.027).*

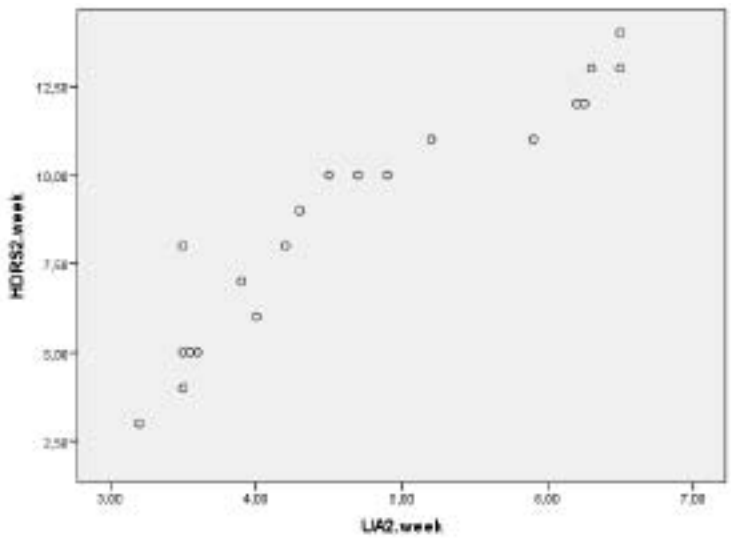

Fig. 4. *Correlation between UA and HDRS in second week (r=0.52, p=0.025).*

(Table III, IV), (Fig. 5, 6).

DISCUSSION

Our findings point to the impairment in purinergic function in all periods of BD, which seems to be related to clinical properties of BD. According to the findings obtained from present study, increased uric acid levels in manic and depressive episodes seem to be associated with the severity of the episode. In cases in remission period, a moderate relation is present between uric acid levels and the age of onset.

While the literature in general present information on increased uric acid levels in mania, (5, 6), Berardis et al. (2008) report that such an increase does not take place in remisson and depression episodes (15). However, according to our findings, uric acid levels are higher in individuals with BD than healthy individuals in all three episodes of the disorder. In this case, it should be asked whether

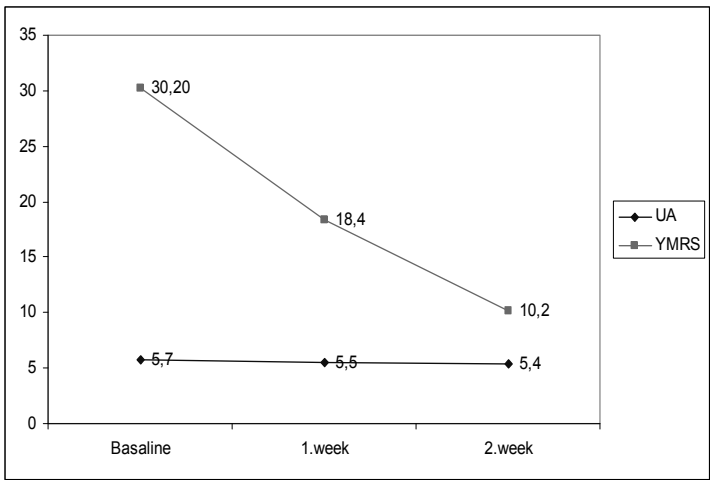

Fig. 5. *Uric acid levels and YMRS scores in manic episode.*

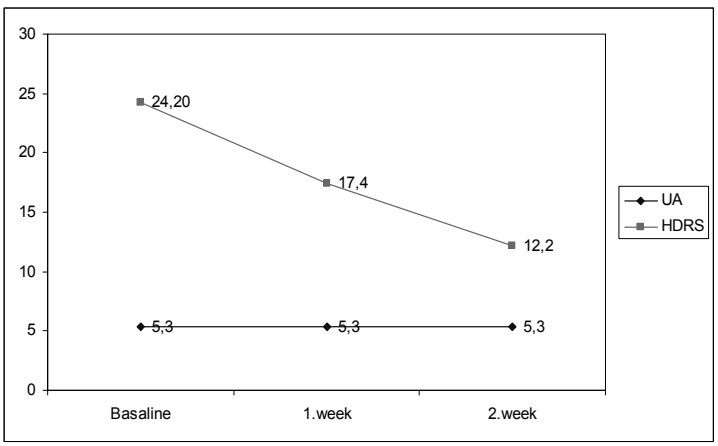

Fig. 6. *Uric acid levels and HDRS scores in depressive episode.*

increased uric acid levels are determinants of a continual predisposition as a trait rather than state marker. In fact, in individuals without any psychiatric disease, increased uric acid levels are associated with temperemantal characteristics such as high impulsivity, hyperthymic and irritable temperament (16). In addition, in other disorders in which uric acid is produced excessively such as, Lesch-Nyhan syndrome, dysinhibition states characterised by impulsivity and irritability are observed. Sutin et al (2013) suggested that higher uric acid levels are associated with impulsivity in both humans and mice (17). Thus, in individuals with hyperthymic and irritable temperament, high plasma uric acid levels may be found irrespective of disease episodes, even of the diagnosis of BD.

In a quite recent study (18) uric acid levels in unipolar cases were found to be lower than those with bipolar disease, schizophrenia and healthy controls and it was reported that uric acid levels returned to normal after 5 weeks of antidepressant treatment. When this findings are evaluated toghether with those of the present study, it may be thought that uric acid levels may be marker for the differentiation of unipolar and bipolar depression. If it is accepted that high uric acid levels are associated with extrovert temperemantal charcateristics, this role of uric acid may be used in the differentiation of unipolar depression from bipolar depression. As it is known, cyclothymic, hyperthymic and irritable temperament is more common in BD than healthy controls and those with unipolar disease (19, 20). High uric acid

levels in BD may, on their own, be related to these temperamental characteristics. In fact, consistent with this interpretation, uric acid levels in cases diagnosed with BD, are different from those in healthy individuals both in first episode cases and chronic cases and in mania, depression and remission periods. However, to confirm this interpratation, the results that found lower uric acid levels in unipolar disease than healthy individuals and higher uric acid levels in bipolar depression than those in healthy individuals should be reproduced by other studies as well.

In the study of Machado-Vieira et al. (2008) in cases in manic episode, clinical remission (decrease of YMRS scores) was found to be associated with the decrease in uric acid levels (6). However no association was shown between the severity of episode (YMRS scores) and uric acid levels. In the study of Salvadore et al. (2010) in first episode mania cases, no relation was found between the severity of episode and uric acid levels (7). In the present study, clinical remisson in manic episode is associated with decrease in uric acid levels, which suggests that increased uric acid levels may be predictive of response to treatment in manic episode. Another finding of the present study was that uric acid levels measured at 1st and 2nd weeks were found to be associated with the severity of disease in manic and depressive episodes. Our results are not consistent with previous studies. At this point, the confounding effects of psychotrope use should be kept in mind. For example, while lithium and carbamazepine decrease uric acid level (21), sodium valproate does not do so (22).

In conclusion, our study is the first one which compares uric acid levels between all episodes of bipolar disorder and healthy controls. Our findings draw attention to the impairment in purinergic function in each periods of BD. This impairment seems to be associated with clinical properties of BD. Our interpretation is that this impairment is trait characteristics rather than state and is associated with affective temperament.

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THYROID HORMONE ACTIVATES RAT LIVER ADENOSINE 5'-MONOPHOSPHATE-ACTIVATED PROTEIN KINASE: RELATION TO CaMKKβ, TAK1, AND LKB1 EXPRESSION AND ENERGY STATUS

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AMP-activated protein kinase (AMPK) is a sensor of energy status supporting cellular energy homeostasis that may represent the metabolic basis for 3,3',5-triiodo-L-thyronine (T_3) liver preconditioning. Functionally transient hyperthyroid state induced by T_3 (single dose of 0.1 mg/kg) in fed rats led to upregulation of mRNA expression (RT-PCR) and protein phosphorylation (Western blot) of hepatic AMPK at 8 to 36 h after treatment. AMPK Thr 172 phosphorylation induced by T_3 is associated with enhanced mRNA expression of the upstream kinases Ca^{2+}-calmodulin-dependent protein kinase kinase-β (CaMKKβ) and transforming growth-factor-β-activated kinase-1 (TAK1), with increased protein levels of CaMKKβ and higher TAK1 phosphorylation, without changes in those of the liver kinase B1 (LKB1) signaling pathway. Liver contents of AMP and ADP were augmented by 291% and 44% by T_3 compared to control values ($p<0.05$), respectively, whereas those of ATP decreased by 64% ($p<0.05$), with no significant changes in the total content of adenine nucleotides (AMP + ADP + ATP) at 24 h after T_3 administration. Consequently, hepatic ATP/ADP content ratios exhibited 64% diminution ($p<0.05$) and those of AMP/ATP increased by 425% ($p<0.05$) in T_3-treated rats over controls. It is concluded that *in vivo* T_3 administration triggers liver AMPK upregulation in association with significant enhancements in AMPK mRNA expression, AMPK phosphorylation coupled to CaMKKβ and TAK1 activation, and in AMP/ATP ratios, which may promote enhanced AMPK activity to support T_3-induced energy consuming processes such as those of liver preconditioning.

Thyroid hormone signaling underlies different molecular mechanisms including (i) actions mediated by direct changes in gene activity through interaction of the thyroid hormone-nuclear receptor complex with specific DNA sequences (1); and (ii) actions that are independent of intranuclear ligand binding of hormone by nuclear thyroid receptors (2). Both genomic and nongenomic mechanisms of thyroid hormone action trigger responses in the low-dose range of thyroid hormone with beneficial characteristics (hormetic responses) and responses at high-dose ranges and/or after prolonged exposure, which are potentially harmful (thyrotoxicosis) (3). Nongenomic, hormetic actions of thyroid hormones having physiological relevance comprise regulatory functions in solute transport and ion homeostasis, mitochondrial respiration, enzymatic activity, specific mRNA translation efficiency, and activation

Key words: Thyroid hormone, liver, AMP-activated protein kinase, CaMKKβ, TAK1, AMP/ATP ratio

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of signal transduction pathways (for specific references see (2).

In the latter case, activation of redox-sensitive transcription factors is triggered by the production of reactive oxygen species (ROS) associated with the calorigenic action of L-3,3',5-triiodothyronine (T_3) administration, which involves increased O_2 consumption rates at the hepatocyte level and Kupffer-cell activation (3, 4). Liver macrophage activation is characterized by both ROS generation (5) and expression and release of cytokines such as tumor necrosis factor-α (TNF-α), a mediator signaling mitochondrial ROS production in hepatocytes (6). The redox activation of these nongenomic signaling pathways by T_3 (i) upregulates the expression of antioxidant enzymes, antiapoptotic and acute-phase proteins, and phase-II detoxification enzymes and phase-III transporters; (ii) promotes hepatocyte and Kupffer cell proliferation (4, 7); and (iii) downregulates pro-inflammatory cytokine and adhesion molecule expression (8), which constitute the basis for T_3 liver preconditioning (PC) against ischemia-reperfusion (IR) injury (7). The hormetic responses underlying T_3- induced liver PC against IR injury (7, 8), which are also observed in a combined T_3 and fish oil protocol (9), are in agreement with those elicited by L-thyroxine (T_4) in the heart (10) of experimental animals, PC strategies with clinical potential (3, 4).

T_3 liver PC against IR injury must confront with a major requirement represented by adequate ATP availability, both to support energy demands for operation of PC mechanisms, namely, synthesis *de novo* of cytoprotective proteins, repair and re-synthesis of altered biomolecules, induction of the acute-phase response, and stimulation of cell proliferation, and to cope with ATP depletion induced in the ischemic phase (3, 4). In this respect, adenosine 5'-monophosphate- (AMP-) activated protein kinase (AMPK) is the downstream component of a protein kinase cascade sensing cellular energy dynamics, which limits anabolism, to prevent excessive ATP utilization, and stimulates catabolism, to increase ATP production (11). Regulation of liver AMPK activity involves direct allosteric activation and reversible phosphorylation in response to energy status, serum insulin/glucagon ratio, nutritional stresses, pharmacological and natural compounds, or cellular ROS generation (11). In view of these considerations, up-regulation of hepatic AMPK signaling was recently proposed to represent the metabolic basis for T_3 liver PC (12). Accordingly, the aim of this study was to test the hypothesis that T_3 administration enhances liver AMPK activation through a Thr172 phosphorylation mechanism, a process that may be mediated by upregulation of the upstream kinases Ca^{+2}-calmodulin-dependent protein kinase kinase-β (CaMKKβ), transforming growth-factor-b-activated kinase-1 (TAK1), and/or liver kinase B1 (LKB1), in addition to the LKB1 stabilizer MO25 (11). Results obtained were correlated with changes in the cellular energy status induced by T_3 through assessment of hepatic AMP/ATP ratios.

MATERIALS AND METHODS

Animal treatment

Male Sprague-Dawley rats (Animal facility of the Institute of Biomedical Sciences, Faculty of Medicine, University of Chile) weighing 180-200g were housed on a 12-h light/dark cycle and were provided with rat chow and water *ad libitum*. Animals received a single intraperitoneal dose of 0.1 mg of T_3/kg body weight or equivalent volumes of hormone vehicle (0.1 N NaOH, controls) at time zero. Studies were performed at zero (controls), 8, 12, 24, and 36 h after hormone treatment. At these experimental times, serum T_3 levels were measured via ELISA (Monobind Inc., Lake Forest, CA, USA), T_3-induced calorigenesis was assessed by the rectal temperature of the animals by means of a thermocouple (Cole-Palmer Instrument Company, Chicago, IL, USA), and the rate of O_2 consumption measured polarographically in liver perfusion experiments as previously described (5). Rats were anesthetized with 1 mL/kg zolazepam chlorhydrate (25 mg/mL) and tiletamine chlorhydrate (25 mg/mL) ip (Zoletil 50; Virbac S/A, Carros, France) and liver samples were taken, frozen in liquid nitrogen, and kept at -80°C for AMPK, CaMKKβ, TAK1, LKB1, and MO25 measurements, in addition to those of AMP, ADP (13), and ATP (14) levels. Experimental animal protocols and animal procedures complied with the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, NIH Publication 86-23, revised 1985) and were approved by Ethics Committee of the Faculty of Medicine, University of Chile (protocol CBA 0440 (FMUCH)).

Western blot analysis of AMPK, CaMKKβ, TAK1, LKB1, and MO25

Cytoplasmatic fractions were isolated from

homogenized liver samples (100-300 mg) using a Nuclear Extraction Kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's instructions. The protein concentration in cytoplasmatic fractions was determined by the absorbance at 590 nm. Soluble protein fractions (50 μg) were separated on 12% polyacrylamide gels using SDS–PAGE (15) and transferred to nitrocellulose membranes (16), which were blocked for 1 h at room temperature with TBS containing 5% bovine serum albumin. The blots were washed with TBS containing 0.1% Tween 20 and hybridized with mouse monoclonal primary antibody for AMPKα1 + AMPKα2 (Abcam, Cambridge, MA, USA), rabbit polyclonal primary antibodies for phospho AMPKα1 + AMPKα2 (phospho T172), CaMKKβ, TAK1 (phospho T187), total TAK1 (Abcam, Cambridge, MA, USA), or rabbit polyclonal primary antibodies for LKB1 and MO25 (Cell Signalling Technology, Inc, MA, USA). In all determinations, rabbit monoclonal antibody for rat α-tubulin (Cell Signalling Technology, Inc, MA, USA) was used as internal control.

After extensive washing, the antigen-antibody complexes were detected using horseradish peroxidase goat anti-rabbit IgG or peroxidase-conjugated affinity goat anti-mouse IgG and SuperSignal West Pico Chemiluminescense kit detection system (Pierce,Rockford, IL, USA). Bands were quantified by densitometry using Gel Documentation System, Biosens SC-750 (Shanghai Bio-Tech Co., Ltd., China). Results are expressed as relative units (individual protein/α-tubulin), with further normalization by the average values obtained in the control groups.

Isolation of hepatic RNA and RT-PCR assay of AMPK, TAK1, CaMKKβ, and LKB1 mRNA expression

Total RNA was isolated from 15-25 mg of frozen liver using an E.Z.N.A Total RNA Kit (Omega Bio-Tek Inc., Norcross, GA, USA) according to the manufacturer's instructions. Quantification of total RNA was performed spectrophotometrically (A_{260}/A_{280} ratio) and RNA quality was checked by electrophoresis on 0.8% agarose gels. The resulting DNAse free RNA was reverse transcribed to cDNA with ThermoScript RT-PCR System reverse transcriptase (Invitrogen Corp., Carlsbad, CA, USA), according to the manufacturer's instructions. The resulting cDNA was amplified in a PCR reaction using Platinum Taq DNA polymerase (Invitrogen Corp., Carlsbad, CA, USA), according to the manufacturer's instructions, and control 18S Classic II (QuantumRNA TM Classic 18S). Enzymes primers were designed according to the Sequence Database www.ensemble.org, using program AmpliX and synthesized by Genytec (Santiago, Chile). Nucleotide sequences for sense and antisense primers used in this study are shown in Table 1. For amplification thermocycler T personal, BiometraH was used.

The amplification was initiated by 5 min of denaturation (94°C), followed by 38 cycles (94°C for 4 min, 37°C for 30 s, 56°C for 30 s, 72°C for 1 min, 72°C for 10 min) for all nucleotide sequences. All amplification products were stored at 4°C before the electrophoretic step. All PCR products were electrophoresed on 1.2% agarose gels containing ethidium bromide (10 μg/mL). The 100 bp DNA ladder (New England Biolabs, Ipswich, MA, USA) was used as a molecular weight standard. Visualized bands by UV-induced fluorescence, and analyzed by densitometry using Gel Documentation System, Biosens SC-750 (Shanghai Bio-Tech Co., Ltd, China). Results are expressed as relative transcript levels (individual mRNA/18S rRNA), with further normalization by the average values obtained in the control groups.

Statistical analyses

Data showing Gaussian distribution according to the Kolmogorov-Smirnov test are expressed as means±standard error of the mean (SEM) for the number of separate experiments indicated.

As required, one-way ANOVA and the Newman-Keuls' test or Students' *t*-test for unpaired data assessed the statistical significance ($p<0.05$) of differences between mean values. To analyze the association between different variables, the Pearson correlation coefficient was used. All statistical analyses were computed employing GraphPad Prism™ version 2.0 (GraphPad Software, San Diego, CA, USA).

RESULTS

T_3 administration enhances liver AMPK mRNA expression and protein phosphorylation

Administration of a single dose of T_3 to fed rats resulted in significant increases in the serum T_3 levels at 8 and 12 h after treatment ($p<0.05$) over control animals, which declined towards control values in the 24 to 36 h time-interval (Fig. 1A).

Concomitantly, T_3-induced calorigenesis was evidenced by the enhancement ($p<0.05$) in the rectal temperature of the animals (Fig. 1B) and in the rate of O_2 consumption by the liver (Fig. 1C), a target organ for T_3 action (1, 3). Furthermore, rat serum T_3 levels were significantly correlated with the rectal temperature of the animals ($r=0.63$, $p<0.05$), whereas rectal temperature was associated with hepatic O_2 uptake ($r=0.86$, $p<0.05$). Under these conditions, T_3 enhanced liver AMPK mRNA expression at 8 and 12 h after treatment, with a peak increase at 24 h ($p<0.05$) and normalization at 36 h (Fig. 1D). In

Table I. *Primer sequences used for gene expression assays.*

Enzymes		Primers
AMPK	Sense	5' CGG CAA AGT GAA GAT TGG AG 3'
	Antisense	5' GGA GTG CTG ATC ACT TGG TA 3'
TAK1	Sense	5' CGA AGT GTT CGA AGG TAG CA 3'
	Antisense	5' CTC TCA ATG GGC TTA GGC AA 3'
CaMKKβ	Sense	5' TGC CCT TTC ATG GAT GAA CG 3'
	Antisense	5' CCA AGG GTG CAG CTT GAT TT 3'
LKB1	Sense	5' GTT TGA CAT TGA GGA CGG CA 3'
	Antisense	5' CCT TCT GGC TTC ACC TTG CT 3'

AMPK, adenosine 5'-monophosphate- (AMP-) activated protein kinase; TAK1, transforming growth-factor-β-activated kinase-1; CaMKKβ, Ca^{+2}- calmodulin-dependent protein kinase kinase-β; LKB1, liver kinase B1.

addition, phosphorylated AMPK/nonphosphorylated AMPK ratios (AMPK-OP/AMPK-OH) were augmented in the 8 to 36 h time-interval after T_3 (Fig. 1E), a parameter that significantly correlated with AMPK mRNA expression (r=0.96, $p<0.005$).

T_3 administration leads to early liver CaMKKβ expression

As shown in Fig. 2A, liver CaMKKβ exhibited a 60% ($p<0.05$) increase in mRNA expression at 8 h after T_3 administration compared to controls, an effect that returned towards control values in the 12 to 36 h time-interval.

Assessment of CaMKKβ protein levels revealed 42% and 25% enhancements ($p<0.05$) at 8 and 12 h following T_3 over controls, respectively, without significant changes being observed at 24 and 36 h (Fig. 2B). CaMKKβ mRNA expression and protein levels were significantly correlated in the studied experimental groups (r=0.91, $p<0.05$) (Fig. 2C), whereas values for protein CaMKKβ and AMPK phosphorylation were significantly associated at zero (controls), 8, and 12 h after T_3 administration (r=0.56, $p<0.02$).

T_3 administration promotes liver TAK1 mRNA expression and protein phosphorylation

T_3 administration upregulated liver TAK1 mRNA expression, with significant 9%, 97%, 130%, and 33% increases over control values being observed at 8, 12, 24, and 36 h after treatment, respectively (Fig. 3A). Ratios of phosphorylated TAK1/nonphosphorylated TAK1 (TAK1-OP/TAK1-OH) were enhanced by T_3 compared to controls, as evidenced by the 33%, 58%, 84%, and 67% increases found at 8, 12, 24, and 36 h ($p<0.05$), respectively (Fig. 3B). mRNA TAK1 levels and TAK1-OP/TAK1-OH ratios exhibited peak effects at 24 h after T_3 (Fig. 3A, B) and were significantly associated (r=0.81, $p<0.05$) (Fig. 3C). Moreover, AMPK and TAK1 phosphorylation were

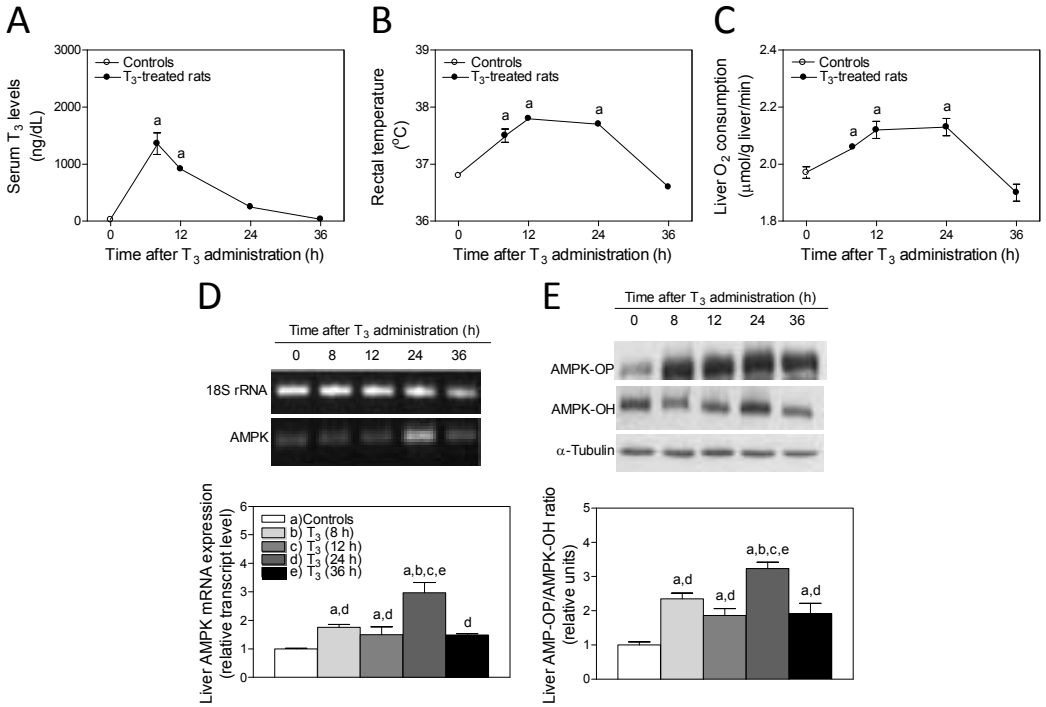

Fig. 1. *T_3-induced calorigenesis and liver AMPK upregulation. (**A**) Serum T_3 levels. (**B**) Rectal temperature of the animals. (**C**) Liver O_2 consumption. (**D**) Representative agarose gel electrophoresis of the RT-PCR products for liver AMPK mRNA (200 bp) and for 18S rRNA (489 bp) in a control rat (time zero) and animals given T_3 at several times after hormone treatment, and the respective densitometric quantification of RT-PCR products of AMPK mRNA expressed as relative transcript levels. (**E**) Representative blots of liver soluble protein fractions for detection of phosphorylated AMPK (AMPK-OP) (62 kDa), non phosphorylated AMPK (AMPK-OH) (62 kDa), and α-tubulin (52 kDa) in a control rat (time zero) and animals given T_3 at several times after hormone treatment, and the respective densitometric quantification normalized by α-tubulin expressed as AMPK-OP/AMPK-OH ratios. Values shown are means ± SEM (n=3-6). Significance studies (one-way ANOVA and the Newman-Keuls' test): $^a p<0.05$ versus control values (in panels **A**, **B**, and **C**); $p<0.05$, indicated by the letters identifying each experimental group (in panels **D** and **E**).*

significantly correlated in controls and T_3-treated animals at the different times studied (r=0.88, $p<0.05$).

Liver LKB1 mRNA expression and LKB1 and MO25 protein levels are not modified by T_3

T_3 administration failed to modified liver mRNA and protein contents of LKB1 in comparison to euthyroid animals (Fig. 4, A and B, respectively). In aggregate, hepatic LKB1 stabilizer MO25 was enhanced by 39% ($p<0.05$) at 12 h after T_3 treatment, without significant changes at 8, 24, and 36 h (Fig. 4C). The protein content of the LKB1 stabilizer STRAD (25) was not detected in the liver of control and T_3-trated animals (data not shown).

T_3 administration alters the content of liver adenine nucleotides with reduction in ATP/ADP and enhancement in AMP/ATP ratios

At 24 h after T_3 administration, liver AMP (Fig. 5A) and ADP (Fig. 5B) contents were enhanced by 291% and 44%, respectively, whereas those of ATP diminished by 64% (Fig. 5C), with no significant changes in total adenine nucleotide (AMP + ADP + ATP) levels (Fig. 5D).

Consequently, T_3-treated animals showed 64% diminution in the hepatic ATP/ADP content ratios over controls (Fig. 5E) (respective ADP/ATP ratios: controls, 0.060 ± 0.011 (n=3); T_3-treated rats, 0.120±0.009 (n=4); 100% increase; $p<0.05$) and

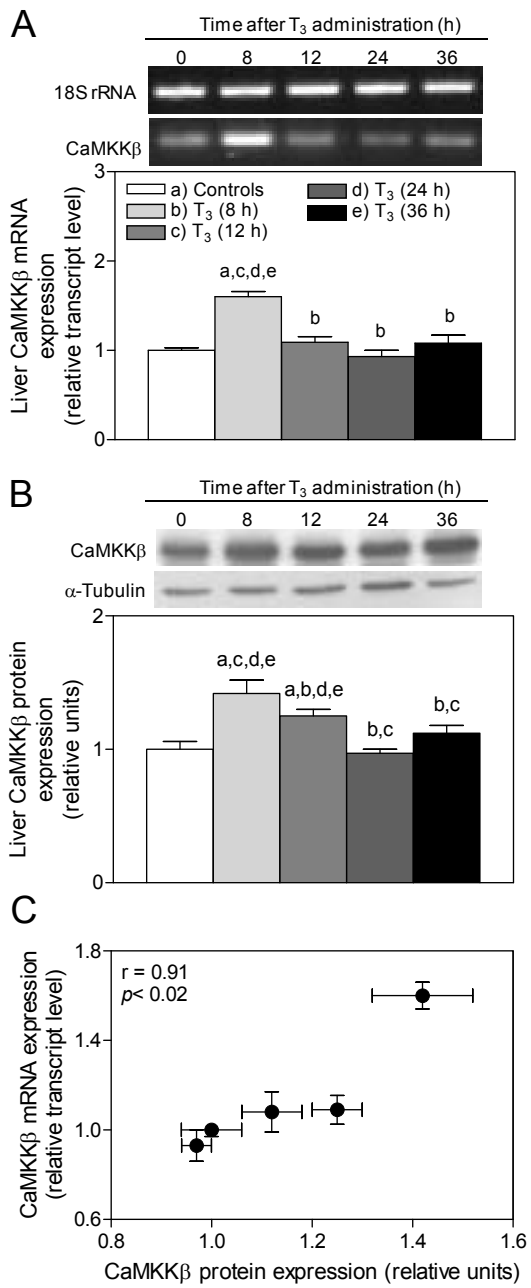

Fig. 2. *T_3 upregulates liver CaMKKβ expression. (**A**) Representative agarose gel electrophoresis of the RT-PCR products for liver CaMKKβ mRNA (200 bp) and for 18S rRNA (489 bp) in a control rat (time zero) and animals given T_3 at several times after hormone treatment, and the respective densitometric quantification of RT-PCR products of CaMKKβ mRNA expressed as relative transcript levels. (**B**) Representative blots of liver soluble protein fractions for detection of CaMKKβ (55 kDa) and α-tubulin (52 kDa) in a control rat (time zero) and animals given T_3 at several times after hormone treatment, and the respective densitometric quantification normalized by α-tubulin expressed as relative units. (**C**) Correlation between CaMKKβ mRNA expression and CaMKKβ protein expression. Values shown are means±SEM (n=5-6). Significance studies (one-way ANOVA and the Newman-Keuls test): p<0.05, indicated by the letters identifying each experimental group (in panels **A** and **B**).*

those of AMP/ATP were increased by 425% (Fig. 5F).

DISCUSSION

Administration of a single dose of T_3 to rats resulted in a functionally and transient hyperthyroid state, with concomitant upregulation of mRNA expression and protein phosphorylation of liver AMPK in the time-period of 8 to 36 h after T_3 treatment over control values, parameters that are significantly correlated. In agreement with these findings in the liver, T_3 administration leads to AMPK induction in skeletal muscle (17) and in heart (18), as a response to physiological energy needs during organ performance. Western blot analysis of AMPK revealed significant increments in Thr 172 phosphorylation as a major activating mechanism of hepatic AMPK (11). This finding is associated with upregulation of the mRNA expression of the upstream kinases CaMKKβ and TAK1, with enhanced protein levels of CaMKKβ and TAK1 phosphorylation, without changes in those of the LKB1 signaling pathway.

Liver CaMKKβ protein expression exhibited an early increase in the time-period of 8 to 12 h after T_3 administration over control values, which correlated with the respective levels of AMPK phosphorylation. Cellular CaMKKβ phosphorylates Thr 172 and activates AMPK by an AMP-independent mechanism requiring increased cytosolic Ca^{2+} concentrations $(Ca^{2+})_c$ (11). This requirement is met by thyroid hormones, as evidenced by earlier observations showing enhanced liver $(Ca^{2+})_c$ measured by fura-2-based fluorescent imaging (19). Proposed mechanisms for this effect of T_3 or T_4 include (i) rapid enhancement of Ca^{2+} uptake into the liver, as assessed in perfused rat liver

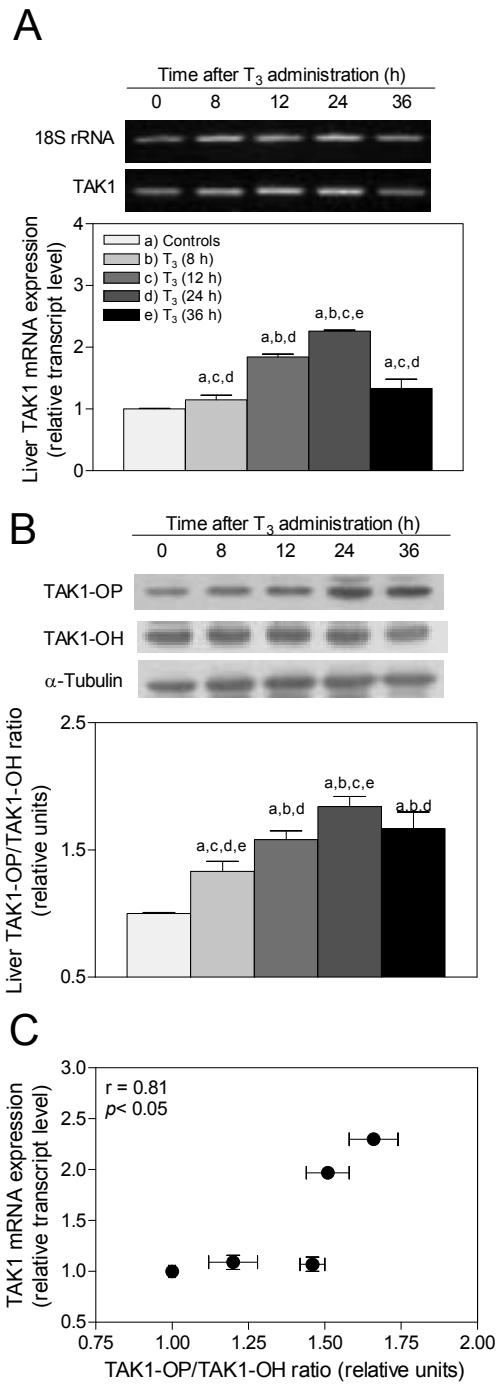

Fig. 3. *T_3 upregulates liver TAK1 expression. (**A**) Representative agarose gel electrophoresis of the RT-PCR products for liver TAK1 mRNA (200 bp) and for 18S rRNA (489 bp) in a control rat (time zero) and animals given T_3 at several times after hormone treatment, and the respective densitometric quantification of RT-PCR products of TAK1 mRNA expressed as relative transcript levels. (**B**) Representative blots of liver soluble protein fractions for detection of phosphorylated TAK1 (TAK1-OP) (67 kDa), non phosphorylated TAK1 (TAK1-OH) (67 kDa), and α-tubulin (52 kDa) in a control rat (time zero) and animals given T_3 at several times after hormone treatment, and the respective densitometric quantification normalized by α-tubulin expressed as TAK1-OP/TAK1-OH ratios. (**C**) Correlation between TAK1 mRNA expression and TAK1-OP/TAK1-OH ratios. Values shown are means±SEM (n=3-4). Significance studies (one-way ANOVA and the Newman-Keuls test): $p<0.05$, indicated by the letters identifying each experimental group (in panels **A** and **B**).*

by a Ca^{2+}-sensitive electrode (19) or in rat liver slices by ^{47}Ca accumulation (20); (ii) opening of inositol 1,4,5-trisphosphate receptor type 1 (IP_3R1) with concomitant Ca^{2+} efflux from endoplasmic reticulum into cytoplasm, a mechanism that is triggered by either increased expression and/or truncation of IP_3R1 by T_3 (21) or T_3/T_4 interaction with plasma membrane integrin aVb3 that is linked to MAPK/phospholipase C activation and IP_3 production gating IP_3R1 (22); and (iii) T_3-induced down-regulation of regucalcin (SMP30), a Ca^{2+}-binding protein activating cellular Ca^{2+} pump enzymes in the plasma membrane, microsomes, mitochondria, and nuclei (23). These findings support the contention that T_3-induced liver $(Ca^{2+})_c$ and CaMKKβ expression may promote early (8 to 12 h) AMPK phosphorylation (Fig. 6), an activation mechanism that relapses at later times (12 to 36 h) due to normalization of CaMKKβ expression.

In addition to CaMKKβ induction, T_3 administration upregulated TAK1 mRNA expression in association with enhanced TAK1 activation via phosphorylation, as evidenced by the significant increases in hepatic TAK1-OP/TAK1-OH ratios over control values in the 8 to 36 h time-interval after T_3 treatment. TAK1 is activated by several inflammatory mediators including TNF-α, interleukin-1 (IL-1), and lipopolysaccharide, via interaction with TNF-α receptor 1 (TNFR1), IL-1 receptor (IL-1R), and Toll-like receptor 4 (TLR4), respectively (24). The TAK1-dependent mechanism of liver AMPK phosphorylation in hyperthyroid state is supported by early data from our group showing redox upregulation of the NF-kB-responsive genes for TNF-α and IL-1α by T_3, with significant increases

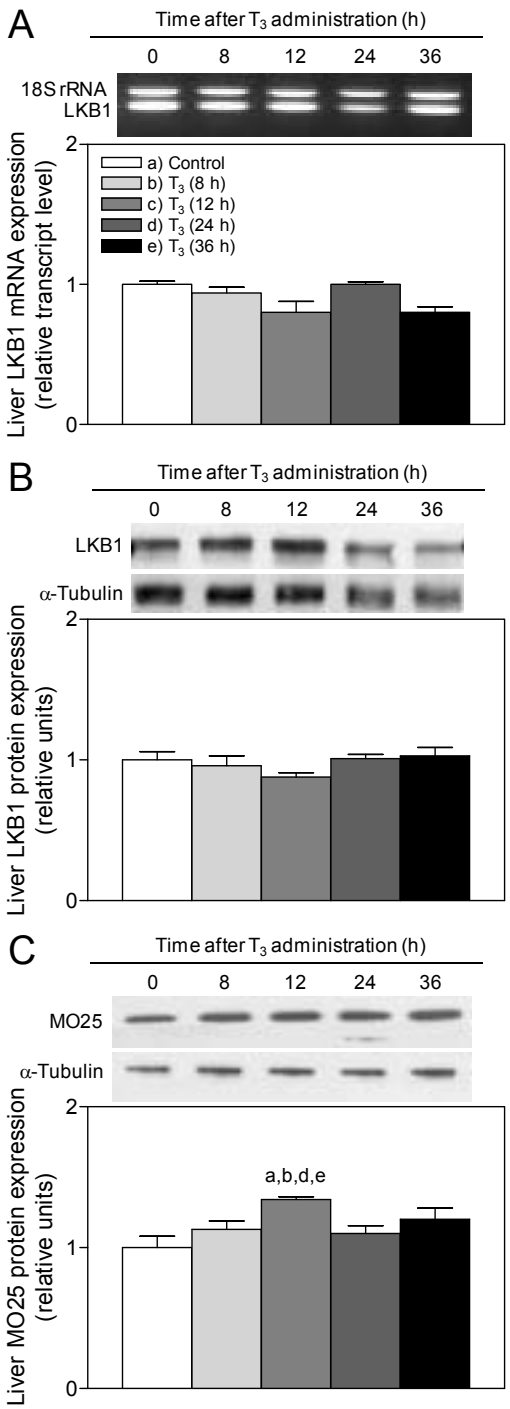

Fig. 4. *T_3 does not influence the expression of LKB1 and MO25 in liver. (**A**) Representative agarose gel electrophoresis of the RT-PCR products for liver LKB1 mRNA (200 bp) and for 18S rRNA (489 bp) in a control rat (time zero) and animals given T_3 at several times after hormone treatment, and the respective densitometric quantification of RT-PCR products of LKB1 mRNA expressed as relative transcript levels. (**B**) and (**C**), representative blots of liver soluble protein fractions for detection of either LKB1 (54 kDa) and α-tubulin (52 kDa) or MO25 (39 kDa) and α-tubulin in a control rat (time zero) and animals given T_3 at several times after hormone treatment, and the respective densitometric quantification normalized by α-tubulin expressed as relative units. Values shown are means±SEM (n=3-4). Significance studies (one-way ANOVA and the Newman-Keuls test): $p<0.05$, indicated by the letters identifying each experimental group in panel (**C**).*

in the serum levels of the cytokines (25). Under these conditions, liver TNF-α/TNRF1 and IL-1α/IL-1R interactions may lead to TAK1 phosphorylation by signaling cascades involving adapter proteins RIP1-TRAF2 and MyD88-IRAK1-TRAF6, respectively (Fig. 6). Furthermore, recent studies provided evidence that activated AMPK has a major role for activating TAK1 (26). This finding supports the suggestion that T_3-induced early liver AMPK phosphorylation by CaMKKβ may contribute to TAK1 activation, thus representing a reinforcing mechanism by which AMPK and TAK1 reciprocally regulate their kinase activities (26) (Fig. 6).

T_3 exerts profound effects on the energy metabolism in target organs including the liver (1,3), which may constitute potential signaling mechanisms modulating AMPK activity. In the absence of significant changes in the total adenine nucleotide (AMP + ADP + ATP) content of rat liver, T_3 administration led to substantial alterations in the cellular AMP/ATP and ATP/ADP content ratios. Data presented indicate that T_3 induced a significant increase in the hepatic AMP/ATP ratio, an effect known to trigger the allosteric activation of AMPK by promoting AMPK Thr 172 phosphorylation and inhibiting dephosphorylation (Fig. 6) (11), a mechanism also reported for higher ADP/ATP ratios (27). Diminution in the ATP/ADP ratio is a well known effect of thyroid hormones, which is related to simultaneous acceleration of the synthesis and consumption of ATP that enhances ATP turnover (28). Under these conditions, increased rates of mitochondrial respiration are achieved, an effect that

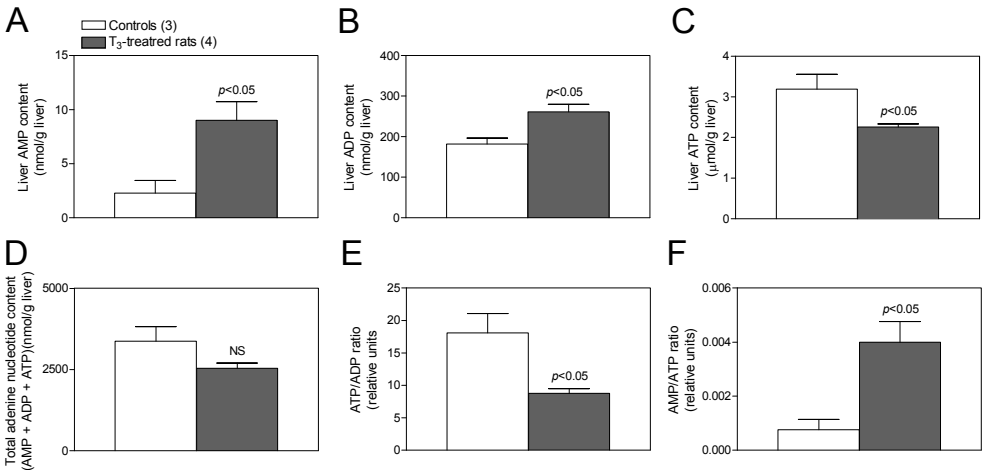

Fig. 5. *Influence of T_3 in the content of liver adenine nucleotides assessed 24 h after hormone administration. (**A**) AMP. (**B**) ADP. (**C**) ATP. (**D**) Total adenine nucleotides (AMP+ADP+ATP). (**E**) ATP/ADP ratio. (**F**) AMP/ATP ratio. Values shown are means±SEM (n=3-4). Significance studies ($p<0.05$) were carried out by Students' t-test for unpaired data (NS, not significant).*

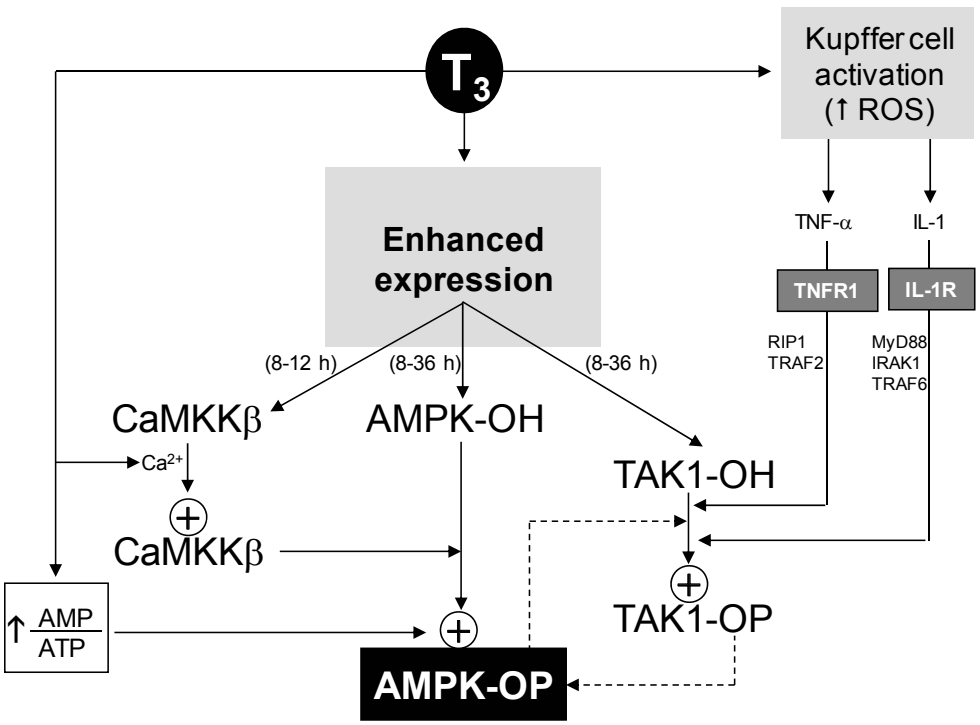

Fig. 6. *Schematic model showing role of liver energy status and of CaMKKβ and TAK1 as upstream kinases in AMPK activation in hyperthyroid state. Mechanisms involve T_3-induced (i) intracellular Ca^{2+} mobilization activating CaMKKβ; (ii) Kupffer cell expression and release of tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) triggering TAK1 phosphorylation; (iii) activated AMPK-dependent TAK1 phosphorylation and reciprocal AMPK-TAK1 interaction; and (iv) direct allosteric activation by AMP/ATP ratio enhancement. Abbreviations: AMPK-OH, AMP-activated protein kinase (AMPK-OP, phospho-AMPK); CaMKKβ, Ca^{2+}-calmodulin-dependent kinase kinase-β; IL-1R, IL-1 receptor; IRAK1, IL-1 receptor-associated kinase 1; RIP1, receptor-interacting protein 1; ROS, reactive oxygen species; TAK1-OH, transforming growth factor-β-activated kinase-1 (TAK1-OP, phospho-TAK1); TNFR1, TNF receptor 1; TRAF2(6), TNF-receptor-associated factor 2 (6).*

has been ascribed to the suppressive action of T_3 and 3,5-diiodo-L-thyronine on the allosteric inhibition of cytochrome c oxidase by ATP, provided that substrate and O_2 concentrations are not rate limiting (29).

In conclusion, T_3 administration to rats upregulates liver AMPK in association with (i) increased AMPK mRNA expression; (ii) higher AMPK Thr 172 phosphorylation coupled to upregulation of the upstream kinases CaMKKβ and TAK1 at early and latter times after treatment, respectively; and (iii) enhancement in the AMP/ATP (ADP/ATP) ratios promoting allosteric AMPK activation. T_3-induced AMPK upregulation may be of importance to cope with the energy requirements of the liver PC against IR observed (7), considering that AMPK activation switches off several anabolic pathways while switching on catabolism as a ATP preserving mechanism (11). This contention, which is currently under study in our laboratory, is in agreement with studies on AMPK activation induced by 5-aminoimidazole-4-carboxyamide-rinonucleoside (AICAR) limiting liver injury due to prolonged ischemia, constituting a target for PC in transplantation medicine (30).

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TYPE III INTERFERON (IFN-λ) ANTAGONIZES THE ANTIVIRAL ACTIVITY OF INTERFERON-α *IN VITRO*

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Type III interferons (IFN-λ) are the most recently discovered members of IFN family. Synergism between different IFN types is well established, but for type I and type III IFNs no conclusive evidence has been reported so far. Possible synergism/antagonism between IFN-α and IFN-λ in the inhibition of virus replication (EMCV, WNV lineage 1 and 2, CHIKV and HSV-1), and in the activation of intracellular pathways of IFN response (MxA and 2'-5' OAS) was evaluated in different cell lines (Vero E6, A549 and Wish cells). The antiviral potency of IFN-λ1 and -l2 was lower than that of IFN-α. When IFN-α and -λ were used together, the Combination Index (CI) for virus inhibition was >1 virtually for all virus/host cell systems, indicating antagonistic effect. Antagonism between IFN-α and -l was also observed for the induction of mRNA for both MxA and 2'-5'OAS. Elucidating the interplay between IFN-α and -λ may help to better understand innate defence mechanisms against viral infections, including the molecular mechanisms underlying the influence of IL-28B polymorphisms in the response to HCV and other viral infections.

Type I (mainly IFN-α and IFN-β) and type II (IFN-γ) IFNs are important components of the innate host immune response to viral infections. Type I IFN comprises a family of about 20 members that bind a common receptor complex: the type I IFN receptor (IFNAR) (1). Type II IFN, namely IFN-γ, is a potent immunoregulatory cytokine, mainly secreted by activated NK and T-cells, that recognizes a receptor complex different from that of type I IFN (2) and has a weaker antiviral potency than type I IFN (3).

A group of cytokines (IFN-λ1/interleukin-29 [IL-29], IFN-λ2/IL-28A, and IFN-l3/IL-28B) has recently been discovered, possessing antiviral effects (4), and has been assigned to a new type of IFN (type III IFN). The biological activity of type III IFN overlaps to some extent with that of type I IFN, and similar subsets of IFN-stimulated genes (ISGs) are induced. Viral infection can trigger both type I and III IFN production, although type III IFN exerts its action through a receptor complex distinct from that of type I IFN, including two subunits: IL-28RA, specific for type III IFN, and IL-10RB, an accessory chain that is also part of the receptors for IL-10 family cytokines, such as IL-10, IL-22, and IL-26 (5). In contrast to the universal expression of IFNAR, there is a more restricted pattern of type III IFN receptor complex, especially that of IL-28RA, suggesting that this novel IFN family has a more specialized role in host antiviral defence (6, 7). Based on scattered presence of receptor complex, the

Keywords: IFN-α, IFN-λ, antagonism, antiviral activity, combination Index, Interferon-stimulated genes

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response to type III IFN is highly cell-type specific: only epithelial-like cells and, to a lesser extent, some immune cells, mount a response to type III IFN (8).

Although type III IFN exerts its actions through a receptor complex distinct from type I IFN, it exhibits a similar antiviral activity (4, 5). In fact, type III IFN reduces viral replication or cytopathic effect of a range of viruses *in vitro*, including DNA viruses such as murine Cytomegalovirus, Hepatitis B virus and Herpes Simplex virus-1 (HSV-1), ss(+) RNA viruses such as Encephalomyocarditis virus (EMCV), West Nile virus (WNV), Usutu virus (USUV) and Hepatitis C virus (HCV), as well as ss(-) RNA viruses such asVesicular Stomatitis virus, Influenza A virus, and Hantaan virus (8-10).

IFNs exhibit direct antiviral activity by inducing an antiviral state through expression of various intracellular genes and, indirectly, by stimulating immune effector functions. Examples of pivotal effector proteins involved in antiviral pathway are the human myxovirus resistance protein 1 (MxA), a family of cytoplasmic GTPase 3 that inhibit the replication of several RNA viruses (11, 12), the double-stranded RNA-dependent protein kinase (PKR) (13) and the members of the family of 2'-5'oligoadenylate synthetases (2'-5'OAS) (14). Several studies have shown synergistic activity of type I and II IFNs in the inhibition of replication of a number of RNA and DNA viruses, including Severe Acute Respiratory Syndrome-associated coronavirus (SARS-CoV) (15,16), HCV (17), Lassa virus (LASV) (18), Varicella-Zoster virus (VZV) (19), Human Cytomegalovirus (hCMV) (20) and HSV-1 (21). Little is known about the effect of type I and type III IFNs combination, as available studies report contrasting information, with synergism reported for some (i.e. HCV) (22), but not for other viruses (i.e. EMCV and HSV-2) (23). In addition, antagonism for the intracellular pathways involved in antiviral activity has been reported (24).

The aim of the present study was to further clarify the interaction between IFN-α and IFN-λ, by investigating the capability of recombinant IFN-α and/or IFN-λ to inhibit the replication of several viruses, either used alone or in combination. To this aim, different cell lines (simian Vero E6 cells, human A549 and Wish cells), whose sensitivity to IFN-λ was previously recognized (9, 10, 25), were treated with increasing amounts of IFN-λ (either IFN-λ1 or -λ2), or IFN-α or a combination of IFN-α with either IFN-λ1 or -λ2, then infected with one of the following viruses: WNV-1 and -2, Chikungunya virus (CHIKV), EMCV and HSV-1, and the extent of virus yield inhibition was established; mRNA levels of MxA and 2'-5'OAS were measured as well.

MATERIALS AND METHODS

Cells, viruses and IFNs

Vero E6 cells were maintained in Modified Eagle Medium (MEM) while A549 and Wish cells in Dulbecco's Modified Eagle Medium (D-MEM), both containing 10% Fetal Calf Serum (FCS) and penicillin/streptomycin at 37°C in a humidified atmosphere of 5%CO_2.

For virus stock preparation, Vero E6 cells were infected with one of following viruses: WNV lineage 1 (Eg 101 strain, kindly provided by H. Feldmann, Rocky Mountain laboratories, Montana, USA), WNV lineage 2 (Nowotny strain, kindly provided by G.L Autorino, Istituto Zooprofilattico Sperimentale Delle Regioni Lazio e Toscana), primary CHIKV and HSV-1 clinical isolates from our laboratory previously described (26). EMCV was propagated on L929 cells. Cell lysates were clarified, aliquoted, and stored at −80°C until use. Virus titration was performed on the respective host cell line by limiting dilution assay, by determining tissue culture infectious dose ($TCID_{50}$) with the method of Reed & Muench. Virus stock titers were: 10^9TCID_{50}/ml for both WNV lineage 1 and 2, $10^{7.25}TCID_{50}$/ml for CHIKV, 10^7TCID_{50}/ml for HSV-1 and $10^{8.5}TCID_{50}$/ml for EMCV.

The following IFN preparations were used: human recombinant IFN-α2b (Intron®; Schering Corp., Kenilworth, NJ, USA; specific activity: 400MIU/mg,1IU corresponding to 2.5pg), human recombinant IL-29/IFN-λ1 (R&D Systems; Inc., Minneapolis, USA; ED_{50} 1-5ng/mL) and human recombinant IL-28/IFN-λ2 (PBL, Interferon source, Piscatway, NJ, USA; $ED_{50:}$ 10-50ng/mL).

Antiviral effect of IFN-α and IFN-λ combinations against EMCV, WNV lineage 1 and 2, CHIKV and HSV-1

A549, Vero E6 and Wish cells were seeded at about 10^5 cells per well in 48-well plates and after 1 day were incubated with increasing amounts of either recombinant IFN-α (1, 10, 10^2 and 10^3IU/mL) or IFN-λ1 (0.01, 0.1, 1 and 10ng/mL) or IFN-λ2 (0.1, 1, 10 and 10^2ng/mL) for 18-20h. In parallel cultures, the cells were incubated with mixtures of IFN-α+IFN-λ1 (1IU/mL+0.01ng/mL, 10IU/mL+0.1ng/mL, 10^2IU/mL+1ng/mL and 10^3IU/mL+10ng/mL, respectively) and of IFN-α+IFN-λ2 (1IU/mL+0.1ng/

mL, 10IU/mL+1ng/mL, 10^2IU/mL+10ng/mL and 10^3IU/mL+10^2ng/mL, respectively). In each plate, 6 wells were filled with 500mL of medium to serve as both virus and cell control. Cell monolayers were then infected with the indicated viruses at a multiplicity of infection (MOI) of 0.01 $TCID_{50}$/cell.

The experimental conditions used in the study (low MOI and overnight incubation, implying multiple replication cycles) were selected in order to maximize the inhibitory effect of IFN, and to obtain a higher resolution in the virus yield inhibition curves. The following virus/cell systems were used: EMCV/A549, /Vero E6 and /Wish cells; WNV lineage 1, WNV lineage 2 and CHIKV/Vero E6 cells; HSV-1/A549 and /Vero E6 cells. After absorption at 37°C for 1h, excess virus inoculum was removed and the wells were filled with 500ml of complete medium with 2%FCS. After overnight incubation of infection, culture supernatants were collected and titrated. Combination index (CI) for constant ratio combinations was calculated through the CompuSyn software, which uses the CI isobologram method of Chou and Talalay (27). The interpretation of CI value in quantifying two drug antiviral interactions is as follows: CI=1, additive; CI>1, antagonism; CI<1, synergism.

MxA and 2'-5'OAS mRNA levels

A549 cells were incubated for 3h, 6h and 24h with increasing amounts of IFN-α (1, 10, 10^2 and 10^3IU/mL), IFN-λ1 (0.01, 0.1, 1 and 10ng/mL) and IFN-α+IFN-λ1 combinations (1IU/mL+0.01ng/mL, 10IU/mL+0.1ng/mL, 10^2IU/mL+1ng/mL and 10^3IU/mL+10ng/mL).

At the indicated time points the cells were harvested and total cellular RNA was extracted using Trizol (Gibco BRL, Grand Island, NY, USA). The quantification of MxA and 2'-5'OAS mRNA was performed by a Taqman real-time RT-PCR method previously established in our laboratory. In particular, for each gene (including β-actin as housekeeping gene) a standard curve was prepared with serial dilutions of a recombinant plasmid containing the region of interest (28). For each experimental condition, the ratio of mRNA for MxA and 2'-5'OAS to β-actin was calculated, and the results were expressed as fold induction over untreated cells.

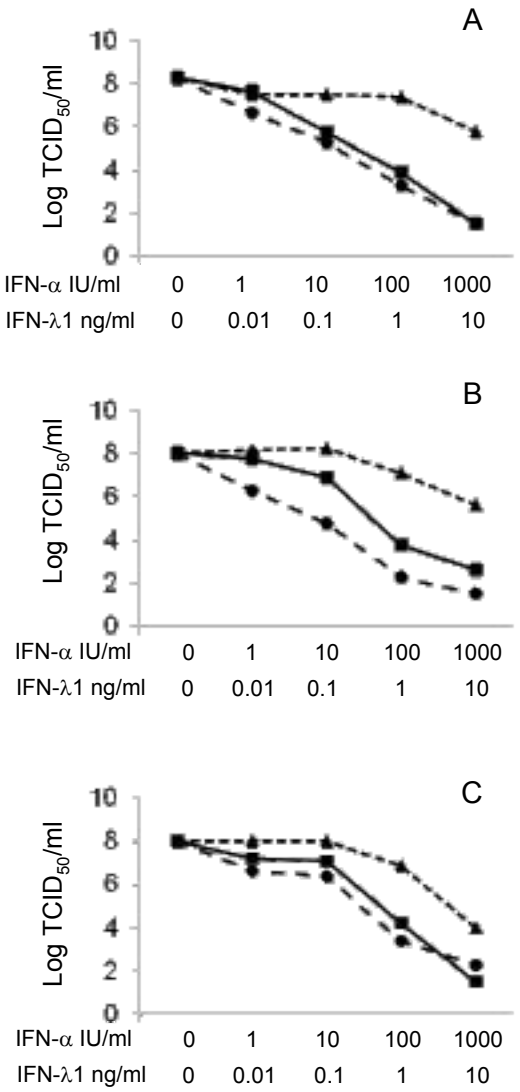

Fig. 1. *Dose-dependent inhibition of EMCV replication by recombinant IFN-α, IFN-λ1 and IFN-α+IFN-λ1 in A459 cells. The cells were treated for 1 day with increasing amounts of either IFN type, alone or in combination, then infected with EMCV at MOI 0.01; infectious virus yield was measured after overnight incubation. Three different experiments are shown (Panel **A, B** and **C**). Dotted lines: IFN-α (●) or IFN-λ1 (▲) used alone; continuous line: IFN-α and IFN-λ1 (■) used in combination.*

RESULTS

IFN-α and IFN-λ show antagonistic activity on the inhibition of EMCV replication

The ability of human recombinant IFN-α and IFN-λ1 to inhibit the replication of EMCV in A549 cells was initially investigated. This virus was chosen because of its well characterized high sensitivity to IFN action. The cells were treated for 1 day with increasing amounts of the above mentioned IFNs, used either alone or in combination, and then infected with EMCV. Although virus yield varied between the 3 experiments performed, the overall trend of the results was uniform, showing a substantially

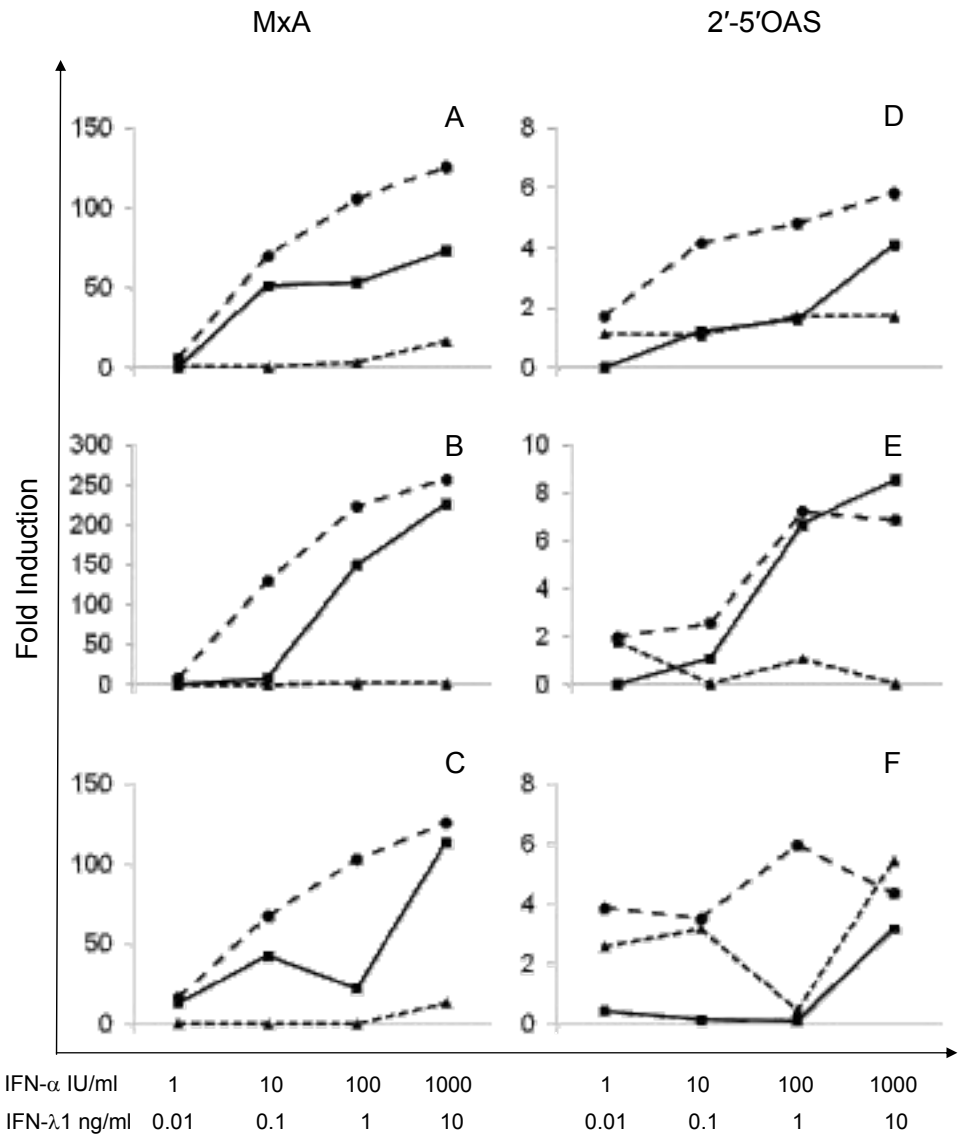

Fig. 2. *Dose-dependent induction of MxA and 2'-5'OAS mRNA levels following treatment with recombinant IFN-α, IFN-λ1 and IFN-α+IFN-λ1 in A459 cells. The cells were exposed to increasing amounts of either IFN type, alone or in combination, then MxA and 2'-5'OAS mRNA levels were measured at different time points (3, 6 and 24h), using β-actin mRNA as housekeeping gene to normalize against RNA content. The results obtained at 3h in three difference experiments for MxA (Panel A, B and C) and 2'-5' OAS (Panel D, E and F) are shown, expressed as fold induction vs treated cells. Dotted lines: IFN-α (●) or IFN-λ1 (▲) used alone; continuous line: IFN-α and IFN-λ1 (■) used in combination.*

different ability of the two IFN types to inhibit EMCV replication. As can be seen in Fig. 1 panel A, B and C, both IFN-α and IFN-λ1 reduced EMCV yield in a dose-dependent manner, being IFN-α more potent than IFN-λ1. In fact, although the EC_{50} values calculated from these experiments were in line with those expected (about 1 IU/ml for IFN-α and 1 ng/ml for IFN-λ1), the dose dependent curves of IFN-α was steeper and reached higher levels of inhibition as compared to that IFN-λ1 (7.25-8 Log $TCID_{50}$/mL reduction as compared to 2.15-4 Log $TCID_{50}$/mL reduction in the 3 experiments). As shown in Table I (representative experiment), IFN-λ2 exerted an even lower antiviral potency as compared to IFN-α.

When considering analysing the antiviral effects of combinations of IFN-α with either IFN-λ1 or

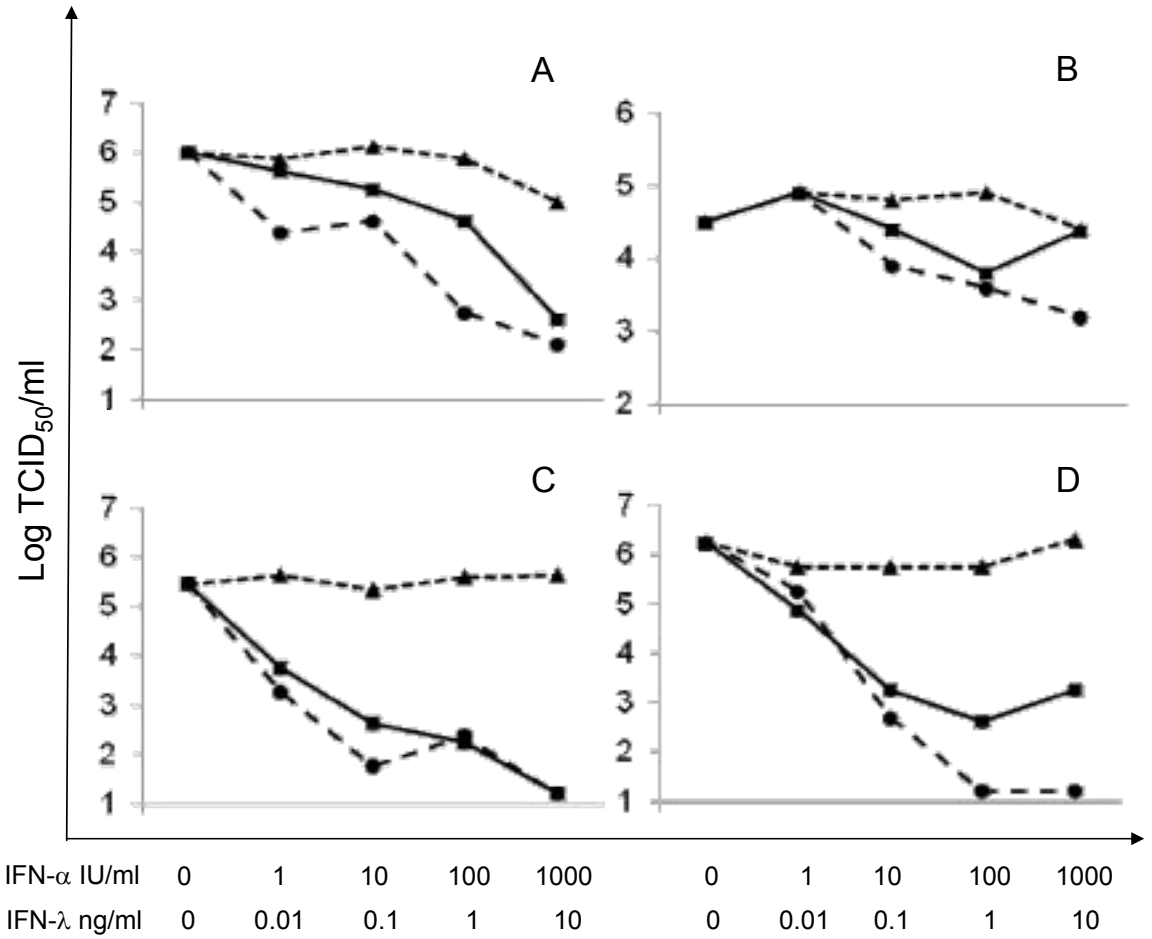

Fig.3. *Dose-dependent inhibition of replication of different viruses by recombinant INF-a, IFN-λ1 and IFN-α+IFN-λ1 in Vero E6 cells. The cells were treated for 1 day with increasing amounts of either IFN type, alone or in combination, then infected with either EMCV (**A**), or HSV-1 (**B**), or WNV lineage 1 (**C**) or WNV lineage 2 (**D**) at MOI 0.01. Infectious virus yield was measured after overnight incubation. Dotted lines: IFN-α (●) or IFN-λ1 (▲) used alone; continuous line: IFN-α and IFN-λ1 (■) used in combination.*

IFN-λ2 for each combination the inhibition of EMCV yield was smaller than that obtained for IFN-α alone (Fig. 1 and Table I), suggesting antagonistic effect between type I and type III IFNs.

The antagonistic effect was statistically confirmed by calculating the CI index IFN-α +IFN-λ combinations. As shown in Table II, CI was >1 virtually for all IFN-α +IFN-λ1 combinations (only one combination in one experiment failed to produce a CI >1). IFN-α+IFN-λ2 combinations behaved in a similar manner, producing almost invariably CI >1 (not shown).

Effect of IFN-α and IFN-λ1 combination on the induction of MxA and 2'-5'OAS mRNA

In order to characterize possible mechanisms underlying the observed antagonism between IFN-α and IFN-λ, we addressed the expression of two well-know IFN-inducible proteins: MxA and 2'-5'OAS. To this aim, A549 cells were treated for 3, 6, 24h with increasing amounts of IFN-α and IFN-λ1, either alone or in combination, and mRNA levels for MxA and 2'-5'OAS, were measured. A dose- and time-dependent induction of both ISGs mRNA was observed with all IFN combinations, but,

Table I. *Reduction of EMCV replication by IFN-α and IFN-λ2 used singularly or in combination in A549 cells.*

		Virus yield reduction ($LogTCID_{50}$/ mL)		
IFN-α (IU/ml)	IFN-λ2 (ng/ml)	IFN-α alone	IFN-λ2 alone	IFN-α + IFN-λ2
1.0	0.1	2.38	0.88	0.90
10.0	1.0	2.13	0.13	0.50
100.0	10.0	4.00	0.13	2.44
1000.0	100.0	4.63	1.00	3.50

Table II. *Combination index for IFN-α and IFN-λ1 against EMCV replication in A549 cells.*

IFN- α	IFN-λ1	Combination Index*		
(IU/ml)	ng/ml)	Exp. A	Exp. B	Exp. C
1.0	0.01	14.29	88.59	2.84
10.0	0.1	2.18	28.34	21.84
100.0	1.0	2.13	1.98	1.55
1000.0	10.0	1.01	4.35	0.54

**The Combination Index (CI) was calculated using the CompuSyn software (Chou, T.-C. and Martin, N. CompuSyn software for drug combinations and for general dose effect analysis, and user's guide. ComboSyn, Inc. Paramus, NJ 2007. [www.combosyn.com]) which uses the method of Chou & Talalay. CI values <1, 1 and >1 indicate synergism, additive effect and antagonism, respectively.*

again, IFN-α was much more effective than IFN-λ1. Furthermore, all the IFN combinations were less effective than IFN-α alone, supporting antagonism between the two IFN types. As an example, in Fig. 2 the dose-response curve observed after 3h of treatment obtained in 3 different experiments is shown.

Overall these results were compatible with the

antagonistic effect observed in the inhibition of the EMCV replication experiments.

IFN-λ1 antagonizes IFN-α antiviral activity also against EMCV, WNV lineage 1 and 2, and HSV-1 on Vero E6 cells

To further investigate whether the antagonistic effect of IFN-α and IFN-λ1, observed on EMCV replication in Vero E6 cells was virus and/or cell-specific, the virus yield reduction experiment was performed using different cell lines and different viruses. As observed on A549 cells, the same pattern of antagonism between IFN-α and -λ1 was observed for EMCV replication on Vero E6 (Fig. 3 panel A) and on Wish cells (not shown). IFN-α was more potent than IFN-λ1 in inhibiting the replication also of WNV lineage 1 and 2 and was antagonized by concomitant treatment with IFN-λ1 (Fig. 3 panels C-D). When using HSV-1 as challenge virus, as expected, IFN-α was generally less effective in reducing viral replication as compared to WNV and EMCV, and the IFN-λ1 antagonism was less evident (Fig. 3 panel B). For CHIKV replication in Vero E6 cells, the antagonistic effect of the combination of IFN-α and IFN-λ1 was only marginal (not shown).

DISCUSSION

The attention to the most recently discovered IFN type (IFN-λ1-3, or IL29, IL28A and IL28B) has been called by the association between some alleles in the regulatory part of the gene and a decreased rate of both recovery from natural infection and response to IFN-α-based therapy in HCV-infected patients (29). Based on these data, it is reasonable to hypothesize some interaction between IFN-α and IFN-λ. Studies addressing this interaction gave unclear and even contrasting data. In fact, combinations of type I and type III IFNs exerted synergistic antiviral effect for HCV replication (22), but not for other viruses (i.e. EMCV and HSV-2) (23). In addition, antagonism for the intracellular pathways involved in antiviral activity has been reported (24). In the present study we observed that: i) IFN-λ is less effective than IFN-α for both the inhibition of virus replication and the induction of MxA and 2'-5'OAS; ii) IFN-λ shows antagonistic activity towards both the IFN-α-driven inhibition of virus replication of and the induction of mRNA of MxA and 2'-5'OAS.

Concerning the first point, it is to be underlined that, when comparing IFN-α and -λ, the differences may be due to differences in specific activities of the preparations, since no internationally agreed scale for specific activity is available for IFN-λ. Nevertheless, our results are in line with previous reports showing that, despite the two IFN types share common intracellular pathways leading to the establishment of antiviral state, IFN-λ exerts a less sustained and less durable signal transducers and activators of transcription (STAT) signalling, leading ultimately to lower antiviral potency (22).

The observed antagonism between IFN-λ and –a was rather unexpected. In fact, the synergism between different IFN types (namely type I and II) in inhibiting virus replication is a well established phenomenon, and has been observed in several virus/host systems. In the present study, the antagonism between IFN-l and –a was observed virtually for all the virus/host cell systems investigated, (although more pronounced in Vero E6 cells infected with WNV lineage 2), and was also appreciated for the activation of pivotal ISGs, i.e. MxA and 2'-5'OAS.

Although possible mechanisms underlying the antagonistic effects of IFN-λ remain to be elucidated, it is possible that the already reported differences in the kinetics of activation of intracellular pathways involved in antiviral activity may play an important role; for instance, the more rapid, but less intense, STAT activation and ISG induction by IFN-λ observed in some studies (22) may lead to a partial desensitization to IFN-α (24). This hypothesis is consistent with previous observation that that HCV-infected patients with increased endogenous levels of ISGs are in a state of desensitization to IFN-α administration. In this context, data obtained in HIV-HCV-infected patients have lead to the hypothesis that reduced response to IFN-α may be the result of decreased expression of IFN type I receptor (30). Detailed studies specifically addressing this issue are necessary. Moreover, it would be interesting to compare the effect of IFN-α combination also with IFN-λ3, whose antiviral potency is in the same order of magnitude of IFN-λ1 and -λ2.

Possible clinical significance of the antagonism between IFN-α and -l are difficult to establish at the moment. The influence of IL28B/IFN-l3

polymorphisms on the response to IFN-α-based therapy in chronic hepatitis C (CHC) suggests that, *in vivo*, the antiviral activities of these two different IFN types are strongly linked, and have spurred intensive studies aimed at understanding the mechanisms involved in the cross-talk between these two IFN types, especially during HCV infection.

Elucidating the interplay between type I and III IFNs will help to better understand innate defense mechanisms against viral infections, adding significant value in the efforts to struggle viral infections in humans. The results will also contribute to elucidate the mechanisms underlying the strong predictive value of IL-28B polymorphisms on the response to IFN therapy in CHC, and, ultimately, provide novel scientific evidence for a more rational planning of available and future treatments.

ACKNOWLEDGEMENTS

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A NEW AID IN TEMPOROMANDIBULAR JOINT DISORDERS' THERAPY: THE UNIVERSAL NEUROMUSCULAR IMMEDIATE RELAXING APPLIANCE

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Among the various treatment options currently indicated to deal with temporomandibular joint disorders (TMD) an important role is played by occlusal devices, which can be used in an individualized or universal manner. A new universal occlusal appliance device was designed and patented at the Clinical Gnathology Service of the Sapienza University of Rome. To assess its validity and efficacy a preliminary study on a sample of 50 patients was carried out. Patients were selected from a cohort of 158 according to the RDC-TMD (SPEC) criteria and randomly assigned to two groups, the patient group (PG), treated with the device, and a control group (CG) without any treatment. The two groups were evaluated by comparing four VAS pain scores: muscular, migraine, cervical and temporomandibular joint (TMJ). On the whole, all VAS pain scores in the PG showed a marked and statistically significant improvement after treatment, decreasing to about 50-80 %, while the control group remained stable. The best improvement was achieved in muscular pain. Age did not affect neither the initial scores, nor the pain response to the treatment. The pain scores tended to slightly increase with time of application (one, two or three months), but this trend was significant only for cervical pain. Overall the results are favourable to the application of this new occlusion device. However, the data should be considered preliminary and require further verification in time and on a higher sample of patients of both sexes.

Temporomandibular disorder (TMD) is usually defined as a generic term that include a number of clinical problems involving the masticatory muscles, the temporomandibular joint (TMJ) and the correlated structures forming the most prevalent clinical entity afflicting the masticatory apparatus (1). Regarding this, it is considered a musculoskeletal disorder, and it is also the first cause of pain of non-dental origin in the oro-facial region including head, face and associated structures.

The aetiology and the pathophysiology of TMD is poorly know, though its multifactorial origin involving a large number of direct and indirect causal variables is generally accepted. Among these, occlusion is frequently reported as one of the major aetiological factors causing TMD. Numerous theories are based on this association supposition. In fact, several therapeutic approaches such as occlusal appliance therapy, anterior repositioning devices, occlusal adjustment, restorative procedures, orthodontic and orthognathic treatment, have been reported as probable causes of TMD (2).

Typical signs and symptoms of TMD are facial pain, clicking/crepitus of the TMJs, limited jaw opening capacity, and deviation in the movement of the mandible (3). As reported in many studies, all these signs are more common in women than in men (4-7). TMD has a chronic nature, especially among women (8). The prevalence of TMD is also related to age, with the highest prevalence occurring at 20-

Key words: pain, temporomandibular disorders, splint, occlusal device, VAS score

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40 years (9-11). As demonstrated by various clinical studies besides local pain, TMD related facial pain could be associated with disorders in different parts of the body (15-18).

The indications and efficacies of therapies for TMD are currently a study topic of great importance. The currently applied treatment models are directly related to the symptoms and the timing over which they have developed, and to some of the factors affecting these symptoms and their development. Moreover, several studies have demonstrated the possibility of treating patients with TMDs using conservative methods that are chosen and individualized over time for each patient and properly modified for any change in lifestyle.

Occlusal splint is considered a conservative and a reversible therapy for patients with TMD, reducing or even eliminating the pain. Nascimento et al. (19) demonstrated a significant reduction of clinical symptoms of TMD in patients with bruxism after 60 days using occlusal splint.

The purpose of this study was to evaluate a new type of occlusal device that is ready-to-use. The technical, mechanical, physical, and construction characteristics of this device, the Universal Neuromuscular Immediate Relaxing Appliance (UNIRA by Rampello), are presented herein, along with the outcome of a preliminary study to assess its validity and efficacy on a sample of treated patients compared with a control group.

MATERIALS AND METHODS

Construction, design, and functional characteristics of the UNIRA

The majority of occlusive immediate splints used for TMD therapy are designed to resist the occlusive forces determined by the strength of the vertical muscles (masseters, internal pterygoids and temporal). In our opinion, in order to obtain a complete functional recovery and pain relief, it is necessary to establish the balance of all masticatory and facial muscles. Thus, we designed a splint that acts not only on the vertical muscles, but also on the horizontal muscles, improving the connection between the tongue, the hyoid bone, and the rachis. The design of the UNIRA stems from a combination of clinical considerations associated with the following technical prerequisites: small dimensions and reduced trauma, good comfort, easy management, and low economic and biological cost.

It was decided that the expansion, thickness, and general structure of this appliance should have a good retention and be firm. Thus, a polyvinyl (polypropylene) material was chosen, which is biocompatible, non toxic, hypoallergenic, and with a hardness of about 60-70 Shore, in accordance with European Union directives (class 1 93-42 CE). After some technical tests including compression, torsion, traction, and cut executed in the laboratory, and from an analysis of similar commercially available devices and the results of a first-phase clinical study with different prototypes over a 2-year period, a first set of UNIRA splints with a thickness of 3 mm in the occlusive active portion and 2 mm in the other parts was constructed, and used in the present study.

The UNIRA standardized device is made of some parts that are considered active and others that have a prevailing "stabilizing" action.

The so-called active parts are:

1) Two lateral genal shields that are vertical and symmetric, and have a right and left-of-oval form.
2) Two inter-occlusive levels with a roughly triangular form, but with round angles; also right and left symmetric.
3) Palatal arch linking the two horizontal, triangular, occlusive levels passing near the palatal vault.

The stabilizing parts are:

1) An arched string connecting the two lateral shields placed in the vestibular fornix inferior and front.
2) Two small vertical and semilunar wings that are detachable at the lower part in a right angle. from the two occlusive horizontal levels, encircling the mouth on the molar and premolar teeth (Fig. 1).

It is also important to note that the connecting parts have an effect upon the tissues that they come into contact with, but for the present description we prefer to divide the different parts of the splint into active and stabilizing.

The aim and functions performed by the described elements are:

1) The two lateral genal shields balance and equilibrate the perioral muscles (horizontal) and stabilize the splint horizontally.
2) The two triangular inter-occlusive and horizontal parts increase the vertical dimension and space the teeth. Indeed, they equilibrate the vertical muscles, protecting the teeth and the temporomandibular structures from inadequate para-functional forces. They also function to stabilize the vertical parts.
3) Particularly innovative in the present model is the palatal arch, which commences from the internal superior pole of the two horizontal inter-occlusive levels and continues crosswise to encircle the palatine vault at the back of the tongue. It has various functions: (i) it links elements 1 and 2, (ii) it retains

the entire splint, opposing its expulsion from the mouth sagittally, and (iii) it induces postural tongue rehabilitation and, as a consequence, rehabilitation of the lower jaw, the hyoid bone, and the cervical column, aiding the correct head posture.

4) The front lower string, the front arch, is positioned in the lower vestibular fornix, between the front lower teeth and the lower lip, linking the lateral 1 and 2 elements to stabilize and retain the splint sagittally.
5) The two small semilunar wings help the retention and intra-oral stabilization of the splint, in order to maintain the two inter-occlusive levels firm between the antagonist teeth.

The surfaces of all described elements are smooth, and thus should cause no trauma. Only the surfaces of the two inter-occlusive levels could eventually develop small holes or retentions, or become rough. Thus, auto-polymerizing and resinous materials can be added to compensate for some issues attributable to the occlusive curves, or to balance and/or individually optimize the splint.

Selection of patients and design

A cohort of 158 patients with temporomandibular articulation pathologies was observed at the Service of Clinical Gnathology of the Head-Neck Department of the Umberto I Polyclinic in Rome, between January and May 2011. These patients were studied using our clinical, anamnestic, and instrumental protocols in order to evaluate the stage of their dysfunctional pathology and/or the presence of any structural deterioration of osteoarticular and muscular components, fulfilling the diagnostic criteria for TMDs. The UNIRA device was designed to address the painful and dysfunctional symptoms associated with TMDs. The final sample selection in the present study, after a careful examination by clinicians, was performed according to the following inclusion and exclusion criteria.

Inclusion criteria:

1. Muscular pain: visual analog scale (VAS) score >30
2. Articular pain: VAS score >30
3. Violent headache and/or migraine – VAS score > 30
4. Nonreducing dislocations of the articular disk in acute cases of Miocene
5. Parafunctions associated with muscular and/or articular pain
6. Limited opening of the mouth of muscular origin
7. Agree to participate in the study

Exclusion criteria:

1. Nonreducing dislocations of the articular disk in the acute form of TMD
2. Consequences of condoyle fractures and/or fracture of another maxillofacial zone
3. Undergone surgery for TMJ
4. In therapy for the same pathologies
5. Articular pathologies of systemic nature (e.g., rheumatoid arthritis, arthrosis, psoriasis arthritis).
6. Well-known pathologies of neurologic and/or psychic nature and other forms of migraine
7. Absence of more than six teeth (three in the inferior arch and three in the superior arch)
8. necessary for the correct function of the splint

Of the 158 patients examined, 92 were excluded for the following reasons: affected by chronic lock (24); consequences of a fracture (8); a pain VAS score <30 (49); absence of more than six teeth necessary for the correct function of the splint (11). Of the remaining 66, 16 declined to participate to the study.

The remaining sample of 50 was divided randomly into two subgroups: the patient group (PG) and the control group (CG). Subjects in the PG, who were fitted with the UNIRA splint, comprised a consecutive series of 25 patients (20 women and 5 men; 20-46 years old, mean±sd 30.9±7.9). Subjects in the CG comprised 25 patients (22 women and 3 men; age 20-45 years old, mean±sd 30.2±7.3) who had similar characteristics as those in the PG, but who were given no pharmacological treatment during the therapy period.

This study was approved by the Ethics committee of the medical faculty at the Sapienza University of Rome Italy, by the number 175/11 and patient consent was also received.

All patients approved the application of their data to this study. The research reported in the paper was undertaken in compliance with the Helsinki Declaration. All patients were trained to use the VAS scale from 0 to 30.

Therapeutic protocol and patient evaluations

The therapeutic protocol for our study of the UNIRA splint was as follows:

1) The splint was applied for a minimum of 1 night, followed by rest to a maximum of 12 h/day (including night and rest) for patients with intense pain.
2) For all patients, the UNIRA splint was the only therapeutic aid used. No other form of therapy was used during the treatment period.
3) The maximum period of treatment was 3 months.

The patients provided informed consent to participate in this study, agreeing to use the UNIRA splint. All patients were aware of the trouble afflicting them and so were able to obtain a real perception of the problem, thus assuring the best compliance.

All patients in the two groups (PG and CG) were compared using a VAS scale. This is a quantitative analysis that uses periodic comparison of mill metric values of mouth-opening fluidity, symmetry, and absence of pain during mandibular movements. To reduce inter-

observer variability, the subjects of the two groups were evaluated by the same two operators who were well trained for this study. The pain levels were measured for the PG at two time intervals: before therapy, and at the end of therapy. For the CG the evaluations were carried out at the beginning of the study period and then again after 4 months**.**

Data analysis and expectations

First, descriptive statistics were computed, then two separate inferential analyses were performed. The first analysis was aimed at testing the effectiveness of the splint (i.e. efficacy in pain reduction before and after treatment in control and patients). The data have been analysed by parametric factorial analysis of variance (ANOVA) considering treatment (CG *vs* PG) as grouping factor (between subjects factor, two levels) and time (before *vs* after treatment) as repeated measure factor (within subjects factor, two levels). Post hoc comparisons were performed using Tukey's Honestly Significant Difference (HSD) test. VAS scores (muscular pain, migraine pain, cervical pain, TMJ pain) were the dependent variables. They did not correlate among them, except for cervical and migraine VAS, which correlated positively (Pearson correlation coefficient before the treatment=0.37, p=0.009). Sex was not taken into consideration because of the low sample size of males (5 males in PG, 3 in the CG), due to the higher incidence in women in the population (see introduction) and thereby weak statistical power. Age was not included, because there was no reason to believe that in the sample considered, which was rather homogeneous, it could affect the variation in VAS scores. Infact, none of the VAS scores before the treatment in the whole sample (both PG and CG pooled) correlated with age (Pearson correlation coefficients between 0.02 and 0.11; Ps between 0.46 and 0.87). Further correlations carried out on the difference between the VAS score before and after the treatment also excluded the possibility that the response to the treatment could be related to age of patients (Pearson coefficients between 0.06 and 0.23; Ps between 0.11 and 0.92). However, a slight non significant trend towards a negative association between cervical pain VAS difference and age emerged: the VAS improvement tended to decrease with increasing age (Pearson coefficient =0.23, p=011).

A second analysis was performed only in the patient group in order to test whether the effectiveness of the treatment differed in relation to time of application (1, 2 or 3 months). The four VAS scores obtained at the end of the treatment were simultaneously analysed by multivariate analysis of variance (MANOVA) with time of application as grouping factor (between subjects factor, three levels. Post hoc comparisons were performed using Fisher's protected least significant difference (PLSD) test.

All hypothesis tests used a=0.05 as criterion for significance. The analyses were performed with the software StatView 5.0.1 (Abacus).

The expected functional responses to treatment with the UNIRA splint were: (i) improvement of muscular and articular pain; (ii) improvement in migraine; (iii) improvement in the quality and quantity of mandibular movement; (iv) absence of real changes in the teeth at the three levels of space perceptible by the patient and the doctor.

RESULTS

Inferential statistics

On the whole, all VAS scores showed a marked decrease in relation to treatment. As explained in the methods, no effect of age emerged and therefore this variable was not considered. Muscular pain VAS markedly improved in the PG group and remained unchanged in the CG group (treatment $F_{1,48}=2.5$; $p=0.12$; treatment x time $F_{1,48}=18.8$; $p<0.0001$, table II). Migraine pain VAS showed a very similar pattern, markedly improving in the PG group and remaining unchanged in the CG group (treatment $F_{1,48}=2.8$; $p=0.10$; treatment x time $F_{1,48}=11.2$; $p=0.002$, table III). Cervical pain also markedly improved in the PG group, while remaining unchanged in the CG; though unfortunately and probably by chance, the CG had significantly lower values than the PG already before the treatment (treatment $F_{1,48}=10.1$; $p=0.003$; treatment x time $F_{1,48}=14.8$; $p=0.0004$, table IV). Finally, TMJ pain VAS showed, although less markedly, a similar profile as the first two VAS (treatment $F_{1,48}=0.07$; $p=0.79$; treatment x time $F_{1,48}=19.6$; $p<0.0001$, table V). Significant post-hoc tests are indicated in the figures.

As regards the effect of time of application, the results of the MANOVA are reported in Table I. For the cervical pain VAS, a significant effect emerged: the score increased significantly with increasing time of application ($F_{2,22}=7.5$; $p=0.003$, table VI). This pattern was similar for the other scores, although not significant. However, these results must be interpreted cautiously because of the low and unbalanced sample size across the three subgroups receiving different application times.

Descriptive statistics

A quantitative improvement in the mandibular

Table I. *MANOVA output for the four VAS scores in the patient group measured at the end of the treatment.*

	DF	Residual	F-value	P-value
VAS muscular	2	22	1.7	0.21
VAS migraine	2	22	2.3	0.12
VAS cervical	2	22	7.5	**0.003**
VAS TMJ	2	22	0.7	0.48

Effect of application time (1 month: N = 4; 2 months: N = 13; three months: N = 8). See also Fig. 5 and method section for details.

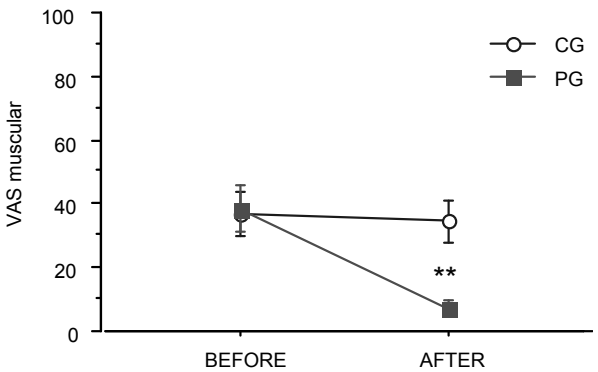

Fig. 1. *Muscular pain VAS score at the beginning and at the end of the evaluation in the patient (PG: N = 25) group, in which UNIRA splint was applied, and in the control (CG: N = 25) group. Data are means±SEM. ** p <0.01. See method section for statistical details.*

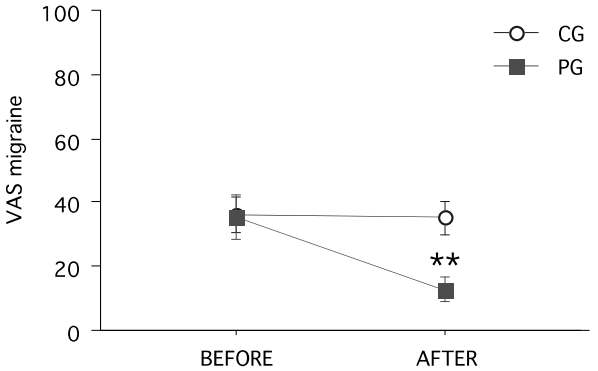

Fig. 2. *Migraine pain VAS score at the beginning and at the end of the evaluation in the patient (PG: N = 25) group, in which UNIRA splint was applied, and in the control (CG: N = 25) group. Data are means±SEM. ** p <0.01. See method section for statistical details.*

movement was observed in three of the five patients who had an initial mouth opening inferior to 30 mm (12% of the entire PG). These patients had an end-of-study mouth opening of 40 mm. The remaining two patients (8%) did experience a slight improvement, but the data remained pathologic (i.e., under 40 mm). The movement quality, with regard to symmetry (as evaluated by the doctor) and fluidity (as evaluated by the patient), improved in 20 PG patients (80%). All 25 PG patients (100%) had no sensation of change in the three space levels of the teeth.

Seven of the 15 patients in the PG (28% of the entire group) who reported muscular pain were cured of that pain after the treatment. An improvement in VAS score was observed in eight PG patients (32%). No change was observed in muscular pain in 14 out of the 15 CG patients who initially reported this symptom (List 4); only 1 CG patient noted any improvement.

In the PG, 14 of the 25 patients (56%) presented with migraine. Of these, 12 (48%) recovered during the study period; there was no change in condition in the remaining 2 patients. In the CG, of the 19 patients who complained of migraine, 17 (68% of the entire group) maintained their condition. In one of the remaining two, migraines became worse, while the condition of the other slightly improved, achieving a high VAS score of 40-60. Overall, there was no significant variation in migraine symptoms between the two groups.

Of the 15 PG patients who presented with cervical pain, an improvement was observed in 10 patients (40% of the entire group), complete recovery was observed in 2 (8% of the entire group), and no change was observed in 3 (12% of the entire group) over the study period. In the CG, those who

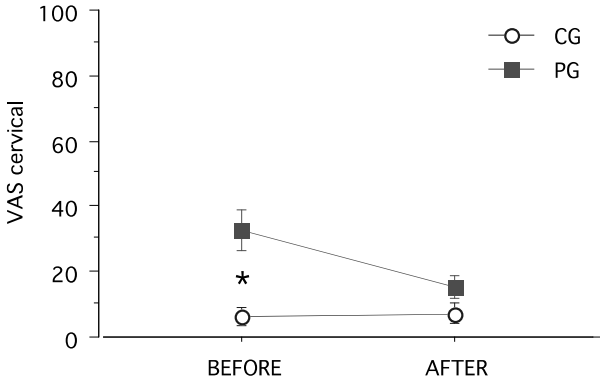

Fig. 3. *Cervical pain VAS score at the beginning and at the end of the evaluation in the patient (PG: N = 25) group, in which UNIRA splint was applied, and in the control (CG: N = 25) group. Data are means±SEM. * p <0.05. See method section for statistical details.*

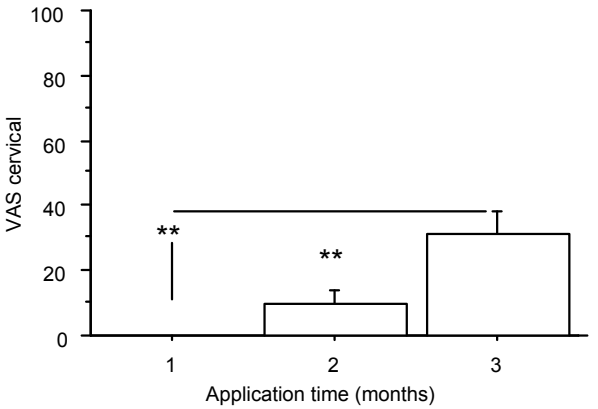

Fig. 5. *Cervical pain VAS score in the patient group in subjects with 1 month (N = 4), 2 months (N = 13) or 3 months (N = 8) of application time of the splint, measured at the end of the treatment. Data are means±SEM. ** p <0.01. At 1 month all patients had score zero. See method section for statistical details.*

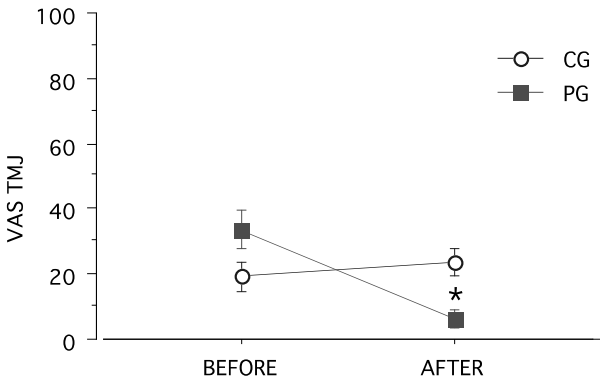

Fig. 4. *TMJ (temporomandibular joint) pain VAS score at the beginning and at the end of the evaluation in the patient (PG: N = 25) group, in which UNIRA splint was applied, and in the control (CG: N = 25) group. Data are means±SEM. * p <0.05. See method section for statistical details.*

presented with cervical pain experienced no change in their symptoms throughout the study period.

Of those patients in the PG who presented with ATM pain, improvement of symptoms was observed in five patients (20% of the entire group), and complete recovery was observed in nine patients (36% of the entire group) over the study period. No change in this symptom was observed in the remaining two patients. The ATM noises experienced by 14 patients in the CG improved during the study period in only 1 patient, while for the remaining 13 patients the symptom was unchanged.

DISCUSSION

We have been treating diseases of the stomatologic apparatus for the past 25 years at our Institute, and we are continually striving to develop diagnostic and therapeutic strategies in order to provide more rapid, comfortable, and less expensive ways of treating our patients. Following this logic, treatment strategies have advanced, allowing us to be consistently up-to date, both clinically and as regards the literature. Patients with TMDs are treated with a combination of occlusive, conservative, behavioural, and physiotherapic solutions, involving cooperation between disciplines and practitioner multidiscipline training. Patients with structural and non-reversible diseases of the osteoarticular components are seen, following diagnosis, by a maxillofacial surgeon, who will apply the correct therapy.

Over time the conceptual basis for using oral devices in treating temporomandibular disorders and Sleep Bruxer has been substantially redefined. Currently, oral appliances are still regarded as useful tools for treating certain kinds of TMD patients, but the emphasis is entirely on their conservative application (20).

The occlusal splint is considered a diagnostic device, done for assist the specialist in the management of patients with TMD. The goal of this removable device is to creating a reversible condition for relieve the pain in neuromuscular deprogramming and consequently, during muscle relaxation, reducing hyperactivity of the masticatory muscles (21-26). For this, it's necessary to obtain a number of dental occlusal contacts, bilateral, uniform and symmetrically distributed. With this in mind occlusal split must be constructed in order to have: presence of excursion guides, good fitting, stability and retention, smooth and polished surfaces.

Due to the multifactorial aetiology of TMD, the influence of occlusal splint in reducing painful symptoms in patients with TMD is a debated subject. However, in cases of bruxism, recent studies (27-30) have shown that its use is valid and rational. In the present study, according to the measurement obtained by the Visual Analog Scale, there was a significant and marked improvement on the pain reported by the patients following the use of the occlusal splint (UNIRA), which ranged from 50 to 80 % from the initial scores. Opdebeeck H. et al. demonstrated the effectiveness of occlusal devices to obtain a significant reduction of pain (31). This reduction may be associated with: changes in the occlusal condition, temporary alterations in the position of previous dental contacts, release the masticatory system from occlusal disharmony, and consequently, reducing the hyperactivity of the mandibular muscles.

As demontrated by previous study TMD patients show an higher prevalence of cervical dysfunction, infact the neck muscle iperactivity can influence the activity of the masticatory muscles; for example a wrong head position, which requires an additional demand on the cervical region, may be able to modify the entire masticatory system, causing pain and spasm (32-33).

Landulpho, et al. (34) demonstrated that an occlusal appliance placed between the dental arches caused a shorter EMG activity in the mandible rest position for the anterior temporalis muscle.

Moreover its very useful to realize an occlusal splint in centric relation, this will contribute firstly for balance muscle position and consequently for relieve dysfunction.

Previous studies have demonstrated that oral appliances are more effective for treating myogenous TMD problems than they are for intra-capsular conditions, but they can be helpful for both in opportunely selected patients (35). With this in mind clinicians will be able to use oral appliances for treating TMD patients conservatively and reversibly, as long as they avoid full-time wear or specific designs that lead to permanent occlusal changes; the worst-case scenario should be nothing more than a failure to relieve symptoms. Indeed, there is no doubt that oral devices can provide protection against excessive attrition between teeth of sleep bruxism patients. They do not stop patients from doing parafunctional activities at night, but they may decrease the duration, frequency, or intensity of those activities. The only negative possibility is development or continuation of morning muscular pain in a small number of patients, which requires a change of strategy for the majority of sleep bruxer patients.

There are many different commercially available, "ready-to-use" appliances for the solution of specific parafunctions. The evaluation of this new type of splint in this preliminary study has revealed that it did not result in worsening of symptoms in any of our PG patients; rather, it resulted in a resolution of symptoms in 24% (these patients are now being followed up every 6 months), and an improvement of symptoms in 64% (these patients are continuing their therapy). Only three of the PG patients experienced no change as a result of using the splint. These three patients were submitted to a diagnostic reevaluation, and a therapeutic program more appropriate to their specific pathology was provided. Following the study period, the CG patients were all entered into a therapeutic multidisciplinary program.

In conclusion, a positive treatment effect of the UNIRA was observed in 22 (88%) of the study PG cohort. The findings regarding improvement of cervical pain are not conclusive, and it is necessary to further analyze this problem. The main limit of this device appears to be mechanical. There are issues with dental anchorage, since there are no supporting elements. It may sometimes be impossible to place the palatal spring optimally in jaws that are particularly arched or particularly low, or those with a large arch. Furthermore, this splint is often prescribed for those_patients with particularly

dysfunctional and painful forms of the disease who have a characteristic tensive musculature, in whom immediate control of pain is necessary. Finally, the sample number of this study is low; further studies are required with a larger sample and also a long-term follow-up.

The benefits of the UNIRA are its immediacy of use by the doctor, the low cost of the appliance, the ease of control by the patient and doctor, the reduction of the sometimes long waiting times in public health systems when it is important to treat the patients as soon as possible. Another benefit is its versatility: it can be used to treat not only patients with TMDs, but also by the doctor for any patient immediately after an odontostomatologic or rehabilitative treatment that requires occlusive protection. The shims we utilize in this splint provide the best resilience to the material, optimize the resistance to forces, and are less traumatic for the oral tissues.

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THE NOSE AND SINUS MANOMETRY: A BIO-PHYSICAL MODEL APPLIED TO FUNCTIONAL ENDOSCOPIC SINUS SURGERY

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The effectiveness of sinus ventilation is due to a regular anatomy of inner nose structures such as the maxillary sinus ostium. With the aid of nose and sinus manometric measurements, it is possible to show that better functional results can be achieved using a conservative surgical technique. The present study compared 30 patients subdivided in two groups. Group A underwent conservative endoscopic sinus surgery whereas group B was operated on using non-conservative endoscopic sinus surgery. Thirty days later, both groups underwent a manometric survey of the maxillary sinus ostium by means of the digital manometry system. The pressure values obtained by nasal and sinus manometry in Group A or Group B patients were referred to those obtained in a Standard Group without nasal-sinus pathologies, calculating a percentage index of functional efficacy (maxillary sinus functional efficacy). The average percentage of the maxillary sinus functional efficacy was 98,35% for group-A patients, and 49,73% for group-B patients. Student t test revealed a statistical difference only between group B patients and standard group patients ($p<0.4$). Patients submitted to a more aggressive endoscopic approach showed inadequate sinus ventilation when compared to the standard reference group.

Sinus cavities (i.e. maxillary, frontal and sphenoid) are located around the nasal cavities and communicate through osties and ducts. An occlusion of these structures, caused by mucosal swelling, will result in a slowly increasing negative pressure inside the sinus cavity (1). The consequence is hypoxia, which is a powerful inducer of nitric oxide synthase, with a consequent increase of NO levels (2). Good sinus ventilation largely depends on the correct conformation of the nasal anatomy, the osties and the ducts, therefore sinus surgery should always be planned with the aim of preserving or restoring the inner nasal structure as much as possible.

By means of a simple biophysical model, it can be shown, that by modifying the effective area of a sinus duct (i.e. by making it wider), the ventilation properties of the related cavity change (3). In this model, the problem of air flow through the ostium-meatal complex coming from the maxillary sinus is studied by means of repeated use of Bernoulli's equation. By this approach one finds that the surgical removal of tissues close to the ostium meatal complex worsens the ventilation functional efficacy of this anatomical structure. Therefore, it can be inferred

Key words: nasal cavities, nasal-sinusal manometry, sinus surgery, biophysics model, nasal endoscopy, maxillary sinus, functional efficacy index, ostium-meatal complex, sinusometry, sinusal pressure

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that, whenever it is necessary to operate because of a sinus pathology, surgery must be planned in such a way to respect, as much as possible, the standard anatomy, in order to achieve optimal functional results.

The model we adopt refers to the ostium-meatal complex shown in Fig. 1. In the axial representation of Fig. 1 the maxillary cavity is taken to be a quasi-spherical cavity (M), in which air at atmospheric pressure is present. The maxillary sinus ostium (I) is modelled by a duct connecting this sphere with the nasal cavity (N). The latter is connected to open air through the vestibulum nasi (V). Air is thus sucked in the nasal cavity through V and proceeds inside, past the maxillary sinus ostium, through the choana (C) (4).

In order to give an account on how the pressure gradient measurement is related to nose ventilation, we may calculate, by Bernoulli's equations (3), the pressure gradient $\Delta p_I = (p_I - p_a)$ in the infundibulum ethmoidalis, by considering a point A well inside the maxillary sinus ostium I, and a point B inside the nasal cavity N. In this way, since the pressure in A is equal to the atmospheric pressure, we can write:

$$p_A = p_I + \frac{1}{2}\rho V_I^2 = p_a, \qquad \text{eq. 1}$$

where V_I is the velocity of air inside the infundibulum aethmoidalis and where we have assumed $V_A = 0$. From **eq.** 1 it follows

$$\Delta p_I = -\frac{1}{2}\rho V_I^2, \qquad \text{eq. 2}$$

which gives the expression of the negative pressure gradient in the infundibulum expressed in terms of the velocity V_I. Therefore, a direct measure of the pressure gradient by means of a digital manometer gives an indirect measure of the degree of ventilation of the nasal structure.

The present work aims to demonstrate that, by modifying the nasal cavity section surgically, by varying its effective section (i.e. removing part of the lateral nasal wall), a reduction of air flow through the maxillary sinus ostium from the maxillary cavity to the nasal cavity during inspiration takes place; this causes a worsening of the sinus ventilation process.

MATERIALS AND METHODS

A functional manometric survey of the middle meatus was initially performed on a homogeneous group of 20 Caucasian individuals, 10 (50%) males and 10 (50%) females , aged from 29 to 79 years (mean age: 42,64 years; standard deviation: 13,1 years) at the Department of Otorhinolaryngology of the "San Leonardo" Hospital (Castellammare di Stabia, Naples, Italy). All these subjects were submitted to a general ENT examination and a nose endoscopy in order to exclude anatomical alterations, such as concha bullosa, paradoxical turbinate, pneumatized agger nasi, septal spur. The subjects also underwent Prick test, P.R.I.S.T and R.A.S.T. in order to exclude either specific or aspecific rhinitis. General medical pathologies were excluded by means of pneumologic, cardiologic and endocrinologic examinations.

Only subjects that did not suffer from any nasal pathology, neither functional nor anatomical were included in this Standard group. All subjects gave their written consent to being examined, on a sheet form approved by the hospital ethic committee.

The manometry was performed using a digital nose and sinus manometer MG1 (patented by Dr. Mario Gamerra) that enable to observe pressure values both at each point and between one point and another one of the naso-sinusal cavity.

The manometer is linked to a rubber tube and a steel probe ending with a knee at right angle, in order to obtain instrumental measurements of pressure gradients inside the sinus maxillary ostium. The probe, realized for this specific purpose, was positioned in the maxillary sinus ostium point which was to be tested. The instrument was first introduced in the middle meatus with the probe pointing upward, then rotated at an angle close to 120° (in clockwise sense in the left nasal cavity and in counterclockwise sense in the right nasal cavity) through the infundibulum toward the maxillary sinus ostium. In this way the probe was placed just behind the uncinate process, and its correct position was confirmed by an endoscopic view for all patients (Fig. 2).The manomenter is linked to a computer with a software for visualization and recording of pressure values. These values , meansured in various points of nasal cavity, enable to verify the efficency of gas exchange , evaluating the functional recovery of nasal cavity after surgery.

The measurements allowed us to observe the pressure values of the key point 1 shown in Tab. I, expressed in mbar, at time 0 (inhalation starts), 1 (after 1 s), 2 (after 2 s), 3 (after 3 s), and 4 (end of inhalation after 4 s). Data were observed at a given temperature and humidity, in a conditioned environment, and with a previous acclimation of the patient, in order to create environmental

requirements for optimal function of the instrument.

Successively 30 Caucasian patients were selected, 15 males (50%) and 15 females (50%), aged from 24 to 68 years (mean age: 43,95 years; standard deviation: 13,05 years) complaining of continuous nasal obstruction and in which nasal polyposis was diagnosed by means of nose endoscopy and CT scan. All patients gave their written consent to surgery and to be included in the study. These subjects were divided in 2 groups: group A and group B, each group consisting of 15 subjects.

Group A patients were operated on through a minimally invasive surgical procedure on the ostium-meatal complex (5, 6). This type of surgical act implies preservation of the anatomical structures forming the ostium-meatal complex, especially with partial resection of the uncinate process and minimal enlargement of maxillary ostium.

Group B was treated by a more aggressive surgical technique, including total uncinectomy, dissection of the bulla, massive enlargement of the maxillary ostium and turbinotomy for the middle turbinate anomalies. In both group nasal polyps were removed by a shaver.

Thirty days after surgery, the two groups of patients underwent a digital manometry survey to evaluate the pressure values induced by the surgical procedure.

These pressure values obtained by nasal and sinusal manometry have been used to calculate the "maxillary sinus functional efficacy" (MSFE), defined as the ratio between pressure values (Δp) observed in Group A or Group B patients and the corresponding pressure values (Δp Standard) of the Standard Group

$$(MSFE \equiv \frac{\Delta p}{\Delta p_{\text{Standard}}} \times 100).$$

Moreover a Student *t*-test was performed in order to compare the pressure value at the time 2 (pressure peak) between group A and the standard group and between group B and the standard group.

RESULTS

No complication was evidenced in any group after nasal and sinusal manometry. Minimal inconvenience was referred by the patients. No differences have been evidenced between the two groups in terms of crusting .

The average pressure values of the standard group were: 1.38 mbar at time 1; 2.75 mbar at time 2; 0.61 mbar at time 3. The standard deviation values were 0.17 at time 1, 0.17 at time 2 and 0.17 at time 3 (Fig. 3).

The analysis of the patients submitted to surgery allowed us to observe the pressure values 30 days after surgery when nasal mucosa was completely recovered and no more scabs or blood was present in the nasal fossa.

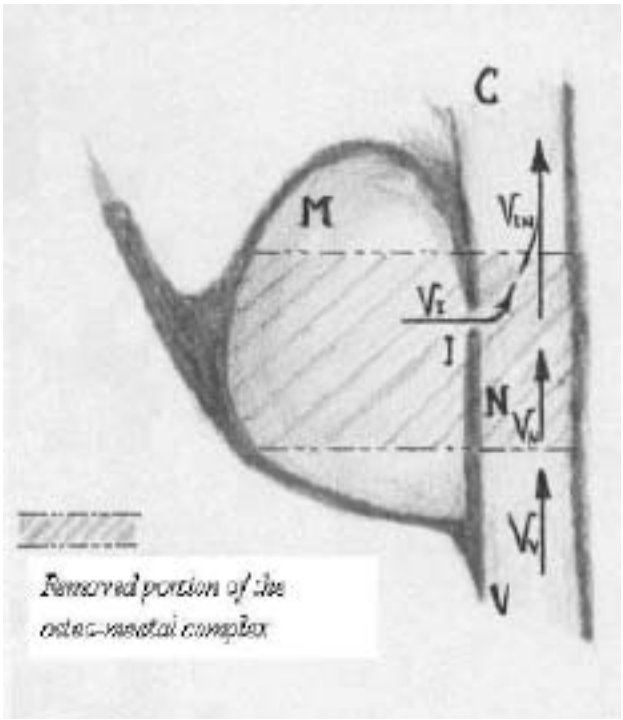

Fig. 1. *Axial representation of the Ostium Meatal Complex of the maxillary sinus. In the maxillary cavity (**M**), still air at atmospheric pressure is present. The infundibulum and the maxillary sinus ostium (**I**) is modelled by a duct connecting this sphere with the nasal cavity (**N**), which is connected to open air through the vestibulum nasi (**V**). Air sucked in through V proceeds past region I in the coana (**C**).*

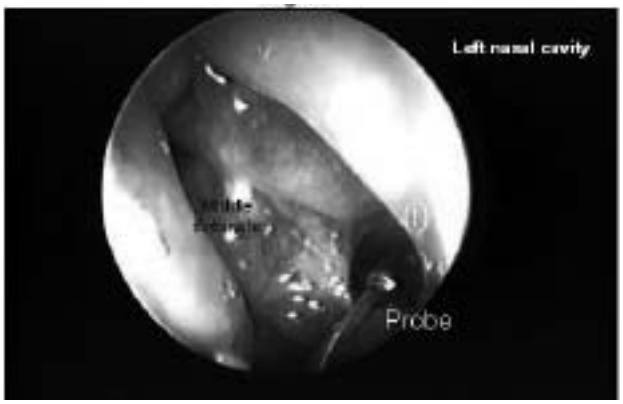

Fig. 2. *Inner endoscopic view of the positioning of the probe in the maxillary sinus ostium.*

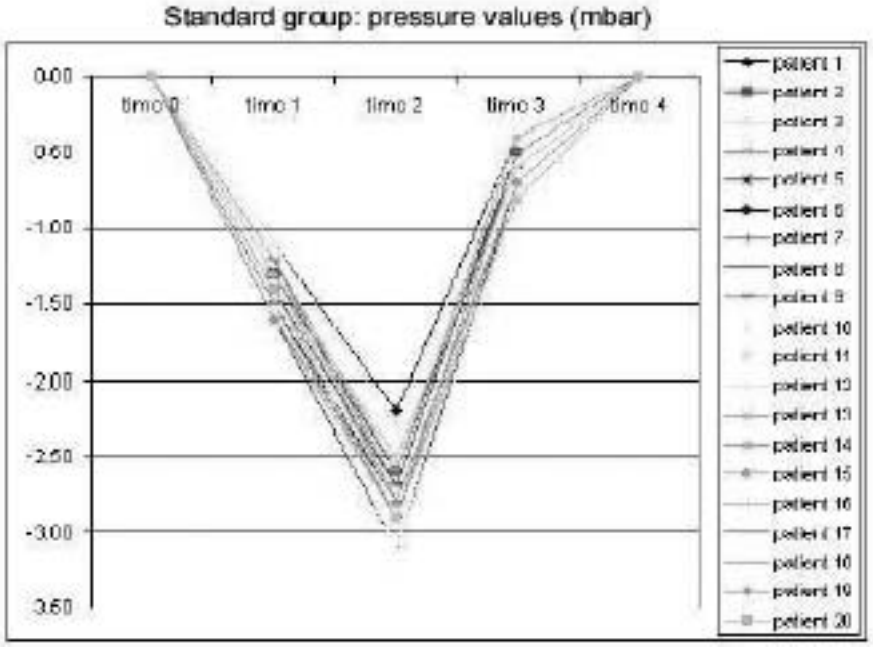

Fig. 3. *Standard pressure values, with average standard deviations, for 20 different subjects.*

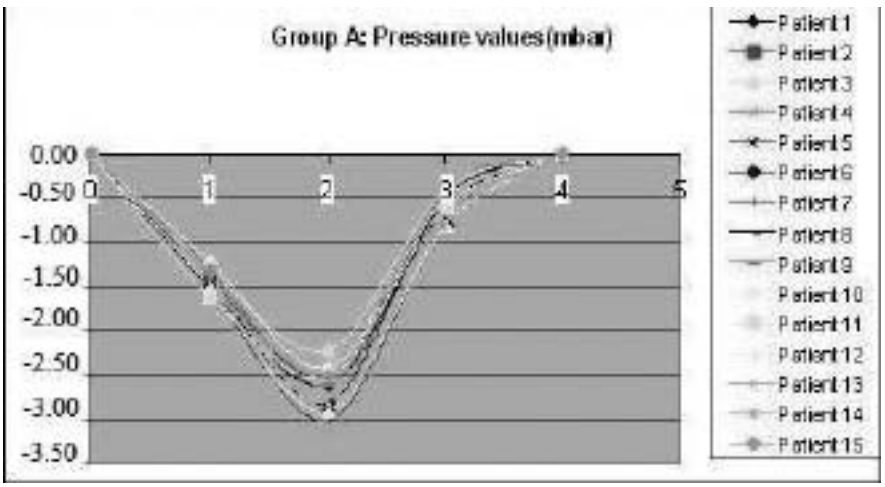

Fig. 4. *Group A pressure values, with average standard deviations, for 15 different subjects.*

The average pressure value (mbar) for group A patients has been found to be: 1.43 mbar at time 1; 2.70 mbar at time 2; 0.61 mbar at time 3. The standard deviation for all three intervals of time is equal to 0.13 mbar (Fig. 4). The "maxillary sinus functional efficacy" (MSFE) was 98.20 % at time 1, 98.80 % at time 2 and 98.00 % at time 3. The average MSFE was thus 98.35 %.

The average pressure value (in mbar) obtained from the patients of the group B was: 0.09 at time 1; 0.11 at time 2; 0.09 at time 3. With a standard deviation of 0.07 at time 1, 0.09 at time 2 and 0.06 at time 3 (Fig. 5). The MSFE was 48.80 % at time 1, 52.14 % at time 2 and 48.25 % at time 3. The

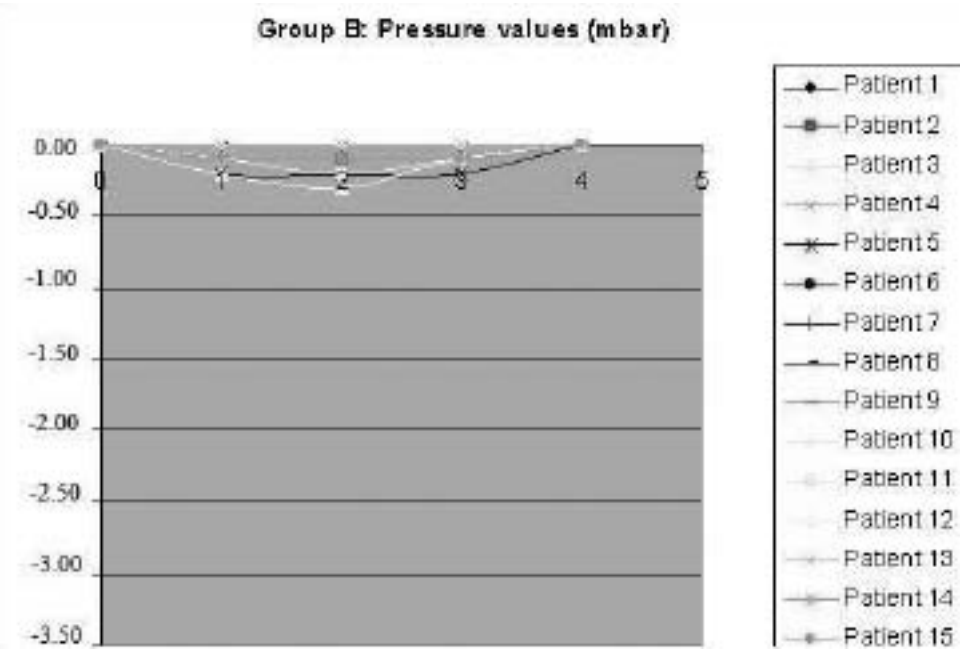

Fig. 5. *Group B pressure values, with average standard deviations, for 15 different subjects.*

average MSFE was finally 49.73 %.

All patients underwent a second digital manometry survey six months from the date of surgery. No patient showed recurrance of nasal polyposis or chronic rhinosinusitis. The data gathered in this second survey were identical to those gathered thirty days from surgery in the first survey.

The pressure peak values observed at time T2 for normal individuals in the standard group have been statistically compared with those of individuals in group A (patients treated with conservative surgery) and in group B (patients treated with traditional surgery).). The results provided by the Student *t*-test in the first case (group A) was a p-value close to 1 resulting that patients operated with the conservative technique had the same statistical characteristics of the patients without nasal disease On the other hand, the comparison with group B gave a value of $p < 0.4$, not resulting in characteristics similar to patients not suffering from nasal disease.

DISCUSSION

The structure and the shape of the ostium meatal complex allows the maxillary sinus and the frontal sinus to ventilate in a "low pressure system" located in the space between the uncinate process - bulla ethmoidalis - middle turbinate (3). Starting from a predefined biophysical model, and applying it to the nose and sinus anatomy, a surgical biophysical standard was developed, so that the operation is not only aimed at the removal of the pathology, but also at correcting the anatomical anomalies and at morphologically regenerating the mucosa, which can then recover its standard functionality as a consequence of optimal ventilation.

The above data show that group A patients, who had been treated with a minimal and conservative surgical technique, presented a 98.35 % average MSFE value. This is approximately an optimal functional activity, if compared with the standard group data, leading to restoration of maxillary sinus ventilation. On the other hand, all group B patients, who had been treated with a non-conservative technique, showed a 49.73 % average MSFE value. The results after 30 days demonstrate that there is an early functional recovery in patients that underwent conservative sinus surgery, in comparison to the patients that underwent non-conservative surgery. This fact by itself does not exclude that there could be a difference in healing

between the two techniques; nevertheless, it confirms that the conservative endoscopic surgery technique (Group A) minimizes variations to the sinus ventilation, unlike non-conservative surgery technique (Group B). Surgery by itself represents a first step in the overall therapeutic strategy of preserving mucous membranes and leads to complete healing of pathological mucosa, thus restoring normal ventilation, in about thirty days. Moreover, since healing of the inner mucosa in a non-conservative surgical operation is attained after a time span going from two to six months (7), it can be inferred that optimal MSFE is attained much earlier in Group A patients (conservative surgical technique).

Other authors have used manometric methods to study some pathological situations. Wide et al. studied the acute disease with frontal sinus trephination and its functional recovery with a method of manometry with saline leading to appreciable results (8).

Kass et al have instead analyzed the sinus pressure in the presence of certain diseases such as mucocele (9) and chronic maxillary atelectasis (10), introducing a 18 gauge needle through the membranous fontanel and connected with an amplified pressure sensitive transducer. In this way the authors have measured the average pressure, detected to be positive in mucocele and negative in chronic maxillary atelectasis. In our work the dynamic pressures that can be measured during the breathing act are considered and the measurement was performed non-invasively through the middle meatus, which is the physiological process through which sinus ventilation takes place.

Bertrand has also testified the validity of the assessment methods in sinusometry of the nasofrontal ductus (11). Other studies have shown the validity of acoustics rhinometry in the evaluation of functional outcomes of endoscopic surgery, due to their good correlation with volumetric computed tomography (12). However, this method can only indicate the changes in nasal geometry, without providing any information on the physiology of the nose and sinus pressure. In fact, it can be argued that an excessive widening of the maxillary sinus ostium effective cross section (evaluated as a success factor of antherior rhinometry) may compromise the physiologic sinus ventilation exchanges, generating mucosal inflammation.

Therefore, in our work, for the first time in literature, the outcome of endoscopic nasal sinus surgery performed with two different approaches (with different degrees of invasiveness) are compared with respect to standard ventilation state giving rise to a set of standard pressure values, measured on a group of healthy patients. All these measurements cannot be measured simply by endoscopic means, nor by rhinometry or rhinomanometry evaluation. Indeed, in our work digital manometry is used to assess the effectiveness of gas exchange at the level of the sinuses (highlighting pressure conditions that might predispose to sinusopaty) and to effectively monitor the success of surgical therapy.

In conclusion, the physical theory suggests that a conservative minimal invasive surgical technique is useful to preserve the biologic mechanisms that regulate the physiology of ventilation of the sinus cavities.

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MODULATION OF MULTIDRUG RESISTANCE P-GLYCOPROTEIN ACTIVITY BY ANTIEMETIC COMPOUNDS IN HUMAN DOXORUBICIN-RESISTANT SARCOMA CELLS (MES-SA/Dx-5): IMPLICATIONS ON CANCER THERAPY

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Multidrug resistance (MDR) in cancer cells is often caused by the high expression of the plasma membrane drug transporter P-glycoprotein (Pgp) associated with an elevated intracellular glutathione (GSH) content in various human tumors. Several chemosensitizers reverse MDR but have significant toxicities. Antiemetic medications are often used for controlling chemotherapy-induced nausea and vomiting in cancer patient. In this *in vitro* study we investigated if the effects of two common antiemetic drugs such as dimenhydrinate (dime) and ondansentron (onda) and a natural compound (6)-gingerol (ginger), the active principle of ginger root, interfere on Pgp activity and intracellular GSH content in order to evaluate their potential use as chemosensitizing agents in anticancer chemotherapy. The human doxorubicin (doxo) resistant uterine sarcoma cells (MES-SA/Dx5) that overexpress Pgp, were treated with each antiemetic alone (1, 10 and 20 μM) or in combination with different doxo concentrations (2, 4, and 8 μM). We measured the intracellular accumulation and cytotoxicity of doxo (MTT assay), the cellular GSH content (GSH assay) and ROS production (DFC-DA assay), in comparison with verapamil (Ver), a specific inhibitor for Pgp, used as reference molecule. We found that exposure at 2, 4 and 8 μM doxo concentrations in the presence of dime, onda and ginger enhanced significantly doxo accumulation and cytotoxicity on resistant MES-SA/Dx5 cells when compared with doxo alone. Moreover, treatment with ginger (20 μM) increased cellular GSH content (>10%) in resistant cells, while ROS production remained below the control values for all antiemetic compounds at all concentrations. These findings provide the rationale for innovative clinical trials of antiemetics or their derivatives as a new potential generation of chemosensitizers to improve effectiveness of the anticancer drugs in MDR human tumours.

Multidrug resistance (MDR) is one of the major clinical problems to successful cancer chemotherapy (1-4). A major cause of MDR is the overexpression of a 170-kD plasma membrane P-glycoprotein (Pgp), an ATP-dependent transporter, which transports various structurally and functionally unrelated chemotherapeutic drugs across the cell membrane, decreasing their intracellular accumulation and

Key words: P-glycoprotein, antiemetics, dimenhydrinate, ondansentron, 6-gingerol, MDR, GSH, ROS, MES-SA/Dx5, doxorubicin

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cytotoxicity in several tumour types (5-10). In addition, high cellular GSH levels observed in resistant cancer cells contribute to drug resistance through its reducing potential (11-14). In recent years, various chemosensitizers have been tested both *in vivo* and *in vitro* in combination with cytostatic drugs to inhibit Pgp activity, but currently none has been used in clinical practice because of their adverse side effects at reversing concentrations (15-18). These problems stimulate the search for novel and highly effective non-toxic Pgp inhibitors.

In our previous in vitro studies we have shown the effectivenees of two side effect-free natural sedatives, flavonoids and honokiol and a natural hepatoprotective compounds, cynarin, to inhibit Pgp activity (19-20). In addition, several drugs (doxorubicin, cisplatin, carboplatin ecc,) used in the intensive chemotherapy regimens can cause nausea and vomiting limiting the use and effectiveness of chemotherapy in cancer patients. So, antiemetic therapy is frequently used during treatment for treating of chemotherapy-induced nausea and vomiting in the management of cancer patients (21-22). The purpose of this paper, that is a continuation of our research conducted in the last years on drugs already in clinical use for supportive care in cancer patients, is to investigate the effects of two antiemetic drugs such as dimehydrinate (dime) and ondansentron (onda), and a natural compound, the (6)-gingerol (ginger), the active principle of ginger root or rhizome (Zingiber officinale Roscoe, Zingiberaceae), on Pgp activity in a human resistant sarcoma cell line (MES-SA/Dx5) that express high levels of Pgp and are resistant to doxorubicin (23-25). Doxo is a well-known P-gp substrate and is frequently used to treat human cancers.

These compounds, besides their antiemetic effects in patients receiving chemotherapy, could act as chemosensitizers modifying Pgp-mediated drug efflux and thus influencing tumour cells sensitivity to anticancer treatment. For this study, doxo resistant sarcoma cells were treated with three different doxo concentrations (2, 4 and 8 μM), alone and in combination with three different concentrations of antiemetic compounds achievable in human serum and intracellular doxo accumulation and cytotoxicity were determined. Furthermore, were have measured the levels of total intracellular glutathione (GSH) and the production of the reactive oxygen species (ROS) after treatment of MES-SA/Dx5 cells with various concentrations of antiemetics. We have used concentrations of doxo which can be achieved in the blood of patients undergoing chemotherapy treatments. The results were compared to those obtained with well-known Pgp inhibitor, Verapamil (Ver), a calcium channel blocker, used as positive control (26).

MATERIALS AND METHODS

Chemicals

Doxorubicin (doxorubicin hydrochloride), Verapamil and 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimenhydrinate, ondansentron hydrochloride, 6-gingerol, dichlorofluorescein diacetate (DCF-DA), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). All chemicals and drugs were freshly dissolved before use, except for doxorubicin, which was stored in a stock solution of 1 mM in PBS at -20°C in the dark.

Cell lines and cell cultures

The doxo-resistant human uterus sarcoma cell line MES-SA/Dx5 was obtained from the European Collection of Cell Cultures (Salisbury, UK) and grown in 25 cm^2 plastic culture flasks (Iwaki Company, Japan) containing 15 ml McCoy's 5a medium (Euroclone, Wetherby West Yorkshire, UK) supplemented with 10% heat-inactivated fetal bovine serum (Euroclone), 2 mM L-glutamine, penicillin (1000 U/ml) and streptomycin (100 μg/ml) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cells were sub-cultured every 3-4 days after treatment with 0.05% trypsin and 0.02 % EDTA in Ca^{++} and Mg^{++} free 1X Phosphate Buffered Saline (PBS). Cells were periodically checked for Mycoplasma contamination.

Intracellular accumulation of doxorubicin

MES-SA-Dx5 cells were seeded on 24-well culture dishes at a density of $1x10^5$ cells/well (Corning-Costar Corp., Corning New York) and incubated for 72 h in growth medium alone at 37°C in an atmosphere containing 5% CO_2 and 95% air to allow cell attachment. At confluence, cells were washed three times with PBS at 37°C and treated with different concentrations of dime (1, 10 and 20 μM), onda (1, 10 and 20 μM) or ginger (1, 10 and 20 μM) at 37°C. After culturing for 24 h, all pre-treated cells were exposed to 3 different doxo concentrations (2, 4 and 8 μM) and the monolayer cultures were then incubated for 3 h at 37°C. In these experiments, Ver at 10 μM was used as a positive control drug. All values were averaged from

at least three wells for each experiment. After incubation, wells were washed three times with ice-cold PBS (pH 7.4) to stop doxo accumulation and cells were solubilized with 250 µl of 4 M NaOH for 2 h and neutralized with 500 µl of 2 M HCl. The concentration of doxo was determined fluorimetrically (excitation, 475 nm; emission, 580 nm) with a fluorescence spectrophotometer (Perkin Elmer LS 45). The amount of doxo accumulated inside cancer cells pre-treated with the antiemetics compounds or Ver was expressed as a percentage of doxo retention in un-pre-treated control cells (doxo alone) at the selected concentrations (2, 4 and 8 µM) and was measured using the following formula: doxo accumulation (%): 100 x (F treated – F control) / F treated, where F treated = mean fluorescence of wells treated with dime, onda, ginger or Ver prior treatment with doxo; F control = mean fluorescence of wells treated with doxo alone.

Cytotoxicity assay

The growth inhibition of MES-SA/Dx-5 cells after treatment with antiemetics alone or in combination with doxo, was analyzed with MTT assay (27). Briefly, cells were seeded into 96-well microplates (Iwaki Company, Japan) at a density of $1x10^4$ cells/well in growth medium and incubated at 37°C for 24 h. The cells were then exposed at different concentrations of doxo (2, 4, and 8 µM) alone or in combination with dime (1, 10, 20 µM), onda (1, 10, 20 µM) or ginger (1, 10, 20 µM) and incubated at 37°C for 72 h. MES-SA/Dx5 cells containing medium alone were used as negative control for optical density (OD). Doxo with 10 µM Ver was used as positive control. The cytotoxicity of each drug itself was also measured. After incubation, wells were rinsed with PBS before adding to each well 100 µl of MTT (0.5 mg/ml) in drug-free medium. After incubation for additional 4 h, the soluble formazan dye produced by metabolically active cells was solubilized in 100 µl of 0.04 N HCl-isopropyl alcohol and quantified at 37°C on a Spectra max 190 Microplate Spectrophotometer reader (Molecular Devices) at the wavelength of 550 nm. Each experiment was performed in triplicate. The percent of cell growth inhibition was calculated as: (1-OD treated well/OD control well) x 100, where OD treated wells corresponded to the mean absorbance values of cells treated with antiemetic compounds or Ver, whereas OD control well corresponded to the mean absorbance values of un-treated malignant cells.

Measurement of intracellular glutathione

The total intracellular GSH levels of MES-SA/Dx5 cells were measured according to Ellman's method (28). Briefly, MES-SA-Dx5 cells were seeded on 24-well culture dishes at a density of $1x\ 10^6$ cells/well (Corning-Costar Corp., Corning New York) and incubated for 72 h in growth medium alone at 37°C in an atmosphere containing 5% CO_2 - 95% air. The monolayers were then exposed for 3 h to different concentrations of dime (1, 10, 20 µM), onda (1, 10, 20 µM) or ginger (1, 10, 20 µM) and Ver (10 µM) at 37° C. For total GSH determinations, the cells were washed with PBS and solubilized with 250 µl of 4 M NaOH for 2 h and neutralized with 500 µl of 2 M HCl. After centrifugation 0.1 ml aliquot of supernatants was added to 0.9 ml of phosphate/EDTA buffer (0.2 M potassium phosphate, 0.01 M EDTA, pH 7.4) containing dithionitrobenzoic acid (DNTB, 0.2 mg/ml) and the increase in absorbance at 412 nm (TNB formation) was monitored with a Cary 3C UV-visible Spectrophotometer for 15 min against an appropriate blank and quantified using molar adsorption coefficient ε=13600 M^{-1} cm^{-1}. Control MES-SA/Dx5 wells containing medium only were used as control. The assay was carried out in triplicate. Cellular GSH concentrations (nmoles for 1 x 10^6 cells) were expressed as percent of control.

Quantification of intracellular ROS

Cellular ROS were measured using the fluorogenic reagent DFC-DA. Briefly, MES-SA/Dx5 cells were seeded on 24-well culture dishes at a density of 1 x 10^4 cells/well and incubated at 37°C in 5% CO_2 as above for 48 h. After this period, the cells were treated with three different concentrations of dime (1, 10, 20 µM), onda (1, 10, 20 µM) or ginger (1, 10, 20 µM), and Ver (10 µM) as control for 24 h at 37°C. Thereafter cells were washed three times with PBS and resuspended with PBS containing 20 µM DCF-DA and incubated for 45 min at 37°C. The DCF-DA is a non-polar molecule that diffuses into the cells and within the cytosol the acetate group is cleaved by intracellular esterase to produce a hydrophobic non-fluorescent molecule that is retained by the cells. This non-fluorescent molecule can be oxidized by ROS to a highly fluorescent compound 2,7-dichlorofluorescein and fluorescence intensity is proportional to the amount of oxidant species produced by the cells (29). For ROS determinations, the cells were washed with PBS and solubilized with 250 µl of 4 M NaOH for 2 h and neutralized with 500 µl of 2 M HCl. Measurements of the change in fluorescence signals were detected using a spectrofluorimeter (Perkin Elmer LS 45; excitation at 485 nm and emission at 530). Cells containing medium alone were used as control. Intracellular ROS levels (DCF) were expressed as percent of control.

Statistical analysis

The results are expressed as mean ±SD and analyzed for statistical significance with the Student's test between the control and compounds. p-values of <0.05 were considered significant.

RESULTS

Effects of antiemetics on doxo accumulation in MES-SA/Dx5 cells

As shown in Fig. 1 pre-incubation of cancer cells for 24 h at 37°C with various concentrations of dime, onda and ginger significantly increased the doxo concentration capacity at all doxo concentrations when compared to those cultures treated with doxo alone ($p<0.05$). In particular, the mean percentage of doxo accumulation capacity after exposure at 2 μM concentration of doxo in presence of dime, onda and ginger at 1, 10 and 20 μM were 19.8±3.2; 38.3±4.7; 44.8±1.9 and 12.5±3.6; 31±6.4; 41±4.7 and 15.7±6.4; 35.1±4.9; 44±1.2, respectively, while in combination with 4 μM doxo concentration were 6.4±2.7; 20.1±5.8; 29.2±2.6 and 4.4±2.0; 12.8±2.8; 20.2±6.5 and 5.7±2.0; 20±3.4; 28.1±4.1, respectively while with 8 μM doxo were 4.1±2.8; 10.6±3.4; 17±3.0 and 1.7±1.1; 2.4±1.4; 11.1±0.8; and 2.5±1.1; 8.4±1.1; 16.2±3.1, respectively. The values of Ver (10 μM) used in combination with 2, 4 and 8 μM doxo were 42.8±0.9; 35.3±2.2 and 16±3.4, respectively (Fig. 2). All values were significant compared to control cells ($p<0.05$).

Effects of antiemetics on doxo cytotoxicity in MES-SA/Dx5 cells

The data reported in Table I show that antiemetic compounds combined with doxo significantly enhanced the sensitivity of MES-SA/Dx5 cells to doxo after incubation of 72 h at 37°C at all doxo concentrations when compared to control cells treated with doxo alone ($p<0.05$). In fact, growth inhibition using 2, 4 and 8 μM doxo concentrations in the presence of dime (1, 10 and 20 μM) increased of 1.45; 2.27; 3.18 (dime plus dx2), 1.4; 1.5; 1.8 (dime plus dx4) and 1.24; 1.3; 1.6-fold (dime plus dx8) respectively, whereas in combination with onda (1, 10 and 10 μM) increased of 1.09; 2.02; 2.54 (onda plus dx2) and 1.19; 1.36; 1.59 (onda plus dx4) and 1.06; 1.17; and 1.48-fold (onda plus dx8) respectively; while in presence of ginger (1, 10 and 20 μM) increased of 1.27; 2.18; 2.9 (ginger plus dx2) and 1.34; 1.5; 1.81 (ginger plus dx4) and 1.2; 1.27; and 1.68-fold (ginger plus dx8), respectively, when compared to the corresponding cells treated with doxo alone. However, results normalized for toxicity of antiemetic compounds reported in Fig. 3 show that ginger was able to significantly increase the percentage of growth inhibition at all doxo concentrations when compared to control ($p<0.05$). In particular at 10 μM concentration, ginger was more efficient that the well-known chemosensitizer Ver and at 20 μM in combination with 8 μM doxo concentration was able to increase significantly the doxo cytotoxicity over 40% when compared with cancer cells treated with doxo alone ($p<0.05$).

Effects of antiemetics on cellular GSH concentrations and ROS production in MES-SA/Dx5 cells

The effects of antiemetics and Ver on cellular GSH content and ROS production in MES-SA/Dx5 cells were shown in Fig 3. In cancer cells treated with dime and onda we observed that mean cellular GSH levels were below the control values at all concentrations (1, 10 and 20 μM) while ginger obtained a slightly higher values compared to control (100%) at 1 and 10 μM and a significant increase of cellular GSH levels (>13%) at 20 μM concentration ($p<0.05$). In contrast, no significant increase of ROS production was observed after treatment of MES-SA/Dx5 cells with all antiemetics at all concentrations (1, 10 and 20 μM), when compared to corresponding control cultures.

DISCUSSION

MDR Pgp-mediated of tumor cells is a major cause of failure of anticancer therapy. A number of chemosensitizers reverse MDR but have significant toxicities. In our present study we found that exposure of Pgp expressing human sarcoma MES-SA/Dx5 cells to group of antiemetic compounds dime, onda and ginger, increase doxo accumulation, when compared to un-treated control cultures (without antiemetics) at all doxo concentrations (2, 4 and 8 μM) (Fig.1). In particular, dime in combination with doxo, produced a significant increase of doxo accumulation at all concentrations tested (up to 45%) compared to control treated with doxo alone ($p<0.05$), while the increase obtained with onda (up to 41%) and ginger (up to 44%) were significant ($p< 0.05$) only when these compounds have been used at higher concentrations (10 and 20 μM) in combination with doxo (2, 4 and 8 μM) (Fig.2).

Table I. *Cytotoxic effects of doxo alone and in combination with the antiemetic compounds or Ver in MES-SA/Dx5 cells.*

	Control	*% G.I.**	*doxo2μM*	*% G.I.*	*doxo4μM*	*% G.I.*	*doxo8μM*	*% G.I.*
Medium	1.21±0.02							
doxo2μM	1.08±0.01	11±1						
doxo4μM	0.95±0.03	22±2						
doxo8μM	0.87±0.02	29±1.7						
Ver10μM	1.1±0.04	9.3±4.1	0.89±0.02	27±2	0.79±0.03	36±2.5	0.74±0.01	40±1.1
dime1μM	1.17±0.02	3±2	1.02±0.07	16±6	0.84±0.04	31±2.8	0.78±0.03	36±3.2
dime10μM	1.13±0.04	6.6±3.5	0.91±0.04	25.±3.7	0.8±0.04	34±3.7	0.76±0.05	38±3.7
dime20μM	1±0.03	17.6±2.8	0.79±0.05	35±4.5	0.71±0.02	41±2	0.62±0.02	49±1.5
onda1μM	1.18±0.01	2.7±0.7	1.06±0.02	13±2.3	0.89±0.05	26±4	0.84±0.01	31±1
onda10μM	1.14±0.03	5.8±2.5	0.95±0.04	22±3.2	0.85±0.02	30±2	0.8±0.02	34±2
onda20μM	1.02±0.04	16±4	0.87±0.05	28±3.6	0.78±0.05	35±4.7	0.69±0.02	43±1.5
ginger1μM	1.2±0.01	1.5±1.1	1.03±0.06	15±5.2	0.86±0.04	29±3.2	0.79±0.05	35±4.5
ginger10μM	1.19±0.01	2.3±1.1	0.93±0.04	24±3.4	0.81±0.04	33±3	0.77±0.06	37±5.2
ginger20μM	1.13±0.05	7±5.1	0.83±0.06	32±5.1	0.73±0.05	40±5.1	0.6±0.07	49±6.1

** Percentage of Cell growth inhibition Each value represents the mean ± SD of three experiments*

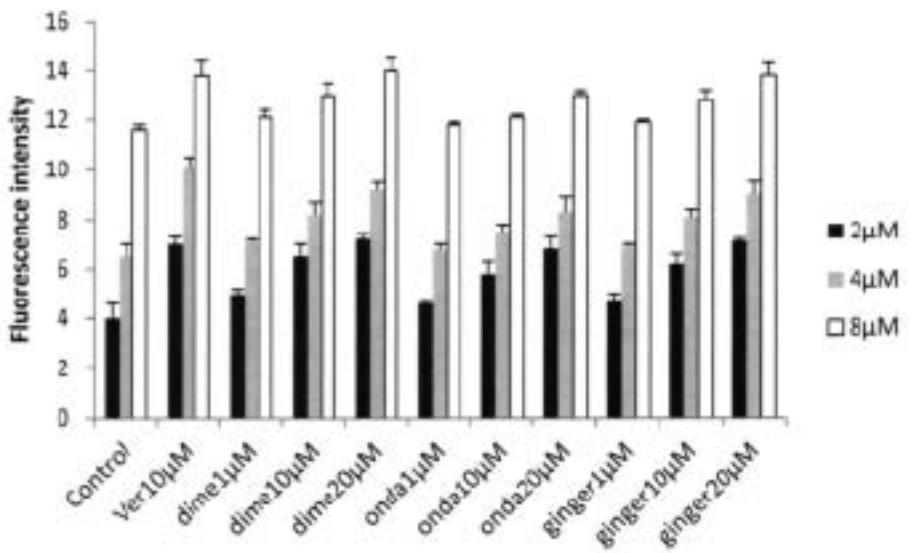

Fig. 1. *Mean intracellular doxo fluorescence intensities retained in MES-SA/Dx5 cells pre-treated for 24 h at 37°C with Ver (10 μM) , dime (1, 10, 20 μM), onda (1, 10, 20 μM) and ginger (1, 10, 20 μM) after incubation for 3 h at 37°C with three different doxo concentrations (2, 4, 8 μM). Cells treated with doxo alone were used as controls. All values were significant when compared to control ($p<0.05$). Date are the mean*±SD *of three experiments.*

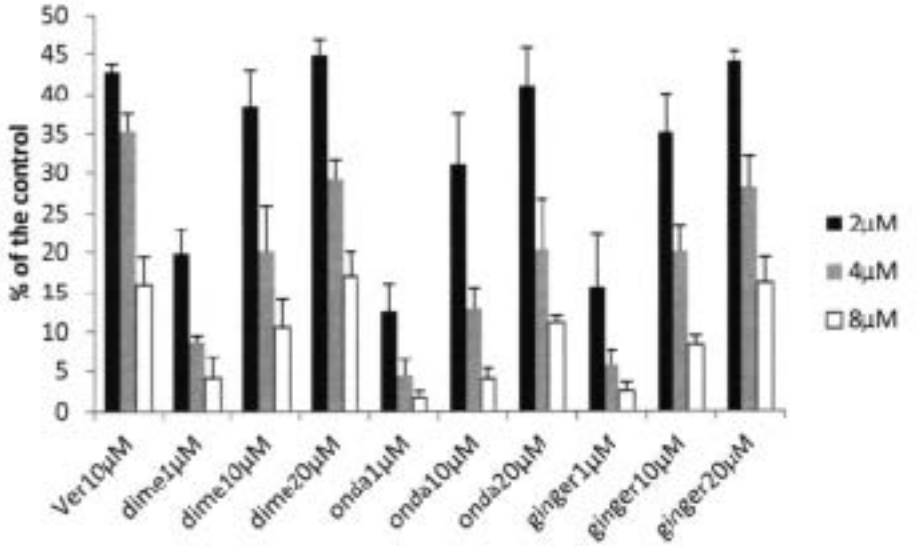

Fig. 2. *Mean percentage of increase of intracellular doxo accumulation in MES-SA/Dx5 cells pre-treated with Ver (10 μM), dime (1, 10, 20 μM), onda (1, 10, 20 μM) and ginger (1, 10, 20 μM) in combinations with three different doxo concentrations (2, 4, 8 μM) relative to un-pre-treated control cultures (doxo alone) at 2,4 and 8 μM calculated as follows: doxo accumulation (%): 100 x (F treated – F control) / F treated. All values were significant when compared with control (p<0.05). Data are the mean*±SD *of three experiments.*

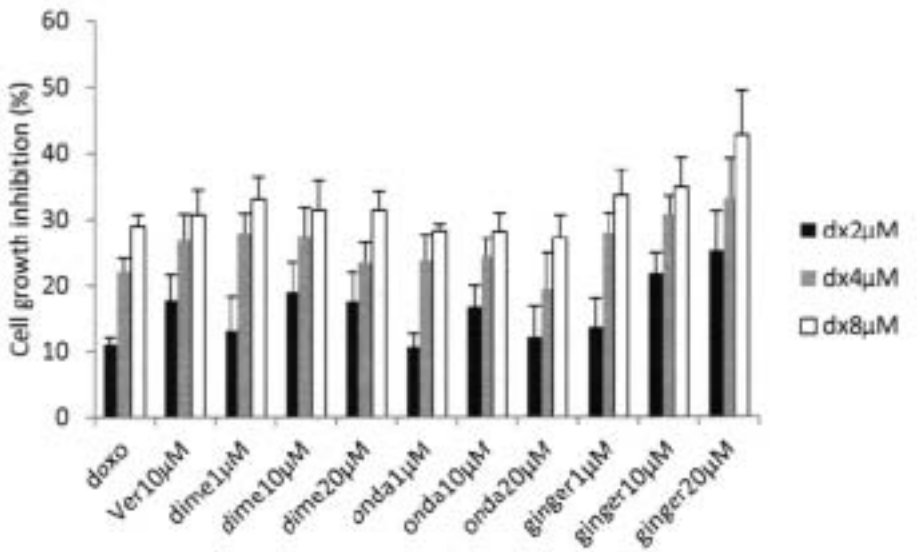

Fig. 3. *Mean percentage of cell growth inhibition determined in MES-SA/Dx5 cells after treatment with doxo alone (2, 4, 8 μM) and in combination with Ver (10 μM), dime (1, 10, 20 μM), onda (1, 10, 20 μM) and ginger (1, 10, 20 μM). The results are normalized for the toxicity of antiemetic compounds or Ver on resistant tumor cells at the indicated concentrations as described in "Materials and Methods". Values obtained with Ver 10 μM, dime 1, 10, 20 μM and ginger 1, 10, 20 μM in combination with all doxo concentrations were significant (p<0.05) when compared to control cells (dx2, dx4 and dx8 alone), while in onda series only onda 10 μM in combination with dx2 μM was significant vs control cells (doxo alone). Data are the mean*±SD *of three experiments.*

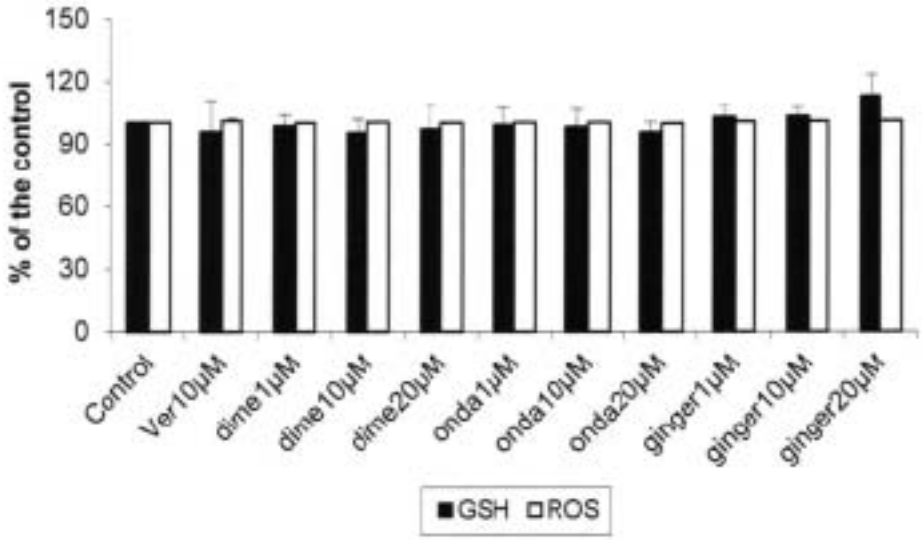

Fig. 4. *Effects of Ver (10 μM), dime (1, 10, 20 μM), onda (1, 10, 20 μM) and ginger (1, 10, 20 μM) on total intracellular GSH concentrations and ROS production. GSH concentration was calculated after 3 h treatment of the cells at 37°C. ROS production was evaluated after 24 h incubation of the MES-SA/Dx5 cells at 37°C. Cells treated with medium alone were used as control (100%). Results are expressed as a percentage of control. Data are means*±SD *of three experiments. A significant increase of cellular GSH level was observed with ginger 20 μM concentration ($p<0.05$). No significant increase of ROS production was observed.*

However, at higher doxo concentrations (8 μM) the capacity to retain doxo dropped drastically for all antiemetics tested while the highest percentage of increase in doxo retention was obtained using a low molecular concentration of doxo (2 μM) in combination with higher concentrations of antiemetics. Therefore, antiemetic compounds at low doxo concentrations could antagonize MDR through competitive inhibition of the Pgp-mediated transport of doxo resulting in a decreased efficiency of the transport of doxo.

In contrast, at higher concentration of doxo MES-SA/Dx5 cells extrude mainly doxo reducing intracellular doxo levels. These results are consistent with an increased sensitivity of doxo resistant sarcoma cells toward doxo treatment as measured by MTT test. In fact, as shown in Tab. I dime, onda and ginger at all molar concentrations (1, 10 and 20 μM) enhanced the cytotoxic effects of doxo in MES-SA/Dx5 cells at all doxo concentrations (2, 4 and 8 μM) and the increase was more elevated in the presence of the lowest molar concentrations (2 μM or 1.1 μg/ml) of doxo (1.45; 2.27; 3.18 and 1.20; 2.02; 2.54 and 1.36; 2.18; 2,9-fold, respectively) that is less than maximal serum concentrations achieved when the standard dose is used in clinical protocols (8 μg/ml).

However, cytotoxic effects of dime and onda themselves on tumour cells alone was about 3-fold higher than ginger as shown in Tab.I. In fact, whether the percentage of growth inhibition was calculated subtracting the cytotoxic activity of both dime and onda alone from the respective values obtained in combination with different doxo concentrations, it is possible to evidence an advantage for ginger at all concentrations (Fig.4). Moreover, ginger is well tolerated and a therapeutic plasma concentration of about 10-15 μM is attainable *in vivo* at clinical dosages while maximal plasma concentration of dime and onda was about 1 μM after a therapeutic oral or i.v. administration (30). Therefore, these findings are particularly important in clinical practice, because a combined therapy of ginger with doxo could enhance the efficacy of doxo-based regimens in the treatment of Pgp-mediated MDR tumors with no severe side effects. In addition, as shown in Fig. 4, exposure to antiemetics induced an

increase in GSH production only for ginger series in resistant sarcoma cells that was only slightly increase at 1 and 10 μM (2.4 and 2.8%, respectively) while was significantly increased (up to 13%) at higher (20 μM) ginger concentration ($p<0.05$). No significant modifications in ROS generation was observed in cancer cells treated with all three antiemetics at all concentrations, when compared to corresponding un-pre-treated control cells (Fig. 3).

These results suggest that dime and onda do not modify significantly the oxidative state of cancer cells while ginger could induce opposite effects in MES-SA/Dx5 cells: on one hand could act as chemosensitizer inhibiting Pgp activity and on the other hand, this natural compound, used at high concentrations, could have an anti-oxidative capacity that could be useful to protect MDR-negative normal cells against the damage caused by generation of free radicals during anticancer treatment while its extrusion from resistant cells via Pgp could reduce the protective effects of cells increasing doxo sensitivity. In conclusion, we found that all three antiemetics tested enhance the cytotoxic effects of doxo in MES-SA/Dx5 cells with effect similar to that of the known Pgp inhibitor, Ver, but with much less *in vitro* toxic effects. In particular, ginger is an effective and safe natural compounds that might be used clinically at high dosages concomitantly with low dosages of anticancer drugs (side effect free) for treatment of Pgp expressing drug-resistant tumours.

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ENIGMATIC QUESTION OF EARLY REACTIVE ARTHRITIS DISCLOSED AFTER RESEARCHES OF *MYCOPLASMAS, CHLAMYDIA TRACHOMATIS* AND ENTEROPATHOGENS FOLLOWING THE HOLISTIC VISION OF "HUMAN BEING"

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An HLA-B27 genetic profile patient, is fully investigated by molecular analyses after an anamnestic assessment of multi-site ecosystems, following the holistic vision of "Human Being". **VDRL and Widal-Wright (WWR) resulted positive, showing at Wright's reaction a title of 1:40. Of all the enzymatic activities measured, only the ALP enzymatic pool activities showed a low increasing value of 297 U/L. Of all later acute phase proteins, Only C_3c protein value (127 mg/dL) and fibrinogen (376 mg/dL) were altered. Cultural and molecular oropharyngeal ecosystem investigation resulted significantly positive to *Mycoplasmas* (*Mh* and *Uu*) and *Chlamydia trachomatis* (*Ct*) together with a spread of saprophytic flora. From an accurate anamnesis, several and severe uro-genital clinical symptomatology emerged from birth until the beginning of rheumatologic symptomatologies that were confirmed by oldest *Mh*, *Uu* and *Ct* silent chronic infections between these ecosystems. The molecular HPV research was negative, while the Thin prep pap-test was indicative of vaginosis and cellular reactive changes associated with inflammation. Parasitological research resulted positive for presence of 5-7 newly-formed *G. lamblia* cysts for microscopic field, while digestibility test was positive for presence of several free fatty acid crystals. The remarkable presence of indigested meat fibre and several mucous dense filaments were observed. The pH value was 6.5, while blood faecal test was positive. The values observed were: ferritin 12 µg/L (10-120), total iron-binding capacity (TIBC) 310 µg/dL (300±20), unsaturated iron-binding capacity (UIBC) 286 µg/dL (200-220) and iron seric level 24 µg/dL (60-130). Faecal research highlighted a very scarce presence of *E. coli*, resulting in 10^2 UFC/g of stool. Of all enteroinvasive pathogens, researched by molecular analyses, only *Yersinia spp*. was positive. After several specific cycles of antibiotic and antinflammatory therapies, the patient improved its general health condition considerably and showed almost complete regression of aching inguinal lymph node inflammation. In a picture of a worsening inflammatory process, produced by pathogens like Mycoplasmas, chronic silent or low grade**

Key words: Chlamydia trachomatis and Yersinia molecular PCR researches, Mycoplasmas, chronic multi-site low-grade inflammations, inflammaging, multi-site microbial ecosystem valuations in HLA-B27 young woman, reactive arthritis, inguinal lymph nodes

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DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

inflammation atypical agents, in young HLA-B27 positive patient, VDRL test resulted positive. This value represents the first non-specific "unique spy" to reveal the precocious immunological signal in order to register the beginning of early innate immune system decay, keeping in mind that mycoplasmal and chlamydial infections are the triggering of cancer in patients genetically susceptible.

A seventeen year old Caucasian woman with a long-standing chronic history of severe pharyngitis associated with mucoid salivation and yellow-greenish coated tongue. Birth history revealed premature rupture of membranes. The remote anamnesis, collected in our clinic in the presence of her mother, showed pharyngotonsillitis episodes and hacking cough during the first month of life, treated with several antibiotic and fluidifying, with partial recovery onlyduring the therapy. These episodes together with bouts of fever continued annually for some years with the same frequency At six years of age, she was subjected to tonsillectomy without any significant improvements. The cultural pharyngeal swabs carried out in that period gave negative results for *S. pyogenes* and *S aureus* findings, while *Mycoplasmataceae* and *Chlamydiaceae* infections were never hypothesized and researched. The development of *S. milleri*, *S. disgalactiae*, *E. faecalis* and *E. coli* by cultural analyses was observed during this period.

After tonsillectomy, the recurrent pharyngeal episodes persisted almost quarterly becoming strongly refractory to antibiotic therapy by penicillin G and amoxicillin with clavulanic acid. These pharyngeal manifestations were always preceded by high fever ranging between 38° and 39°C without any apparent reason, resistant to antibiotic and betamethasone aerosol therapies. At the age of ten, the patient began to manifest burning urination, becoming ever more frequent. Cultural urinalysis checks never highlighted any significant bacterial growth, remaining always between 1.0 x 10^4 and 4.0 x 10^4 UFC/mL of changeable saprophytic flora. After same initial urogenital problems, they became ever more serious in spite of the young age, while the patient showed the first clinical signs like arthralgia with diffused itching accompanied with irregular and mucoid intestinal evacuation.

At the age of 10, the young patient matured the first clinical signs of migrant articular pains, persistent itching and dermographism positivity. During the following years the patient underwent periodic oro-pharyngeal check-up in other regional hospitals without no improvement after antibiotic and antinflammatory therapies. The pharyngeal swabs analyzed for common germs carried out during these periods often were negative, or occasionally positive for saprophytic flora, changing after complete and selective cycles of specific antibiotic therapy (amoxicillin-clavulanic acid 1g x 2 times/die for 7 days), obtaining a temporary recovery only. After these microbiological results the patient was also treated with a short cycle of methylprednisolone emisuccinate aerosol in order to try solving this atypical chronic pharynx inflammation, without any success. Lacking remission of the specific symptomatology, the patient observed an exacerbation of the fever just 5-7 days after the specific antibiotic and steroid therapies were completed, ending 10-15 days later without apparent cause. These atypical pharyngitis episodes, resistant to non-specific antibiotic therapy, were associated with systematic clinic worsening, that are migrant arthralgia symptoms, muscle aches, tiredness and chronic fatigue in spite of early age. Despite the use of several cycle of antibiotic and steroid-based therapies, her mother referred no remission of the specific symptomatology but, on the contrary, she observed a few subsequent periods of relapsing exacerbation just when the therapy was finished.

During the second session, conducted without her mother, on the basis of our internal questionnaire, the patient declared various personal urogenital problems.

Multidisciplinary clinical observations

On her first visit in the consulting room of the University ENT Clinic of Chieti, the patient signed an informed consent and together with her mother, provided a detailed anamnesis. On clinical oropharynx examination, signs of chronic pharyngitis associated with mucoid salivation and yellow-greenish coated tongue were evident.

Furthermore we focused our attention on the symptomatology of all ecosystems evaluated following the holistic vision of "Human Being".

On her second anamnestic visit, she explained in detail her other problems in the presence of the physician's expert team.

The ocular and conjunctival problems observed, ranging from light redness to burning and dry eyes, attributed by the patient to the continuous use of TV and computer (Fig. 1). On the visit to the Emergency Service of the University Eye Clinic of Chieti, the patient was studied according to the declaration of Helsinki and granted written informed consent. The patient, in addition to clinical signs, attributable to initial conjunctivitis, had a Schirmer's test without anaesthetic drops (Schirmer I°) with a weak lachrymal functionality (16 mm), Break Up test (<11'').

The urogenital and gynaecological clinical signs exhaustively described by the young patient showed very old problems, never correctly investigated before and never solved. The patient reported a precocious dysuria with 8-13 urination episodes daily, increasing from infancy to the present. The previous urinalysis and common cultural researches were always without any significance and without any important bacterial charge under 1.0 x 10^5 UFC/mL. During late adolescence (14-17 years), the urological signs were accompanied by various gynaecological symptoms. These were represented by initial spotting followed by vulvar itching, leucorrhoea and vulvodynia. During the late teens metrorrhagia, light pelvic inflammation disease and lastly dispareunia were present along with the existing clinical symptomatology after sexual intercourse with a coetaneous boyfriend. The young patient declared no oral sexual experience. Furthermore the presence of five lymph nodes in both sides of inguinal region were noted; the dimension of these varied from 7.0 to 13.0 mm.

MATERIALS AND METHODS

Specimens

Ocular cotton swab and conjunctival scraping were performed for cultural and molecular analyses respectively. After a conjunctival scraping, the patient received a few of chloramphenicol/tetracycline ointment, 1 time/night for three nights for preventive aim purpose (1).

Pharyngeal swab, lingual and pharyngeal scraping were collected following the procedures already described (2-4). All ecosystems pH measures were carried out by digital portable pH-meter, after dispersion of this cellular material into 1.0 mL of sterile saline solution. The double sampling mode was collected and each one dispersed separately on CPS and CAN Columbia agar with 5% sheep blood, according to BioMerieux Manual procedures. The *Mycoplasmataceae* germs, like Ureaplasma urealyticum (Uu) and Mycoplasma hominis (Mh) were cultured by Mycoplasma IST 2, following the BioMerieux instructions, as reported (3, 4). *Chlamydiaceae* (*Ct* and *Cp*) were researched by PCR analyses following the same programs already reported (3, 4).

Faecal samples from three different and consecutive daily evacuations were collected and processed for faecal occult blood testing (FOBt), qualitative digestion functionality, parasitological, and molecular examinations. A 1.0 g of each sample previously collected was dispersed immediately with 10.0 mL of sterile saline solution and processed for microbiological culture and molecular entero-invasive pathogens researches, as already described (5-7). Aliquot of 1.0 mL was enriched in Selenite F broth and incubated at 37°C. 1.0 µL of new faecal suspension was dispersed directly onto each SS, EE and SM ID 2 agar plates for culture analysis at 37°C, according to BioMerieux Manual procedures.

Some drops of the first void urine sample (50.0 mL) were used for fresh cytobacteriological microscopic observation while 1.0 µL was used for cultural analysis. The first void urine sample (50.0 mL) was centrifuged at 4000 rpm for 10 m at 4°C and the pellet collected by 1.0 mL of the same supernatant. An aliquot of 50.0 µL was used for common cultural and mycoplasmas analysis, while the remaining suspension was used for chlamydia trachomatis research after a new centrifugation at 5000 rpm for 5 m at 4°C.

The middle urination (100 mL) was collected for common cultural analysis and centrifuged at 3000 rpm for 10 m at 4°C and the pellet was observed with optical microscope for sediment evaluation. The pH value was measured by using a digital portable pH-meter.

Urethral material was collected by cellular scraping with Dacron swab, introducing it for 2.5 cm and then revolving it quickly. The intense yellow cellular material collected was used for *Chlamydia trachomatis* research.

Cervical specimen was collected by cervex-brush following the Cytyc procedure and dispersed into PreservCyt solution; after 24 h, 1.0 mL of this suspension was centrifuged for 10 m at 4°C and the pellet was taken for HPV and Ct molecular analyses, while the remaining suspension was used to prepare the ThinPrep Pap test microscope plate for cervical cytology.

Vaginal yellow-marked mucoid exudate was collected by three cotton swabs around the vaginal fornix to culture all common bacteria, parasite, micetes and mycoplasmas researches. This material was dispersed

into 1.0 mL of sterile saline solution and used for the fresh cytobacteriological microscopic examination and cultural analyses. The pH value was measured. A drop of this suspension was used to prepare a slide, drying up in thermostat at 37°C and stained with Gram's method.

Two intestinal checks were ordered to verify the therapeutic efficacy at one and three months from the end of the therapy, while a check of all ecosystems investigated was carried out at the end of the 7th and 12th month.

RESULTS

Clinical biochemistry

After a visit to the Consulting Service of Predictive Medicine, routine blood analyses from hepatic and renal functionalities and all electrolytes were carried out. Erythrocyte blood count was normal, while a ferritin 12 µg/L (10-120), total iron-binding capacity (TIBC) 307 µg/dL (280 ± 320), unsaturated iron-binding capacity (UIBC) 283 µg/dL (200-220) and iron seric level 24 µg/dL (60-130) values were measured (Table I). White blood cell count (WBC) was 8.700 leucocytes/µL, with an increasing shift to lymph-monocyte line (50% neutrophils, 37% lymphocytes, 10% monocytes, 2.0% eosinophils, 1.0% basophils). Erythrocyte sedimentation rate (ESR) was 17 mm/h. All enzymatic activities (CPK, CPK-MB, GOT, GPT, LDH, γ-GT) were approximately average values. Amylase activity (AMS) was 86 U/L, showing an increasing trend near higher values (90 U/L), while alkaline phosphatase activity (ALP) was 297 U/L (90-270 U/L).

Routine panel of acute-phase proteins (APP_s), including α_1-GPA, α_1-AT, ceruloplasmin, haptoglobin, fibrinogen and C_3c complement fraction were determined by nephelometry; each protein presented a medium value, with the exception of the C_3c: 127 mg/dL (45-83), and the fibrinogen: 376 mg/dL (180-350) (Table II).

The rheumatoid factor (RF), antistreptolysin O titre (ASOT) and streptozyme test were negative. The antibodies against *H. pylori* (Ab-Hp) and the antibodies (IgG/IgA) against *C. trachomatis* resulted negative. The antibodies anti-extractable nuclear antigen (anti-ENA), anti-nuclear (ANA), anti-neutrophil cytoplasmic antibodies (ANCAs), anti-DNA, anti-phospholipids (aPL), anti-cardiolipin (aCL), were all negative. Only VDRL was positive despite the fact that the Treponema pallidum haemoagglutination test was negative (TPHA). The Widal-Wright reactions were positive, resulting in a 1:20 titer for H antigen of *S. paratyphoid B* and a 1:40 titer for Wright's reaction (Table III). The patient's blood, tested for HLA-B27 by PCR analysis, was positive.

Faecal analyses results

The observation with an optical microscope highlighted the presence of 5-7 neoformed cysts of *G. lamblia* only; no other parasites or their eggs were demonstrated. A specimen of each stool sample was analyzed for searching blood traces, resulting all positive; also the digestibility tests, performed on the same samples presented pH values equal to 6.79, no neutral lipid drops, while several free fatty acid crystals were observed. The conspicuous presence

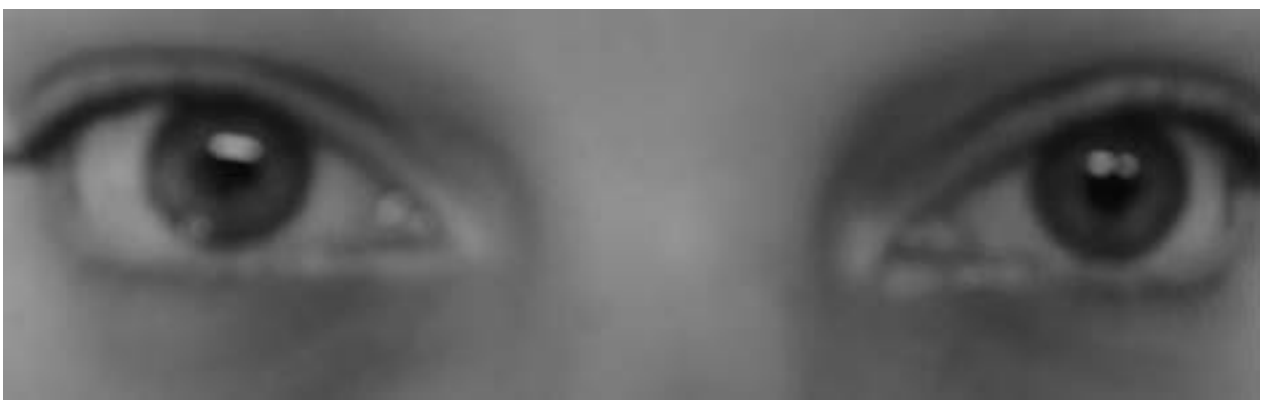

Fig 1. *In this photo the initial clinical condition is visible before the Schirmer's test; molecular Ct negative research at first control became positive at seventh month, after abundant chlamydicidal therapy, indicating a probably initial chlamydial "resistent form" phase, but highlighted after several oral efficacious antibiotic cycles.*

Table I. *Altered parameters of iron metabolism in young patient HLA-B_{27} affected by chronic chlamydial infection from birth.*

Parameter Reference range	IRON (60-130 µg/dL)	TIBC (280 ± 320µg/dL)	UIBC (200-220 µg/dL	Ferritin (10-120 µg/L)
Value obtained	24	307	283	12.0

Table II. *Biochemical enzymatic activities, faecal occult blood test and APP_s indicating a temporary* "steady state" of *chronic inflammatory process.*

Parameter Reference range	AMS < 90 U/L	ALP 90-270 U/L	FOBt negative	Fibrinogen 180-350 mg/dL	C_3c fraction 45-83 mg/dL
Value obtained	86 U/L	289	positive	376	127

An increasing trend for AMS and an elevated value for ALP activities of isoenzymatic pools represent the enterocytes chronic inflammation supported by the FOBt positive test and by Yersinia infection. The movement of these APP_s focalize an altered "steady state" due to chronic inflammatory processes present in several ecosystems investigated.

Table III. *Serological parameters of a young HLA-B27 patient presenting different immunological meaningfulness for different microbial agents, indicating for VDRL result a silent immunological reaction to Mycoplasma infection triggering autoimmune disease.*

Serological parameter reference	Streptozyme negative	VDRL negative	R. Widal negative	R. Wright negative	Ab-Chlamydia IgG/IgA absent
Value obtained	negative	positive	1:20	1:40	absent

of mucous condensate filaments were observed together with indigested meat fibre, while no presence of starch granules was noted.

Cultural and molecular results of different ecosystems: Oropharyngeal ecosystem

The oropharyngeal ecosystem was examined evaluating the presence of atypical bacteria, like the *Chlamydiaceae* (*Ct* and *Cp*) and *Mycoplasmataceae* (*Mh* and *Uu*) families, and microbiological pathogens by molecular and cultural researches. The saliva pH value was 7.83. In Table IV the cultural and molecular microbiological results are reported. Different microbiological patterns resulted on both

Table IV. *Culture and molecular analyses of normal, common pathogens and atypical bacteria from oro-pharyngeal and ocular specimens collected by different sampling modalities.*

Microrganism	Lingual swab	Lingual scraping	Pharyngeal swab	Pharyngeal scraping	Ocular swab	Conjunctival scraping
Staphylococci	absent	absent	absent	absent	absent	absent
S. pyogenes	absent	absent	absent	absent	absent	absent
Streptococci	S. faec. 100%	S. faec. 70%	S. milleri 40%	S. milleri 50%	absent	absent
Enterobacteria	absent	absent	absent	absent	absent	absent
Corynebacteria	absent	C. ulc. 30%	C. ulc. 60%	C. ulc. 50%	C. ulc.100%	C. xerosis100%
Yeast	absent	absent	absent	absent	absent	absent
M. hominis	absent	$>10^4$UCC/mL	absent	$>10^4$UCC/mL	n.d.	n.d.
U. urealyticum	absent	$>10^4$UCC/mL	absent	$>10^4$UCC/mL	n.d.	n.d.
C. trachomatis(*)	absent	Present	absent	Present	absent	absent
C. pneumoniae (*)	absent	n.d.	absent	absent	n.d.	n.d.

(*) the molecular analyses were performed in duplicate.

Table V. *Cultural and molecular urological ecosystem valued from three different and sequential sampling modalities.*

Microrganism	*FVU*	*Middle emiction*	*Urethral scraping*
Streptococci group	n.d.	absent	n.d.
Staphylococci group	n.d.	absent	n.d.
Enterobacteria group	n.d	absent	n.d.
Corynebacteria group	C. ulcerans 100%	C. ulcerans 100%	n.d.
Yeast	n.d	absent	n.d.
T. vaginalis	n.d.	absent	n.d.
M. hominis	10^3 UCC/mL	n.d.	10^3 UCC/mL
U. urealyticum	10^3 UCC/mL	n.d.	10^4 UCC/mL
C. trachomatis (*)	absent	n.d.	highly present
HPV (*)	n.d.	n.d.	absent

(*) the molecular analyses were performed in duplicate.

lingual samplings. In the first case, the saprophytic flora consisted of 100% *S. faecalis* grown from cotton swab material compared to 70% *S. faecalis* and 30% *C. Ulcerans* resulted from the same source but collected by cellular scraping. Noteworthy differences were observed when the results for urogenital mycoplasmas and chlamydia trachomatis from both sampling modalities were compared. The presence of >1.0 x 10^4UCC/mL *M. hominis*, >1.0 x 10^4UCC/mL *U. urealyticum* and *C. trachomatis* resulted from the scraping material rather than to cotton swab source. *C. pneumoniae* research was negative in both the different samplings. Identical behaviour was observed from pharyngeal source for atypical bacteria by both sampling modality. No significant differences were observed from

Table VI. *Culture and molecular analyses of normal, common pathogens and atypical bacteria from cervical and vaginal different sites collected by different sampling modalities.*

Microrganism	*Cervical source (scraping)*	*Vaginal source (cotton swab)*
Staphylococci group	n.d.	absent
Streptococci group	absent	E. faecalis 30%
S. agalactiae	absent	absent
Enterobacteria group	absent	absent
Corynebacteria group	absent	C. ulcerans 70%
L. acidophylus	n.d.	3 x10^5 UFC/mL
Yeast	n.d	absent
T. vaginalis	n.d.	absent
M. hominis	absent	10^4 UCC/mL
U. urealyticum	10^3 UCC/mL	>10^4 UCC/mL
C. trachomatis (*)	highly present	absent
HPV (*)	absent	n.d.

(*) the molecular analyses were performed in duplicate.

Table VII. *The table represents the therapeutical six-months flow-chart pattern.*

THERAPY	I° month	II° month	III° month	IV° month	V° month	VI° month
Oropharyngeal therapy	AZT, DOX, Cycles	_____	Rifampicin and AZT	_____	AZT, DOX, Cycles	_____
Lymph nodes therapy	steroid therapy I° and XVI°day		steroid therapy I° and XVI°day	_____	steroid therapy I° and XVI°day	_____
Gynaecological therapy	_____	Gynaecological therapy cycles (*)	_____	Gynaecological therapy cycles (*)	_____	Gynaecological therapy cycles (*)
Urethral therapy	Sunday morning of each week		Sunday morning of each week		Sunday morning of each week	
Immunosuppressive and anti-inflammatory therapy	MTX 7°, 14°, 21°, 28° day	COX-2 inhibitor II° and III° week	MTX 7°, 14°, 21°, 28° day	COX-2 inhibitor II° and III° week	MTX 7°, 14°, 21°, 28° day	COX-2 inhibitor II° and III° week

The patient received monthly and cyclically antibiotic therapies, immunosuppressive and anti-inflammatory for different anatomical inflammatory sites. (*) During antibiotic cycle the patient received milk enzymes, multivitamin administrations and dietary supplements orally and daily, while two different vaginal gels and Lactobacillus suppository were administered, on alternate days, to stabilize the pH value in physiological range, to provide the ecosystem of enough glycogen and to restore the normal ecosystem conditions. These nutrient therapy was suspended only during the menstrual phase.

same source for saprophytic flora by two different samplings.

Ocular ecosystems

In spite of the ocular and conjunctival problems, also clinically evaluated (Fig. 1), the *C. trachomatis* researches were negative in both source. The presence of *100% C. ulcerans* and 100% *C. xerosis* (Table IV) were present in ocular swab and conjunctival scraping respectively.

Urological ecosystems

The fresh cytobacteriological microscopic examination on first voiding urine (FVU) revealed a count of 50 erythrocytes (reference value <500 cells/mL), a count of 100 leucocytes (reference value <500 cells/mL) and a count of 10^3 germs (reference value <250 germs/mL).

The cultural analyses for mycoplasmas on material from FDV showed a growth of *M. hominis* 1.0 x 10^3 UCC/mL and of *U. urealyticum* 1.0 x 10^3 UCC/mL; Ct-DNA molecular research was negative (Table V). The urinalysis sediment of middle emiction showed an evident presence of bacterial cells in contrast to the rare presence of erythrocytes and polymorphonucleates; the urine pH value was 7.81 and an abundant presence of bacteria was present, while no any significant results were evident from the remaining physical-chemistry analysis.

The cultural analysis of middle emiction only showed the growth of *C. ulcerans* >1.0 x 10^6 UFC/mL.

The cultural and molecular researches carried out on urethral scraping source highlighted the presence of 1.0 x 10^3 UCC/mL for *M. hominis* and of 1.0 x 10^4 UCC/mL for *U. urealyticum*, while Ct-DNA research showed an intense fluorescent band at 156 pb (data not shown). On this source the HPV-DNA research (Table V) was negative.

Cervical and vaginal ecosystems

The cervical ecosystem, evaluated by the thin prep pap-test, showed an inflammatory condition with shift of bacterial flora, compatible with a vaginosis, while the Ct-DNA research presented a high positive fluorescent band to 156 pb; HPV-DNA research was negative.

The vaginal ecosystem, evaluated at optic microscope revealed no "clue cells" formation, while a number of 30-40 leucocytes/yield were present. Some of these were aggregated to form pus. *T. vaginalis*, *G. vaginalis* and *Micetes* researches gave both negative results at the cultural analyses. A very low presence of for *L. acydophylus* (3.0 x 10^5 UFC/mL) was observed (physiological age related condition: >1.0 x 10^8 UFC/mL), while the pH vaginal value was 5.8 (physiological range: 3.8-4.4). The Total Microbial Charge/mL (TMC/mL) was around 1.0 x10^8 UFC/mL (physiological range: 1.0 x 10^{10} - 1.0 x 10^{12} UFC/mL). The cultural analyses for common bacteria and *Mycoplasmas* (Mh and Uu) showed a growth of 70% C. ulcerans and 30% *E. faecalis*, while *M. hominis* and *U. urealyticum* were both 1.0 x 10^4 UCC/mL (Table VI).

Gastrointestinal ecosystems

The three cultural faecal results highlighted the absence and a very scarce *E. Coli* microbial growth representing 100% of the TMC/mL equal to a count colony ranging among 0.0 - 10^2 UFC/g of stool. The molecular searching by PCR for enteroinvasive pathogens showed the presence of *Yersinia* group. These faecal results were doubly repeated on each new sample preparation.

Intestinal therapy and therapeutic efficacy

The neoformed *G. lamblia* presence was treated with 4 tablets of tinidazole 500 mg in a single dose repeated again on the third day. The *Yersinia* infection was treated by 250 mg of ciprofloxacin pills, administered in the morning and in the evening, for eight days, together with milk enzymes and multivitamin administered four hours later. After three days from the antibiotic suspension, the therapy was followed by 200 mg of ceftibuten pills, for six days following the previous procedure together with milk enzymes and multivitamin administration. The last antibiotic cycle for *Yersinia* infection consisted of doxycycline 100mg x 2 times/day for 5 days. These antibiotic choices were made based on Medical Literature indication. The milk enzymes and multivitamin were administrated during all the next antibiotic therapies. A blood check up, carried out after the end of the third month from the beginning of intestinal therapy for all biochemical parameters evaluating the physiological functionality, was within reference ranges. Noteworthy was the improvement of

haematic crasis with a return to the reference values of iron, iron-binding capacity and ferritin. AMS and ALP values were 70 U/L and 257 U/L, respectively. FOBt and parasitological examinations, repeated on three samples were all negative; the qualitative digestion functionality resulted normal together with the pH value (7.8). The faecal cultural analyses highlighted only saprophytic flora, where *E. coli* growth represented the 80% of TMC/mL, corresponding to about $1x10^8$/g of stool. Molecular control of faecal *Yersinia* research resulted negative. Also the second intestinal cultural and molecular checks performed at the fourth month given an intestinal bacterial charge improvement and molecular *Yersinia* negative result. Furthermore, it was established that the intestinal ecosystem would had been verified again in the next years.

Therapy

Mycoplasmas and *Chlamydia*, due to their ability to escape the immune innate system, and to survive the specific therapy, cause severe health problems due to silent and/or low grade inflammatory state and the absence of a direct immunological response. On the basis of results present in all ecosystems investigated, evaluating the drugs congruity and their pharmacokinetics for each ecosystems, we suggested to the patient a therapeutical set plan (reported in Table VII) and a clinical and check control after seventh month, one month after the all therapy end.

Articular hand pains were initially treated by ibuprofen lysine salt gel until normal condition was recovered.

Ocular therapy

Although the conjunctival molecular results were negative for Ct infection, we recommended the patient to treat the conjunctival abrasion by scraping with chloramphenicol/tetracycline ointment, 1 time/night on the seventh day and then with 2 times/day for 6 days with azithromycin ophthalmic drops; this treatment was proposed to prevent conjunctival light abrasion caused by scraping modality sampling and environmental contamination.

Oropharyngeal therapy

The initial chlamydicidal and mycoplasmicidal polycyclic oropharyngeal antibiotic administration was done following this therapeutical protocol: the first month consisted of oral administration of 500 mg azithromycin (AZT), 2 times/day for 3 days. After a 10-day pause, the patient began a second cycle with oral administration of 100 mg doxycycline (DOX) x 2 times/day for 5 days, followed by a second 10-day pause. In the morning and in the evening, during the first three months, the patient provided for his oral hygiene monthly alternating mouthwash with tincture of iodine, 0.074% diclofenac acid and 0.2 % chlorhexidine solutions. This oral treatment was repeated also during the second quarter. During the third month, the oropharyngeal therapy consisted of a bottle of 600 mg of rifampicin administered in two halves in the morning and in the evening by aerosol for the first 7 days**.** On the 15th and 30th day the patient received 500 mg azithromycin (AZT), 2 times/day. The oropharyngeal antibiotic posology is shown in Table VII. The patient received multivitamin and dietary supplements, enriched of reducing factors and microelements to nourish the infected cells, to stabilize the reticulate bodies (RBs) and to satisfy chlamydial and human nutritional cellular increased requirements (1, 4, 7). Further, the patient received a vaginal lactobacillus to prevent the worsening of dismicrobism of vaginal ecosystem.

Lymph nodes therapy and urethral therapy

The lymph node anti-inflammatory therapy consisted of 0.5 mL of injectable triamcinolone acetonide (40mg/mL) administration around each of the five lymph nodes. This therapy was administered on the first and the sixteenth day of the 1st, 3rd and 5th, while the 6th and 7th month they were kept on observation without any further injections. The urethral therapy was performed, introducing for about 2.5 cm an adequate chloramphenicol/tetracycline ointment by a sterile equipment after morning urination, each Sunday during the 1st 3rd and 5th month. The specific urological symptomatology (dysuria and burning emiction) was absent already during the 5th month.

Cervical and vaginal therapy

The first day of the second month she was administered an oral pill/each 14 days of fluconazole 150 mg for preventive purpose. The antibiotic therapy consists of three cycles of azithromicin,

doxycycline and newly azithromicin, following the protocol in Table VII. The first antibiotic cycle at the beginning of the first menstrual cycle day consisted of oral administration of 500 mg azithromycin (AZT), 2 times/day for 3 days, followed by a 7-days pause; the patient began the second cycle with oral administration of 100 mg doxycycline (DOX) x 2 times/day for 5 days, followed by a second pause of 5-days. The third cycle consisted of oral administration of 500 mg azithromycin (AZT), 2 times/day for 3 days, followed by a 5-days pause.

Immunosuppressive and anti-inflammatory therapies

Following the indication reported by Colmegna et al. (8), the 10mg/mL Methotrexate solution (MTX) was given intramuscularly and twenty-four hours later the patient assumed an oral 10mg folinic acid tablet. This therapy was carried out from the 6th day of the week for the 1st, 3rd and 5th months, following Table VII.

Anti-inflammatory COX-2 inhibitor therapy was administered following this program: 200 mg capsule of celecoxib, in the morning and in the evening after the first week pause, fourteen days after the last MTX injection. The anti-inflammatory therapy with Celecoxib was carried out from the eighth to the twenty-first day of the second month and repeated at the fourth and sixth months. A biochemical check was performed to verify haematological crasis, hepatic and renal functionalities at the end of third month, without any significant changes in reference ranges, together with APPs normalization and a significant decrease of ALP activity (234 U/L), while the AMS activity value was perfectly recovered (67 U/L).

DISCUSSION

It is well-known that the oxidation process bring to cellular death for nutritional deficiency factors and energetic unbalances. It is also reported that *Chlamydiaceae*, parasiting the cells, consumes energy (ATP) and cellular sustenance for own perennial metabolic cycles, transforming oneself into "*persistent form*" during iron restriction and adverse nutritional conditions (9). Studies carried out on HeLa cells highlighted the central role of chlamydial infection to alter and to modulate homeostasis iron (10). Cellular redox potential (GSH/GS-SG) plays a pivotal role on both life and death decision of cells, because an intimate relationship between these pathogens, oxidative stress and iron metabolism exists (11). In addition, it was also reported that the expression of IL_{10} in Ct-infected HeLa cells was regulated by ferritin (12). Further, Chlamydia trachomatis can transform oneself into "*penicillin form*" (13, 14) and "*aberrant form*" (15) as a consequence of penicillin and quinolones antibiotic therapies, but starting again in reproductive phase just the mutate environmental condition are favourable. Several studies demonstrated that apoptosis inhibition was carried out by Chlamydia infection, whilst other Authors reported that apoptosis induction by this atypical pathogen was due to different mechanisms (16, 17). It is recently reported that *C. pneumoniae* causes apoptosis through the imbalance glutathione redox state (18). It is also described a male infertility case resolution after treatment by means antibiotic therapy and glutathione infiltration in patient with asymptomatic prostatitis (1, 7). Recently we reported a case of a patient affected by chronic pharyngitis with jugulodigastric lymph node inflammation solved after oral treatment of suitable quantities of vitamins, supplementing with glutathione and dietary supplements to nourish the infected cell, in order to satisfy the chlamydial and mycoplasmal nutritional needs and delaying the RBs transformation in EBs (4), so favouring the persistence of chlamydial form sensible to chlamydicidal and mycoplasmicidal therapy with azythromycin and doxycycline.

According to Waites KB et al. (19), the Mycoplasmas, being primarily found in the respiratory and/or urogenital tracts, exist, as a consequence of this, also into intestinal habitat together with chlamydial EBs (20). So the "*Human Genus*" is the primary host for these atypical bacteria. Mycoplasmas, reach the survival through a surface parasitism of cellular mucous membrane, living on internal of it, requiring a tight nutritional dependence on guest cell (19), exteriorizing the phospholipids membranes, are responsible of VDRL positivity sign. Actually, VDRL is the unique immunological parameter assessing the response of innate immune system, stressed for decades by these silent agents causing chronic inflammation. So, the trigger of autoimmunity phenomenon highlights itself, several years later, in subjects genetically susceptible, introducing their prematurely to inflammag-

ing phenomenon, as recently reported (4, 7). Into the persistent inflammatory scenery, LPS and MALP-2 molecules, would be the biochemical agents causing, throgout the increased proinflammatory cytokines, the Reactive Oxygen Species (ROS) and Oxide Nitric (NO) that produce the unbalance of glutathione potential redox (18). These phospholipid derivative molecules, would have a low immunogen power to cause of their hydrolytic enzymatic cascading degradation and their following use of simple precursors for repairing membrane processes. From their natural intestinal habitat, Mycoplasmas and Chlamydia trachomatis are responsible, passing through natural (21-24) and sexual reciprocal contamination from of chronic oro-pharyngeal infections (3, 4, 7, 25, 26), chronic lung disease and bronchopulmonary dysplasia (23, 24, 27-29), urogenital tracts (30), asymptomatic and chronic prostatitis (1, 31, 27, 28) and sexual transmitted diseases (32).

Following the holistic vision of "Human Being", as a "*one system*" thermodynamically opened to continuative energetic and bacterial exchanges with external environment, on the basis of the clinical evidence of all different ecosystems investigated, supported by an unequivocal clinical anamnesis and by few early, but significative altered immunological and biochemical parameters, reinterpreting few precocious alterations (4,7) how an irreversible condition from her innate immune system, we studied this young patient HLA-B_{27} positive, presenting same initial health problem from the birth, as already reported (21, 23, 24, 27, 28). The patient, after the very likely initial contamination, never adequately highlighted, continually stressed by Mycoplasmas and Chlamydia trachomatis, in spite of the guidelines of Infectious Society Disease of America (ISDA) elaborated by Stamm on results of urine cultural analysis indicating the obligatoriness of Ct research in presence of urination stimuli, was never investigated.

The patient presented some biochemical unclear variations (C_3c, fibrinogen, ALP activity) and unclear positivity of reaction Wright's test, developed the VDRL positivity in presence of TPHA test negative. In light of this, considering the early "*fatigue chronic*" symptomatology presented by the young patient together with precocious arthritic signs, already described (4, 7, 21), we have reconsidered the results from initial health problems present from birth.

The apparent normal white blood count (8700 leucocytes/μL) with an increased percentage of lymph-monocytes cellular lines (47%) was evaluated as constant stimulations of immune innate system (4, 7) by *Mycoplasmas, Chlamydia trachomatis* and *Yersinia* agents, infecting the patient at the time of the birth (21, 23). The presence of iron and vitamin deficiencies, due to intestinal blood losing caused by Yersinia infection, for lacking of intestinal absorption due to increasing peristalsis and for presence of chronic chlamydial and mycoplasmal infections (9-12, 19) were solved after the administration of vitamins and nutritional supplements.

The fibrinogen and C_3c complement fraction, two proteic markers of later APPs were evaluated as a fluctuating "*steady state*" condition, generated from the initial disability of innate immune system to confront these atypical infections, able to escape by several strategies (11) from its control. Alternatively, it is possible to consider the increased level of plasmatic fibrinogen (376 mg/dL) like an early condition for the production of antibodies against cyclic citrullinated peptides (Abs-CCP). It has been reported that Abs-CCP is more sensitive and specific marker in the diagnosis of RA than RF (33), like in this case, showing a RF negative result. Further, it is described how the response therapeutic treatment get decline serological level of Abs-CCP in early phase of rheumatic disease (33), according with fibrinogen normalization after antinflammatory and antibiotic treatments obtained in this case. VDRL positive result, reveals a key role for a precocious mycoplasmas infections diagnosis (4, 7) if compared with more specific but later LAC, aPL and aCL immunological parameters, as already reported (7). The increased activity of ALP pool isoenzymes, from cellular intestinal injury, might indicate a long time course depending "*release/repair*" from chronic intestinal *Yersinia sp.* presence, happened surely after childhood, being a bacterium responsible of food poisoning. The survival of this bacteria, in subclinical conditions, into enterocytes and M cells of the follicle-associated epithelium of Peyer's patches, would permit a periodic or continuous release and of their own enterotoxins, so altering the intestinal physiological permeability and nutritional functions, stressing them continuously to produce mucoproteins and altering the biochemical concentration of

these "*gene muc*" products, making to decrease their protection functions. Considering the low title of the Wright's reaction without significance for *Brucella* infection, but on the contrary, an indirect sign of *Yersinia* presence, the FOBt positivity, the alterated digestibility test represents an increasing intestinal motility condition. Moreover the remarkable presence of mucous condensate filaments, the lack of an adequate intestinal charge of normal gut flora resident, the presence of an alterated pH value, indicate an intestinal state resulting from incapacity of innate immune system to contrast subsequently and effectively the Yersinia penetration into the intestinal mucosa. A common sign of all altered and investigated ecosystems, showed from the patient, was indicated also by each altered pH value, indicating the presence of high dismicrobism caused by pathological bacteria., The altered biochemical concentration of mucoproteins, caused by increased intestinal motility with dilution of these mucoproteins, could not allow to play their protective role, also depending on the "*muc*" gene variability. This study suggests the first triggering role of these atypical pathogens to cause a chronic low grade autoimmune response against the tissue/organ target, causing "*inflammaging*" phenomenon in young patient with chronic latent infection by *Yersinia type*, leading to reactive arthritis, in patient HLA-B_{27} positive

No alterations were observed after a blood check up for all biochemical parameters, physiological functionality evaluation, whole stools evaluation and Yersinia molecular control. Negative results of initial alterations would be controlled in the future to evaluate the VDRL negative result far from immunosuppressive therapy.

During chlamydicidal and mycoplasmicidal polycyclic antibiotic therapy, the patient received constantly multivitamin and dietary supplements, enriched carnitine, creatine, tryptophan, adenosyl-methionine, glutathione, selenium and microelements to nourish the human cells of infected ecosystems (1, 4, 7). Our opinion was that this dietary supplements supplied a chronic deficiency of intracellular ecosystem environment caused by development of chronic chlamydial and mycoplasmal infections, like it is a "*thermodynamically opened system*" to entry of these bacteria. If on the one hand, the "*human being*" with its metabolism needs constantly a correct and balanced diet to produce "ATP-*energy*" for sustaining ours physiological functions, on the other hand these "*atypical bacteria invaders*", deplete the cellular environment of essential nourishments, causing the apoptosis with their endocellular parasitism and apparent "*false*" commensalism. Further, these dietary supplement introductions and in particular a major thiol groups availability could stabilize "*in vivo*" the reticulate chlamydial forms (RB_s), exposing themselves to a major chlamydicidal and mycoplasmicidal antibiotic therapy effects for a major time.

Although several infectious agents, like virus and bacteria, as reported in the Literature cause the chronic pharyngitis, seldom these agents resist for decades to antibiotic, non-steroid anti-inflammatory drugs (NSAID) and steroid-based periodic or continuative therapies. Also if the oropharyngeal episodes, uro-genital and the inguinal lymph nodes inflammation were regressed after antibiotic and anti-inflammatory therapies, we informed the patient that it's important have 2 annual controls. In the setting of an evolutive chronic inflammatory process, generated by these atypical pathogens at low grade inflammation, in HLA-B27 positive young patient, VDRL positive value together with TPHA negative test, represent actually the unspecific "unique spy" to reveal the precocious immunological signal of survival and the beginning of early cellular decay of the innate immune system.

Considering no more rare the chlamydial and mycoplasmal oro-pharyngeal chronic infections (3, 4, 26) and the other possible human ecosystem contamination (23), we want to underline here the importance of VDRL test to screen and to unveil the old "Mycoplasmas" infections (1, 4, 7). The precocious and long antibiotic azythromycin treatments, also for antinflammatory effect (34) improving the general health condition of patients on the time, could oppose themselves to slow but progressive inflammaging of innate immune system causing more severe diseases, as reported (35). To sustain these our hypothesis, further complete studies will be necessary following the holistic vision of "*Human Being*".

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LETTER TO THE EDITOR

ALLERGEN IMMUNOTHERAPY MAY EXERT AN EXTRA-ANTI-ALLERGIC ACTIVITY IN CHILDREN

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Allergic patients frequently suffer from infections. Allergen immunotherapy (AIT) usually improves respiratory symptoms, mainly in allergic rhinitis (AR). This study was aimed at evaluating the possible impact of AIT on extra-allergic outcomes in a cohort of Italian children with respiratory allergy patients. The study was performed on 77 children (43 males, mean age 10.5 years) with AR. The kind and the number of prescribed allergen extracts, type of diagnosis, severity of symptoms, and use of drugs were evaluated at baseline and after 2 year AIT. Globally 40 patients were treated with AIT, the remaining 37 children served as control. AIT-treated children had lower symptoms, drug use, and less severe extra-allergic surrogate markers of infection in comparison with children untreated with AIT. In conclusion, this study provides the first evidence that 2-year SLIT is able of exerting an adjunctive anti-allergic activity in AR children.

Allergic rhinitis (AR) is a very common disorder as it concerns up to 40% of paediatric population (1). Social and economic costs are impressive; school attendance and performance, and quality of sleep may be also significantly affected (2).

The AR immunopathology is characterized by T-helper-2 (Th2)-dependent inflammation with a Th1-response reduction. Thus, it has been pointed out that allergic patients could present higher susceptibility to contract upper respiratory infections (URI) rather than non-allergic subjects (3). In this regard, it has been evidenced that allergic children have more numerous and severe respiratory infections (RI) than non-allergic children (4).

AR management includes patient education, allergen avoidance, and treatment. The treatment of AR may be classified as: preventive, symptomatic, and allergen immunotherapy (AIT). Preventive therapy is theoretically the simplest, but it is rather difficult to obtain. Symptomatic therapy is based on the prescription of drugs; it is usually effective, but it does not cure AR, as symptoms recur immediately after its suspension. In contrast, AIT treats all AR, including ocular, symptoms by tackling the underlying cause of allergy. AIT is effective and exerts long-term preventive activity also after discontinuance. AIT is indicated in patients with AR when symptoms are surely IgE-dependent. The rationale of AIT

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is that administration of high-dose allergens may reduce and prevent IgE responses restoring the clinical tolerance of the causal allergen, so its inhalation does not induce symptoms.

Sublingual immunotherapy (SLIT) efficacy in patients with AR has been clearly evidenced by several meta-analysis studies (5). In addition, it has been previously reported that AIT was able to reduce URI in adult patients treated with AIT in comparison with patients treated only with symptomatic drugs (6). Therefore, the aim of this study was to evaluate the effect of 2-year AIT course on this issue, in a cohort of children suffering from AR.

MATERIAL AND METHODS

Study design

The study was named RINOBIT after the Latin words "RINitis OBservatio ITalica" (in English: Italian observation of rhinitis). The study was conducted in 10 Allergy and Rhinology Centres homogeneously distributed in Italy. It was designed to include samples representative of the pediatric AR population and to have the ability to identify the new diagnosed cases. The study was approved by the Review Board of each participating center and an informed consent was obtained from the parents of each patient.

Subjects

A total number of 77 children (43 males, mean age 10.5 years) with AR were prospectively and consecutively enrolled. A detailed clinical history was taken and a complete physical examination was performed. The patients were included in the study on the basis of AR diagnosis.

Skin prick tests (SPT) were performed as stated by the European Academy of Allergy and Clinical Immunology (7). The panel consisted of: house dust mites (*Dermatophagoides farinae* and *pteronyssinus*), cat, dog, grasses mix, mugwort, ragweed, *Parietaria officinalis*, birch, hazel, olive tree, cypress, *Alternaria tenuis*, *Cladosporium*, *Aspergillus* mix (Stallergenes, Milan, Italy).

The AR diagnosis was made on the basis of a history of nasal symptoms and positive SPT according to the validated criteria proposed by ARIA document (2).

The parents reported their perception of both the severity of symptoms and the use of anti-allergic drugs (such as oral antihistamines and intranasal corticosteroids) by a visual analogue scale (VAS) according to validated criteria (8). For symptoms, VAS must assess a global evaluation including all symptoms (for eye itching, tearing and redness, for nose itching, sneezing, rhinorrhea and obstruction).

In addition, 4 adjunctive parameters were considered as surrogate markers for respiratory infections, including sore throat, headache, productive cough, and antibiotic consume. Each item was scored as follows: 0: no symptom or no use of antibiotics; 1: mild symptoms or rare use of antibiotics (1 course/year); 2: moderate symptoms or use of antibiotics (2-4 courses/year); and 3: severe symptoms or frequent antibiotic use (>4 courses/year).

Patients were further subdivided in two subgroups according to the presence (or absence) of AIT prescription. AIT-treated children assumed SLIT.

Patients were evaluated immediately before SLIT (Visit 1: V1) and after 2 years of SLIT course (Visit 2: V2).

SLIT

Forty subjects undergoing SLIT received allergen commercial extracts (Staloral 300, Stallergenes, Milan, Italy).

SLIT schedule was continuative course for 2 consecutive years. The dose during the maintenance phase was five drops 3 times per week.

Statistical analysis

Continuous and/or discrete parameters were reported as mean, SD, third quartile, and frequency. Categorical parameters were reported in contingency tables. Homogeneity of data was evaluated by χ^2 Fisher exact test. The significance of the values concerning the principal parameters between V1 and V2 was calculated by Student's *t*-test for paired data and by non-parametric Wilcoxon test for continuous parameters. Confidence Interval at 95% (CI) was reported for the mean of the differences between V1 and V2. McNemar test was used for categorical or discrete parameters. The *p* value concerning the statistical significance was set at 0.05. Statistical analysis was performed by statistical package BMDP Dynamic produced by BMDP Statistical software, Inc (Los Angeles, CA, USA).

RESULTS

Assessment at baseline

Number of sensitizations

Globally, the mean number of sensitizations was 3.0. There was no significant difference between subgroups as AIT-treated patients had a mean of 2.9, whereas patients without AIT prescription had on average 3.2 sensitizations.

Severity of symptoms

Symptoms, assessed by VAS, and use of symptomatic drugs were significant higher in

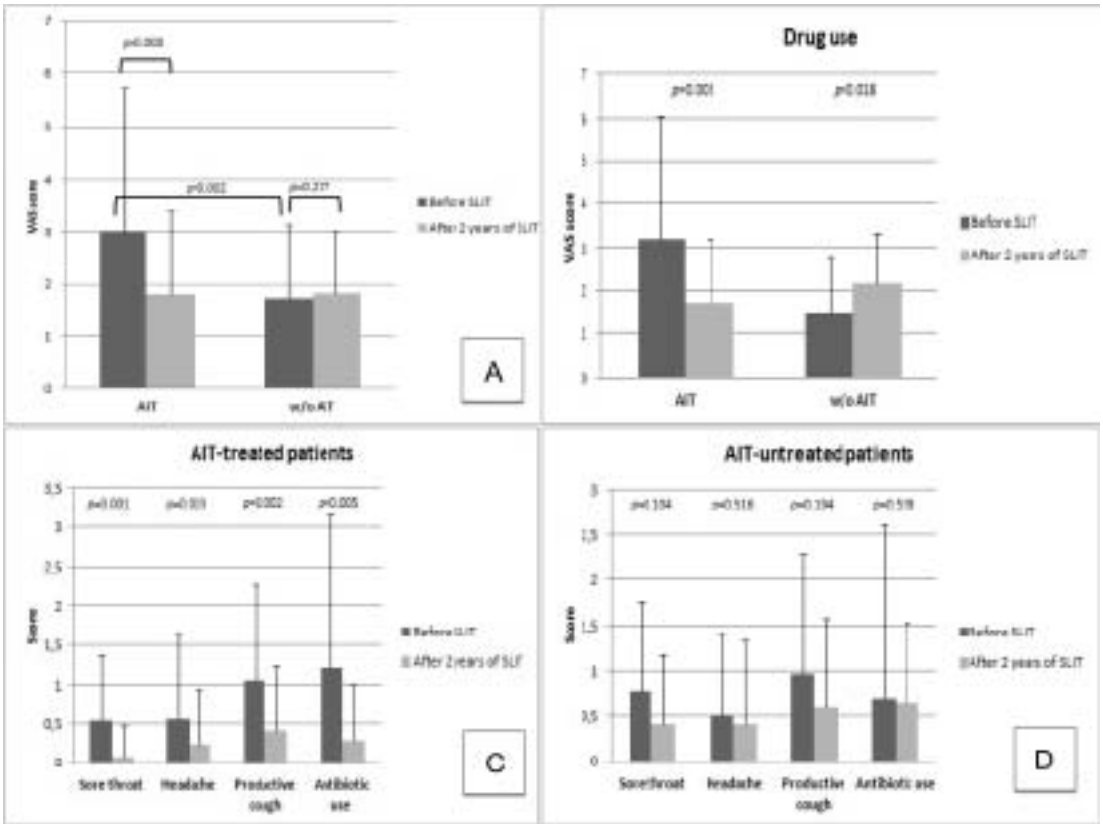

Fig. 1. ***1A****: Perception of symptoms assessed by VAS in patients treated (AIT) or not (w/o AIT) before and after 2 years;* ***1B****: Perception of drug use assessed by VAS in patients treated (AIT) or not (w/o AIT) before and after 2 years;* ***1C****: Respiratory symptom scores in AIT-treated;* ***1D****: Respiratory symptom scores in AIT-untreated patients before and after 2 years.*

children treated with AIT as reported in Fig. 1A. There is no significant difference about sore throat, headache, productive cough, and antibiotic use (Fig. 1B).

Sensitizations in AIT patients

The most relevant allergen is house dust mite: 25 patients were sensitized and 20 treated with its extract (80%); the second allergen was grass pollen: 13 sensitized patients, 8 of them (63.6%) treated with its extract; the third was Parietaria: 11 sensitized subjects and 7 treated with its extract (63%). In addition, 9 children were allergic to olive (3 treated with AIT, such as 33%), 5 were allergic to birch (2 treated with AIT=40%), 5 allergic to dog and 4 to cat (nobody of them was treated withAIT).

Assessment after 2 year SLIT

Severity of symptoms

Symptom severity and anti-allergic drug use (assessed by VAS) significantly diminished in AIT-treated children in comparison with children treated with drugs alone (Fig. 1C).

Extra-allergic parameters

AIT-treated children had significantly less soar throat, headache, productive cough, and antibiotic use in comparison with children treated with drugs

alone (Fig. 1D).

DISCUSSION

Previously, it has been evidenced that allergic patients have a two-fold increase of RI risk, and duration almost 3 weeks longer of the symptoms than non-allergic subjects (9).

Allergic reaction is indeed characterized by mucosal inflammation predisposing to infection. In addition, allergic subjects express ICAM1, an adhesion molecule involved in inflammatory events as ligand of LFA1, expressed on leukocytes. ICAM1 is also the main receptor for rhinovirus (3). Moreover, allergic patients present a typical Th2-polarization with a consequent reduced Th1-response that is supposed to adequately fight infections mainly through IFN-γ. It is also to underline that the relationship between allergy and URI is of considerable interest as there are few studies over this topic and findings are conflicting. In this regard, it has been demonstrated that experimental rhinovirus cold in atopic subjects heightened susceptibility to the detrimental effects of colds both concerning immunological and clinical aspects (10). Another study reported that atopic asthmatics suffer from more frequent lower-respiratory-tract infections and have more severe and longer-lasting lower-respiratory-tract symptoms than normal subjects (11).

On the other hand, specific immunotherapy exerts profound effects on immune response, mainly concerning the balance between Th1 and Th2 cells. Particularly, immunotherapy induces an increased IFN-γ production (12). Therefore, this study aimed at evaluating whether 2-year SLIT may affect URI in allergic children.

Actually, SLIT-treated patients showed both a reduction of symptoms, putative for infection, and antibiotic use in comparison with untreated patients. A possible explanation of this relevant effect might be due to modulation of Th2-polarized immune response in SLIT-treated patients. Indeed, it has been recently evidenced that SLIT is able of increasing IFN-γ production (13). However, this preliminary study is an open study, recruiting a relatively restricted number of patients, and should be followed by controlled trials on this issue. Moreover, we have to consider that another important drawback of this study is the absence of cultural, viral or bacterial, determination.

In conclusion, this study provides the first evidence that 2-year SLIT is able of exerting extra-anti-allergic activity in AR children.

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LETTER TO THE EDITOR

CHANGES IN THE CONTENT OF SHORT, MEDIUM AND LONG-CHAIN FATTY ACIDS IN ISOLATED HEPATOCYTES INCUBATED IN THE PRESENCE OF MAGNESIUM IONS AND/OR ETHANOL

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Magnesium is one of the commonly used dietary supplements. Therefore, this study was to evaluate the content of short, medium and long-chain fatty acids and their esters in isolated rat hepatocytes induced by magnesium and/or ethanol. Isolation of hepatocytes was carried out by the Seglen's enzymatic method using collagenase. To thus prepared samples ethanol and/or $MgCl_2$ solution were added, respectively, so that their concentrations were as follows: 150 mM/dm^3 ethanol and/or 2 mM/dm^3 $MgCl_2$, 4 mM/dm^3 $MgCl_2$. The contents of short, medium and long-chain fatty acids and those of ester-bound acids were determined. The statistical evaluation of the experiment was made by comparing the area normalized for the analysed fatty acids in hepatocytes incubated for 5 h in the presence of the test substances. The effect of magnesium ions on the content of fatty acids and their esters in isolated hepatocytes incubated for 5 h depended on their concentration in the medium. A normalizing effect of magnesium ions on ethanol-induced changes in the content of C14–C17, C18-C20 and C21-C24 fatty acids was demonstrated. A normalizing effect of magnesium on ethanol-induced changes in the content of ester-bound fatty acids in hepatocytes was not confirmed.

Isolated animal or human hepatocytes [1-5] are the model recommended to trace "in vitro" the influence of exogenous factors on the metabolism in the cells. The study of changes in the content and quantity of fatty acids allows for assessing whether the metabolism rate is a normal one. As components of cell membranes and cell organelles fatty acids determine their properties, and in particular the permeability to nutrients. The role of fatty acids and their metabolism are essential for the proper functioning of cells and the whole body (6-10).

Short chain fatty acids (SCFA) have a significant effect on the body by local energy delivery to the epithelial cells of the colon, lowering pH, increasing the absorption of calcium, iron and magnesium, and the impact on the metabolism of glucose and protein in the liver. They inhibit the synthesis of cholesterol and triglycerides in the liver cells (6, 11).

Medium-chain fatty acids (MCFA) are an excellent source of energy. They are transported to the liver, where they undergo beta-oxidation. In the liver they are bound to albumin to a lesser extent, so they do not contribute to fatty degeneration of the organ. Medium-chain fatty acids can be replaced by long-chain fatty acids. There are reports that medium-chain fatty acids prevent liver damage and

Key words: fatty acids, magnesium ions, ethanol, hepatocytes

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stimulate the re-synthesis of triglycerides and also improve the lipid transport system (6, 12).

Long-chain fatty acids (LCFA) are esterified in the liver into triglycerides and phospholipids outside the mitochondria, and oxidation to CO_2 and ketone bodies in the mitochondria (6, 13).

Magnesium is one of the commonly used dietary supplements. Therefore, this study was to evaluate the content of short, medium and long-chain fatty acids and their esters in isolated rat hepatocytes induced by magnesium and/or ethanol.

MATERIALS AND METHODS

Isolation of hepatocytes was carried out by the Seglen's enzymatic method using collagenase (14). The hepatocytes in the suspension were counted in a Fuchs-Rosenthal chamber. Before being counted, the cells were stained in a 0.4% trypan blue solution. A microscopic image showed, besides normal hepatocytes, few dead cells of a more intense blue colour. The obtained samples of isolated hepatocytes containing over 95% of live cells were transferred for further tests.

The resulting samples of isolated hepatocytes with more than 95% viable cells were introduced into the culture medium (Hepatocyte Medium) in an amount of 9 cm^3. To ensure aseptic conditions during the incubation, 100 IU/cm^3 of penicillin and 100 mg/cm^3 of streptomycin was added. To thus prepared samples ethanol and/or $MgCl_2$ solution were added, respectively, so that their concentrations were as follows: 150 mM/dm^3 ethanol and/or 2 mM/dm^3 $MgCl_2$, 4 mM/dm^3 $MgCl_2$ (15).

The composition and content of fatty acids were determined in incubated hepatocytes. The contents of short, medium and long-chain fatty acids and those of ester-bound acids were determined. The presented results are the mean of three assays.

The statistical evaluation of the experiment was made by comparing the area normalized for the analysed fatty acids in hepatocytes incubated for 5 hours in the presence of the test substances. The incubation time of 5 h resulted from the fact that *in vitro* tests on isolated hepatocytes are performed by numerous operators for 3-5 h. This time provides for careful observation of changes occurring in the liver. Furthermore, this time is taken into account in liver transplant surgeries.

The possibility of differences in the volumes of the test samples compared to the control sample volume was assumed. Therefore, in order to compare the obtained results normalization of the area under the chromatographic peaks of the test samples to the surface of the control sample was carried out. The statistical assessment of the *in vitro* experiment was performed by comparing the normalized areas for analysed fatty acids and their esters contained in hepatocytes incubated for 5 h with the test substances. This resulted from the fact that it had been assumed that there was a possibility of differences in the volumes of the tests samples as compared to the volume of the control sample. Hence in order to compare the obtained results the areas under the chromatographic peaks of the test samples were normalized to the peak area of the control sample.

RESULTS

Table I shows the proportion of C14-C17 SCFA, C18-C20 MCFA and C21-C24 LCFA in hepatocytes incubated in the presence of ethanol and/or $MgCl_2$ solution. Table II shows the proportion of esterified C14-C17 SCFA, C18-C20 MCFA and C21-C24 LCFA in hepatocytes incubated in the presence of ethanol and/or $MgCl_2$ solution.

DISCUSSION

Analysing the total whole-cell fatty acids with different carbon chain lengths it was found that the test hepatocytes samples differ in this regard (Table I).

The control hepatocytes contained primarily acids with C18-C20 carbon chain lengths, the content of C14-C17 fatty acids was half lower, and that of C21-C24 fatty acids was the lowest. The most significant changes in the content of the analysed fatty acids were observed in hepatocytes incubated in the presence of ethanol. A multiple increase in the content of total fatty acids was found, which in turn gave rise to increased quantities of fatty acids.

The increase in the content of fatty acids in hepatocytes induced by ethanol is probably a consequence of their accumulation, due to inhibition of the Krebs cycle and beta-oxidation in hepatocytes (15-18).

Examining the effect of magnesium ions on the changes caused by the addition of ethanol it was shown that they have a stabilizing effect which is dependent on the concentration of magnesium ions. This is evidenced by the changes in the content of fatty acids of all chain lengths in hepatocytes incubated with a mixture of ethanol and 2 or 4 mM/dm^3 $MgCl_2$ solution, with a stronger stabilizing effect induced by a lower concentration of magnesium ions

Table I. *The proportion of fatty acids: C14-C17, C18-C20 and C21-C24 in hepatocytes incubated in the presence of the test substances for 5 hours (normalized areas under the chromatographic peaks).*

Simple	C14-C17	C18-C20	C21-C24
control hepatocytes	208	494	68
hepatocytes incubated with 2mM/dm^3 $MgCl_2$ solution	181	424	58
hepatocytes incubated with 4mM/dm^3 $MgCl_2$ solution	196	470	66
hepatocytes incubated with ethanol	2359	5758	516
hepatocytes incubated with ethanol and 2mM/dm^3 $MgCl_2$ solution	205	485	71
hepatocytes incubated with ethanol and 4mM/dm^3 $MgCl_2$ solution	176	263	52

Table II. *The proportion of esterified fatty acids C14-C17, C18-C20 and C21-C24 in hepatocytes incubated in the presence of the test substances for 5 hours (normalized areas under the chromatographic peaks).*

Simple	C14-C17	C18-C20	C21-C24
control hepatocytes	212	499	43
hepatocytes incubated with 2mM/dm^3 $MgCl_2$ solution	208	484	41
hepatocytes incubated with 4mM/dm^3 $MgCl_2$ solution	160	413	39
hepatocytes incubated with ethanol	201	466	63
hepatocytes incubated with ethanol and 2mM/dm^3 $MgCl_2$ solution	72	165	8
hepatocytes incubated with ethanol and 4mM/dm^3 $MgCl_2$ solution	134	306	24

in a mixture with ethanol.

The normalizing effect of magnesium ions with regard to the fatty acid content of all chain lengths in hepatocytes incubated in culture medium with ethanol and magnesium ions is probably due to the fact that the presence of magnesium ions alone in the culture medium resulted in a lower fatty acid content in those hepatocytes as compared to the hepatocytes in the control sample.

To determine which of the processes (beta-oxidation, esterification) was the cause of the changes in the fatty acid content, the content of ester-bound fatty acids in the test hepatocytes was determined.

The content of ester-bound fatty acids in hepatocytes varied across the samples. In all hepatocyte samples, esterified C18-C20 fatty acids prevailed, the content of C14-C17 fatty acids esters was half lower, while that of C21-C24 fatty acids esters was the lowest.

In the control hepatocytes, esters of medium-chain fatty acids prevailed, the content of short-chain fatty acids was half lower, with the lowest content of C21-C24 fatty acids esters (Table II). The most significant (when compared to the control group) variations in the amount of esterified fatty acids of all chain lengths were demonstrated in hepatocytes treated with a mixture of 2mM/dm^3 ethanol and $MgCl_2$ solution. In comparison with the control hepatocytes, lower contents of C21-C24 ester-bound fatty acids and ester-bound fatty acids of other chain lengths were observed. Similar trends were observed when the incubation medium contained ethanol with 4mM/dm^3 $MgCl_2$ solution (Table II).

The study showed that the content of ester-bound fatty acids in all test hepatocytes decreased when compared to the control hepatocytes (with the exception of C21-C24 fatty acids). Thus, the changes in the fatty acids content in hepatocytes were not due

to estrification, but they were probably a result of their oxidation or peroxidation.

It is commonly known that fatty degeneration (hyperlipidemia) resulting from the accumulation of fatty acids and their esters is the effect of ethanol on the cells of the liver (18, 19).

These results allow for suggesting a hypothesis that the effect of magnesium ions on the content of fatty acids and their esters in isolated hepatocytes incubated for 5 hours depended on their concentration in the medium.

Concurrent hypomagnesaemia due to the fact that they form complex compounds with fatty acids has been confirmed in people with alcoholism. The presence of Mg ions in the medium confirmed the stabilizing effect on the profile of C14–C17, C18-C20 and C21-C24 fatty acids present in the test hepatocytes. Such a normalizing effect of magnesium was not found when the profile of ester-linked fatty acids was analyzed.

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LETTER TO THE EDITOR

FIRST CASE REPORT OF VANADIUM METALLOSIS AFTER CERAMIC-ON-CERAMIC TOTAL HIP ARTHROPLASTY

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The development of metallosis as a complication following rupture of a hip replacement is known to occur as a result of contact with metal components of the prosthesis (1). In such cases, high cobalt (Co), chromium (Cr) and molybdenum (Mo) levels in the blood have been reported by several Authors (2). Recently, it has been stressed that the clinical investigation should focus on general reactions to high circulating metal levels, such as toxicity for the central nervous system (CNS) and the immune system (3). Despite the increasing interest of literature in ceramic-on-ceramic hip arthroplasty (4), little is known about these complications, and in particular of metallosis. To our knowledge this is the first description of a condition of extensive metallosis and radiographic signs presenting as a result of wear of a ceramic-on-ceramic prosthesis.

Case report

In November 2010 a 69-year-old Caucasian male agricultural worker resident in the Apulian region (Italy), who had been fitted in 2005 with a ceramic-on-ceramic non cemented arthroprosthesis of the right hip, came to our observation complaining of coxalgia and progressive functional impairment. The femoral component of the hip replacement was made of titanium-alumina-vanadium (Ti6Al4V) (Table I).

Radiography showed areas of periacetabular osteolysis, an eccentric position of the head of the prosthesis and an anomalous shape at the level of the lateral portion of the neck of the femur. Anteroposterior projections revealed the "cloud sign", a radiographic thickening of the periprosthetic tissues caused by deposits of metal debris (Fig. 1).

In the following months the patient's clinical conditions worsened and a fistula developed, secreting abundant, dense greyish-black matter, as well as skin dyschromia with blackish marks around the mouth of the fistula. The patient complained of a reduced sensation and strength in both legs. In addition, a greyish-green stain became evident on the central portion of the tongue. Further clinical-instrumental and toxicological tests led to the diagnosis of a peripheral polyneuropathy of the legs, as well as bilateral neurosensorial hypoacusia (Table II), attributed mainly to the absorption of vanadium in view of the particularly high blood and urinary values demonstrated (Table III).

Surgical revision of the prosthesis was performed, and during the procedure an ample infiltration of

Key words: Metallosis, vanadium, hip arthroplasty, ceramic-on-ceramic

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Table I. *Components of the first prosthetic implant and of the subsequent surgical revision parts.*

	First implant	Second implant (after surgical revision)
Neck	*	long modular head 8° A/R
Head	size 28 mm-ceramic biolox	size 36 mm-ceramic biolox
Stem	Antega size 13(B.Braun Aesculap AG & CO KG-Tuttlingen-Germania)-Ti6Al4V	size 13 (Wright-Medical-Technology Inc.- Arlington-USA)- Ti6Al4V
acetabulum	size 54 uncemented with ceramic liner 52-54 x 28mm (B.Braun Aesculap AG & CO KG-Tuttlingen-Germania)-Ti6Al4V	size 64 uncemented with polyethylene liner UHMWPE (Wright-Medical-Technology Inc.- Arlington-USA)- Ti6Al4V

Table II. *Laboratory and instrumental tests supporting the diagnosis of metallosis.*

Tests	Findings
Blood chemistry	All blood tests including liver and renal function markers, within normal range except for ESR=64mm/h (range 1-15 mm/h)
Culture of the fistula matter	Negative for bacteria and mycetes
CT	Air bubbles around the proximal third of the femoral stem, rupture of the ceramic component; no clear image of whether the prosthetic head and neck are intact.
US scan	multiple subfascial cysts containing matter, with a hyperintense wall and hypointense content; the largest measured about 10 cm in max diameter
Electroneurography	Moderate-severe sensory-motor axonal neuropathy of the peroneal and sural nerves bilaterally
Audiometry	bilateral neurosensorial hypoacusia

Table III. *Values of heavy metals in biological fluids before surgical revision and 3 months later (blood and urine, determined by Zeman effect atomic absorption spectrophotometry.*

	VANADIUM (µg/L) (Reference Values)		ALUMINA (µg/L) (Reference Values)		TITANIUM (µg/L) (Reference Values)	
	BEFORE SURGICAL REVISION	3 MONTHS LATER	BEFORE SURGICAL REVISION	3 MONTHS LATER	BEFORE SURGICAL REVISION	3 MONTHS LATER
BLOOD	6.1 (range 0.03-0.20)	2.6 (range 0.03-0.20)	*	*	*	*
SERUM	5.8 (range 0.03-0.10)	1.9 (range 0.03-0.10)	18.0 (range 1.0-6.0)	11.5 (range 1.0-6.0)	*	*
URINE	56.0 (range 0.05-0.20)	15.7 (range 0.05-0.20)	78.0 (range 1.5-6.0)	61.0 (range 1.5-6.0)	35 (range 0.05-0.50)	13.4 (range 0.05-0.50)

The values were compared with the reference range for the Italian population (SIVR 2011) (no values available).*

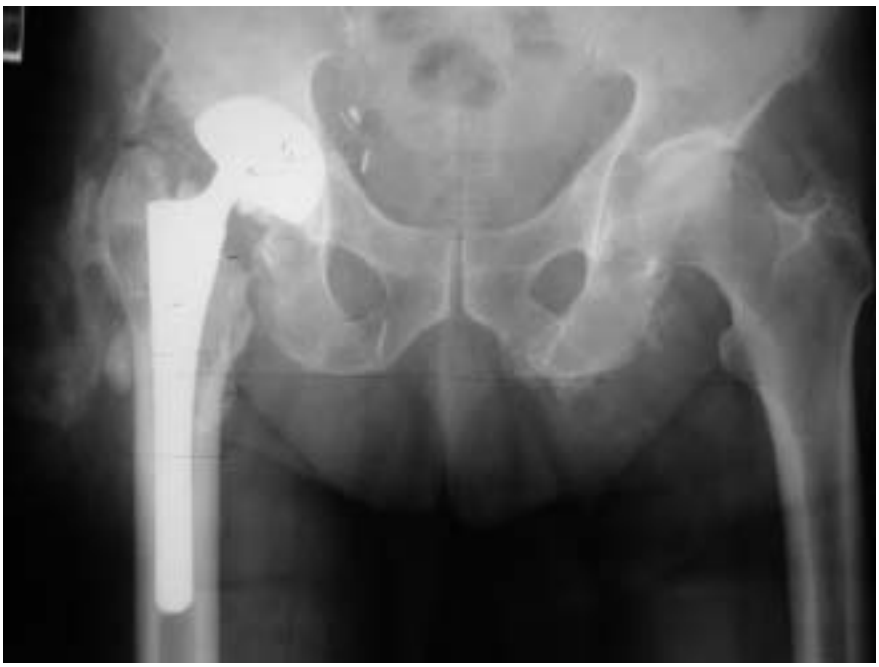

Fig. 1: *X-ray of the right hip, antero-posterior projection, showing the "cloud sign", radiographic thickening of the periprosthetic tissues caused by deposits of metal debris.*

Fig. 2. *At surgical revision, during removal of the prosthesis severe periprosthetic infiltration of blackish matter was found in the subcutaneous and subfascial planes, due to diffuse metallosis.*

blackish fluid was demonstrated in the subcutaneous and muscle fascia planes, due to diffuse metallosis (Fig. 2), as well as multiple cysts, the largest of which measured 10 x 5 cm. There was evident rupture of the ceramic acetabular liner and scattered fragments could be seen within the joint, as well as an elliptic deformation of the ceramic head. The femur neck appeared to be intact whereas the metal back showed an ample zone of erosion in the upper external portion (Fig. 3).

Toxicological analysis of the fluid contained in the periprosthetic cysts demonstrated concentrations of V: 2465 µg/L, Al: 3982µg/L and Ti: 21850µg/L; these values reflected the same proportions as those present in the metal alloy (Ti6Al4V), as also those previously described in other cases of metallosis (5).

Fig. 3. *The retrieved prosthetic components showed evident rupture of the acetabular liner and an elliptic deformation of the ceramic head.*

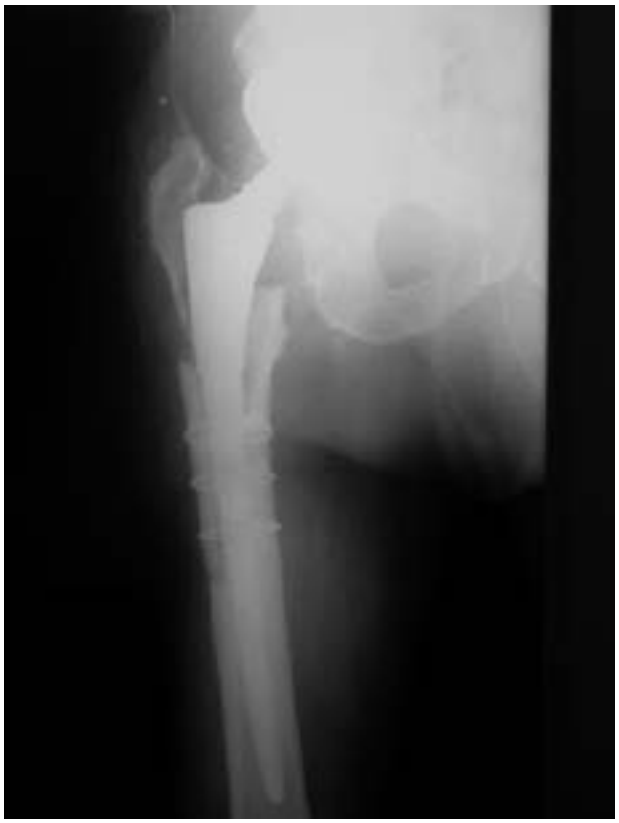

Fig. 4. *Postoperative control good positioning of the second implant components.*

The patient underwent removal of the prosthesis components and positioning of autologous bone grafts to support an uncemented cup size 64 (Wright-Medical-Technology Inc.- Arlington-USA) fixed with 3 25 mm screws and a polyethylene UHMWPE liner. The femoral component, removed according to Wagner's technique, was replaced with a new stem size 13, with a long modular neck 8° A/R, and a ceramic head with a 36 mm diameter (Wright-Medical-Technology Inc.-Arlington-USA) and 3 metal rings (Stryker Corporation Srl-EUROPA). The new prosthetic components were made of a titanium alloy (Ti6Al4V). At postoperative X-ray controls, the components were shown to be well positioned (Fig. 4).

Already 3 months later, toxicological tests showed a substantial reduction in the metal elements levels in all the biological assays (table III). At subsequent follow-up visits no signs were found of metal intoxication by the elements of the alloys. At 12 months after the surgical revision, complete recovery of the joint function was demonstrated, with good radiographic positioning of the prosthetic elements, and complete resolution of the peripheral neuropathy, confirmed by electroneurography (fig.5). Instead, the hypoacusia was still present at audiometry, while the greyish-green stain on the tongue was gradually becoming less marked and extensive.

DISCUSSION

The onset of metallosis as a complication of total hip replacement surgery has a reported incidence of about 5.3% (6). The condition is attributable to wear of the prosthetic surface due to fretting, that alters the biomechanical properties. This phenomenon is caused by prosthesis malposition in fact acetabular cup was set at an angle of 50° before the revision. With ceramic-on-metal or polyethylene-on-metal

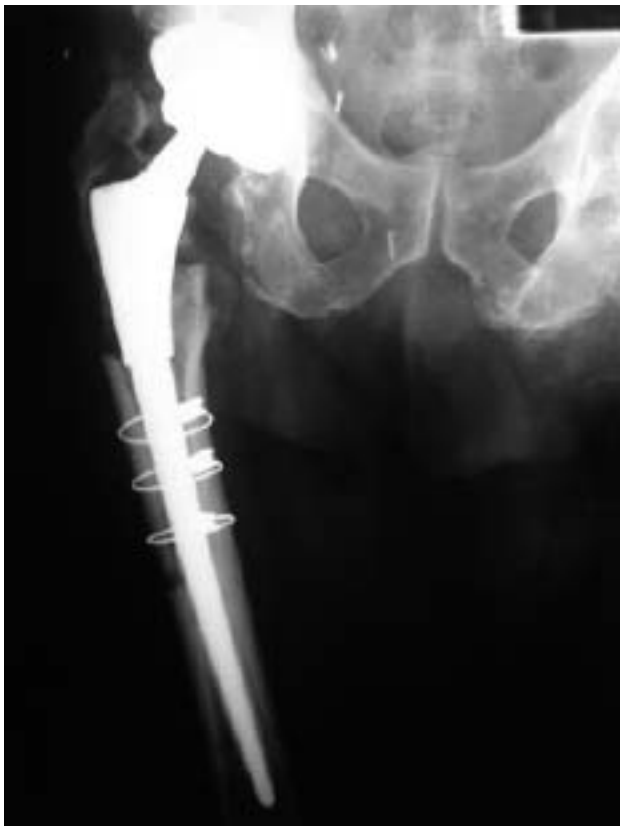

Fig. 5.*One year later the patient had a stable radiograph appearance.*

implants, instead, it is caused by rupture of the polyethylene or ceramic liners (7). To the best of our knowledge, this is the first case of onset of metallosis in a patient with a total hip replacement with ceramic-on-ceramic bearing surfaces to be reported in the literature. The use of ceramic guarantees lesser wear over time and hence a lower risk of metallosis (8). However, ceramic can fret and break and so cause the bearing surfaces to fail to fit properly, causing the stem to impinge on the metal back (9). The patient's physically demanding job may also have been a factor in the genesis of the fracture of the ceramic liner, due to functional overload, and of the ensuing friction between the head and the metal back causing hence a chronic inflammatory reaction (10). An intrinsic fragility of the liner is to be excluded, since the implant was a Biolox forte, a third generation ceramic that has an extremely low incidence of fracture (0.004%) (11).

In any case, the main problem was the metallosis caused by continual infiltration of metal debris. This type of event can induce intoxication by heavy metals (3, 12). The metal ions can deposit in organs inducing toxic effects (13, 14).

Some metal ions are well known to have toxic effects, especially Cr and Co, used to produce metal-on-metal and metal-on-ceramic implants (15).

In our case, V is responsible for peripheral neuropathy and tongue stain, Al for central neurological effects while Ti for local tissue reaction. Although Ti is correlated with a high incidence of metallosis, it is considered an inert metal with major biocompatibility, and not generally toxic (16). Nevertheless, the periprosthetic osteolysis is imputable to the Ti particles and ions (17). Meanwhile, alumina is associated with toxic effects on the CNS (18), while few studies have been made of the effects of intoxication by vanadium , most of which were focused on cases of inhalation of this metal due to occupational exposure. In such cases the main effects were of local, irritant type (19).

In the case we report, because of the manifestations of neuropathy, ototoxicity and a greyish-green stain on the tongue, toxic effects induced by Ti and Al were excluded and the clinical picture was judged compatible with a diagnosis of metallosis due to V. Our case allowed us to make an in-depth study of the systemic effects resulting from release of this heavy metal. In fact, in previous reports of cases of metallosis due to prosthetic implants made of Ti-Al-V, signs of severe necrobiosis and necrosis were described, but no clinical manifestations of specific intoxication by V (16).

This unique case emphasizes the possibility of association of metallosis with a failed ceramic-on-ceramic hip arthoprosthesis, as well as the importance of a correct positioning of prosthetic components, respecting the morphometric planes. A better knowledge of the specific clinical manifestations of intoxication by the metals employed in prostheses may help to monitor the onset of metallosis in patients fitted with a total hip replacement.

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LETTER TO THE EDITOR

NITRIC OXIDE SYNTHASE ISOENZYME EXPRESSION IN HUMAN ORAL LICHEN PLANUS

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The roles of nitric oxide (NO) synthase (NOS) enzyme in pathological mechanisms of the oral cavity are still incompletely understood. The aim of this study was to investigate the expression of the endothelial, neuronal and inducible isoforms of NOS (eNOS, nNOS and iNOS) in oral lichen planus (OLP) development in humans. OLP and healthy oral mucosa biopsies were taken for mRNA and protein analysis of NOS isoenzymes by RT-PCR, western blot and immunohistochemistry. The mRNA and protein levels of eNOS and nNOS were present in all samples, with a significant increase only for eNOS in OLP. The normal oral mucosa exhibited only small amounts of iNOS mRNA and protein, while it showed a significant rise in OLP samples. These results were confirmed by immunohistochemical analysis. Our findings suggest that NO produced by increased eNOS and iNOS expression may have circulatory and immune functions in the development of OLP.

Nitric oxide (NO) is a reactive nitrogen free radical produced by the NO synthase (NOS) isoenzymes; it affects a plethora of downstream physiological and pathological processes(1, 2). The past three decades have seen an explosion in the understanding of the role of NO in biology and pathology, highlighting various protective and damaging modes of action. NOS isoforms use the N-guanidine group terminal of L-arginine to produce NO and L-citrulline (3, 4). Much of the controversy surrounding the role of NO relates to the differing concentrations generated by the three isoenzymes of NOS, endothelial NOS (eNOS), neuronal NOS (nNOS) and macrophage or inducible NOS (iNOS) (5-7). The eNOS and nNOS are constitutive isoforms that can quickly synthesize small amounts of NO via increased intracellular Ca^{++} and Ca^{++}–calmodulin-NOS interaction following receptor stimulation (6, 8). The iNOS is independent of the intracellular Ca^{++} levels and is mainly involved in the inflammatory processes (9, 10). Endotoxins of Gram-negative bacteria and/or pro-inflammatory cytokines trigger resident and immigrant inflammatory cell population and induce iNOS formation. iNOS produces large amounts of NO for sustained periods of time (11). High NO output produced by iNOS plays a role in a non-specific immune response, while acting as a cytostatic and cytotoxic agent in fungal, bacterial

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and protozoal infections and in tumours (12).

NO has been implicated in the pathomechanism of a variety of diseases including oral lichen planus (OLP) (6, 13, 14). Lichen is a chronic autoimmune disease of unknown etiology that affects the skin and mucosae (15). In almost half of the cases of lichen, the mouth is involved, and is often the only area to be affected. Lichen in the mouth appears as white striations, white papules, white plaques, erythema, erosions, or blisters affecting predominantly the buccal mucosa, tongue and gingiva. It may not cause any symptoms or only be sore occasionally (17, 18). The etiopathogenesis of OLP is complex, with the involvement of T lymphocytes, histiocytes, mast cells, intercellular adhesion molecule-1, major histocompatibility complex class II antigens and NO (6, 16, 19). Current data suggest that OLP is a T-cell–mediated autoimmune disease in which autocytotoxic CD8+ T cells trigger apoptosis of oral epithelial cells (20, 21). However, the precise cause and pathomechanism of OLP remains obscure (22, 23).The purpose of this study was to determine the expression of eNOS, nNOS and iNOS in OLP by using immunohistochemical, western-blot and rt-PCR techniques.

MATERIALS AND METHODS

Fifteen patients (10 female and 5 male, between 30 and 60 years) with reticular lichen planus on the buccal mucosa were controlled in the study after the signed informed consent. After the systemic and oral anamnesis and clinical inspection of the patients, biopsies were taken from the lesions for diagnostic certainty and for biochemical analysis. Five samples from clinically healthy buccal mucosa were also taken for control (3 female and 2 male). The samples were immediately immersed in a gelatine substance, Bio-optical KILLIX, and then immediately frozen at –80° for 24-48 h.

For the histopathologic analysis sections from each patients were coloured with haematoxylin-eosins.

ENOS, nNOS and iNOS immunohistochemical localization

NOS isoforms were localized by immunohistochemical staining of the mucosa with monoclonal anti-eNOS, anti-iNOS or anti-nNOS primary antibodies (1:100, Saint Cruz Biotech Inc., Santa Cruz, California) with use of the Vectastain ABC kit (VectorLaboratories, Burlingame, CA, USA) as published earlier.

ENOS, nNOS and iNOS protein analysis by Western-blot analysis (WB)

Determination of the expressions of NOS proteins were performed as described in detail in Felaco et al. and Di Nardo Di Maio et al. B-actin was used as a control. The densitometric analysis of WB was performed using a computerized densitometric system (Bio-Rad Gel Doc 1000, Milan, Italy)

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) for eNOS, nNOS and iNOS

Total RNA was extracted using 1 ml ULTRASPEC-RNA (Biotech, Lab., Inc. Huston, TX, USA), as recommended by the manufacturer. RNA was dissolved in diethyl pyrocarbonate (DEPC)-treated water and quantified spectrophotometrically at 260 nm. First-strand cDNA was generated by adding RNA (1µg) to a mixture containing 1 mM deoxy-nucleoside- triphosphates (d-NTP), 1U/µl RNase inhibitor, 2.5 U/ µl moloney murine leukemia virus reverse transcriptase, 2.5 µM oligo-dt, 5 mM $MgCl_2$, 10x PCR buffer in a final volume of 20 µl. Reverse transcription was performed at 42°C for 1 h followed by heat inactivation of reverse transcriptase at 92°C for 10 min. 18S was amplified from the same amount of RNA to correct for variation of different samples. PCR amplification was performed using a Programmable Thermal Controller (MJ Research, Inc. Massachusetts, USA) at the following annealing tenperatures: 58°C for 60 s for eNOS, 56°C for nNOS and 55°C for iNOS. The PCR solution contained 10 µl of first-strand cDNA, 4 µl 10x PCR buffer, 2 mM $MgCl_2$. The following primer pairs were used: sense 5'-CTC TCC AGG CAC TTC AGG C-3' and antisense 5'-TGT CTG TCT GCT GCT CCT AG-3' for human eNOS, sense 5'-CGT TTG GCA TGG GGG AGT GAG C-3' and antisense 5'-TTG GGG GCC TGG GAT TTC TGG-3' for human nNOS and sense 5'-CGT AAA GAC CTC TAT GCC AA-3' and antisense 5'-AGC CAT GCC AAA TGT CTC AT-3' for human iNOS. 2U Termophylus Acquaticus (Taq) DNA polymerase (Celbio, Milan, Italy), and water to a final volume of 50 µl. PCR products were run on 2% agarose gel electrophoresis and photographed after ethidium bromide staining under UV light. B-actin was used as a control.

Densitometric analysis of bands of mRNA and protein levels of eNOS, nNOS and iNOS

Bands on the gel were scanned using a computerized densitometric system (Bio-Rad Gel Doc 1000, Milan, Italy) and were quantified by measuring their integrated optical density (I.O.D.) with ISO Transmission Density image analysis system (Koak CAT 152-3406, Eastman Kodak Company, Rochester, USA).

Statistical evaluation

All results are expressed as mean±standard deviation. We performed repeated measures to compare means between groups. Probability of null hypothesis of less than 5% was considered as statistically significant.

RESULTS

Histological analysis of the lesions confirmed the presumed clinical diagnosis of OLP.

Localization of eNOS, nNOS and iNOS immunoreactivity

Immunohistochemical analysis showed increased eNOS immunereactivity in endothelium of OLP samples (Fig. 1A) compared to the healthy controls (Fig. 1B). No differences were observable in nNOS immunoreactions of nerve fibres between normal oral mucosa (Fig. 1C) and OLP samples (Fig. 1A). iNOS was strongly present in the OLP samples (Fig. 1A) while there was virtually an absence of iNOS in the healthy oral mucosa (Fig. 1D).

ENOS, nNOS and iNOS protein levels detected by WB analysis

To assess the amounts of eNOS, nNOS and iNOS in healthy and OLP mucosa we investigated their

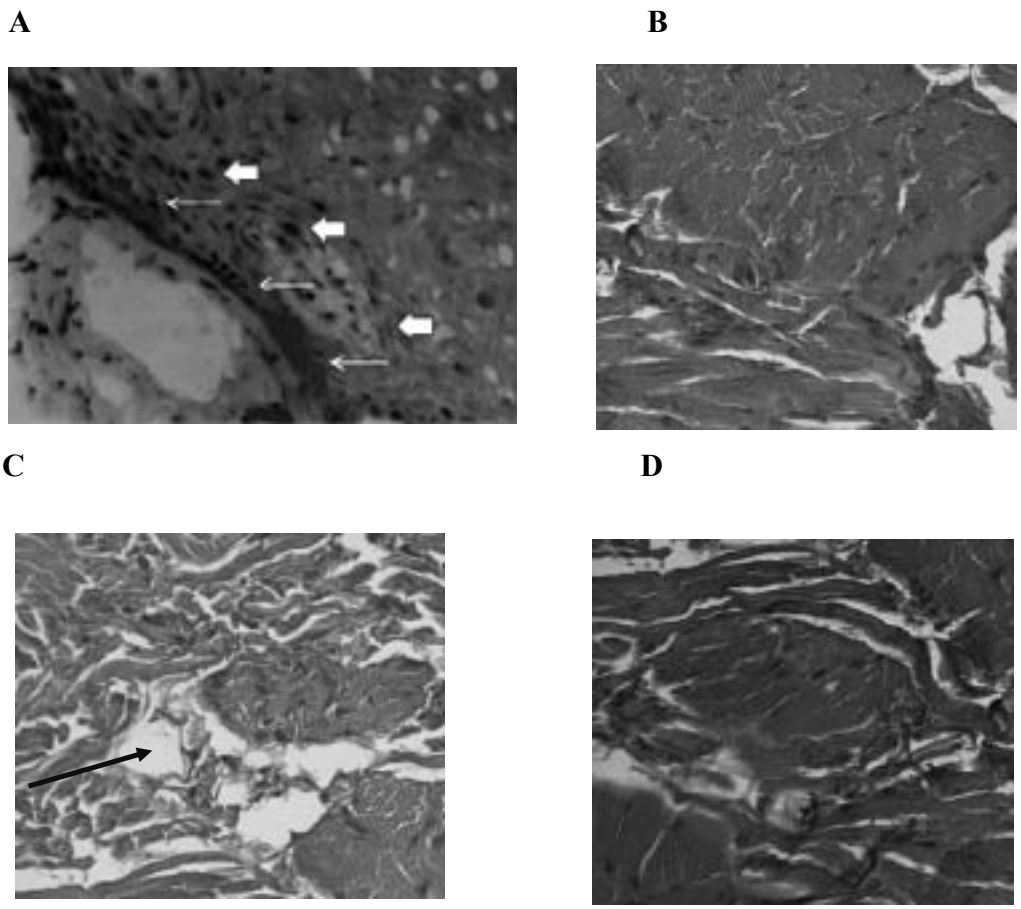

Fig. 1. *Immunohistochemical localization of eNOS, nNOS and iNOS in healthy and oral lichen planus (OLP) affected oral mucosa. eNOS immunopositive endothelial cells (red arrows) were found in the vessels of normal mucosa (**1B**), but their reactivity (thin arrows) is significantly increased in OLP (**1A**). No reactivity difference was found in nNOS immunostaining of nerves (black arrows) between normal oral mucosa (**1C**) and OLP samples(medium arrows) (**1A**). Virtually, there was an absence of iNOS immunoreactions in normal oral mucosa (**1D**), but iNOS was strongly present in lymphocytes (large arrows), macrophages (large arrows) and mast cells (large arrows) under basement membrane (v) of OLP sections (**1A**).*

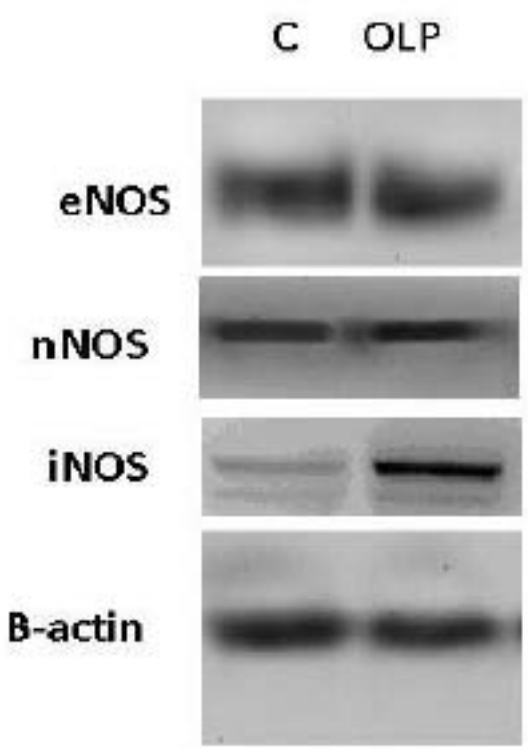

Fig. 2. *Western blot detection of eNOS, nNOS and iNOS proteins obtained from normal (**C**) and oral lichen planus (**OLP**) affected oral mucosa. B-actin was used as a control.*

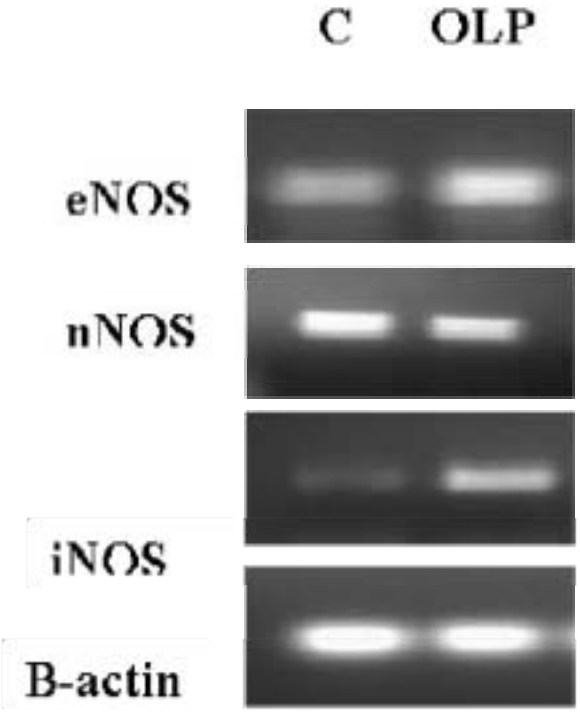

Fig. 3. *RT-PCR detection of eNOS, nNOS and iNOS mRNA obtained from healthy (**C**) and oral lichen planus (**OLP**) affected oral mucosa. B-actin was used as a control.*

protein levels (Fig. 2). The eNOS protein level was significantly elevated in OLP compared to normal oral mucosa (Table I). There was no difference in nNOS protein level between the groups (Table I). The iNOS protein was hardly detectable in normal oral mucosa, but OLP affected mucosa showed a significant increase of iNOS protein (Table I).

eNOS, nNOS and iNOS levels by RT-PCR analysis

To evaluate the transcription of eNOS, nNOS and iNOS in healthy and OLP mucosa we analyzed their mRNA levels (Fig. 3). The mRNA of eNOS and nNOS were present in all samples, but we found a significant increase only for eNOS in OLP (Table I). The normal oral mucosa exhibited only hardly observable mRNA of iNOS, but it showed a significant rise in OLP (Table I).

DISCUSSION

Several pathological features indicated that OLP is an immunologically mediated inflammatory response, including the fact that a band like infiltrate of predominantly T-lymphocytes appears subjacent to the epithelium (1). The basal epithelial cells are the target for immune destruction by cytotoxic T lymphocytes (22, 20).

A lymphocytic infiltrate formed on the sub-epithelial lamina propria as well as a characteristic fibrinogen deposit at the basal membrane level (11). Chronic inflammatory disease has been clinically associated in OLP lesions with development to oral cancer (17). Infection and chronic inflammation are inclined to carcinogenesis through inflammation-related mechanisms (21).

NO has a dynamic role during physiological and pathological human oral processes, including immune-pathogenesis cells, neo-angiogenesis, apoptosis, T-cell and macrophagic reaction (12). Localization of 8-nitroguanine and inducible nitric oxide synthase (iNOS) was found in oral epithelium of OLP and oral squamous cell carcinoma (OSCC)

Table I. *Densitometric analysis of protein and mRNA bands of eNOS, nNOS and iNOS performed by western blot and RT-PCR technique.*

eNOS	**control (I.O.D)**	**OLP (I.O.D.)**
western blot	3 ± 0.05 (n=5)	4.2 ± 0.2 * (n=15)
RT-PCR	2 ± 0.1 (n=5)	3.5 ± 0.5 * (n=15)

nNOS	**control (I.O.D.)**	**OLP (I.O.D.)**
western blot	1.2 ± 0.1 (n=5)	1 ± 0.2 n.s. (n=15)
RT-PCR	1.8 ± 0.4 (n=5)	1.5 ± 0.5 n.s. (n=15)

iNOS	**control (I.O.D.)**	**OLP (I.O.D.)**
western blot	0.02 ± 0.04 (n=5)	1.4 ± 0.2 ** (n=15)
RT-PCR	0.01 ±0.02 (n=5)	1.3 ± 0.1 ** (n=15)

Data are presented as integrated optical density (I.O.D.) and are expressed as means±SEM of mucosa samples for control and oral lichen planus (OLP) groups $p<0.05$, $p<0.01$**, n.s. not significant.*

(15). Levels of NO were measured in the saliva of patients affected by OLP and aphthous ulcerations (RAU) (12, 15, 19). Salivary NO_2 concentrations, the breakdown product of NO, from patients with OLP and RAU showed a significant increase compared to those of the control group (1, 12-14). An increase in NO_2 levels was reported in the saliva during lemon juice stimulation (15, 19, 24). This suggests that production of NO_2 in the salivary glands is controlled by neural mechanisms (10, 25). Furthermore, it has recently been found that salivary NO was increased in patients with Sjögren Syndrome if compared to healthy control patients[26]. Immunohistochemical analysis showed an increase nNOS expression of nervous fibre of the labial salivary glands of patients affected by Sjögren Syndrome (14, 26, 27).

Therefore, the salivary glands could increase the expression of nNOS in patients affected by OLP and RAU but further studies are necessary to obtain confirmation (10, 13).

Moreover, psychological stress caused the liberation of NO in correlation with the increase of nNOS activity (19, 28). This could also explain the increased production of NO in patients with OLP and RAU (10, 27). Free radical NO is a high reactive molecule that could play an important role in the cytotoxic action of T-cells during oxidative stress intracellular reaction (29, 30). It can act directly or indirectly by its interaction with superoxide radicals, oxygen, thiol groups and transitional metals in proteins with the production of nitrous-thiols (R-SNO) (31, 32) toxic molecule like peroxi-nitrite (ONOO-) and nitrites (14, 23, 25). Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are considered

to play a key role in inflammation. ROS can generate 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), an indicator of oxidative DNA damage (8, 15, 19) it has been demonstrated that NO can damage cellular DNA, proteins and lipids, inducing cellular death and tissue injury (30).

In our research, during RT-PCR analysis, Oral Lichen Planus surgical specimens showed high increases of mRNA levels of eNOS and iNOS compared to the healthy control group. Low nNOS levels were observed in all samples of oral lichen planus and healthy mucosa.

Western-blot analysis confirmed the results observed with RT-PCR examination with an higher intracellular activity of eNOS and iNOS protein and low expression of nNOS protein into samples compared to the control.

Immunohistochemical analysis found a strong presence of eNOS and iNOS in all samples of Lichen planus around the area of increased vasodilation of the inflammatory process (3, 10, 14). Erythrocyte cells increased and vasculogenesis was most evident around sub-epithelial vesicles (3). While nNOS monoclonal antibodies showed low samples on staining and no differences between normal oral mucosa and lichen planus specimens (14, 19).

Such results with himmunohistochemical, RT-PCR and Western Blot analisys confirm the presence of nitric oxide activity in Oral lichen planus. The different expressions and localizations of eNOS, iNOS and nNOS isoforms showed the interactions and different functions of the proteins.

Endothelial Nitric Oxide Synthase (eNOS) and inducible nitric oxide synthase (iNOS) strongly increased in all specimens and high intracytoplasmic expression with himmunohistochemical, RT-PCR and Western Blot analisys showed the central role of these mediators during the pathogenesis and cronical development mechanisms of oral lichen planus.

The results show that an increased quantity of Nitric Oxide may indicate patho-physiological implications in the development, maintenance and evolution of Oral Lichen Planus lesions.

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LETTER TO THE EDITOR

DIMENSIONAL AND MICROBIOLOGICAL *IN VITRO* ANALYSIS OF A DENTAL IMPLANT LOCKING TAPER CONNECTION

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The present study was carried out to compare the differences in contact, height and contact area between the implant-abutment interface and the implant-healing cap interface of an implant system featuring a locking tapered connection by using X-ray micro-tomography. It was also conducted to test *in vitro* whether the implant-healing cap tapered interface is capable of preventing bacterial leakage from the implant well to the external environment. The images of the samples, acquired by the X-ray micro-tomography, after being processed with a dedicated software, showed a greater contact height (CH) in the implant-abutment sample (3.57 mm) compared to the implant-healing cap sample (2.52 mm). This was also true for the contact area that was equal to 40.63 mm^2 in the implant-abutment sample and 25.14 mm^2 in the implant-healing cap sample. No bacteria were detected both in the nutrient of the test group and of the negative control after 24 h. An increased contact height and contact area in a tapered connection, between the implant and the abutment, have demonstrated to offer mechanical and biological advantages, in a implant-healing cap tapered connection. The major concern regards the microbiological aspects of this connection. The implant-healing cap tapered connection provides an hermetic barrier to microbial passage *in vitro*, even though such connection features lower contact height and contact area compared to the implant-abutment connection of the same implant system.

In order to determine the main factors associated with the achievement of dental implant success and survival, several studies have been focused on the analysis of the interface between implant and abutment and the biological implications due to the presence of a microgap that is formed at the level of this interface.

This area may be colonized by bacteria in the mouth, which are then able to infiltrate the microgap and move into the implant well, which will become a bacterial reservoir (1, 2). Most importantly however, the microgap will also be permeable to the movement of bacteria, from the implant well, where they have been able to reproduce undisturbed, to the surrounding peri-implant tissues.

This has been demonstrated by numerous *in vivo* and *in vitro* studies on different implant systems (3-5). The inflammatory response of the tissues surrounding the microgap may, in some cases, lead to a consequent loss of bone combined with the apical migration of epithelial junction (6). The size of the peri-implant tissues seems therefore to be profoundly influenced by

Key words: implant, abutment, connection, healing cap, microbiological anaysis

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the presence/absence of a microgap between implant and abutment and by its vertical position relative to the bone crest (7, 8). The size of the microgap is significantly different depending on the type of connection available, in an external hex connection; microgap size varies from 21 μm to 60 μm. Internal hex connections, feature a microgap that ranges from 2 μm to 15 μm. Finally, in systems with locking tapered connection between implant and abutment, microgap size is greatly reduced and ranges from 0.5 μm to 1.5μm (9, 10). *In vitro* studies, carried out on implant systems with this type of connection demonstrate contrasting results (4, 11). Several clinical studies have analyzed long term peri-implant bone remodeling around implant systems with a locking taper connection, the results have shown a minimal bone loss after a follow-up of 6 to 8 years (12-15). Moreover further studies revealed biomechanical advantages when using a tapered connection, due to the more homogeneous way in which the mechanical stress deriving from occlusal load is distributed to the implant component and peri-implant bone (16-18). In order to study the mechanical and physical characteristics of the implant-abutment connection researchers have used finite element models and/or the use of Micro-tomography x-ray scanning devices (19, 20).

The purpose of this study was to investigate on this kind of interface, both from a dimensional point of view, analyzing it with a micro-tomography x-ray 3D scansion device and comparing it with the corresponding implant-abutment interface, and from a microbiological point of view, testing the capability of the implant-healing cap tapered connection of preventing bacterial leakage from inside to outside.

MATERIALS AND METHODS

In order to assess the different contact heights and contact areas between the implant-abutment interface and implant-healing cap interface, two implants were used (Exacone™, Leone SpA, Sesto Fiorentino, Italy). The implant system analyzed in this study presents an internal conical connection featuring a 1.5° taper, which is often referred to as a locking Morse taper connection. It is a screwless connection, where implant-abutment mating occurs by friction between the opposite surfaces: thanks to the Morse taper principle, in fact, the high friction developed between the surfaces of two equal conical elements creates a solid connection between them. In order to activate the connection, an impact load acting along the implant axis has to be applied onto the abutment/healing cap: this way microscopic interferences between the parts will lock the component inside the implant, thus creating a very tight connection.

One sample 4.1 x 10 mm was connected to the standard abutment provided by the manufacturer and after applying a perpendicular force with a dedicated instrument, the connection between the two pieces was activated, according to the manufacturer's protocol. One sample 4.1 x 10 mm was connected to the healing cap and the connection was activated in the same way described above. The two samples were then mounted on resin blocks in order to stabilize them in a perpendicular position and avoid movements, which would create artifacts in the images acquired by the SkyScan. The x-ray microtopographic scansions were acquired using the Sky Scan model 1072 (SkyScan, Kartuizersweg 3B, 2550 Kontich, Belgium). Each sample underwent 5 consecutive acquisitions by Skyscan 1072. The following acquisition parameters were used for both samples:

- rotation step = 0.45°
- total rotation angle = 180°
- power source 100 kV/98 microA
- filter thickness 1 mm (Al)

A dedicated reconstruction software was then used to process all the images acquired from the Sky Scan, in order to create exact 3D duplicates of the examined implant samples. The 3D model can be virtually investigated in all its parts through the acquired sections, with no need to cut, modify or alter the sample (27, 28). In order to measure the contact height and contact area of the tapered interface between the implant and the external component, in sample 1 an abutment, in sample 2 a healing cap, sequential analysis of all reconstructed axial sections was carried out, in order to determine precisely where contact between the implant and the external component began and where it ended. Once the contact area was determined the software calculated the contact height in mm and contact area in mm^2 between the implant and the external component.

In order to test the capability of the seal to prevent microbial infiltration in vitro, 5 implants with the same locking taper connection, were used (4.1 x 14 mm). Each implant was mounted on a resin support and the 5 samples were successively divided into the following 3 groups:

-Test Group: composed of 3 implants, in which 1 μl of a bacterial agar mix at a 2% concentration was inoculated inside the implant body using a micro-pipette. The bacterial agar mix was obtained by mixing 100 μl of bacterial mixture in Brain Heart Infusion or BHI (consisting of Streptococcus oralis and Actinomyces Odontoliticus with an initial bacterial concentration of 3 x 10^6 CFU / ml) with

100 ul of 4% soft agar and 1 µl of Trypan Blue (total volume 201 µL). After inoculating the bacterial agar mix the healing cap was applied and the connection was activated using a dedicated instrument.

-Positive Control: consisting of 1 implant, inside which, 1 µl of the 2% agar bacterial mix previously described was inoculated. This implant was left without healing cap in order to assess the viability of microorganisms over time.

-Negative Control: 1 implant was left bacteria-free and the healing cap was connected. This sample was necessary to determine if cross-contamination occurred.

Each sample was immersed in an individual 15 mL Falcon tube, each containing 10 ml of BHI and were subsequently incubated for 96 h anaerobically at 37°C. After the first 24 h 20 µl of BHI were taken from each tube. Each sample was then individually plated on an agar plate, numbered in correspondence with the sample analyzed. The 5 dishes were in turn incubated anaerobically for further 5 days at the end of which it was visually assessed if bacterial colonies had formed. In this case the passage of microorganisms from the implant well to the external BHI would be proved. The drawings of BHI from tubes were repeated at 48, 72 and 96 h for a total of 4 samples and 20 agar plates evaluated.

RESULTS

During sequential analysis of all reconstructed axial sections, obtained by the Skyscan, three levels were identified for each sample. A first level (L0) was determined and identified where the implant and the external component were still not in contact and the gap between them was visible in the images, meaning it was greater than 10 µm (Fig. 1). The following level (L1) identified the initial contact between the implant and the external component, at this point no gap was visible between the two surfaces, meaning that less than 10 µm divided them (Fig. 2). The last level (L2) identified the area where the implant and the external component lost contact again and a thin radiolucency was visible, meaning that the gap was once again greater than 10 µm (Fig. 3).

The mean values for contact height and contact area of the two samples, obtained by Micro-CT scanning and data processing with a dedicated software are shown in Table I. The implant-abutment interface showed greater contact height and contact area compared to the one present between the implant and the healing cap.

The microbiological *in vitro* study, which evaluated the possibility that bacteria, positioned within the implant lumen and sealed with a healing cap, could be able to infiltrate the interface and reach the outside, showed the following results. The first samples of BHI taken after 24 hours,

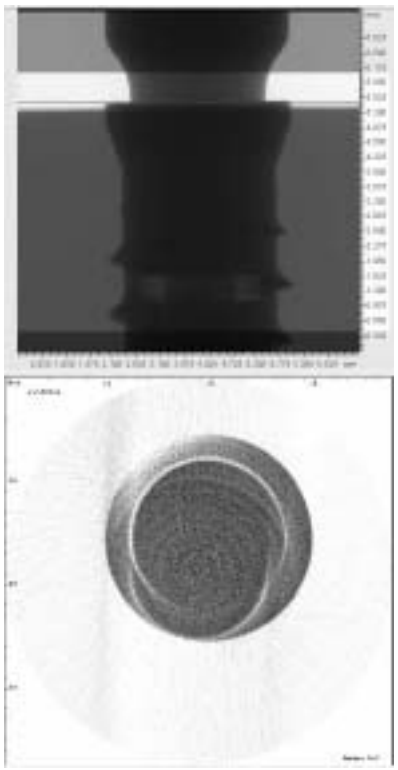

Fig. 1. *Level 0 (L0) identifies the area where the implant and external component, in this case the abutment, are about to come in contact.*

Table I. *Micro-CT scanning values for contact height and contact area.*

External Component Connected	Contact Height (mm)	Contact Area (mm^2)
Abutment	3.57	40.63
Healing Cap	2.52	25.14

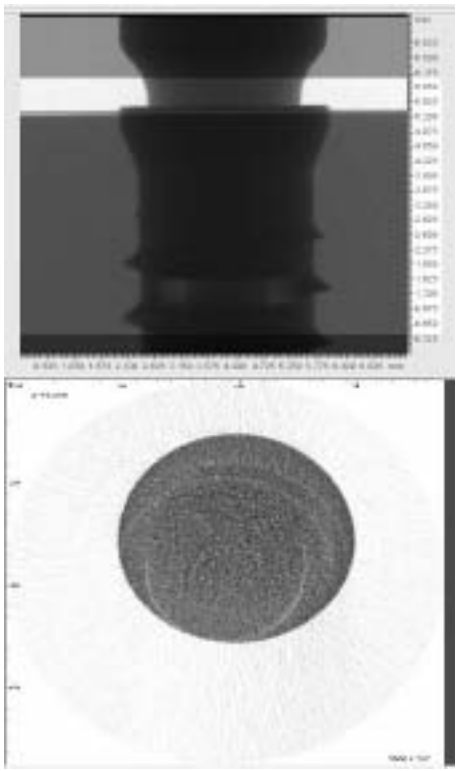

Fig. 2. *Level 1 (L1) location of first implant-abutment contact.*

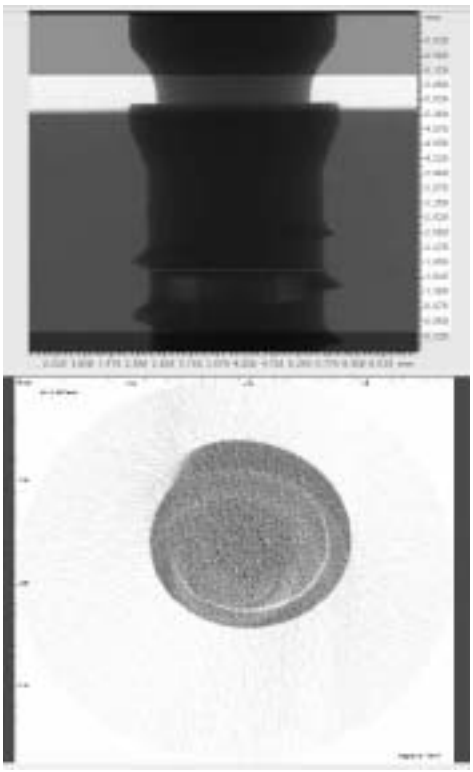

Fig. 3. *Level 2 (L2) Area where the microgap between implant and abutment is once again visible, identifies the position where the two pieces lose contact.*

resulted in absence of bacterial growth in the agar plates corresponding to the test samples and in the negative control, and bacterial growth in the agar plate corresponding to the positive control. This result suggests that the bacteria inoculated within the 3 implants of the test group (Sample 1-3) were not able to infiltrate the microgap between the implant and healing cap during the first 24 hours.

The samples of BHI taken subsequently, at 48, 72 and 96 hours resulted in no bacterial growth after being plated on individual agar plates. This was also true for the agar plate corresponding to the positive control. This result suggests that the microorganisms inoculated inside the implants have lost their viability between the first sampling at 24 hours and the second one at 48 hours. Therefore the absence of bacterial growth in the agar plates corresponding to the test group samples cannot be considered valid results, for the assessment of bacterial passage through the microgap, since the microorganisms might not have been any longer viable already at 48 hours. The only valid result, which shows the absence of bacterial infiltration from the implant well to the external environment, is the one at 24 hours when bacteria were still viable, as evidenced by the agar plate corresponding to the positive control.

DISCUSSION

When a microgap or a misfit in the implant-abutment interface is present, biological and/or mechanical complications may arise. *In vitro* studies have shown the possibility that bacteria may penetrate this microgap, colonize the implant lumen and release bacterial endotoxins into the surrounding tissues, causing inflammatory processes which may lead to bone resorption. It is therefore necessary to understand more in depth the physical properties of this interface and whether it is able to prevent bacterial infiltration. The results of the x-ray micro-topographic analysis show how the implant-healing cap connection results in a lower contact height and

contact area compared to the connection between the same implant type and the abutment. This result was predictable as the abutment has a longer inner portion and therefore is further in contact with the implant body. A greater contact area between the implant and its external component offers a greater surface on which the mechanical stresses can be distributed. However this mechanical advantage is less important when considering the implant-healing cap interface, as it is not supposed to withstand mechanical stresses. Moreover the healing cap is a temporary device and therefore requires to be easily removable. The main issue concerning the implant-healing cap interface is whether it is able to prevent bacterial infiltration of the microgap even though it offers a lower contact height and therefore a "shorter" barrier to bacteria. The results obtained in the second part of this study suggest that the Morse taper implant-healing cap interface, offers an intimate connection, which does not allow bacterial penetration in 24 hours. This is the only valid result obtained, as the data obtained at 48, 72 and 96 hours is not reliable since the microorganisms present in the positive control sample, left without healing cap, had lost their viability. The causes of this event cannot be determined with certainty: possibly during the preparation of the bacterial soft agar gel the amount of nutrients was insufficient, or the initial bacterial concentration was too low. However, the use of agar at 4% and the initial bacterial concentration of 3 x 10^6 CFU / ml were not random choices, but were the same that had been used in previous studies where microorganisms remained viable for 72 hours (14).

The interface between an implant and its healing cap (or healing screw) is of fundamental importance, as it is the first one to be exposed to the oral cavity and this may occur during the most delicate phase of osseointegration. The implant system analyzed in this study features a locking taper connection between the implant and both the abutment and the healing cap. When connected to the abutment greater contact height and contact area are present between the two pieces than those present between the implant and the healing cap. This difference in contact height and contact area does not seem to negatively influence the microbiological characteristics of the implant-healing cap interface, which is able to prevent in the first 24 hours any kind of microbial penetration.

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LETTER TO THE EDITOR

EXHALED BREATH CONDENSATE, NASAL EOSINOPHIL CATIONIC PROTEIN LEVEL AND NASAL CYTOLOGY DURING IMMUNOTHERAPY FOR CYPRESS ALLERGY

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Interest in cypress allergy is widely rising: an increasing number of studies have pointed out the efficacy of immunotherapy to reduce cypress-related symptoms and drug use. Cypress immunotherapy is well tolerated, but there are few studies dealing with its sub-clinical effects on the airways. The aim of this investigation is to assess the effects of immunotherapy on airways by the analysis of exhaled breath condensate (EBC), nasal lavage fluid (NAL) and nasal cytology. Fifteen mono-sensitized to cypress pollen patients have been observed, among them 9 have been treated with sub-cutaneous immunotherapy (SCIT), 3 with sub-lingual immunotherapy (SLIT) and 3 which were not treated underwent EBC, NAL and nasal cytology out of the pollen season. 8-isoprostane in EBC, Eosinophil cationic protein (ECP) and inflammatory cells in nasal cytology were also evaluated. The median value of 8-isoprostane in EBC was 18.58 pg/ml in patients who did not undergo immunotherapy, 49.38 pg/ml in SCIT patients and 13.41 pg/ml in SLIT subjects. The median value of ECP in nasal lavage was higher in non- treated subjects (27.3 mg/l) than in those treated with SCIT (1 mg/l)($p < 0,05$) or SLIT (2.6 mg/l). All nasal cytology specimens did not show any sign of inflammation. In conclusion SLIT seems to be well tolerated and to reduce significantly the levels of ECP in nasal lavage. In addition the levels of 8-isoprostane in EBC among SCIT patients were unexpectedly high and need to be further evaluated.

Recently it has been reported an increased prevalence of cypress allergy. In addition the clinical features of cypress allergy seem to increase in duration and severity (1). Therefore in the last years several studies have been carried out on cypress immunotherapy, showing efficacy and safety of common allergen extracts (2). Assumed the clinical efficacy and safety of subcutaneous (SCIT) or sublingual (SLIT) cypress immunotherapy, little is known about its immunological effects on the airways. Moreover, even if some patients could experience rhinitis or asthma symptoms during

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pollen immunotherapy, the inflammatory parameters during treatment have not been evaluated.

Traditionally airways inflammation is indirectly studied by lung function tests, rhinomanometry, rhinometry, whereas a direct assessment can be made only by invasive techniques such as biopsies and bronchoscopy. In the last decades new non-invasive methods to evaluate the inflammatory changes in the respiratory tract have been proposed. Analysis of exhaled breath condensate (EBC), nasal lavage fluid (NAL) and nasal cytology are considered safe and reliable methods to investigate some features of the inflammatory response of the airways (3).

The aim of this pilot study is to evaluate the inflammatory response of respiratory mucosae during SCIT or SLIT immunotherapy in patients mono-sensitized to *cupressacee* by the analysis of 8-isoprostane in exhaled breath condensate (EBC), eosinophil cationic protein (ECP) in nasal lavage and nasal cytology.

MATERIALS AND METHODS

Subjects

The population studied consisted of 15 patients (8 females and 7 females; mean age 48 years with a range of 29-68 years), all non smokers, with persistant moderate-severe allergic rhinoconjunctivitis according to ARIA guidelines (4), and mono-sensitized to cypress. None of the patients had symptoms of asthma. The sensitization was confirmed by skin prick test and Cap-RAST. Nine subjects were on SCIT, eight of them practised SCIT since three years, one since a year and three were on SLIT (all of them were at the third year of treatment) at the time of immunological studies. Three further patients mono-sensitized to cypress were not receiving immunotherapy.

All samplings of exhaled breath condensate for 8-isoprostane, nasal lavage for ECP and nasal cytology were performed 8-12 weeks before the cypress pollen season (absence of pollen ascertained by pollen count). At the time of investigation none had respiratory symptoms, infections or was using anti-inflammatory drugs. All patients provided a signed and informed consent to the study.

SCIT or SLIT treatment

As for the SCIT, a standardized extract of Juniperus ashei absorbed onto aluminum hydroxide phosphate (Stallergènes Sa, Antony, France) has been used. Sampling of EBC, NAL and nasal cytology were performed in the maintenance phase, about 15 days after the monthly injection. SLIT was carried out with a standardized extract of Juniperus ashei, administered as a glycero-saline solution (StaloralR; Stallergènes Sa, Antony, France 300IR/ml). The sublingual treatment was self-administered by each patient at home. Both treatment has been used according with standard product characteristics. The concentration of the major allergen Jun a 1 was equal to 300 μg/ml. Sampling of EBC, NAL and nasal cytology were carried out three years after starting SLIT and performed the day when the patients did not take SLIT.

Skin prick test (SPT)

A positive control (histamine, 10 mg/l) and a negative control (glycerinated saline) with a standard panel of allergens (pollens: Parietaria, olive, Compositae, and Graminaceae; inhalants: Dermatophagoides pteronyssius and D. farinae, Alternaria and Aspergillus, dog and cat fur from Bayer, Milan, Italy) were administered and interpreted in accordance with the European Academy of Allergology and Clinical Immunology (EAACI) Subcommittee on Allergen Standardization and Skin Tests (5). The response was considered positive if the diameter of the wheal is more than 3 mm larger than the one provoked by the negative control.

Exhaled Breath Condensate

Exhaled breath condensate (EBC) was collected using a condensing chamber (Ecoscreen Jaeger, Hoechberg, Germany) as previously described and following the recommendations of the ATS/ERS task force on standardization of Exhaled Breath Condensate collection (6). Subjects were invited to wash the mouth with purified water and rinse it before the collection. After this passage they had to breath tidally through a mouthpiece connected to the condenser with a nose-clip for 15 minutes. An average exhaled breath condensate of 1.5 ml was collected for each patient, immediately stored at -80°C and analyzed within 2 months. There was no significant variability in EBC volume among the subjects. 8-Isoprostane in EBC samples was measured with a specific radioimmunoassay as described previously (7). The detection limit was 12 pg/mL. The interassay coefficient of variation of the test was <2%; the interassay coefficient was <3%. Samples below the detection limit (2 subjects) got half of the detection limit value (8).

Nasal lavage fluid

Nasal lavage fluid was collected by a modified version of the "nasal pool device" as described previously (9). In order to obtain immediate nasal lavage fluid, 10 ml of saline was inserted by means of a syringe connected to a commercial Foley catheter with the cuff adapted in the vestibulum nasi. The saline was recovered from

the nasal cavity after 10 minutes and the samples were then centrifuged to avoid cellular contamination. After the centrifugation samples were stored at -80°C and then analyzed within 2 months after collection. The eosinophilic Cationic Protein (ECP) was measured using a fluoroimmunoassay (Pharmacia ECP CAP System FEIA; Pharmacia AB, Uppsala, Sweden). The detection limit was 2 μg/l. As for 8-isoprostane in EBC samples below the detection limit (7 subjects) got a half detection limit value.

Nasal cytology

Nasal cytology was performed by anterior rhinoscopy. Scrapings of the nasal mucosa were collected from the middle portion of the inferior turbinate using a Rhino-Probe® (10). The samples were placed on a glass slide, fixed by air drying and then stained by the May-Grunwald Giemsa method (Carlo Erba®, Milan, Italy). This staining can identify and distinguish all cellular components of the nasal mucosa (including neutrophils, eosinophils, mast cells, lymphocytes), bacteria, spores, and fungal hyphae. The slides were observed under a Nikon E600 light microscope (Nikon, Canada); 50 microscopic fields were read at a magnification of 1,000×. Cell counts, bacterial and fungal analysis were carried out by a semi-quantitative grading, as proposed by Meltzer and Jalowayski (11), from grade 0 to 4.

Statistical analysis

Median values were compared by the Mann-Whitney U test and Kruskal Wallis test. The Spearmann's coefficient rho was used for correlations. The results were considered statistically significant when $p<0,05$. For the statistical analysis the software SPSS 12.0 has been used.

RESULTS

The median value of 8-isoprostane in EBC was 18.58 pg/ml (range 15.99-18.85 pg/ml) in patients who did not undergo immunotherapy, 49.38 pg/ml (range 6-106.9 pg/ml) in SCIT patients and 13.41 pg/ml (range 6-24.01 pg/ml) in SLIT subjects; these differences were not statistically significant (Fig. 1). The median value of ECP in nasal lavage (Fig. 2) was higher in non- treated subjects (27.3 mg/l; range 17.4-31.1 mg/l) than in those treated with SCIT (1 mg/l; range 1-7.4 mg/l)($p < 0,05$) or in those treated with SLIT (2.6 mg/l; range 1-3.1 mg/l), even if this last difference is not statistically significant.

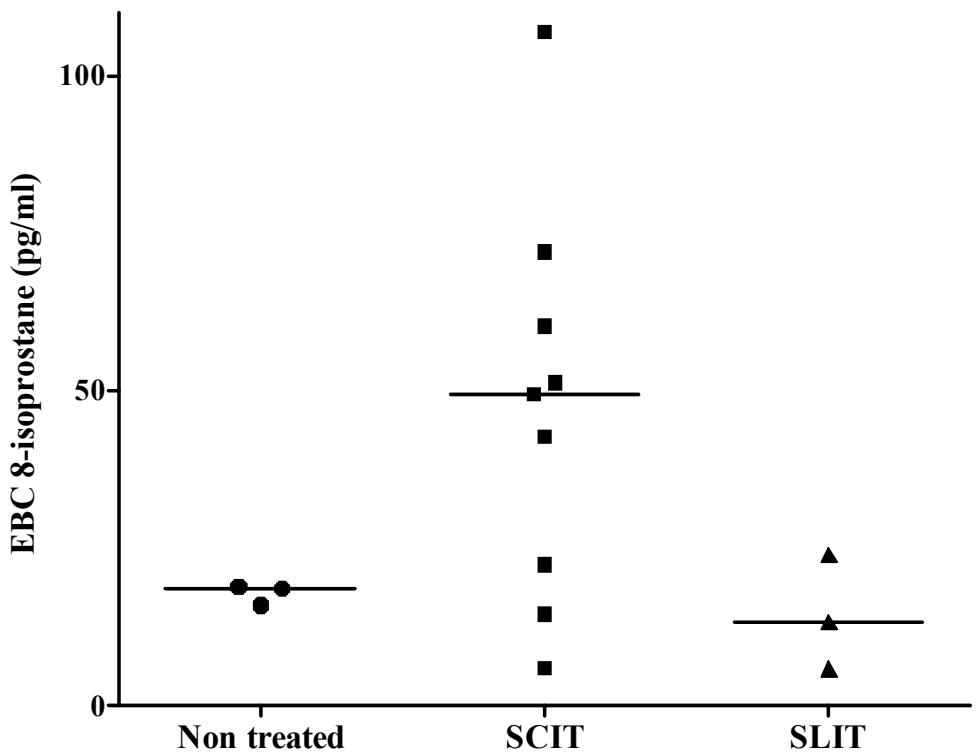

Fig. 1. *Concentration of 8-isoprostane in exhaled breath condensate of allergic patient non treated, treated with sub-cutaneous immunotherapy and sub-lingual immunotherapy.*

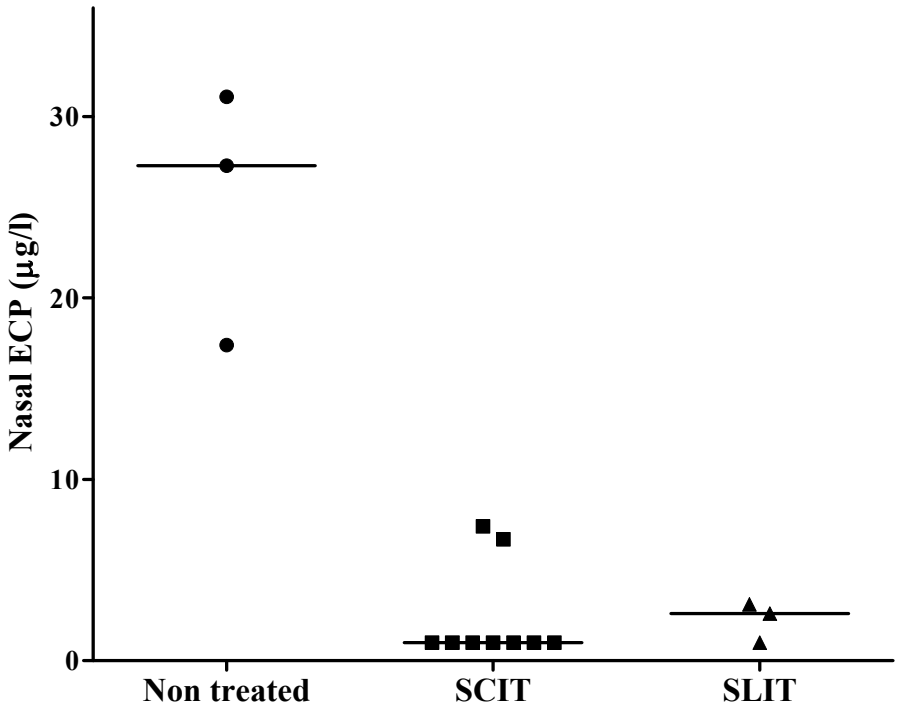

Fig. 2. *Concentration of ECP in nasal lavage of allergic patient non treated, treated with sub-cutaneous immunotherapy and sub-lingual immunotherapy. ($p<0.05$ for ECP values in treated patients in comparison with not treated).*

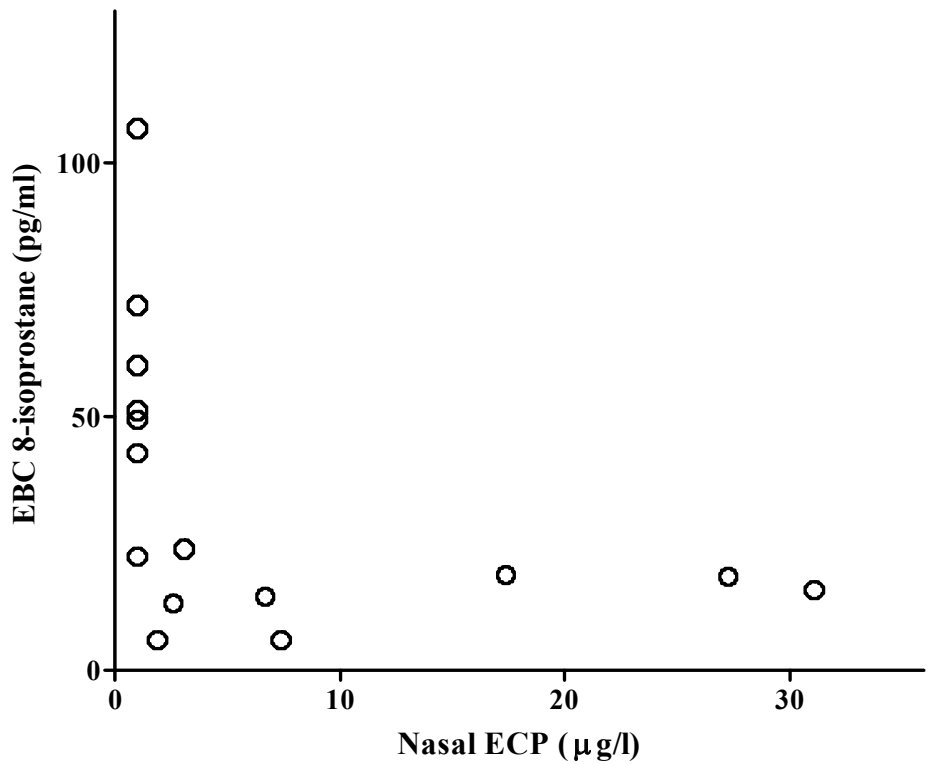

Fig. 3. *Correlation between 8-isoprostane in exhaled breath condensate and ECP in nasal lavage.*

All nasal cytology specimens were negative for inflammatory cells, and in particular no eosinophils were found in the groups after nasal cytology. In 4 samples there were few bacteria and in 2 samples epithelial cells showed signs of mild suffering. ECP in nasal lavage did not correlate with the concentration of 8 isoprostane in EBC (Fig. 3).

DISCUSSION

This study analyses for the first time the sub-clinical effects of the allergen immunotherapy combined with the exhaled breath condensate, nasal lavage and nasal cytology in a group of mono-sensitized to cypress pollens patients. 8-isoprostane in EBC was higher in subjects undergoing SCIT than in the other groups, even if the difference was not significant. Interest in cypress allergy is widely rising in Europe and Japan as a consequence of the diffusion of *cupressacee* as ornamental plants during the '80s and '90s and the subsequent increasing number of patients complaining cypress allergy related symptoms (1). One third of the scientific papers dealing with cypress allergy have been published in the last 6 years.

Although the availability of a standardized extract of *cupressacee* pollen is recent, several studies have been aimed to reduce symptoms, cutaneous reactivity and anti-allergic drug use (12, 13) through immunotherapy. Previous studies have assessed the immunological effects of immunotherapy. Glück et al. (14), found a decrease in $CD8^+$ T lymphocytes and their expression of IL-4 and IFNγ, after one year of treatment with SCIT, whereas Marcucci et al. (15) reported no significant change in eosinophils and mast cells in oral biopsies during SLIT.

Eight-isprostane is a marker of oxidative stress and thus indirectly of inflammation, besides it is increased in EBC of patients with allergic rhinitis during the pollen season (16) and after allergen challenge in asthmatics (17). The levels of 8-isoprostane found in the SCIT group were comparable to the concentrations obtained from parietaria judaica allergic patients out of the pollen season before starting SCIT and just finishing immunotherapy (18). Moreover similar concentrations of 8-isoprostane in EBC have been found in other models (16, 17). Hence we could speculate that the administration of SCIT could result into some increase of oxidative stress, even if not a significant one. Meaningfully, subjects undergoing SLIT had lower levels of 8-isoprostane, similar to those of not treated patients and close to the levels found in allergic patients out of the pollen season (16) and in healthy subjects (17).

Nasal lavage fluid is a suitable medium to find bio-markers of inflammation (19, 20). ECP has been measured in nasal lavage as a marker of allergic inflammation (21). In a previous study we found higher levels of ECP in nasal lavage of allergic subjects compared with healthy subjects, and the concentration decreased significantly after immunotherapy (22), suggesting a potential anti-inflammatory effect of SCIT and SLIT. Similar results were obtained in subjects allergic to dust mites treated with SLIT (23). In this study ECP concentrations in nasal lavage of patients treated with SCIT or SLIT were mostly below the detection limit, while the concentrations were higher in non-treated patients and similar to patient with allergic rhinitis in pollen season (24), although they were not exposed to cypress pollen and no inflammatory cells were found in nasal cytology.

In this study the low concentrations of ECP in patients treated with SCIT or SLIT confirmed that immunotherapy reduces the ECP levels in nasal lavage out of the pollen season and does not cause eosinophils' recruitment in the nose. Finally nasal cytology is a well established method to study nasal inflammation (19). As expected, nasal cytology was substantially negative, as previously suggested (25) since this study has been conducted out of the pollen season.

The main limitation of this study remains the small number of subjects. Another limitation is that there are not previous patients' randomization to SCIT, SLIT or none, so the different results might be due to different patients' characteristics. On the other hand, in order to have a sufficiently clean model, monosensitized subjects were mandatory, and unfortunately such patients are relatively rare. However several studies on 8-isoprostane in EBC are characterized by a small number of subjects (16), because of the high cost of the analysis, especially when sensitive methods, as radioimmunoassay, are used. a relevant number of samples were expected with ECP below the detection limit, since we were

assessing non-symptomatic no-smokers. Regrettably, the result of an allergic subject non-treated with high ECP level in nasal lavage is difficult to explain.

In conclusion this is the first study that evaluates several non-invasive tools to assess the inflammatory response related to SLIT and SCIT. Because of the small number of subjects under investigation, no final conclusion can be made. The high levels of 8-isoprostane in EBC among SCIT patients were unexpected and need to be further evaluated.

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