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"Development of health models in pediatric infectious diseases"

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Content

Introduction

The quality of health processes

The quality of the delivered pediatric health care is very variable, and about 50% of the indicated care is currently delivered in both chronic and acute diseases[1]. The health sector might be expected to be a finely tuned system, able to use corporate knowledge in a constant process of critically reviewing and improving its activities and processes.

A health system could be defined efficient when good measured health quality, perceived health quality and sustainability are achieved. This can be obtained conceptualizing the health system as a learning organization. A learning organization accesses for analytic purposes operational data, which become also the basis of understanding changes in both demand and delivery process[2].

The learning process can be synthesized with the Plan-Do-Study-Act dynamic model. This model supports the combined application of multiple strategies to reach objectives that are clearly stated and quantitatively monitored, through well-defined actions. The barriers that prevent a goal to be reached are identified, described, and resolved before new actions to overcome those barriers being put forward to reach the goals. This continuing process allows a progressive improvement of health care[3].

The role of research in this scenario is capital: basic and clinical research is essential to identify evidence-based practice, on the other hand methodological research applied to public health is the instrument through which the outcome are measured and used to adjust the health processes.

From research to health processes: the translational approach

The most popular vision of the translational research is that of a scientific research that facilitates the translation of findings from basic science to practical applications that enhance

human health and wellbeing. In this basic science-centered conception, two major areas have traditionally been identified: *a) from laboratory findings to clinical practice*, in other words the "bench to bedside and back" approach, and *b) to the community and back*, focusing at actively involving community practitioners in making research and at exploiting scientific results to improve public health[4]. More recently, the term has acquired a more comprehensive meaning, and "translational" has become a kind of research that facilitates the translation of the scientific evidence obtained at different levels of research into the clinical practice[5]. This is particularly true in pediatric research, as pediatric medicine has always had a strong identity of social and preventive medicine. In this perspective, many pediatric scientific societies have made the spreading of scientific knowledge to the community one of their statutory principles, and recognized it as a resource to translate research to the every day children's care, to implement evidence based clinical practice and ultimately to sharp high quality health policies in pediatric care.

Beside the aforementioned translational approach to optimize the existing research, a translational way of conceive and programming research is arising, that aims at promoting research suitable for translational application.

A scientific innovation can be very far from the clinical application when it is conceived and when its efficacy is shown in an experimental model. This distance depends – among the other factors – on the chosen model (*in vitro*, *ex vivo*, *in vivo*, human vs animal tissues and cells), on the risks associated to the technology, on the existing treatment for the studied disease and thus on the need for innovation. However, the transition from research to the health process imply efforts not only in newly produced evidence but even in the case of documents providing the standards of care such as metanalysis and clinical practice guidelines, where diverse barriers to the implementation have to be overcome to allow effective application. Even if rigorously constructed and well summarized in straightforward recommendations,

clinical practice guidelines remain often unapplied unless implementation interventions warrant spreading, tailoring and focused education at the local level. Implementation of clinical practice guidelines is probably the example of translational research with the largest measurable impact on target populations. In the experience of Albano et al. with the guidelines on the management of acute gastroenteritis in children, a brief implementation intervention to primary care pediatricians led to less violations to the guidelines and shorter duration of the diarrhea[6].

Regardless the scientific evidence to be "translated", a meticulous evaluation of what is needed to successfully reach the final target should be integral part of a scientific project.

Planning effective health processes in chronic and acute diseases

Chronic and acute diseases are very different settings to conceive quality-assuring health processes. Chronic diseases provide a the optimal model to design studies of quality improvement, for different reasons: high rate of scientific innovation for diagnosis and treatment, availability of databases, the variety of feasible interventions, the social and economic aspects of such diseases. Recently, Denson reviewed the bundle of translational studies providing new insights into pathogenesis, disease behavior, and treatment responses in pediatric Inflammatory Bowel Disease (IBD)[7].

The Pediatric Infecious Disease Unit of our Department is a reference center for children with HIV and tuberculosis. These two populations have represented in the recent years an important part of the health care demand in Campania region.

Pediatric HIV is a unique model to study the interaction between organic impairment, functioning, psychosocial disabilities and family structure. Children and adolescents living with HIV experience the most of their disability as related to areas related to access to social services, including school and health services or to social stigma, rather than to clinical issues.

In this context, there is a need of a process of care that takes into account the psychological and social functioning and the conventional approach based on the sequence pathogen (virus)-lesion (CD4+ decrease)-illness fails to provide a definite health status. The biopsychosocial model fits with this kind of disease.

Tuberculosis (TB) in children offers another example of the social implication of a disease. TB often occurs in contexts of poverty, immigration and low cultural background. This is a major problem in children depending on their parents to receive adequate treatment, where lack of language comprehension and economic problems could affect the disease outcomes. TB care is based on the application of protocols, due to diagnostic issues that makes it a probabilistic model, but TB health process should be tuned on local socio-epidemiologic factors. Health care professionals involved in TB care are largely unaware of this need.

Acute diseases are a good example of health models in which translational approach of quality improvement fits well, especially if the disease has a high socioeconomic burden, as in the cases of flu-like syndrome and acute gastroenteritis. In these conditions, the health processes are impaired by an overuse and a misuse of economic resources: such conditions as are often over treated and account for an inacceptable number of unnecessary hospitalizations with related costs. Approximately 45% of all the hospitalizations in children in Campania region are related to acute infectious diseases: according to estimates of the Italian Ministry of Health pooled with local data, inappropriate hospitalizations related to acute gastroenteritis and acute respiratory illness are expected to cost more than 10.000.000 € per year in this Region[9,10]. This trend is observed also in other European countries. Guidelines for acute gastroenteritis are available, but are scantily applied, and improving the health process would essentially mean to effectively implement existing evidence oriented at doing the least. Education through straightforward messages and Europe-wide interventions is needed to improve health policies and reduce costs in acute gastroenteritis.

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Aims

Aim of this thesis was to study the barriers to effective health processes in different domains and settings of Pediatric Infectious Disease medicine, and to design and apply interventions to improve health processes in this field. Namely, chronic and complicated disease, such as HIV infection and tuberculosis, as well as an acute common disease such as acute gastroenteritis were object of the different studies in the following chapters.

Results – 1

The biopsychosocial approach to complex/chronic infectious diseases

Pediatric HIV and tuberculosis are challenging conditions. Both conditions often occur in clusters with poverty, marginalization, discrimination, and immigration. There is a growing concern about these social and economic problems, and health care providers have to address these barriers to deliver high quality assistance.

A specific element of discomfort in pediatric HIV care is the unawareness of HIV status by vertically infected children, which should to be disclosed before the adolescence. This is a delicate process that should actively involve the parents/caregivers, requires psychological support and cannot be ignored by caring physicians. The process outcome affects in turn the disease perception, the adherence to the care plan, the overall disability. In this setting of care, we identified the need for parental support and addressed it through a family group psychotherapy intervention, as described in the 1.a session.

Similarly, pediatric tuberculosis occurs in families with social and economic disabilities. Although almost all the children with tuberculosis followed at our center are born in Italy, half of the cases are from foreign-origin families. In the 1.b session, we analyzed social and economic factors affecting tuberculosis management and outcomes, and found difference that should be taken into account when tailoring protocols to the everyday medicine.

1.a

Family group psychotherapy to support the disclosure of HIV status to children and adolescents

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INTRODUCTION

Positive mental health encompasses diverse aspects related with quality of life and general well-being, and can be considered as the achievement of emotional resilience[1]. Resilience is the psychological process developed in response to intense life stressors that facilitates healthy functioning, playing a major role in response to illness and other life adversities[2]. The needs of children living with HIV infection are progressively shifting from those strictly clinical to those related to psychosocial issues[3]. However, this is in contrast with the established model of care of HIV-infected children, which is characterised by a close physician-patient relationship. Such a model is largely the consequence of the social stigma with the need of hiding the disease. However, this often results in delayed disclosure to children and adolescent[4,5].

Our Unit has been providing care to children, adolescents and young adults with HIV infection since the onset of AIDS epidemics in agreement with the guidelines of the National Institutes of Health[6]. In parallel with the evolving aspects of HIV epidemics, we progressively developed a comprehensive approach integrating psychological, social and biomedical support to the management of HIV. This model provides in- and outpatient care by the medical staff of the reference center, and it also includes home care provided by physicians and nurses working in the hospital.

Using the International Classification of Functioning, Disability and Health (ICF), a specific instrument to evaluate the disabilities and dysfunctions in children, we showed that

environmental factors and psychosocial issues had a major negative impact on the quality of life of HIV infected children and their families[7]. Interestingly, the greatest impairment reported by parents was related to the need of hiding the HIV infection status to their children because of social stigma. This was associated with delayed disclosure[7], which in turn generated a vicious cycle of increased anxiety ultimately leading to further functional disabilities in HIV-infected children. Therefore, disclosure has a key role in coping with HIV infection. Current recommendations regarding the disclosure of HIV infection to children are based on lessons learned from pediatric oncology[8]. Similar to trends observed in oncology, many parents and care providers of HIV-infected children thought they need to protect children from emotional burdens and social prejudices associated with their disease. With the advent of new therapies in the mid 1990's and the dramatic improvements in the mortality and morbidity of HIV-infected children, changes in disclosure practices began to take place, and the disclosure of HIV infection is now a step of care[5]. Disclosure is not only related to psychosocial aspects and eventually resilience, rather it also affects therapy and its outcome.

In a recent qualitative study, caregivers reported that their children became more adherent to antiretroviral medications following disclosure[9]. In an interview involving 120 families in a resource-limited setting, disclosure was perceived as a step towards self-sufficiency, but also with potential negative social effects[10]. On the contrary, other studies have indicated that children aware of their HIV status may be less likely to adhere[11]. The conflicting relationship between disclosure and adherence may be explained by the age-related patterns of relationship between the child's age, the type of communication and the support to it. Children and adolescents frequently perceive limited communication before, during, and after disclosure, which is a discrete event rather than a process[12]. Once children are made aware of their HIV status, their carers expect them to become able to self-manage treatment, without supervision and reminding[13]. However, we previously showed that adherence was strongly

related to caregivers more than any other factor, such as child's age or disease status[12]. Disclosure is therefore an important step that has a broad effect on HIV infection course and needs to be accompanied by psychological support.

These issues led us to plan a family group psychotherapy (FGP) with the families of children with HIV infection, with the aim of removing barriers that prevent disclosure and to ultimately increase resilience in the caregivers. The basic concept was to work with small groups of caregivers and to discuss common problems and feelings in order to build competence and self-reliance in families and patients so that parents and their children could effectively manage their own health.

The intervention was incorporated in our HIV care planning. Specific objectives of the intervention were the following: to provide information for sound management of the disease; to offer an empathetic understanding of the problems and to discuss problems and solutions; to focus on HIV disclosure in a timely and appropriate manner, acknowledging the need for a close interaction between parents of children, the medical staff and the team of psychologists.

METHODS

Children with HIV and their families, seen at our HIV reference center for the management of pediatric HIV infection, were enrolled. Inclusion criteria were the following: age 1–16 years; diagnosis of vertical HIV infection obtained > 1 year before enrollment; unawareness of HIV infection status; therapy according to the criteria reported in the NIH guidelines for the Use of Antiretroviral Agents in Pediatric HIV Infection[6]. To quantitatively estimate adherence, each caregiver was asked how many doses of the total prescribed antiretroviral therapy had been omitted in the previous 4 days, and children were defined as non-adherent if they had taken less than 95% of all prescribed doses of antiretroviral therapy in this period. This method

provides a reliable estimate of adherence to antiretroviral therapy in children[14]. Patients and caregivers were randomly assigned to either FGP intervention or control group. Parents decided whether the mother, the father or the caregiver would participate in the support group. Written informed consent was obtained for every participant.

The intervention was structured through meetings between parents of children and the team of psychologists of the reference center. Eight 2-hour group sessions took place once a month. The first and last meetings were longer to allow test administration. The content of the meetings is described in the Appendix. Families in the control group received complete health assistance but no psychological support and they were not aware of the intervention.

The following tests were administered before and after the support group meetings:

- The Psychological General Well-Being Index (PGWB-I)[15] that assesses the psychological and mental health status of an individual through 22 items. The scale consists of 6 domains: anxiety, depression, sense of positive and well-being, self-control, general health and vitality. The overall score ranges from 0 to 110 (worst-best states possible). Scores 91-110 are consistent with high level of well-being; scores 71-90 with good level of well-being; scores 51-70 with medium level of well-being; scores 31-50 with low level of well-being; scores 0-30 with very low level of well-being[16]. Changes in psychological well-being index were defined according to these ranges.
- The Short-Form State-Trait Anxiety Inventory (Sf- STAI)[17] is a six-item version of the Spielberger STAI for the assessment of anxiety state[18]. It is a validated instrument for measuring anxiety in adults. It clearly distinguishes between a temporary condition of anxiety in a specific situation and anxiety as a general trait. The results of the scale range from 20 to 80. A score ≥ 60 indicates an elevated level of anxiety; a score between 50-59 indicates a moderate level of anxiety; a score ≤ 49 indicates absence of anxiety[17]. Changes in anxiety were

defined according to these ranges. A final self-assessment through rating was obtained after the last meeting.

Both PGWB-I and Sf-STAI have proven reliable in chronic conditions, with a high test/retest coefficient [19,20].

Results were expressed as number/percent or as mean ± SD. The Student t-test, the Wilcoxon matched-pairs signed rank test and the χ^2 method, or exact Fisher's test when appropriate, were performed to compare the variables and $p < .05$ was the cut-off for significance. Data were analyzed with the SPSS package version 20.

RESULTS

A total of 17 parents of as many children (mean age 11.7 ± 3,7 years, range, 4.2–18.0; 7 boys) were enrolled, 13 of them being biological and 4 foster parents. Ten children were single orphan, one was double orphan and entrusted to a caregiver; 10 children were of Italian origin and 7 were of non-EU origin. Ten caregivers were randomly assigned to support group intervention and the other 7 to controls. The corresponding children populations were age-matched (Table 1). The intervention group had a higher viral load, due to the presence of three patients with > 100 copies/ml. However, all patients had < 1000 viral copies/ml. Of note, viral load was reduced in two children following the intervention (in one patient the viral load was associated with poor compliance and in another with viral resistance).

Psychological Well-being

Baseline PGWB-I scores differed between FGP and controls (57.4 ± 15 vs 79.7 ± 7.3, $p = .04$). The average PGWB-I increased in the intervention group (T0: 57.4 ± 15; T1: 72.5 ± 21, $p = .22$) and decreased in controls (T0: 79.7 ± 7.3; T1: 48.1 ± 9, $p = .01$) (Fig 1, A). Psychological well-being improved in 70% of the caregivers in the FGP group vs none of the control group; it

decreased in 20% of FGP parents vs 71% of the controls (p = .01) and finally remained stable in 10% and 29% of the FGP and controls, respectively (Table 2). An association was observed between parental schooling and changes in parental well-being (p = .02) but not with other social and economic factors (Table 3).

Anxiety

The baseline Sf-STAI scores were not different between FGP and controls. The average Sf-STAI decreased in the intervention group (T0: 51.7 ± 4; T1: 43 ± 4, p = .22) while it increased in controls (T0: 40.6 ± 6; T1: 57. ± 3, p = .01) (Fig 1, B). Anxiety was significantly diminished in 60% of caregivers in the intervention group vs none of the controls (p = .03) (Table 2). Similarly to PGWB-I, changes in anxiety were also associated with parents' education (p = .01) (Table 3).

Disclosure of HIV infection to children and adherence to antiretroviral therapy

Disclosure took place in six of 10 (60%) of the group support caregivers within 12 months following the intervention. Comparatively only 1 of 7 (14%) of controls achieved this step (p = .59). However, disclosure was correlated neither with PGWB-I (p = .15) nor with Sf-STAI (p = .45), nor with any other social, cultural or economic features (Table 4).

Changes in adherence to HAART of children were not correlated to intervention (Table 2) nor to the disclosure of the HIV status to children by parents.

Assessment of the perceived benefit and efficacy of the intervention

A final self-assessment of the benefits perceived from the intervention was obtained after completing all sessions at the last group meeting: caregivers answered the question 'To what extent did the group help you understanding issues concerning HIV?', and the level was 7.1 ±

2 on a score 0-9 (not helpful-very helpful). A final self-assessment of the empathetic support received was provided by caregivers answering the questions 'How helpful was to meet other parents of children with HIV?' and 'To what extent did attending the group help you in feeling less isolated with regard to your child's HIV infection?'. On a score 0-9 (not helpful-very helpful), the perceived helpfulness by empathetic support was 9 ± 0 and 9 ± 0, respectively. Only one meeting was missed by two participants.

DISCUSSION

Our results show that an intervention based on group psychotherapy delivered to families of HIV-infected children improves the quality of life, increases their general well-being and reduces their anxiety state, thereby increasing their resilience. Our observations, even with the limitation of a wide age range of the patients and of the heterogeneity of the type of families, show that the support group effectively breaks the isolation and creates opportunities for carers, by sharing psychological resources and experiences. These results also depend on parents' education, as suggested by the significant association between carers' schooling and their psychological improvement in terms of both general well-being and anxiety. It is well known that the education strongly affects the capacity of living with chronic diseases and that supportive interventions are more effective in high culture settings. This is demonstrated for diseases such as diabetes or chronic obstructive pulmonary disease: positive effects on depression, health-related quality of life, feelings of mastery, and self-efficacy are confined to patients with high education, while those with only a primary education do not benefit from supportive interventions[21]. Our data obtained in a small population of families of low educational level indicate that interventions tailored to those specific conditions, with simple peer communication are effective and contribute to resilience.

Although there are limited data on psychological support groups in pediatric settings, interventions involving families of children with HIV are generally delivered with the aim of improving patients' care beyond strictly clinical aspects. However, the effects of interventions are rarely measured. We did that and also applied disclosure as a secondary outcome parameter of the intervention. The latter promoted the disclosure of HIV status from carers to children. However, the relationship between psychological support and disclosure process by the caregivers is complex, and this effect could be related to cognitive gain rather than to psychological effect. Moreover, not all the studies clearly showed an impact of the disclosure on quality of life, as well as the acceptance of disclosure appears to be higher in foster parents[22,23]. We did not find any association between disclosure and type of parents (biological vs foster), but this might be due to the heterogeneity of our populations. The group therapy fostered the information about HIV infection in carers, enabling them to disclose to their children a troublesome truth. Awareness is a prerequisite to self care and it is a fundamental leading force in the construction of personal future[24]. Current recommendations by the American Academy of Pediatrics support disclosure to children as young as 8 years of age and to all adolescents[25]. From this perspective, the support by the group provided important motivations to self-management and resilience.

Looking at clinical features, our population was largely made of children and adolescents with virologically and immunologically well-controlled disease, reflecting the high standards of clinical care in pediatric HIV. No association was found between either HIV viral load or number of CD4+ lymphocytes and primary or secondary outcomes of our intervention, and only three patients had detectable viral load at baseline.

Unawareness and psychological discomfort could affect adherence and hamper effective management of the disease, especially in adolescents, in whom the lack of responsibility prevents an effective therapeutic alliance. We previously described a changing pattern of

adherence over time in HIV-infected children and adolescents, and demonstrated that psychosocial features of caregivers and children play a major role in adherence. Interestingly, children of foster parents had the highest level of adherence and it may be expected that depressed and debilitated parents have limited ability in sustain optimal adherence in their HIV-infected children[14]. However, adherence is the result of a complex interplays of determinants and it is impossible to establish a direct link with psychotherapeutic interventions. In our sample, only 65% of all children showed an optimal adherence to HAART at baseline. We observed that adherence increased over the period of observation. However, this was also the likely consequence of the higher attention paid by physicians and other health care professionals to this outcome during the study.

In contrast with the physical well-being, psychological discomfort was high in the families enrolled, and the support group was well accepted and recognised as effective by families. The mutual empathetic support among the participants was perceived as very helpful. These interventions should be integral part of the standard care for HIV-infected children and adolescents. Psychosocial issues in young patients should be carefully considered to ensure clinical success[26]. Management of psychological impairment through a biopsychosocial model of care may reduce anxiety, depression and social isolation by lowering physical tension, increasing a sense of control and self-efficacy, ultimately increasing resilience in the parents of infected children[27]. The caregivers experienced a sense of relief and expressed positive feelings for being treated in a non-judgemental way. As previously described, the possibility to freely talk about their own emotions without being censored is a path of growth for HIV-positive subjects[28].

In conclusion, attention needs to be paid to both psychosocial and biomedical aspects of pediatric AIDS. It is important to involve caregivers and family members who are in close contact with the HIV-infected child. With little effort and easy to perform and sustainable

interventions, the bio-psycho-social state of HIV-infected children and their families may be substantially improved.

Appendix: psychotherapy sessions.

First meeting

A psychologist expert in HIV guided the meeting; a younger psychologist and a social worker were also actively involved. All the group members met in a friendly context. Parents were invited to introduce themselves and their families and share their expectations. The aims of the group support were presented, and the participants were asked to fill the standardized cognitive instruments. Parents reported their fears and feelings of isolation in relation to their and/or their children's illness. Some of the parents reported fear for their own disease. A major issue was related to the disclosure of the HIV status.

Second meeting

An overall increased acknowledgment of the pain and the distress related to the issues raised by parents was recorded and openly discussed. Most interventions focused on the sense of guilt for having vertically transmitted the infection, the fear of illness and death, the uncertainty about the future. Reasons supporting early disclosure to children were gently introduced.

Third meeting

The inner resources of the parents/carer and the family resources were explored under the guide of therapists. The barriers to disclosure were often related to the perceived misconception of the HIV infection in the 'outside world'. The fear of death was strongly rooted in those participants who already had experienced a death for HIV in the family and was contained by an element of life expressed by others. The risk of family break up by a member of the group was counterbalanced by someone else's confidence as well.

Fourth meeting

A conscious sense of guilt arose for the transmission of the infection, in association with the idea of having betrayed their task to be bearers of life rather than death.

Fifth meeting

In this session, the attendees were invited to take advantage from available social services. Particularly, a social worker provided information on local resources as well as financial resources to support HIV-infected subjects. The physicians of the medical Service joined the group and parents were able to directly and openly ask questions and make comments. The physician stressed the efficacy of HAART in fostering a full control of the disease, preventing HIV progression and allowing a full normal life, if optimal adherence to treatment was reached.

Sixth meeting

This session was named 'Not when, rather how' and addressed HIV disclosure, because therapists spoke about the scattered experiences and built a roleplaying to give simple explanations to young children or information about the nature and consequences of illness to older children.

Seventh meeting

In this session, the focus was on consolidation of earlier meetings in order to help the caregiver to find confidence and language competences to talk to the children

Eighth meeting

In the final session tests were administered to caregivers in order to obtain a feedback on empathetic support and cognitive advance.

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Table 1. Baseline psychological outcomes and socioeconomic features of patients and families of family group psychotherapy group (intervention) and control group.

		Intervention (n=10)	Controls (n=7)	*p*
Sex n (%)	F	5 (50)	5 (71)	.622
	M	5 (50)	2 (29)	
Family n (%)	Natural	7 (70)	6 (86)	.603
	Adoptive	3 (30)	1 (14)	
Age mean ± SD		12 ± 4	11 ± 2	.114
Orphanity n (%)	One	5 (50)	5 (71)	.784
	Both	1 (10)	0 (0)	
	None	4 (40)	2 (29)	
Origin n (%)	Italian	5 (50)	5 (71)	.622
	Extra EU	5 (50)	2 (29)	
CG education n (%)	Middle school or less	4 (40)	6 (86)	.134
	High school	5 (50)	1 (14)	
	Graduation	1 (10)	0 (0)	
Income n (%)	One	6 (60)	4 (57)	.59
	Both	0 (0)	1 (14)	
	None	4 (40)	2 (29)	
Mother transmission n (%)	Sex	3 (30)	5 (72)	.486
	Blood transfusion	2 (20)	0 (0)	
	Drug	2 (20)	1 (14)	
	Adoptive	3 (30)	1 (14)	
Adherence to HAART n (%)	Adherent	6 (60)	5 (71)	.627
	Non adherent	4 (40)	2 (29)	
CD4 lymphocytes/uL mean ± SD		795 ± 415	604 ± 233	.124
HIV RNA copies/ml mean ± SD		92 ±117	20 ± 0,7	.001

HAART, highly active antiretroviral therapy.

Table 2. Changes in psychological and clinical features after the family group psychotherapy.

		Intervention (n=10)	Controls (n=7)	*p*
CG PGWB-I n (%)	Improved	7 (70)	0 (0)	.017
	Stable	1 (10)	2 (29)	
	Worsened	2 (20)	5 (71)	
CG Sf-STAI n (%)	Improved	6 (60)	0 (0)	.037
	Stable	2 (20)	2 (29)	
	Worsened	2 (20)	5 (71)	
HIV Disclosure n (%)	Yes	6 (60)	1 (14)	.134
	No	4 (40)	6 (86)	
Change in adherence to HAART n (%)	Improved	3 (30)	2 (29)	.784
	Stable	5 (50)	5 (71)	
	Worsened	2 (20)	0	
Adherence to HAART T1 n (%)	Adherent	7 (60)	7 (71)	.228
	Non adherent	3 (40)	0 (29)	
CD4 lymphocytes/uL T1 mean ± SD		863 ± 379	665 ± 271	.677
HIV RNA copies/ml T1 mean ± SD		92 ± 150	20 ± 1	.06

CG, caregiver; PGWB-I, Psychological General Well-Being Index; Sf-STAI, Short form of State-Trait Anxiety Index; HAART, highly active antiretroviral therapy; T1, after the intervention.

Table 3. Change in psychological general well-being and anxiety of caregivers after the intervention according to parental schooling.

CG education (n)		Middle or less (10)	High (6)	Grad (1)	*p*
PGWB-I n (%)	Improved	2 (20)	4 (67)	1 (100)	.021
	Stable	1 (10)	2 (33)	0 (0)	
	Worsened	7 (70)	0 (0)	0 (0)	
Sf-STAI n (%)	Improved	2 (20)	4 (67)	0 (0)	.013
	Stable	1 (10)	2 (33)	1 (100)	
	Worsened	7 (70)	0 (0)	0 (0)	

CG, caregiver; PGWB-I, Psychological General Well-Being Index; Sf-STAI, Short form of State-Trait Anxiety Index.

Table 4. Rate of disclosure of HIV status to children by families according to psychological outcomes of the intervention.

HIV Disclosure (n)		Yes (7)	No (10)	*p*
PGWB-I n (%)	Improved	5 (71)	2 (20)	.156
	Stable	0 (0)	3 (30)	
	Worsened	2 (29)	5 (50)	
Sf-STAI n (%)	Improved	4 (57)	2 (20)	.456
	Stable	1 (14)	3 (30)	
	Worsened	2 (29)	5 (50)	

PGWB-I, Psychological General Well-Being Index; Sf-STAI, Short form of State-Trait Anxiety Index.

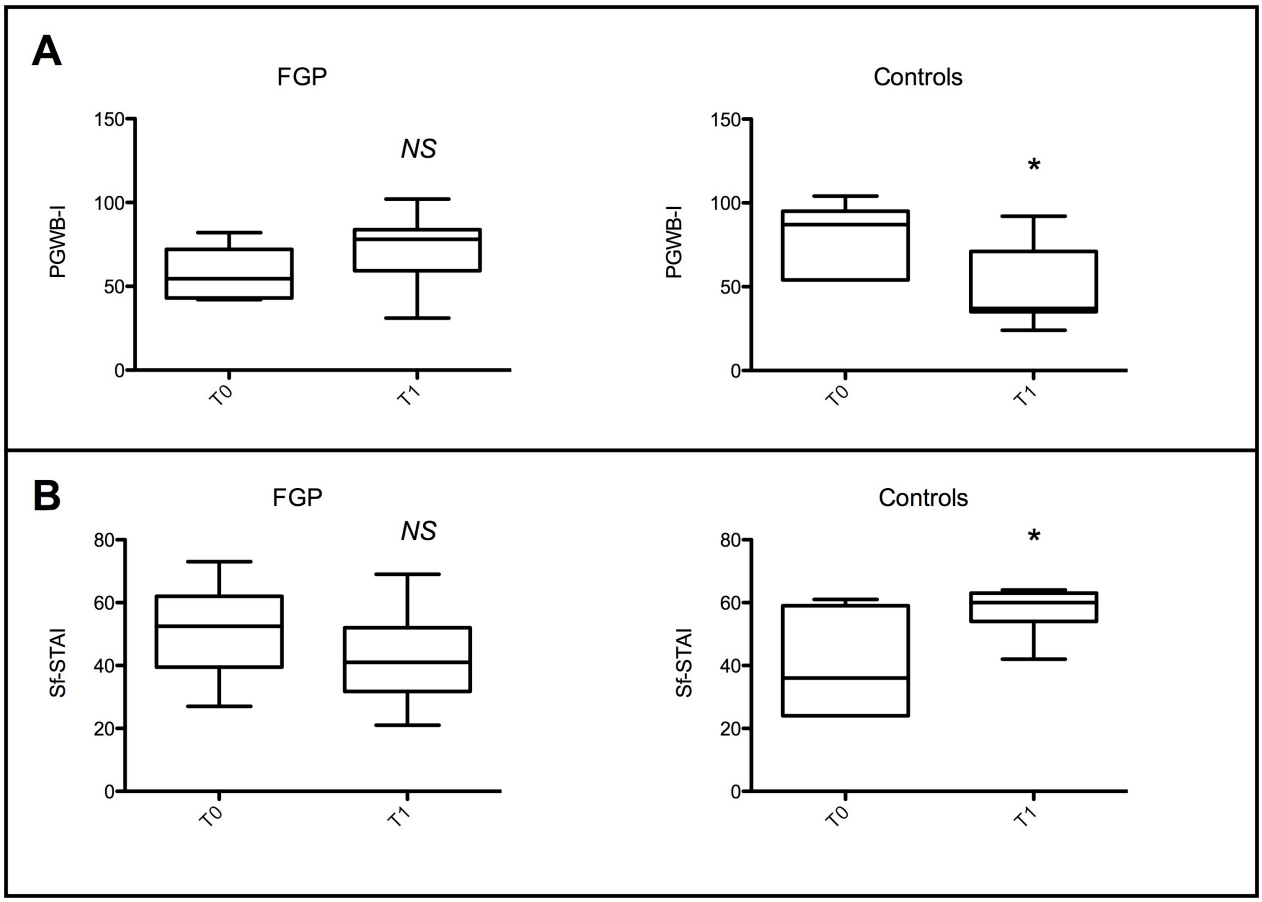

Figure 1. Psychological general well-being (A) and anxiety (B) scores before and after family group psychotherapy. FGP, family group psychotherapy; PGWB-I, Psychological General Well-Being Index; Sf-STAI, Short form of State-Trait Anxiety Index; *$p < .05$.

1.b

Drug resistance and socioeconomic factors affect the outcome of tuberculosis disease in children in Italy

Article in writing

INTRODUCTION

Tuberculosis (TB) is the leading curable cause of death worldwide with a disproportionate burden between low and high-income countries and settings. In 2012, 8.6 million subjects developed TB disease and 1.3 million died worldwide [1]. In Europe there is a long-term decreasing trend and 72,334 cases were reported in 2011 [2]. Childhood TB has a relatively low burden, with approximately 40,000 notified paediatric cases between 2000 and 2009 [3]. Similar to other low incidence countries, in Italy a slight increase in the incidence of TB has been observed in children and adolescents in the last 10 years [4]. Social and economic factors may play a major role in the disease. In developing countries, low income, ethnic factors and education have been associated with TB incidence [5 6]. In this setting, lower education and unemployment are associated with delayed access to care and poorer outcome in adults with TB [7-9]. In European countries, little is known on the social, economic and ecological factors that may affect the risk of infection and disease as well as the disease outcome, and even less is known about children and TB. Paediatric TB provides a reliable measure of the epidemiology and of local risk factors due to the shorter lag time between infection and the onset of clinical disease. In an ecological study in California, paediatric cases were selected and linked to census data, showing that census tracts with lower median incomes, higher ethnic heterogeneity, and more immigrants had higher rates of paediatric tuberculosis [10]. In low prevalence countries, TB is tightly linked to immigration: about 30% of children diagnosed with TB disease in the US are foreign-born, but two thirds of the US-born children

have at least one foreign-born relative [11]. The impact of familial socioeconomic profile is virtually unknown in this setting although it is well possible that it plays a role in affecting the course of a disease, which requires a long-term therapy and a close follow up. We comparatively studied the familial social and economic profile in immigrants and resident families in children with TB seen at a reference centre in Italy and investigated the relationship between these factors and disease management and outcome.

MATERIALS AND METHODS

We evaluated the clinical records of children and adolescents with TB disease referred at our tertiary care centre for paediatric infectious disease, between January 2009 and February 2013. The centre sees all children below 14 years old and most of those below 18 years old of Campania (approximate population 5 millions). Patients with comorbidities were excluded. The following social and economic data were collected: parental origin and education, family income and employment, number of households, presence of elderly or immunosuppressed households or households with chronic cough. A score from 1 (very poor) to 5 (excellent) was given to estimate knowledge of Italian or English language. The disease course was considered the primary outcome. According to the WHO definitions [1] and to previous studies [12 13] TB was defined complicated if culture positivity or clinical and/or radiologic findings of active TB were *i)* persistently present after 3 months of therapy or *ii)* relapsed within 6 months after the end of the treatment or *iii)* treatment was omitted for at least two months. We measured and considered as secondary outcomes of case management the following variables: length of hospital stay; missed (no show) medical visits scheduled for follow-up; adherence to anti-TB treatment, measured with the 4-question Morisky scale [14]. According to such scale, parents or adolescents were defined non adherent if they responded "yes" to at least one on the following questions in three repeated tests: 1) Do you ever forget to take your

medicine? 2) Are you careless at times about taking your medicine? 3) When you feel better do you sometimes stop taking your medicine? 4) Sometimes if you feel worse when you take the medicine, do you stop taking it?

Drug resistance was assessed by standard drug susceptibility tests.

The Student t-test, the χ^2 method, or exact Fisher's test were performed when appropriate for statistical analysis; $P < .05$ was the cut-off for significance. Data were analysed with the SPSS package version 20.

RESULTS

Sixty children and adolescents (30 boys; median age 4.2 years; range 0.2-17.8 years) with active disease were diagnosed in 5 years, 40% of which in the last year of observation. The paediatric TB incidence normalized for total admissions at our Paediatric Department showed a trend to increase, from 73.9 (2009) to 219/100,000 hospital referrals (2013) (Figure 1). Thirty-eight (63.3%) cases were symptomatic and the remaining children had a positive chest radiography. There was no difference in the outcome measures between symptomatic children and those diagnosed by radiology. Extrapulmonary manifestations were observed in 18 (30%) patients. In Table 1 the disease localization and symptoms reported are listed. Of the 33 patients in whom cultures were obtained, 60% yielded positive results.

Twenty-eight children (46.6%) had at least one foreign-born parent, although only 9 (15%) children were born in a foreign country. Considering that the foreign citizens account for only 2.8% of the total population in Campania [15], the observed incidence was about 24-fold higher in the foreign-origin than in the Italian-origin population. The geographical origin of children's families is reported in Table 2. Notably, 14 families were from Eastern Europe, 10 of which were from Romania, and 6 were of Roma ethnicity.

In table 3, the socioeconomic features of the children's families according to their origin are

shown. A comparative evaluation showed that 32 Italian and 28 foreign-origin families did not differ in parental education, family income and employment rates. However, language knowledge was significantly different, with half of foreign parents having a poor understanding of either Italian and English (*P* <.0001). Although the socioeconomic profile was similar, the outcome indicators in children were different in the two groups (Table 4). The total hospital stay was significantly longer in children of foreign origin than in those from Italian families (12±13.1 vs 5.1±6.5 days, *P* =.012), and the percentage of missed scheduled visits was also higher in foreign origin patients (15.7±16 vs 8.6±9.6, *P* =.042). Overall, six children (10%) had a complicated course, 5 of them being from foreign origin families; in 3 of them the reason was a relapse related to the presence of a previously unidentified MDR infection. In one child with poor compliance to treatment, chronic cough and weight loss persisted for more than three months after the treatment onset. Overall, drug resistant TB strains were detected in 6 (21.4%) of foreign origin children and in only 1 (3.1%) Italian child (OR: 8.45, 95CI: 0.95-75.3, *P* =.043). Of the 7 children harbouring resistant strains, 5 (83.3%) were from Eastern Europe (4 Roma children) and 4 of them were MDR-TB. The resistance pattern is shown in Table 5.

All children did eventually well. The overall proportion of children adherent to treatment was around 68% with no difference according to family origin. Non-adherent children and those harbouring resistant strains were more likely to have a complicated course [33.3% of non-adherent vs 4.9% of adherent (OR: 9.75; 95CI: 1.5-62.6, *P*=.019) and 42% of patients with resistant TB vs. 5.7% of patients with susceptible TB (OR:12.5; 95CI:1.9-83.3, *P*=.017)]. Multivariate analysis showed that harbouring resistant strains rather than the family origin was a risk factor for a complicated course [(OR: 8; 95CI: 1.1 -58.8; *P*=.039) vs (OR: 4; 95CI: 0.39-41.9; *P*=.24)]. Adherence was associated with mother education: 45% of children from mother with primary school education or less were non adherent in comparison with only

8.3% of those from mother with higher education (secondary school or more) (OR: 9, 95CI:0.97-83.6, *P* =.050). No correlation was found between family income or language skills and adherence to treatment, missed appointments, total hospital stay and any other outcomes. According to the local epidemiologic surveillance protocol, case tracking was routinely applied in all TB cases and yielded positive results in 24 (85.7%) and 18 (56.2%) of the immigrant and Italian families, respectively (*P* =.020).

DISCUSSION

The epidemiology of childhood tuberculosis has a different pattern in high and low incidence countries, but in the latter immigration plays a pivotal role [16]. Unlike the observed decrease in high incidence regions, paediatric notifications have increased between 2000 and 2009, by 6.1% for children below 1 year of age and 7.4% for those aged 1-4 years in low incidence countries, where about 30% of all cases are of foreign origin [3]. Our data indicate an even more stringent pattern, with a 79% increase in the incidence of cases/100,000 hospital referrals between 2009 and 2012 and almost half of children with active TB coming from families with at least 1 foreign parent. Prevention and control of TB is hampered in several European countries by the lack of specific measures to be applied in at risk population such as immigrants [11 17 18]. We looked for possible risk factors related to geographical origin and socioeconomic features. Except for language skills, the socioeconomic features of resident and immigrant population were similar, and confirmed a major role of deprivation as a general risk of TB. Although deprivation was similarly distributed in Italian and non-Italian families of affected children, a higher risk of mismanagement was detected in foreign-origin children. Our data indicate that children from foreign-origin families are more likely to harbour drug resistant strains and that drug resistance ultimately account for the complicated course of these cases. Recently, a multicentre study has shown that the risk of drug resistance is about 4

to 20 times higher in immigrants than in residents in Italy [19], a pattern similar to other low incidence settings [20 21]. Our data support this feature in children in Italy. Multidrug resistance was detected in 26% of foreign origin cases and children harbouring drug-resistant strains had a more severe course, as judged by their relapses and persisting symptoms and longer hospital stays. It is of note that in three out of seven drug resistant cases, culture was not primarily attempted and multidrug resistance was not recognised before a relapse occurred. This suggests the need for routine determination of drug susceptibility, which should be routinely obtained in children of foreign origin.

In addition to the higher incidence of drug resistance, foreign patients tended to miss scheduled medical visits. This may contribute to the poorer outcome of the disease in foreign children, although there is no direct proof of such a link. Low adherence to treatment is a risk factor equally distributed in Italian and non-Italian children, and it can be speculated that the disease was perceived as mild by the parents of many affected children. In the absence of drug resistance, the most frequent cause of poor response to treatment is non adherence to therapy, and education and counselling by nurses have proved to be more effective than counselling by physicians in improving adherence [22 23]. Health care professionals should be aware of the frequent intrafamilial pattern of transmission, especially in immigrants. The high rate of infected children detected through case tracking in foreign origin families reflects the importance of looking for source cases in this setting, allowing optimal disease control and prevention, translation of drug susceptibility information from the source case to the affected child and the opportunity of immunization.

In conclusion, in foreign origin children the incidence of TB is markedly increased, mismanagement occurs more frequently and the disease course is more complicated compared to indigenous population. This complicated course is linked in part to the high rate of multidrug resistant strains. On the other hand, in immigrant families there is a lower

efficacy of medical care as judged by the longer hospital stay and more missed visits as a consequence of social and cultural background, so that cultural and epidemiologic factors act synergistically in this frail population. Adequate medical and social protocols should be developed for at risk children to achieve a better control of TB. These should include a closer interaction between health care and social services, the presence of cultural-linguistic mediators, and the implementation of integrated strategies for effective management. This should include routine microbiological investigations with routine drug susceptibility tests, more frequent clinical controls with close monitoring of adherence to therapy, check of recovery and case tracking.

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Table 1.

Symptoms reported and localization of TB disease in 60 children.

Localization	**n (%)**	**Symptoms**	**n (%)**
Pulmonary	51 (85)	Fever	23 (38.3)
Lymph node	11 (18.3)	Chronic cough	20 (33.3)
Osteoarticular	3 (5)	Lymphadenopathy	14 (23.3)
CNS	2 (3.3)	Weight loss	6 (10)
Intestinal	2 (3.3)	Haemoptysis	3 (5)
Miliary	1 (1.6)	Night sweat	3 (5)
Cutaneous	1 (1.6)		
Laryngeal	1 (1.6)		

Table 2.

Region of origin of the families of 60 children and adolescents with TB disease.

Region of origin	**n (%)**
Italy	32 (53.3)
Eastern Europe *Romania* *Ukraine* *Russia* *Poland* *Bulgaria*	14 (23.3) *10 (16.6)* *1 (1.6)* *1 (1.6)* *1 (1.6)* *1 (1.6)*
Africa	8 (13.3)
Asia	4 (6.6)
South America	2 (3.2)

Table 3.

Characteristics of the families of 60 children with TB disease according to origin.

		Italian	Foreign origin	
Age at diagnosis (years, m±SD)		6.6±4.7	5.3±5.1	.315
Households (n, m±SD)		4.8±1.6	4.1±1.1	.059
Gender n (%)	M	17 (53.1)	13 (46.4)	.605
	F	15 (46.9)	15 (53.6)	
Hospital stay (days, m±SD)		5.1±6.5	12±13.1	.012
Missed scheduled visits (%, m±SD)		8.6±9.6	15.7±16.3	.042
Lag time from source identification (days, m±SD)		33.2±42	30.4±16	.849
Mother education n (%)	Primary or less	13 (56.5)	9 (60)	.832
	Secondary or more	10 (43.5)	6 (40)	
Father education n (%)	Primary or less	18 (75)	10 (66.6)	.718
	Secondary or more	6 (25)	5 (33.3)	
Employment n (%)	Yes	27 (84.3)	19 (67.8)	.220
	No	5 (15.7)	9 (32.2)	
Income n (%)	< 20.000 €/year	19 (79.2)	20 (87)	.477
	≥ 20.000 €/year	5 (2.8)	3 (13)	
Language skills n (%)	Adequate	32 (100)	14 (50)	<.0001
	Inadequate	0 (0)	14 (50)	

Table 4.

Impact of family origin on the outcomes of TB disease

	Foreign Origin	Italian	OR* (95CI)	*P*
Complicated disease course§	5 (17.9)	1 (3.1)	6.7 (0.7-61.7)	0.88
Presence of drug resistance	6 (21.4)	1 (3.1)	8.45 (0.95-75.3)	.043
Non adherent patients	6 (23.1)	6 (22.2)	1 (0.3-3.8)	.94
Positive case tracking n (%)	24 (85.7)	18 (56.2)	4.7 (1.3-16.6)	.013

* OR vs. Foreign origin

§ Culture positivity or clinical and/or radiologic findings of active TB persisted after 3 months of therapy or relapsed within 6 months after the end of the treatment, or treatment omitted for at least two months.

Table 5.

Features of the seven drug resistant TB cases.

Gender/age (years)	Country	TB site	Culture	rpbO PCR (RIF)	Resistance (patient)	Resistance (adult source)	Treatment	Outcome
F/5.6	Romania	CNS	-	+	-	INH, RIF, SPM, PZA	AMK, MFX, LIN, CS, EMB	Improved
M/12.2	Ivory Coast	Lung (cavitary)	+	-	INH	-	INH, RIF, PZA, EMB, PZA	Relapse, improved
F/1	Romania	Lung	+	+	INH, RIF	INH, RIF, SPM, PZA	INH, PZA, EMB, AMK, MFX	Relapse, improved
M/3.8	Romania	Lung	-	+	-	INH, RIF	INH, PZA, EMB, AMK, MFX	Improved
F/2	Peru	Lung	+	nd	INH, RIF, EMB	-	PZA, EMB, KAN, ETH, MFX, CS	Improved
M/1	Romania	Lung	-	+	-	INH, RIF, SPM, PZA	EMB, AMK, MFX, LIN	Relapse, improved
F/14	Pakistan	Lung	-	nd	-	RIF	INH, PZA, EMB, MFX	Improved

INH, isoniazid; RIF, rifampicin; EMB, ethambutol; SPM, streptomycin; PZA, pyrazinamide; AMK, amikacin; MFX, moxifloxacin; LIN, linezolid; CS, cycloserine; KAN, kanamycin.

Results – 2

Improving the health care performances through education: an e-learning program for acute gastroenteritis

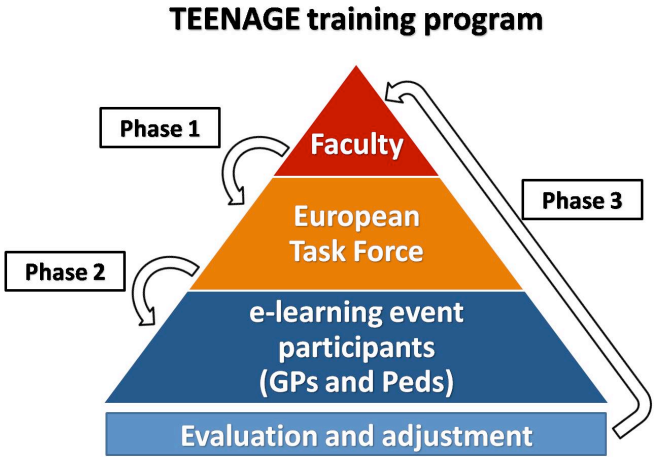
Acute gastroenteritis is a public health problem worldwide. The excess of costs related to unnecessary interventions and hospitalizations in Western countries are opposed to an inacceptable mortality in developing areas. Educational programs have been launched, but a permanent and universally accessible resource has not been realized.

The European Society for Pediatric Gastroenterology Hepatology and Nutrition (ESPGHAN), in partnership with the United European Gastroenterology (UEG), has led an initiative aimed to test e-learning for the quality improvement in the management of acute gastroenteritis in children.

With this initiative, we had the opportunity to apply the Plan-Do-Study-Act methodology to the field of medical education also exploring the power of information technology. This project, called TEEN-AGE (Tutorial European Electronic Network on Acute Gastroenteritis), has eventually stimulated the development of a wider editorial initiative by ESPGHAN.

Constructing an educational event for the implementation of health processes in acute gastroenteritis: the TEEN-AGE project

The Tutorial European Electronic Network on Acute Gastroenteritis (TEEN-AGE) program aims at providing a model for e-learning-based dissemination and implementation of evidence-based Clinical Practice Guideline (CPGs) in Europe, using the ESPGHAN/ESPID CPGs for acute gastroenteritis (AGE). Although the quality of CPGs is increasing, their implementation in clinical practice is often impaired by local factors. Traditional implementation methods (publications, courses, etc.) are of difficult access to practitioners from rural or developing European regions. E-learning can effectively disseminate and implement CPGs. In fact, it can be tailored to learner-specific needs, participants have flexible access to training but also receive immediate feedback on performance and it removes access barriers for practitioners in less advantaged, remote and rural areas. Implementation very much depends on local practice patterns (cultural and legal standards, organizational limitations) that may affect CPGs applicability. In addition, implementation of CPGs requires a multifaceted process that includes characterization of the target audience, tailoring and dissemination. Our program consisted in a 2-phase training intervention: 1) a workshop to train a European task force on innovative e-learning strategies for CPGs implementation in pediatric gastroenterology; 2) the e-learning implementation initiative targeted to a wider audience.



The Workshop entitled "**Online Strategies for the Implementation of European CPGs in Pediatric Gastroenterology**", held in Naples on 17-18 September 2012, was the first step of the TEEN-AGE project.

The workshop, held in Naples, Italy, was aimed at presenting the features of the TEEN-AGE project to representatives (Pediatric Gastroenterologists) from 15 European countries that will act as local tutors. The topics were:

- ✓ training in guidelines implementation and e-learning methodology
- ✓ contents of the *e-learning intervention* that will be offered in each country
- ✓ target of the intervention and modes of enrollment
- ✓ measuring the outcomes of the intervention

Faculty

- Alfredo Guarino
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  Coordinator of the project
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- Andrea Lo Vecchio
  PhD student, University of Naples Federico II, Naples, Italy

1. Attendees

Country	*Attendee*	*Affiliation*
Germany	Christine Prell	University of Munich
Portugal	Marta Tavares	University of Porto
Scandinavian Area	Malin Ryd-Rinder	Stockholm South General Hospital
Slovenia	Jernej Dolinsek	University of Maribor
Netherlands	Merit Tabbers	University of Amsterdam
Poland	Chmielewska	University of Warsaw
Russia	Elena Doroshina	University of Moscow
Belgium	De Bruyn	University Hospital of Brussels
Greece	Alexandra Papadopoulou	University of Athens
UK	Mohamed Mutalib	University College of London
Turkey	Pinar Urenden	University of Istanbul
Romania	Tudor Pop	University of Cluj

The content organization of the e-learning event was established: 1) Introduction, definition and epidemiology; 2) Assessment of dehydration; 3) Indications to medical visit and hospitalization; 4) Lab investigations; 5) Nutritional interventions; 6) Oral rehydration; 7) Active treatment (probiotics and antidiarrheal drugs); 8) Indications to antibiotics; 9) Antiemetics; 10) Appendix: treatment for inpatients.

Participants to the workshop were introduced to the procedures of enrollment of the study population (i.e., the participants to the e-learning course). The participants (final users) enrolled in the project are called “teenagers”, from the acronym of the project. The “teenagers” are physicians (both pediatricians and general practitioners) working with in- and out- patients who are expected to take the e-learning course and to undergo a pre- and post- course evaluation.

The outcomes of the intervention were identified:

a. *Knowledge outcomes*

Pre- and post- e-learning course multiple-choice questionnaires have been tested by the tutors. The modifications were introduced in both the questionnaires and the course structure.

b. Clinical practice outcomes

A database for data entering clinical cases before (baseline) and after the e-learning course cases was provided to each tutor. Two slightly different databases have been realized, for inpatients and outpatients. Inpatients are defined as patients managed in a hospital, no matter if emergency room, emergency department or regularly admitted patients.

E-learning is effective in implementing guidelines for acute gastroenteritis in European children and improves knowledge and clinical practice

Article in submission

INTRODUCTION

Clinical practice guidelines (CPG) are systematically developed statements that assist healthcare practitioners in making decisions about appropriate care for specific diseases based on evidence[1 2]. The overall quality of CPG is increasing and their development, quality control and evaluation protocols are well established[3 4]. However, a major limit of guidelines is their implementation[4]. Integration of recommendations into clinical practice is a complex and largely unknown process, and there is a growing interest to design effective implementation strategies. The latter include multiple approaches such as dissemination, outreach visits, audits, mass-media strategies, interactive meetings, financial incentives[5]. However, traditional implementation methods may fail because they are cumbersome, time-consuming and expensive, especially to the practitioner[6 7]. In addition, there is no information on their impact on practice, since only the impact on knowledge is usually evaluated. In a previous study, the administration of a course to physicians improved adherence to guidelines and resulted in improved outcome of children with acute gastroenteritis (AGE)[8]. AGE is a major problem in paediatrics. CPG for AGE are available, but poorly applied and most children receive unnecessary interventions[9 10]. This leads to an excess of referrals, hospitalizations and drug prescriptions, ultimately resulting in high costs, with little benefits for patients[11]. Low adherence to recommendations for AGE has been reported both in developed and developing countries[12 13]. Compliance with the recommendations for AGE may improve children's clinical outcomes and reduce complications and costs[8].

E-learning is being explored as a tool for education in medical science with promising results. E-learning was effective in improving paediatric prescribing skills of junior doctors, and outcomes were maintained over a 3-month period[14]. Residents, registrars and nurses receiving an e-learning program on paediatric cardiopulmonary resuscitation achieved a significant improvement in basic and advanced life support techniques[15]. However, the potential use of technology in medical education and transfer of knowledge to practice is not fully exploited and the impact on patient outcomes following e-learning courses needs to be investigated[16].

AGE is an ideal candidate condition for CPG implementation through e-learning, due to its huge burden and broad interest as well as a recognized target for implementation. Recently, the Federation of the Societies of Paediatric Gastroenterology Hepatology and Nutrition (FISPGHAN) indicated e-learning programs as an educational priority that should be exploited to decrease AGE-related mortality worldwide[17].

We aimed at assessing the impact of an e-learning course on the management of AGE in children of Europe based on the CPG jointly produced by the European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN)/European Society for Paediatric Infectious Diseases (ESPID)[18] on the knowledge as well as on clinical practice of European paediatricians and general practitioners.

METHODS

The study was approved by the Education Committee of ESPGHAN and conducted with the technical partnership of the United European Gastroenterology Federation (UEG) as part of the Tutorial European Electronic Network on Acute Gastroenteritis (TEEN-AGE) initiative. Physicians from 11 European countries were randomly enrolled as described below, and requested to sign a written informed consent. Each participant, after registered into the UEG

website, received a personal voucher to access the section where the study version of the e-learning course was hosted[19] and clinical data of enrolled patients were loaded.

E-learning course design and production

The e-learning course included 5 learning modules addressing the 5 key areas of AGE management based on ESPGHAN/ESPID guidelines. These were: 1) Introduction and definitions; 2) Clinical assessment and management; 3) Oral rehydration and active treatment; 4) Other treatments; 5) Appendix: treatment of inpatients. All the learning material (video, slides, evaluation questionnaires, figures, web references, checklists) was reviewed by the Scientific Committee of the TEEN-AGE initiative for content and format and approved for the final version. The course is freely available and only requires physician's registration.

Study design

We designed a pre/post single-arm intervention study. Forty hundred and fifteen paediatricians from 11 European countries were directly invited to participate in the study. They were identified either through regional/national databases or through national scientific societies, hence this was considered a randomly enrolled sample from each country. All participant physicians were asked to provide personal data (age, country, spoken languages, previous experience with e-learning) and other information about their practice (specialty, years of activity, inpatient/outpatient work setting). Each physician had a baseline and a post-course questionnaire measuring his/her knowledge, which included questions from a large pool of calibrated items on AGE (epidemiology, clinical assessment, treatment, use of antidiarrheal agents, antiemetics, antibiotics, use of laboratory tests). In addition, each physician reported his/her decisions on management of 3 to 5 consecutive children of <5

years of age referred to his/her inpatient or outpatient practice because of AGE. AGE was defined as a decrease in the consistency of stools (loose or liquid) and/or an increase in the frequency of evacuations (>3 in 24 hours) with or without fever or vomiting[18]. Clinical cases were recorded at the end of the visit or at the time of discharge in case of inpatients by each learner and loaded into an anonymous electronic Case Report Form (CRF). The CRF included 5 domains: child and family data, clinical features, home management, reasons for admission and hospital management. In addition, the presence of underlying chronic conditions and/or concomitant acute illnesses was recorded in the CRF to provide a correct interpretation of the outcomes according to case-specific risk factors.

Once completed the baseline phase, the physicians had one month-period to take the 5 learning modules course. They were subsequently invited to load information on 3 to 5 consecutive cases of AGE using the same CRF. Finally they completed the post-course test of knowledge.

Definition of inappropriate interventions

Inappropriate interventions in the management of AGE were identified by comparing the reported clinical data or medical interventions, including prescriptions and procedures applied, with the CPG recommendations, for each of the following domains:

1. Evaluation of the main signs/symptoms to assess dehydration (Did the physician report the capillary refill time, skin turgor, respiratory pattern, etc.?)
2. Concordance between the objective assessment of dehydration and the physician's estimate (Was the physician able to adequately assess the reported signs?)
3. Nutritional interventions (e.g., withdrawal/changes in milk formula or in any feeding)
4. Prescription of blood tests (other than electrolytes)
5. Rehydration route (e.g., oral, nasogastric or intravenous)

6. Prescription of microbiological investigations

7. Prescription of probiotics (indications and strains)

8. Prescription of antiemetics (indications and drugs)

9. Prescription of antibiotics (indications and drugs)

10. Prescription of other anti-diarrheal drugs

Inappropriate interventions were divided into major and minor violations. A major violation was defined as: *a)* an active medical intervention not included in CPG recommendations that might negatively affect the course of the disease and/or might be associated with unnecessary costs, or *b)* any violation to "high grade" recommendations in the guidelines (recommendations supported by strength of evidence I and II according to the Muir-Gray score in the CPG). A minor violation was defined as: *a)* an intervention that did not substantially change the outcome but was generally considered inappropriate, or *b)* any violation to "low grade" recommendations in the referral guidelines (strength of evidence III, IV and V according to Muir-Gray).

To obtain a quantitative estimate of adherence to the AGE CPG in our model, any major violation reduced the overall adherence by 10% and any minor violation by 5%; the final score (percentage) was calculated by the sum of the results reported for each domain, with an ideal maximum of 100%. Scores >90% were considered full adherence .

Primary outcomes of the e-learning intervention were the proportion of participants fully adherent to guidelines based on their medical interventions and their performing scores to the knowledge questionnaires (number of correct answers on 15 total questions).evaluation of knowledge also included the time to complete the test as recorded in the e-learning platform as an indirect proof of improved knowledge.

Statistical analysis

Statistical analyses were performed with the statistical computing environment R (version 3.0.1; R Foundation for Statistical Computing, Vienna, Austria). Data for continuous variables were expressed as means ± standard deviation. Data for categorical variables were presented as frequencies and percentages.

In univariate analysis, the differences in the theoretical knowledge of CPG recommendations and in the average adherence score, before and after the course, were evaluated using the Wilcoxon signed-rank test for paired samples.

To examine the impact on adherence of factors relating to either physician's and patient's level, a two-level random intercept multilevel logistic regression analysis (MLRA) was used to properly account for clustering of AGE cases among physicians. MLRA was applied separately on the pre-education group (PreEG) and post-education group (PostEG) data in order to investigate whether factors associated to non-adherence to CPG prior to the e-learning course also led to inappropriate interventions after the course. Adjusted odds ratio and corresponding 95% confidence intervals were obtained with the MLR method.

All tests were two-tailed, and *P* values <0.05 were considered significant.

RESULTS

One hundred and forty-nine physicians signed up to the e-learning course and were considered enrolled. Ninety (60%) were excluded because they did not complete all the modules within the established deadline. Fifty-nine (40%) of the enrolled physicians (45 females, median age 40 years, range 26-59) completed the course and were considered for further analysis and their baseline characteristics are shown in Table 1. Twenty-seven of 59 participants (46%) had less than 10 years of activity. They were from Slovenia (12), Greece (11), Netherlands (9), Portugal, Romania, Russia (5 each), Turkey, Italy (3 each), Belgium and Germany (2 each). Among the participants, there were 39 physicians (66%) working in

hospital setting and 20 (34%) in outpatient clinic. No difference in age, gender, years and setting of activity, previous experience with e-learning and previous knowledge of CPG was observed between the physicians who completed the course and those who did not.

Data of 545 children with AGE (342 females; median age 21 months, range 1-62) were collected by the participants, 281 before (PreEG, 51%) and 264 after taking the course (PostEG, 49%). Three hundred and forty-three patients (65%) were managed in hospital and 192 (35%) in outpatient setting. The presence of an underlying chronic disease or a concomitant acute illness was reported in 45 (8%) and 67 (12%) cases, respectively. A total of 25 out of 545 children (5%) presented with severe dehydration according to physician's estimate (Table 2). There was no significant difference in clinical features between PreEG and PostEG at onset of AGE, with the exception of stool output (p=0.016).

Knowledge about the CPG on AGE increased after the e-learning course as judged by the 15-question knowledge test scores before (8.6±2.7 points) and after the course (12.8±2.1 points, p<0.001). The response time also decreased after the course (579±379 vs 878±503 seconds, p<0.001) in the 59 enrolled physicians (Figure 1).

The proportion of patients managed in full adherence (no inappropriate interventions or only one minor violation) increased from 33.6±31.7% to 43.9±36.1% (p=0.037). Similarly, the average adherence increased from 87.0±7.7% to 90.6±7.1% (p=0.001) (Figure 2).

The mean proportion of patients managed with inappropriate interventions in each domain was calculated. The most common violations to the CPG were the prescription of stool cultures in the absence of bloody diarrhoea and/or underlying chronic diseases, dietary changes and inconsistent estimate of dehydration compared with objective parameters. As shown in Figure 3, the e-learning course reduced inappropriate interventions in all the domains. Hospital admission was not appropriate in 22% of the PreEG and 15% of the PostEG (p=0.2). Indeed, the proportion of hospitalized children with >5% weight gain at discharge

was only 25% among the PreEG and 26.5% among the PostEG (p=0.841), indicating a low proportion of children with AGE with at least moderate degree of dehydration in hospitals, and this data did not change after the e-learning course.

The MLRA model is shown in Table 3. With such model, we assessed the link between specific factors related to physicians or, respectively, to clinical features of children, and the discrepancies with the guidelines recommendations. We also investigated whether these factors were still associated with inappropriate interventions after the course. Physicians who had a previous knowledge of the guidelines were more likely to be adherent with the CPG [OR=0.29 (95%CI=0.1-0.86), p=0.026]. However the e-learning course filled the gap between those who already know the CPG and those who did not [OR=1.92 (95%CI=0.58-6.37), p=0.289]. Coming to the patients' features, children in the PreEG with bloody diarrhea [OR=5.75 (95%CI=1.39-23.89), p=0.016] and abdominal pain [OR=1.88 (95%CI=1.1-3.24), p=0.02] were more likely to receive inappropriate interventions, but this risk was abolished after the course [OR=1.9 (95%CI=0.46-7.84), p=0.37 and OR=0.61 (95%CI=0.25-1.49), p=0.279, respectively, in the PostEG]. Instead, frequent vomiting episodes (>5/day) remained associated with inappropriate management either before [OR=4.07 (95%CI=1.39-11.89), p=0.01] and after the course [OR=5.22 (95%CI=1.64-16.69), p=0.005]. Chronic diseases were a protective factor against non-adherence only in the PreEG group [OR=0.24 (95%CI=0.07-0.86), p=0.028].

DISCUSSION

E-learning is a logical strategy to improve practice, due to its universal availability, asynchronous accessibility, interactivity, presence of implementation tools (such as checklists, web references, etc.), and the low costs for the learner[20 21]. However, there is no proof of its efficacy in improving clinical practice. Many studies used surrogate outcomes to

predict such changes, such as drug and test prescriptions[14], simulations of resuscitation procedures[15] and structured clinical examination tests[22].

We investigated the efficacy of an e-learning educational intervention on AGE on knowledge and also its direct impact on clinical practice. The e-learning course increased the theoretical knowledge about appropriate management as judged by the questionnaire. This was supported by the rates of correct answers but also by the reduced time to fill the questionnaire. Translated into clinical practice, better knowledge is supposed to reduce the "time to effective interventions", an added value in the physician's daily practice.

The overall adherence to CPG significantly improved, as judged by both average adherence of medical interventions and the mean proportion of children managed according to the recommendations. However, we not only investigated the specific gaps and the discrepancies with the application of recommendations, but also evaluated their determinants through a specifically developed logistic regression model and examined the role of both physicians' and patients' features. Logistic regression showed that the e-learning course filled the gap between physicians who had a previous knowledge of CPG and those who did not. According to this model, the presence of abdominal pain and bloody diarrhoea were major determinants of non-adherence before the e-learning intervention, but this changed after the course, indicating that the intervention reduced mismanagement triggered by clinically alarming signs. Before the intervention, but not after, noncompliance was less common in children with chronic diseases. This can be an indirect measure of efficacy, because unnecessary blood tests and stool cultures, which are indiscriminately performed in otherwise healthy children, are recommended in children with chronic conditions.

We investigated the possible different domains of inappropriate interventions and measured the effectiveness of the medical practice in specific areas. Notably, when comparing the PreEG and the PostEG, a decrease of the violations was observed in all the domains. It is to note that

the discrepancies observed consisted in were an excess of medical interventions, such as unnecessary drug prescriptions and requests for tests non needed.

E-learning education was highly effective in reducing inappropriate requests for microbiological investigations, which were highly prescribed in hospitalized patients. The e-learning course also reduced dietary changes, improved the estimate of the dehydration degree, decreased the use of not recommended probiotics and the inappropriate use of antiemetics and antibiotics.

A major exception with the successful impact of e-learning is that the presence of frequent vomiting in children with AGE remained unchanged as a cause of unnecessary interventions after the cause. This mismanagement of vomiting originated by the prescription of antiemetics in children with AGE, which is not recommended in CPG. Antiemetics were not recommended in the 2008 AGE guidelines, but a matter of debate and selected guidelines including ondansetron suggesting their possible role in paediatric emergency departments to reduce the number of admission. [23-26].

We found that a decreased urine output was a risk factor for discrepancy in the estimate of dehydration, probably due to overestimation. This finding probably represents a bias, because urine output was not included in the parameters considered by the validated scoring systems (such as clinical dehydration scale or CDS and Gorelick score)[27 28] but it still is an important sign of dehydration. On the contrary, trained physicians were more likely to correctly rehydrate a child in the presence of reduced urine output, probably due to a better awareness of the importance of this hallmark of dehydration

This study has some limitations, such as the scattered country distribution of participating physicians and the fact that we did not consider in- and outpatients separately. However this is the first study assessing the ultimate impact on practice of e-learning education in paediatrics and provides the first demonstration that this tool is effective not only in

improving knowledge about CPG, but also in increasing the consistency of clinical interventions with those recommended. Although e-learning demonstrated to be a "smart tool" for education in medicine, the acceptance of this approach is still poor, as suggested by the high drop-out of the enrolled trainees. It should be noted however that the limited time to complete the course and the initial request of enrolling cases were the major causes of dropout, but were related to the trial. As in other areas of information technology and web-based education, large scale and targeted information is needed to attract interest by the desired audience. These successful results have led ESPGHAN, in close collaboration with UEG to implement its e-learning program and to use e-learning for education and training at European level and to set up a dedicated editorial project to shape specific competences in delivering high quality and widespread education in paediatric gastroenterology, hepatology and nutrition.

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Table 1. Baseline characteristics of the enrolled European physicians.

M/F	n/N (%)	14/45 (24)
Age (years)	mean±SD	39.5±7.57
Years of activity n/N (%) - < 10 yr - ≥ 10	n/N (%)	 27/59 (46) 32/59 (54)
Setting - Inpatient - Outpatient	n/N (%)	 39/59 (66) 20/59 (34)
Previous experience with e-learning. n/N (%)	n/N (%)	32/59 (54)
Previous knowledge of guidelines	n/N (%)	34/59 (57)

Table 2. Characteristics of the 545 children enrolled with AGE.

		PreEG	PostEG	*p*
Total	N	281	264	
M/F		152/129	144/120	1
Mean age±SD (months)	mean±SD	23.04±15.46	23.38±16.24	0.98
Weight-for-age SD-scores (mean±SD)	mean±SD	0.03±0.9	0.029±0.092	1
Children managed as inpatient	n/N (%)	184/281 (65)	164/264 (62)	0.423
Children managed as outpatients	n/N (%)	97/281 (35)	100/264 (38)	0.423
Chronic underlying disease	n/N (%)	20/281 (7)	25/264 (9)	0.352
Concomitant acute illness	n/N (%)	35/281 (12)	32/264 (12)	0.054
ORS at home	n/N (%)	128/281 (46)	148/264 (56)	0.0164
Children with severe dehydration	n/N (%)	13/281 (5)	12/264(5)	0.286

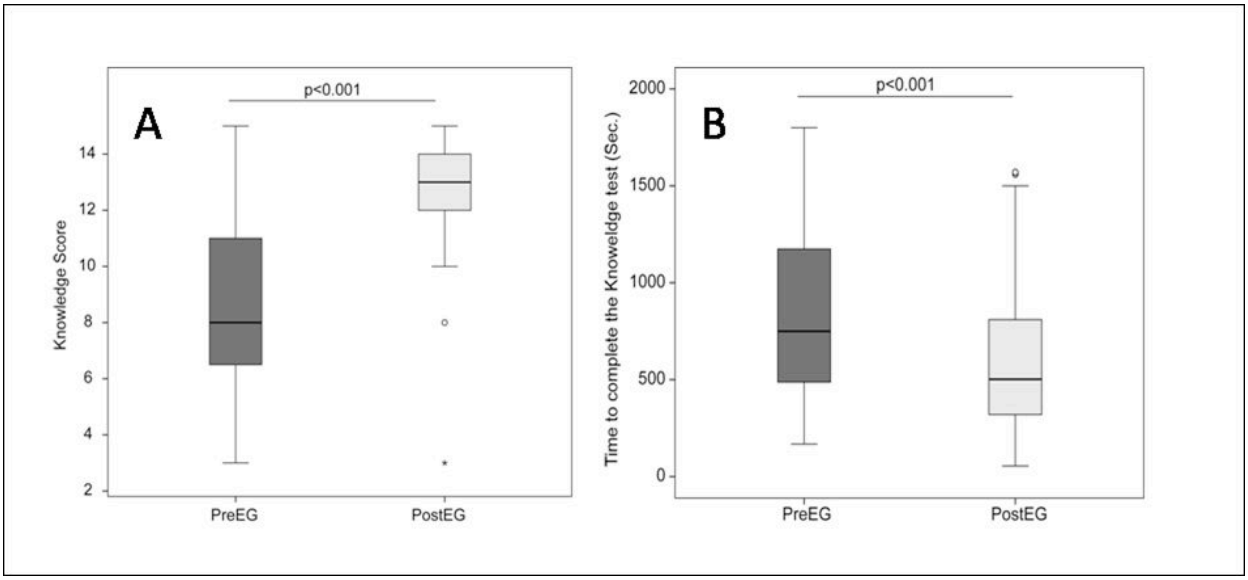
Pre-EG, pre-educational group; PostEG, post-educational group; ED, emergency department; ORS, oral rehydration solution.

Table 3. MLRA model for the estimate of the risk of non-perfect adherence.

	PreEG		PostEG	
	Odds Ratio [95% C.I.]	p	Odds Ratio [95% C.I.]	p
Learners' characteristics				
Gender (F vs M)	0.77 [0.23 to 2.59]	0,674	0.6 [0.15 to 2.41]	0,472
Age (Years)	1.04 [0.95 to 1.15]	0,404	1.02 [0.9 to 1.14]	0,776
Previous experience with e-L	1.26 [0.44 to 3.62]	0,67	1.67 [0.5 to 5.62]	0,406
Years of activity (≥10 vs <10)	0.62 [0.16 to 2.33]	0,477	0.21 [0.04 to 1.12]	0,068
Specialty (Paediatrician vs GP)	1.01 [0.17 to 6.05]	0,992	1.73 [0.19 to 16.23]	0,63
Setting (Outpatient vs Inpatient)	0.56 [0.18 to 1.81]	0,335	0.31 [0.08 to 1.18]	0,086
Previous knowledge of CPG	**0.29 [0.1 to 0.86]**	**0,026**	1.92 [0.58 to 6.37]	0,289
Patients' characteristics				
Gender (F vs M)	1.1 [0.58 to 2.11]	0,765	0.88 [0.43 to 1.8]	0,728
Age (Months)	1 [0.97 to 1.02]	0,844	1.01 [0.98 to 1.03]	0,66
Chronic disease (yes vs no)	**0.24 [0.07 to 0.86]**	**0,028**	0.92 [0.22 to 3.82]	0,911
Concomitant acute illness (yes vs no)	1.2 [0.43 to 3.35]	0,725	1.55 [0.48 to 5.05]	0,465
Episodes of vomiting				
3 to 5 vs. <3	1.2 [0.52 to 2.74]	0,668	0.44 [0.17 to 1.14]	0,092
>5 vs. <3	**4.07 [1.39 to 11.89]**	**0,01**	**5.22 [1.64 to 16.69]**	**0,005**
Abdominal pain (yes vs no)	**1.88 [1.1 to 3.24]**	**0,022**	0.61 [0.25 to 1.49]	0,279
Diuresis (decreased vs normal)	0.83 [0.37 to 1.86]	0,651	0.68 [0.27 to 1.69]	0,403
Duration of symptoms (days)	1.13 [0.92 to 1.37]	0,24	1.14 [0.91 to 1.43]	0,248
Stool output				
3 to 5 vs. <3	0.96 [0.37 to 2.54]	0,942	2 [0.71 to 5.66]	0,191
>5 vs. <3	1.36 [0.5 to 3.75]	0,548	0.79 [0.25 to 2.48]	0,688
Bloody diarrhoea (yes vs no)	**5.75 [1.39 to 23.89]**	**0,016**	1.9 [0.46 to 7.84]	0,377

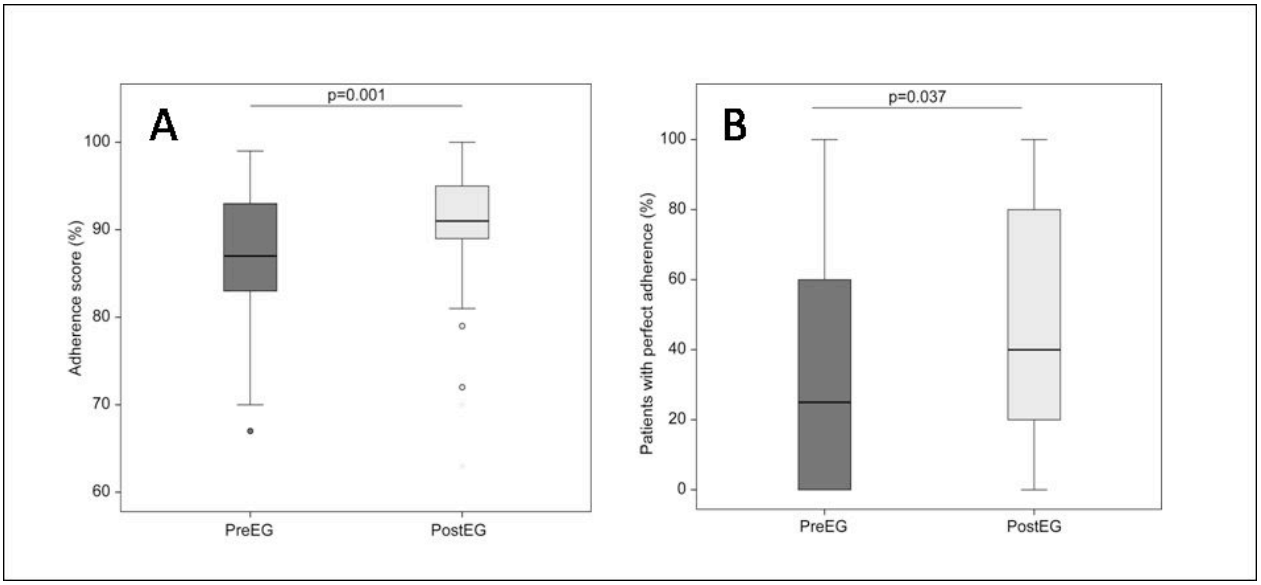
Pre-EG, pre-educational group; PostEG, post-educational group; GP, general practitioner.

Figure 1.



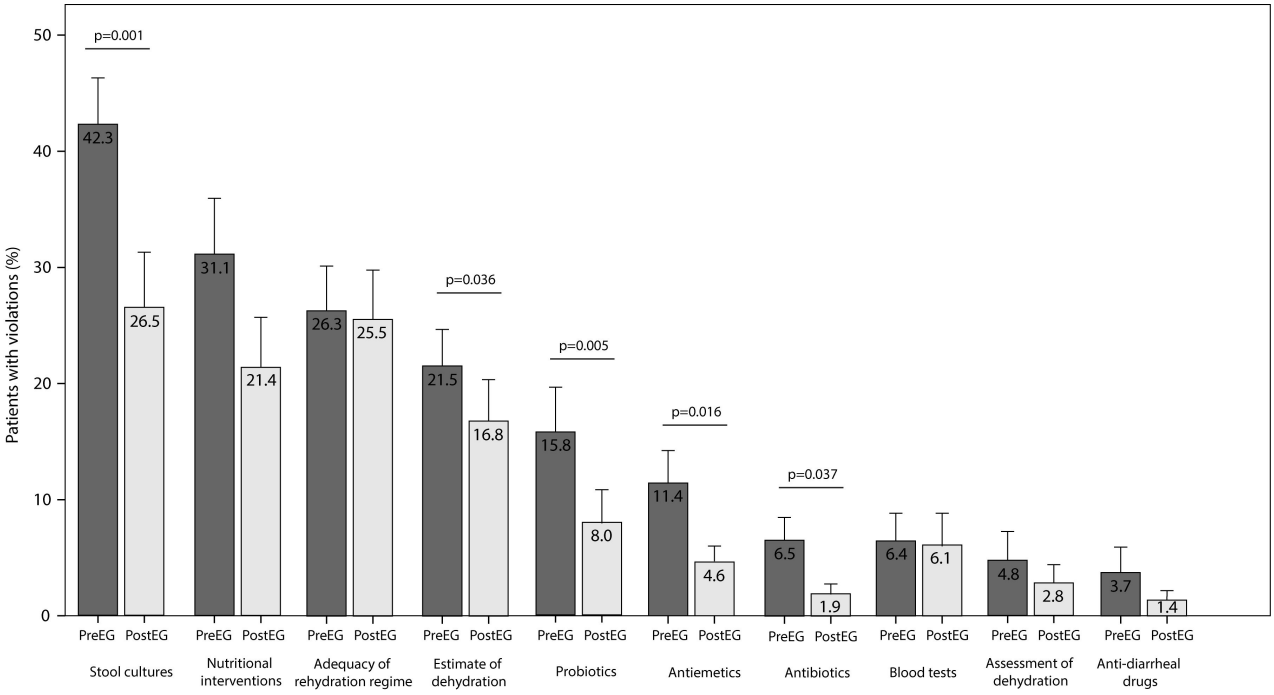
Impact of e-learning on knowledge about management of acute gastroenteritis in children before (Pre) and after the e-learning intervention (Post): A) learners' scores and B) time to complete the 15-question evaluation tests (as recorded by the e-learning platform).

Figure 2.



Adherence to clinical practice guidelines for acute gastroenteritis in 545 children <5 years managed before (Pre) and after (post) the e-learning implementation intervention: A) average adherence percentage score and B) proportion of patients managed in full adherence with the guidelines.

Figure 3.



Changes in inappropriate interventions for acute gastroenteritis in children <5 years managed before (Pre) and after (Post) e-learning.

Results – 3

Preclinical models of disease: microbes and gut

The classical meaning of "translational research" is that of the development of a disease model from the bench to the bedside. The two original works described in this session follow that direction and try to respond to clinical questions with a pure experimental approach. Both works were conducted in an in vitro model of human intestine.

In the 3.a session the role of the HIV Tat protein in the human intestine, trying to explain intestinal and systemic inflammation, growth and metabolic disturbances observed in HIV children in the everyday practice.

In the 3.b session the role of autophagy in Rotaviral gastroenteritis was explored, and vitamin D was evaluated as an autophagy rescuer and thus as an antidiarrheal drug in this common condition.

The HIV-1 transactivator factor (Tat) induces enterocyte apoptosis through a redox-mediated mechanism

This work was published in PLoS One in 2011

INTRODUCTION

The intestinal mucosa is a functional barrier against pathogens being both a physical obstacle with columnar cells linked together by tight junctions, and the site of mucosal immunological cells. HIV infection is mainly initiated on the intestinal mucosal surface through heterosexual or homosexual transmission [1,2] and HIV acutely induces infiltration of the gut mucosa thereby resulting in the release of activated effector memory CD4+ and CD8+ T cells, damage to the intestinal barrier and increased epithelial apoptosis [3]. Clinical data support a relationship between chronic HIV infection and intestinal dysfunction including increased permeability, altered nutrient absorption, diarrhea and reduction of the absorptive surface [4–10]. Acquired immunodeficiency syndrome (AIDS) enteropathy is an idiopathic, pathogen-negative diarrhea and is associated with an increase in inflammation [11], mucosal immune activation, villous atrophy and crypt hyperplasia that may be observed in all stages of HIV disease even in the absence of HIV virus [12]. The detection of viral proteins and/or nucleic acids in enterocytes and in goblet cells indicated that HIV virus plays a direct pathogenic role at intestinal level [13,14]. Kotler et al. detected HIV DNA, RNA and protein antigens in lamina propria mononuclear cells and epithelial cells of gastrointestinal tract from HIV patients [14]. However, several effects induced by HIV are not mediated by lytic propagation of viral particles, but rather by viral factors that are released by infected cells [15]. We previously demonstrated that the viral protein Tat induces ion secretion in Caco-2 cells and in human colonic mucosa, and inhibits intestinal cell proliferation. Tat-induced ion secretion is associated with an increase in intracellular Ca2+ as a result of extracellular Ca2+ entrance and

mobilization of intracellular stores [16]. A similar effect is induced by Tat in neurons [17]. In addition, Tat causes an imbalance in reactive oxygen species (ROS) generation in neurons, which is neutralized by antioxidants, thereby implicating perturbation of the intracellular redox status in the pathogenesis of HIV-induced cell damage [18].

Oxidative stress is implicated in the pathogenesis and morbidity of HIV infection [19,20]. An increase of ROS and an alteration of antioxidant defenses have been reported in HIV-infected patients [21] associated with decreased levels of antioxidants [22]. The mechanisms involved in HIV-induced oxidative stress are unknown, but HIV-1 proteins gp120 and Tat have been implicated in this process [23] because both induce oxidative stress and cause apoptosis in brain endothelial cells [23].

Antioxidant defenses are also impaired in HIV-infected patients and, in particular, glutathione metabolism is altered [24]. Reduced glutathione (GSH) is the main intracellular thiol molecule responsible for ROS scavenging and for the maintenance of oxidative balance. It is also involved in the protection of DNA and nuclear proteins from oxidative damage. Intracellular GSH depletion triggers ROS production thereby inducing an arrest in the intestinal cell cycle [25]. GSH levels are depleted in plasma, in epithelial lining fluid of the lower respiratory tract, in peripheral blood mononuclear cells and in monocytes in HIV-infected patients [26]. Antioxidant deficiency leads to severe degeneration of intestinal epithelial cells, and even a mild intracellular redox imbalance inhibits enterocyte proliferation [27].

Interestingly, GSH levels progressively decrease as the HIV-1 viral load increases [28]. A fall in GSH during HIV infection may result from reduced GSH synthesis or increased catabolism. Tat blocks transcription of manganese superoxide dismutase, an enzyme that prevents oxidative stress, and decreases the activity of glucose-6-phosphate dehydrogenase, a key enzyme in pathways that maintain GSH in its reduced state [29]. Moreover, Tat induced oxidative stress in an immortalized endothelial cell line from rat brain capillaries [30].

In this scenario, we hypothesized that the enteropathogenic effects induced by Tat are associated with an imbalance of the redox state in the intestine.

METHODS

Intestinal cell line cultures

Caco-2 cells were used as small intestinal cell model [53]. At 15 days post-confluence, cells exhibit a well-differentiated brush border on the apical surface and tight junctions with typical small- intestinal microvillus hydrolases and nutrient transporters. Caco-2 cells were grown in Dulbecco's modified Eagle minimum essential medium (DMEM; Gibco, USA) with a high glucose concentration (4.5 g/L) at 37uC in a 5% CO2 atmosphere. The medium was supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 1% non-essential amino acids, penicillin (50 mU/mL) and streptomycin (50 mg/mL), and changed daily. To verify some data obtained in Caco-2 cells, we used HT-29 as a supplementary intestinal cell model. HT-29 were grown in RPMI 1640 cell culture medium supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 100 mU/ml penicillin and 100 mg/ml streptomycin.

To evaluate the polar nature of the effects induced by Tat on Caco-2 cells, monolayers were grown on polycarbonate filter (Corning Incorporated, NY USA) for 15 days post-confluence and then exposed to Tat 0,5 nM for 1, 24 and 48 hours at apical or basolateral side and then GSH/GSSG ratio and caspase-3 activation were evaluated as previously described. Controls were stimulated with the same volume of medium without Tat.

Fluorescence microscopy

Cells were grown on the chambered cover glass for 3 days. Cells were exposed to 20 mM 79-dichlorofluorescein diacetate (DCFH- DA, D6665; Sigma-Aldrich, St. Louis, MO) for 1 hour at 37uC in the dark. Then, they were treated with Tat (0.5 nM), adding the protein directly to the

cell culture, and incubated at 37uC for 1 hour to detect the ROS. Cells were washed in PBS and after mounting, the fluorescence images from multiple fields of view were obtained using a Nikon Eclipse 80i microscopy. The images were analyzed using NIS Elements D imaging software. A positive control was obtained by incubating cells with H2O2 10 mM for 5 min.

Reactive oxygen species production

The production of ROS was measured using the DCFH-DA fluorometric method, which is based on the ROS-dependent oxidation of DCFH-DA to DCF. Caco-2 cells were grown in 6- well plates for 15 days post-confluence. Cell monolayers were treated with Tat 0.05, 0.1 and 1 nM for 15 min or with Tat 0.5 n M for 15, 30, 60 and 120 min at 37uC. Medium was removed and cells were washed by PBS. Then, cells were treated with DCFH-DA (20 mM) for 30 min at 37uC in the dark. Intracellular ROS production was measured with a spetrofluo- rometer (SFM 25; Kontron Instruments, Japan). A positive control was obtained by incubating cells with H2O2 10 mM for 5 min. Neutralization experiments were performed incubating Tat 0.5 nM with 30 ng/ml of anti-Tat polyclonal antibodies (Tecnogen, Piana di Monte Verna, Italy) at +4uC overnight with gentle shaking. The preparations were then used to stimulate cells.

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nM with 30 ng/ml of anti-Tat polyclonal antibodies (Tecnogen, Piana di Monte Verna, Italy) at +4uC overnight with gentle shaking. The preparations were then used to stimulate cells.

Antioxidant defenses evaluation

GSH and GSSG intracellular levels were measured by method described by Allen et al. with few modifications [54]. Briefly, cells were exposed to Tat alone or in combination with NAC and then lysed with Triton X-100. Protein was precipitated with 1%sulfosalicylic acid and supernatants used to measure in parallel total and reduced glutathione. Oxidated glutathione (GSSG) was determined by subtracting the reduced form from total glutathione. GSH and GSSG values were normalized for protein content. All assays were performed three times. Catalase activity, a well-established indicator of oxidative stress, was measured with an assay kit from Calbiochem (Gibbstown, NJ) and normalized for protein content. Neutralization experiments were performed incubating Tat 0.5 nM with 30 ng/ml of anti-Tat polyclonal antibodies (Tecnogen, Piana di Monte Verna, Italy) at +4uC overnight with gentle shaking. The preparations were then used to stimulate cells. gp120 (ab69717) and goat polyclonal anti-gp120 antibody (ab21179) were from Abcam plc (Cambridge, UK).

Oxidative stress evaluation in HIV-positive patients

An aliquot of rectal dyalisis, urine and serum samples from 20 patients infected with HIV and 6 control subjects aged 26–228 months were obtained during a routine check-up. All HIV patients had been on HAART for more than 3 years. Immediately after collection, samples were separated into 2 mL aliquots and stored at 280uC until analysis. We used levels of 8-hydroxy-29-deoxyguano- sine (8-OHdG), as biomarker of DNA oxidative stress. 8-OHdG was measured in duplicate using a highly sensitive ELISA kit (Li StarFish Srl, Milan, Italy). The length of dialysis bag is 3 cm and the concentration of oxidative marker was normalized for

the exact volume inside the bag. Differences were evaluated by the Mann- Whitney test. The Pearson's correlation coefficient was applied to investigate the correlation between 8-OHdG and viral load.

Actin staining

One step of fixation and permeabilization was performed with 4% paraformaldehyde and 0.2% Triton X-100 for 30 min at +4uC. After three washes in PBS, the cells were treated with a 50 mg/ml solution of fluorescein isothiocyanate-phalloidin (Sigma- Aldrich, Milan, Italy) in PBS for 40 min. Nuclei were stained with Hoechst 5 mg/ml (Sigma-Aldrich, Milan, Italy) for 5 min at +4uC. The cells were washed three times with PBS and were mounted with Mowiol (Invitrogen S.R.L, San Giuliano Milanese, Italy). The monolayers were examined using a Nikon Eclipse 80i epifluorescent microscope (FITC filter). The images were analyzed using NIS Elements D imaging software.

Caspase-3 activity assay

We used caspase-3 as a marker of apoptosis [55]. An apoptosis assay kit was used to determine caspase-3 activity, according to the manufacturer's instructions (Biovision, Mountain View, CA). Caspase-3 activity was investigated in Caco-2 cells by the release of the chromophore pNA after substrate cleavage. Modifications of caspase-3 activity were determined by comparing the sample optical density (OD) with the control.

Immunoblotting

Total cells lysates were obtained by homogenization of cell pellets in cold lysis buffer (20 mM Tris, pH 7.5 containing 300 mM sucrose, 60 mM KC1, 15 mM NaC1, 5% (v/v) glycerol, 2 mM EDTA, 1% (v/v) Triton X-100, 1 mM PMSF, 2 mg/ml aprotinin, 2 mg/ml leupeptin and 0.2%

(w/v) deoxycholate) for 1 min at 4uC and further sonication for additional 30 sec at 4uC. Cytosolic, microsomal and mitochondrial fractions were prepared with the Qproteome Mitochondria Isolation Kit (Qiagen). Equal amounts of protein were subjected to 10% (v/v) SDS-PAGE and transferred to a PVDF membrane (Millipore). The membrane was blocked with 5% (w/v) skim milk and incubated with primary antibody, followed by incubation with an HRP-conjugated secondary antibody. Proteins were visualized with an ECL detection system (GE-Healthcare). The following antibodies were used for Western blot analysis: rabbit polyclonal anti-Tat antibody (Tecnogen, Piana di Monte Verna, Italy), mouse monoclonal anti-caspase3 antibody (full length protein), mouse monoclonal anti- PARP antibody, goat polyclonal cytochrome c antibody, mouse monoclonal anti-COX IV antibody, mouse monoclonal anti- tubulin antibody, mouse monoclonal anti-GAPDH antibody (Santa Cruz Biotechnology). gp120 (ab69717) and goat polyclonal anti-gp120 antibody (ab21179) were from Abcam plc (Cambridge, UK).

Co-immunoprecipitation

A total of 800 mg of protein lysate from the Caco-2 cells exposed to Tat 0,5 nM 24 hrs were precipitated with 1 mg of tubulin antibody. Protein A/G agarose beads (Santa Cruz) were used to collect the immunoprecipitated complexes and the beads were washed with PBST before SDS-PAGE and Western blot analysis with anti-Tat antibody.

Experiments in human small intestinal specimens

Biopsies from the distal part of the duodenum were obtained from 5 children seen at the Department of Pediatrics and undergoing endoscopy (84–192 months of the age) for intestinal disorders. All biopsies were from macroscopically normal areas, and intestinal histology was subsequently reported to be normal. Tissue samples were transported to the laboratory in culture medium and processed within one hour. Duodenal mucosa specimens

were obtained from 4 HIV-negative children and from one HIV-positive child. Specimens were washed and observed by stereomicroscope to exclude tissue necrosis. Organ culture was performed in DMEM with a high glucose concentration (4.5 g/L) supplemented with 0.5% FCS, 1% non-essential amino acids, 2% penicillin (50 mU/mL) and streptomycin (50 mg/mL) and incubated in 5% CO2/95% air for one hour before treatment. Short-term experiments were run using high Tat concentrations to maximize the cytotoxic effect before spontaneous tissue disruption. Specimens were exposed to Tat alone (0.1 mM) or preincubated with NAC (10 mM for 4 h). Short-term experiments with an higher Tat concentration were performed to maximize the effect before spontaneous tissue disruption. After stimulation, samples were homogenized and lysed in RIPA buffer: 100 mM Tris-HCL pH 7.5, 300 mM NaCl, 2% NP40, 1% Na deoxycholic acid, 0.2% SDS, 100 mg/ml PMSF, 5 mg/mL aprotinin, 1 mg/mL leupeptin, 0.7 mg/ml pepstatin. Whole extracts were centrifuged and protein content was determined by the Bradford assay. GSH/GSSG intracellular levels and caspase-3-cleaved protein were evaluated as described above. For western blot assay we used the mouse monoclonal anti-caspase-3 cleaved protein (Cell Signaling Inc., Danvers, MA). The experiments were undertaken with the understanding and written consent of each child's parents and the study methodologies conformed to the standards set by the Declaration of Helsinki. The study protocol was approved by the Ethics Committee of the School of Medicine, University of Naples Federico II, Italy.

Statistical analysis

We used GraphPad Prism Software (San Diego, CA) to evaluate the two-tailed unpaired Student t test and a 2-tailed paired Student t test to evaluate statistical significance. An alpha value of 0.05 was set for statistical significance. p-Values for each analysis are indicated in figure legends.

RESULTS

HIV-1 Tat induces intestinal epithelial oxidative stress thereby increasing reactive oxygen species and impairing antioxidant defenses

To evaluate whether an altered redox state could be responsible for the effects induced by Tat, we measured the intracellular levels of ROS and of two main intracellular antioxidant defense systems, catalase and glutathione, in intestinal epithelial cells. Fluorescence microscopy showed that ROS levels were increased in Caco-2 cells exposed to 0.5 nM of Tat for 1 hour as judged by the fluorescence green signal produced by the interaction between dichlorodihy- drofluorescein diacetate (DCFH-DA) and ROS (Fig. 1A). ROS production was also increased in HT-29 cells stimulated with HIV-Tat under the same conditions (Fig. 1B). As a positive control, cells were treated with H2O2, and cells treated with the same volume of media without Tat protein served as negative control. Increasing Tat concentrations (0.05–1 nM) were added to Caco-2 cells. DCFH-DA was used for ROS quantification and measured 15 min after Tat stimulation.

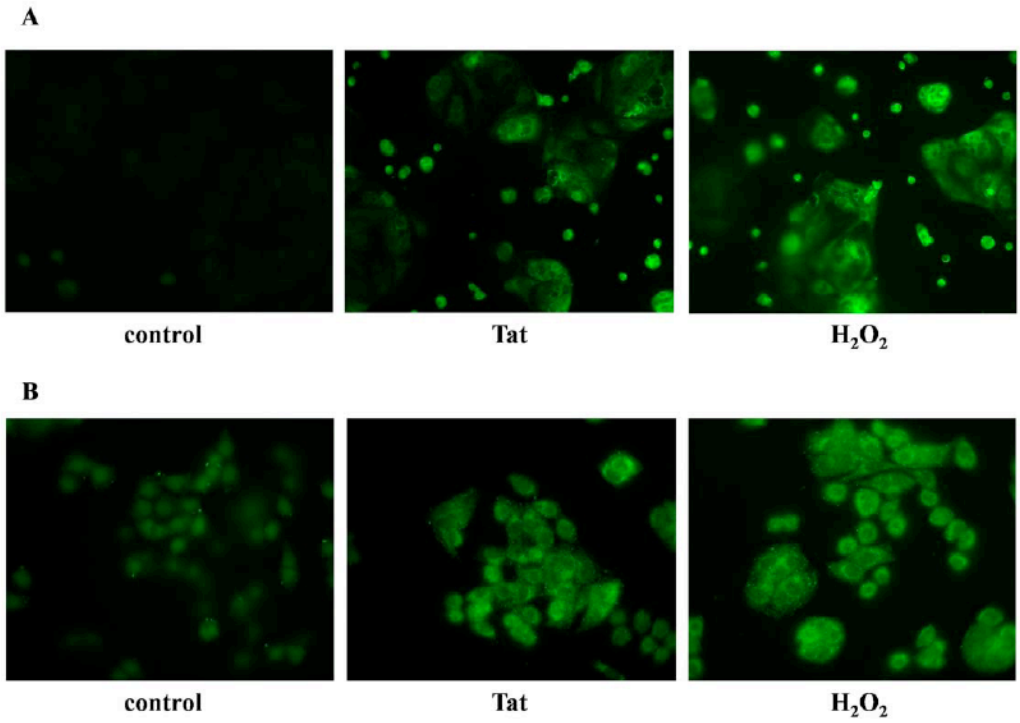

Figure 1. Influence of HIV-Tat protein on ROS generation in Caco-2 cells (A) and HT29 cells (B). Immunofluorescent staining of ROS by DCFH-DA after Tat exposure were compared to H_2O_2- and untreated cells (control). Representative staining is shown at 1 hour post-exposure. Magnification: 200×. Data are representative of 3 separate experiments with 3–4 replicates for each experimental condition.

Exposure to Tat protein resulted in a dose-dependent increase of ROS (Fig. 2A). Since ROS generation is usually rapid after a toxic stimulus, we performed time-course experiments in Caco-2 cells exposed to Tat for 15, 30, 60 and 120 min (Fig. 2B). A ROS increase was evident as early as 15 min after exposure to Tat; levels returned to control values after two hours. This suggests that the antioxidant defenses may be activated to counteract oxidative stress. We carried out neutralization experiments to ascertain whether ROS generation is a specific effect induced by Tat. As shown in Fig. 2C and D, anti-Tat polyclonal antibodies inhibited the increase of ROS intracellular levels in Caco-2 and HT-29 cells. To see whether Tat enters into the cells, we stimulated Caco-2 cells from the same culture with Tat at 1, 24, 48 and 72 hrs and, in parallel, we evaluated ROS concentrations and Tat intracellular levels. In these conditions, Tat was detected inside the cells 1 hour after stimulation and until at least 72

hours post-exposure.

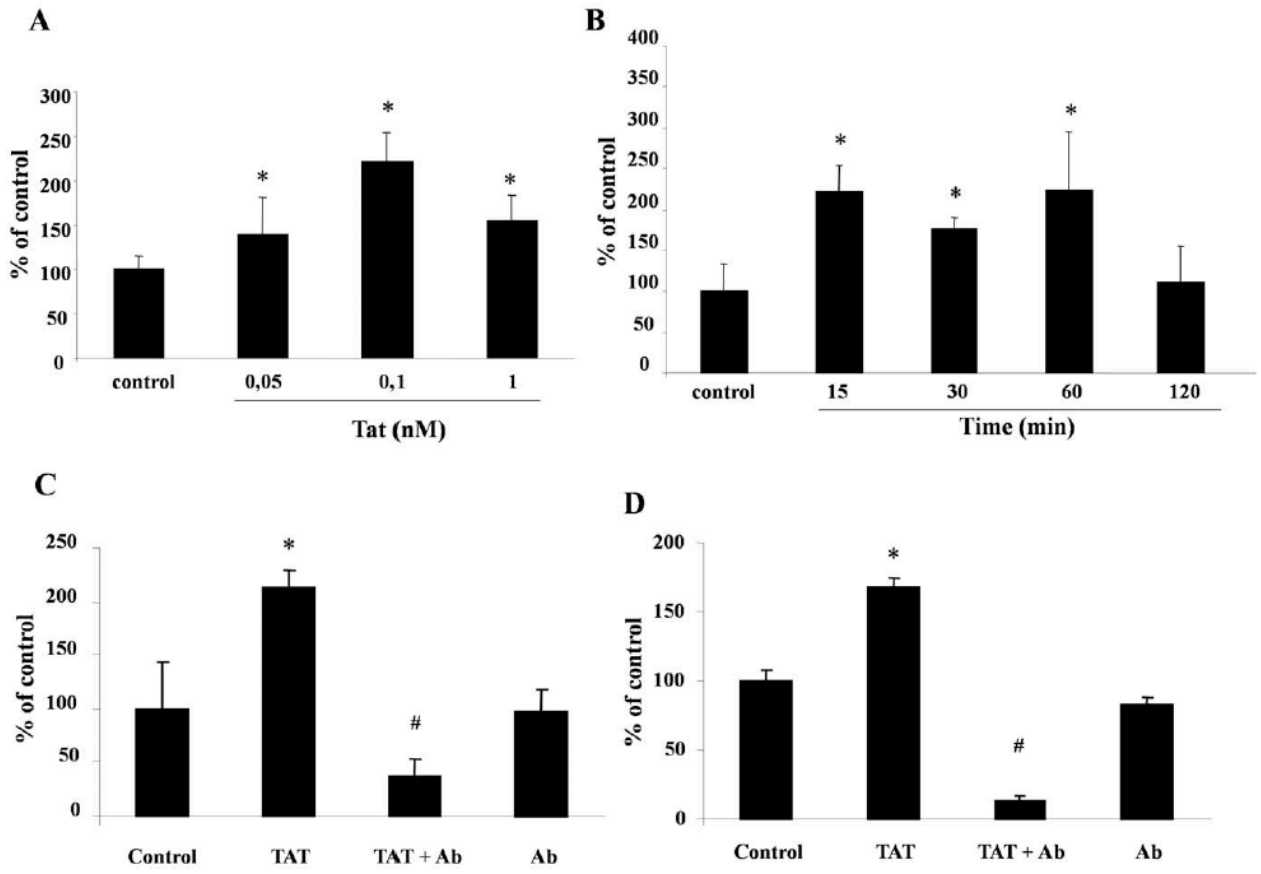

Figure 2. Tat-induced ROS generation is dose- and time-dependent. Caco-2 cells were exposed to different concentrations of Tat for 1 hour (A) and to 0.1 nM for 10, 30, 60 and 120 min (B), and ROS intracellular levels were evaluated by the DCFH-DA fluorometric method. To evaluate the specificity of the effect, Caco-2 cells (C) and HT-29 (D) were incubated with Tat with or without the anti-Tat polyclonal antibody. Data are representative of 3 separate experiments. *p<0,05 vs control; #p<0,05 vs Tat.

We next investigated whether Tat-induced ROS generation was associated with a decrease of antioxidant defenses by measuring the levels of glutathione, one of the major intracellular ROS scavengers. Glutathione is an important cellular antioxidant, and it plays a major role in protecting cells against oxidative stress. In fact, the intracellular balance between the reduced (GSH) and oxidated (GSSG) glutathione forms in healthy conditions was reported to show a predominantly reducing state being GSH about 80–90% and GSSG 10–20% [31]. We found that the GSH/GSSG ratio was lower in Caco-2 cells exposed to Tat than in controls and that the effect was dose- and time-dependent (Fig. 3A and B). The reduction of GSH was significant between 0.1 and 1 nM of Tat (Fig. 3A) and was already evident at 1 hour post- exposure. GSH remained low at 24 hours and returned to basal level at 48–72 hours (Fig. 3B). Also in this

case, anti-Tat polyclonal antibodies completely inhibited the Tat-induced GSH/GSSG imbalance in Caco-2 (Fig. 3C) and in HT-29 cells. The decreased activity of the enzyme catalase, another component of the antioxidant defense, after 1 hour of exposure to Tat (Fig. 3D) indicates that antioxidant defense is a target of Tat.

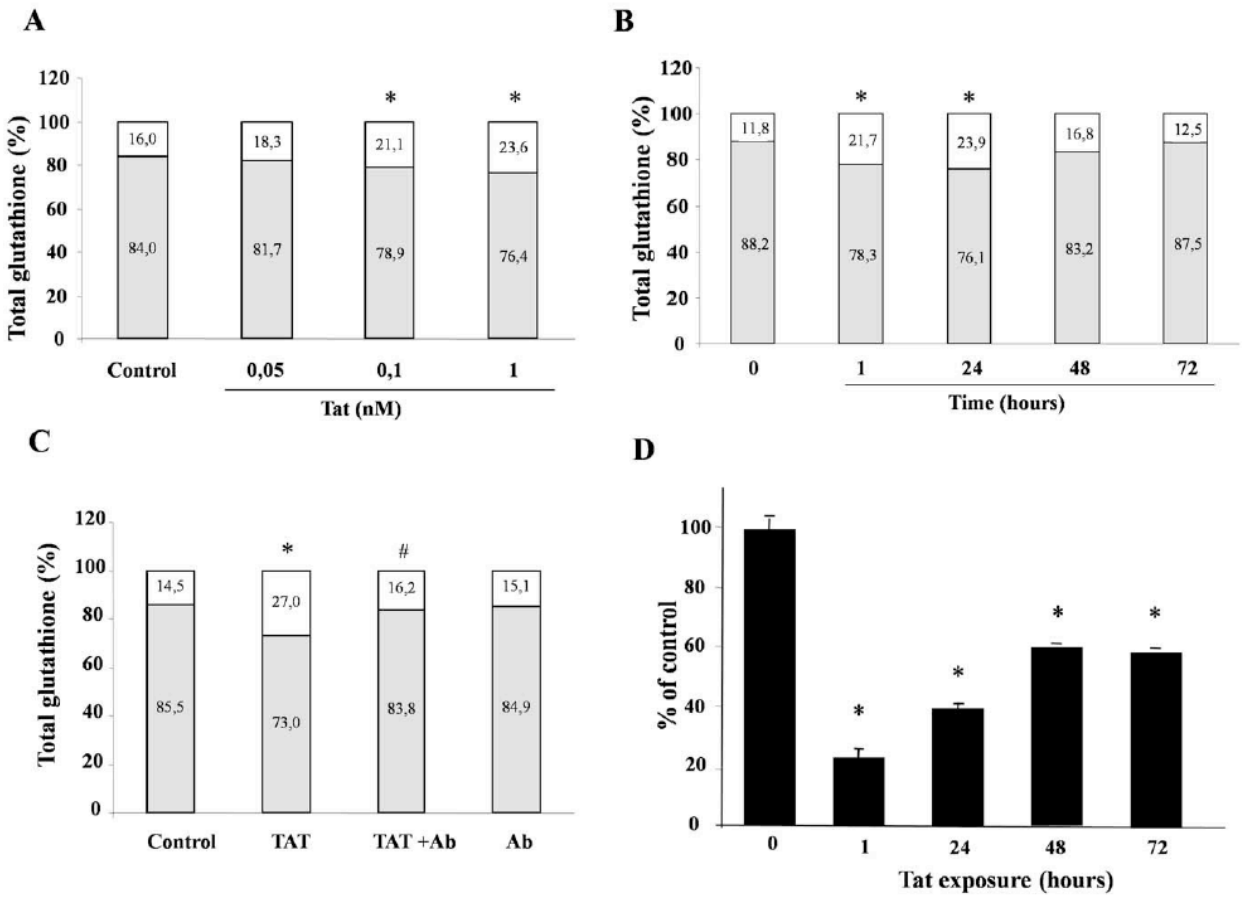

Figure 3. Tat induced alteration of intracellular antioxidant defenses. Caco-2 cells were exposed to different concentrations of Tat for 1 hour (A) and to 0.1 nM for 10, 30, 60 and 120 min (B), and the percent of GSH (grey) and GSSG (white) was evaluated as described under "Methods" in the Method section. To evaluate the specificity of the effect, Caco-2 cells (C) were incubated with Tat with or without the anti-Tat polyclonal antibody. *p<0,05 vs control; #p<0,05 vs Tat. (D) Catalase activity was evaluated in Caco-2 cells after different times of Tat exposure. Data are representative of 3 separate experiments. *p<0,05 vs time 0.

Intestinal oxidative stress is increased in HIV-infected children

In nuclear and mitochondrial DNA, 8-hydroxy-29-deoxyguanosine (8-OHdG) is one of the predominant marker of free radical-induced oxidative damage and is therefore widely used as biomarker for oxidative stress in clinical samples [32–34]. To investigate whether HIV-infected patients are affected by intestinal oxidative stress, we measured 8-OHdG concentration in a small amount of rectal dialysis solution from 20 HIV-positive children

treated in our tertiary care center for patients with AIDS (Fig. 4A). The characteristics of patients are shown in Table 1.

Table 1. Characteristics of the HIV-positive patients.

Age (months)	
Mean ± standard deviation	134.8±53.3
Range	26–228
Sex	
Male	6
Female	14
CD4+ (cells/ml)	
Mean ± standard deviation	963.85±496.43
Range	176–2027
HIV RNA	
Patients with HIV RNA <40cp/ml	11
Patients with HIV RNA >40cp/ml	9
Mean viral load (cp/ml)± standard deviation	3934.89±6776.77
Range	48–14500

The concentration of 8-OHdG was higher in HIV-infected children than in serum-negative children (Fig. 4B). To investigate whether intestinal oxidative stress was related to virus replication, we divided the HIV-positive patients in two groups related to plasma viral load. We identified 9 children with a viral load higher than 40 copies/ml and 11 children with undetectable HIV RNA (,40 copies/ml). The mean 8-OHdG concentration was higher in children with HIV RNA .40 copies/ml than in children with HIV RNA ,40 copies/ml and in serum-negative children (Fig. 4C), which suggests that in children with chronic HIV infection, oxidative damage measured in rectal mucosa may be linked with viral replication. However, 8-OHdG concentrations in urine and serum did not differ between patients and HIV-negative control children, suggesting that the oxidative stress is localized in rectal mucosa. Finally, a significant correlation was detected between 8-OHdG and viral load ($r = 0.4653$; $p = 0.0009$).

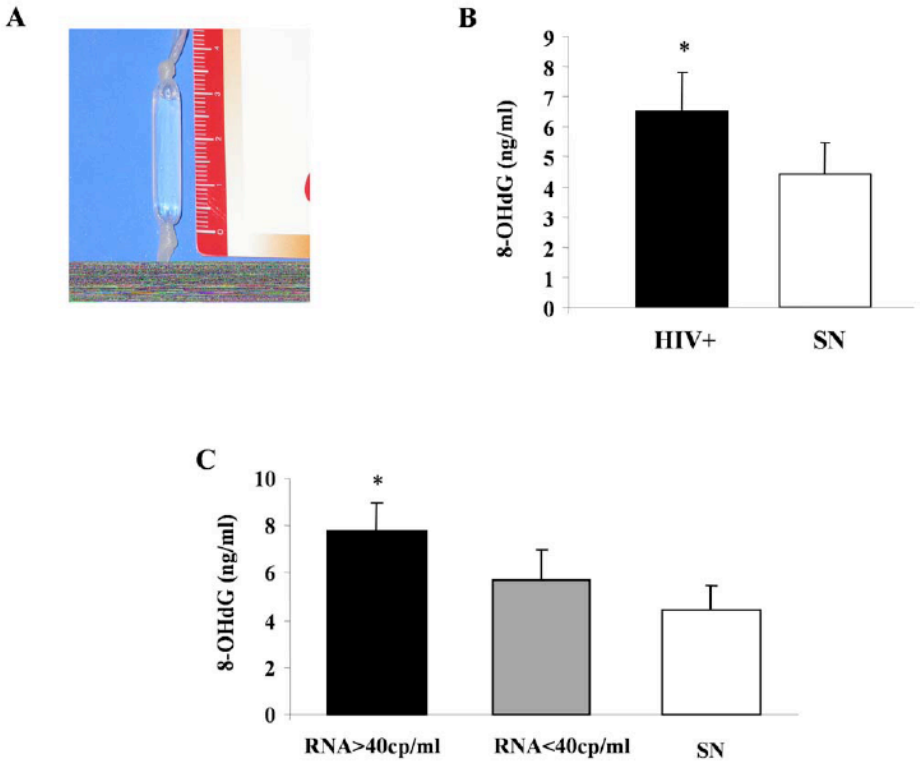

Figure 4. 8-hydroxy-2′-deoxyguanosine (8-OHdG) concentration in rectal dialysis in HIV-negative and positive children. The determinations of rectal 8-OHdG production were performed using a dialysis bag (A) in serum-negative (n = 6, SN) and positive (n = 20, HIV+) children (B). *p<0.05 vs SN. (C) HIV+ children were divided in two groups and compared with controls (n = 6, white): HIV RNA >40cp/ml (n = 9, black), HIV RNA <40cp/ml (n = 11, grey). *p<0.05 vs RNA <40cp/ml and SN groups.

Activation of caspase-3 and cytochrome c release in the cytosol indicates that Tat induces apoptosis through involvement of the intrinsic pathway

Several viruses induce apoptosis as a strategy to spread the infection or to induce the breakdown of infected cells, thereby favoring viral dissemination. To investigate whether Tat induces intestinal apoptosis, we studied caspase-3 signaling, which is activated by two fundamentally distinct signaling cascades, namely the extrinsic and intrinsic (or mitochondrial) pathways [35]. We found that caspase-3 activity was higher in Caco-2 cells exposed to Tat for 72 hours than in control cells (Fig. 5A). Caspase-3 is a critical effector of apoptosis and is responsible for the proteolytic cleavage of many key proteins including the nuclear enzyme poly (ADP ribose) polymerase (a-PARP). In a western blot time-course experiment, we found that the levels of the full-length forms of caspase-3 protein and a-PARP were reduced after Tat exposure compared with control cells thereby indicating activation of the apoptotic molecular mechanisms (Fig. 5B).

Proapoptotic stimuli, including ROS and calcium overload, are able to activate the intrinsic pathway of apoptosis by inducing mitochondrial membrane permeabilization and the release of cytochrome c in the cytosol. Because we previously found that Tat induced an increase of cytosolic calcium from intracellular stores [16], we used western blot analysis to test the effects of Tat on the release of cytochrome c. Twenty-four hours after Tat exposure, cytochrome c levels in the cytosol of cells were very high and they continued to increase up to 72 hours (Fig. 5C upper panel). Cytochrome c was not detected in the cytosol of control cells. The same filter was reprobed with a monoclonal antibody against cytochrome oxidase subunit IV (COX IV), which is a mitochon- drial marker, and then with anti-tubulin antibody, which is a cytosolic marker. The absence of COX IV in the cytosolic samples confirmed the absence of mitochondrial contamination in the cytosolic fraction (Fig. 5C middle panel), and the absence of tubulin in mitochondrial samples indicates that the mitochondrial fractions were free from cytosolic content (Fig. 5C lower panel).

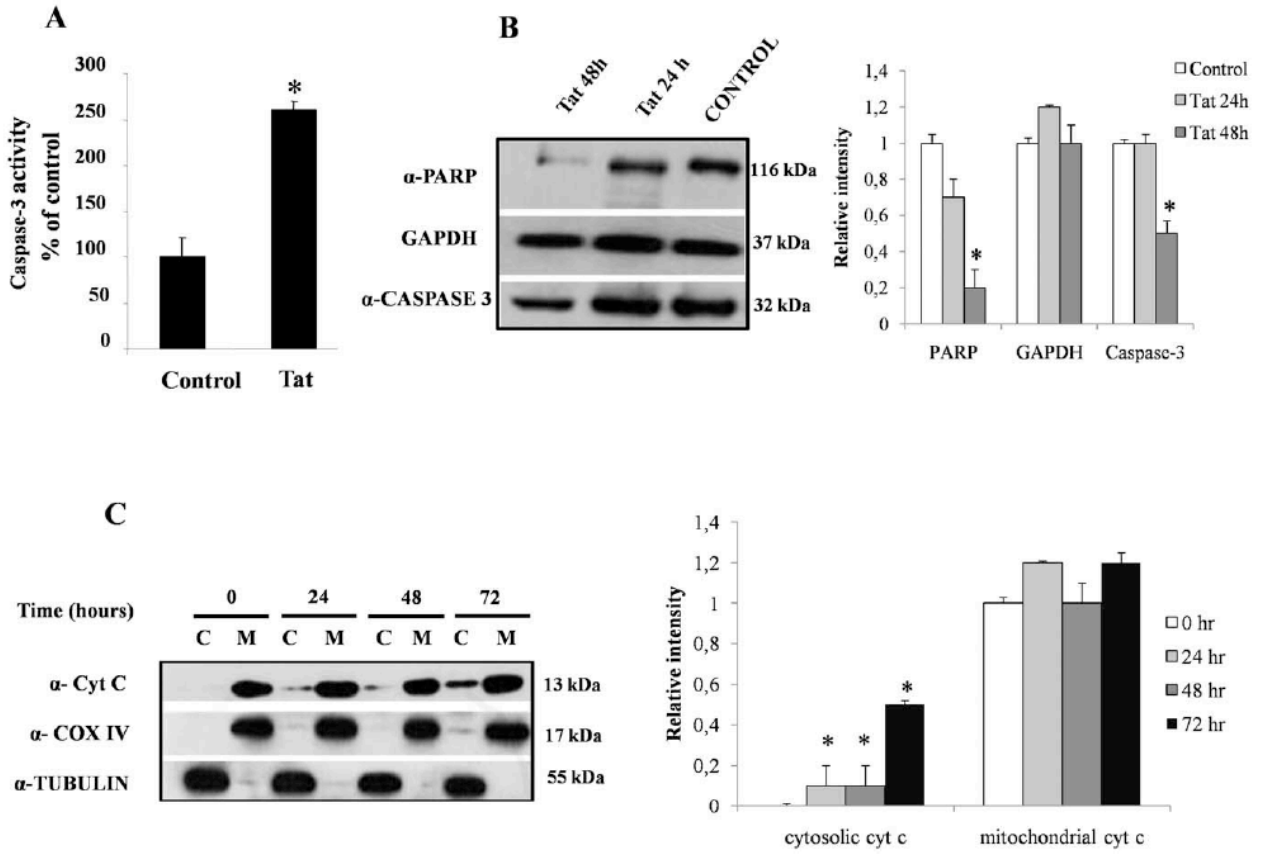

Figure 5. Influence of HIV-Tat protein on apoptosis in Caco-2 cells. Caspase-3 activity (A) and full-length protein (B lower panel) were evaluated in Tat-treated Caco-2 cells. To verify that Tat induced apoptosis, cleaved PARP was evaluated in the same western blot used to evaluate the activation of caspase-3 (B upper panel). Normalization of western blot was performed with GAPDH in all experiments (B middle panel). *p<0.05 compared with untreated control cells. Protein extracts from cytosol (C lanes) and mitochondria (M lanes) assayed for cytochrome c by western blot analysis (C); tubulin was used as cytosolic marker, and COX IV as mitochondrial marker. Densitometric acquisitions are shown from three separate experiments. *p<0,05 vs time 0.

Because oxidative stress disrupts the cytoskeleton, we investigated the intracellular actin architecture. The architecture of actin cytoskeleton was normal in Caco-2 cells, but it became instable and fragmented after Tat exposure. Disruption of the actin cytoskeleton was dependent on the time of Tat exposure (Fig. 6A). To investigate the effects of Tat on microtubules in Caco-2 cells, we performed experiments of co-immunoprecipitation of Tat and tubulin. The protein extracts from Caco-2 cells exposed to Tat were subjected to co-immunoprecipitation with an antibody against tubulin or with control IgG, and subsequently analyzed through Western blotting with the anti-Tat polyclonal antibody. Tat coprecipitated with tubulin indicating that it binds to the microtubule cytoskeleton (Fig. 6B).

Polar effects by Tat on the apical and basolateral side of the Caco-2 cell monolayers

To test the hypothesis that Tat induces different effects depending on its addition to the apical or basolateral side of the epithelium, Caco-2 cell monolayers were exposed to Tat 0.5 nM for 1, 24 and 48 hours at apical or basolateral side (Fig. 7). In these conditions Tat reduced the GSH/GSSG ratio at 1–24 hours, with a more potent effect at the basolateral than the apical side (Fig. 7A). In addition, the activation of caspase-3 was observed following basolateral but not apical stimulation (Fig. 7B). The polar effect by Tat is in agreement with our previous data showing chloride secretion induced by

basolateral addition of Tat [16].

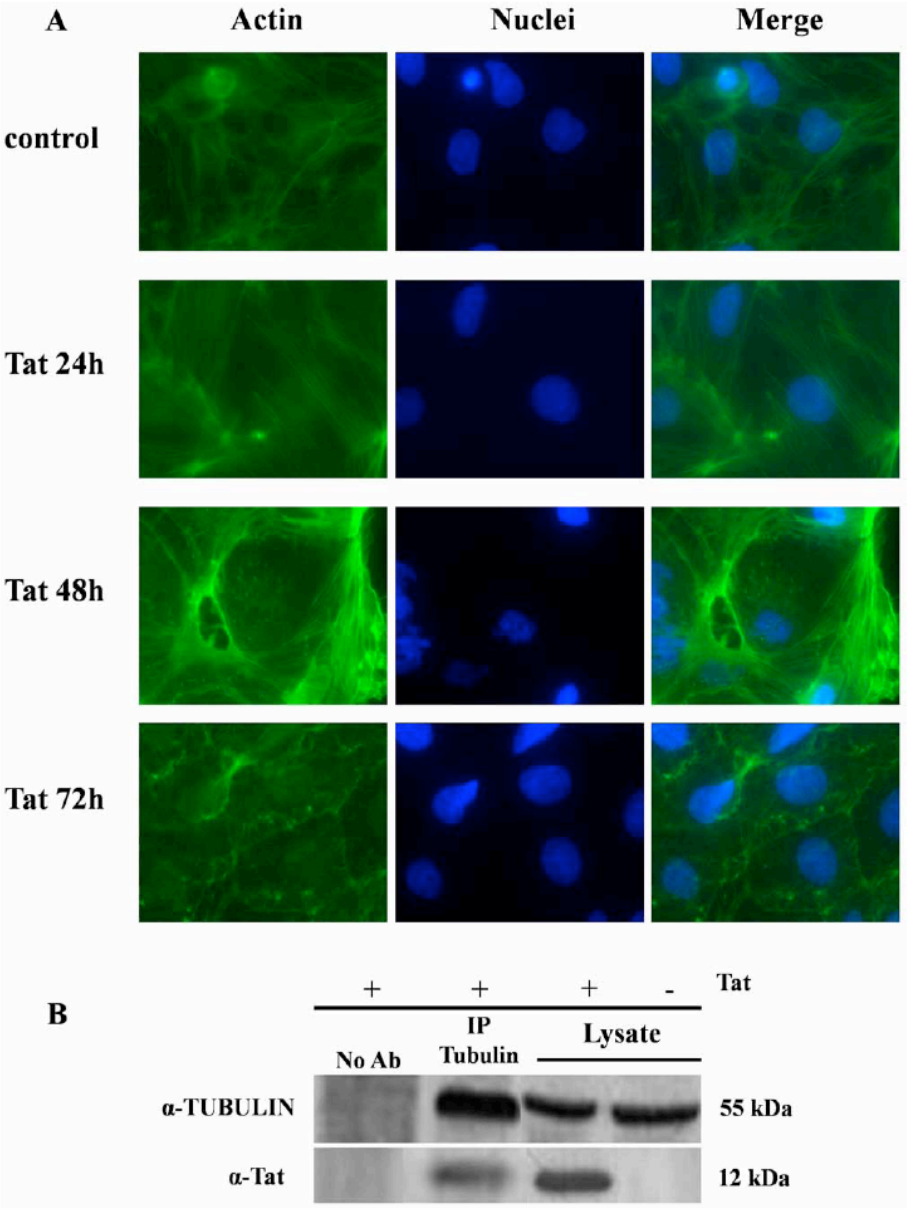

Figure 6. Tat-induced disruption of the actin cytoskeleton. (A) Direct immunofluorescence of actin staining by FITC-conjugated phalloidin (green). The nuclei were stained with Hoerst (blue). Caco-2 cells were exposed to Tat 0.5 nM for 72 hours. Magnification: 1000×. Data are representative of 3 separate experiments with 3–4 replicates for each experimental condition. (B) Cell lysates of Caco-2 cells were stimulated with Tat 0,5 nM for 24 hours and subjected to immunoprecipitation using anti-tubulin antibody or control IgG. Tat protein was detected from immunoprecipitates by Western blotting with anti-Tat antibody.

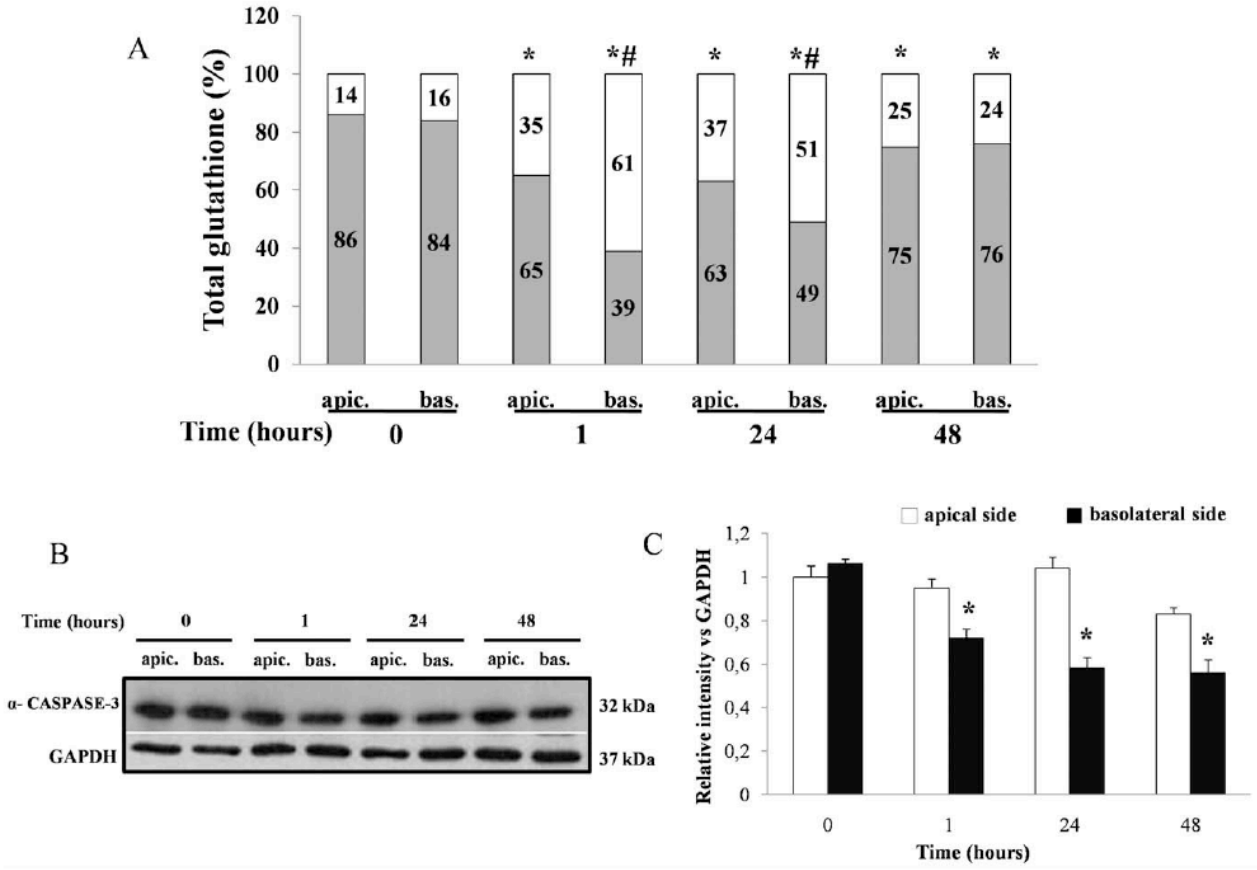

Figure 7. Polar effects by Tat on the apical and basolateral side of the Caco-2 cell monolayers. Tat was added to apical or basolateral side of Caco-2 cell monolayers and GSH (grey)/GSSG (white) ratio (A) and caspase-3 activation (B) were evaluated after 1, 24 and 48 hours. *p<0,05 vs control; #p<0,05 vs apical Tat stimulation at the same time. Normalization of western blot was performed with GAPDH in all experiments. Densitometric acquisitions are shown from three representative separate experiments.

Effects by gp120 on oxidative stress and apoptosis in Caco-2 cell monolayers

Previous studies investigating the effects of HIV-1 on epithelial barrier function, demonstrate that both Tat and gp120 (the latter being a surface envelope glycoprotein) directly increase perme- ability of brain endothelial cells through a redox-dependent mechanism [23,30]. In order to investigate the similarities and differences between the effects induced by gp120 and by Tat, we evaluated the effects induced by gp120 on oxidative stress and apoptosis in our experimental system. An imbalance in GSH/ GSSG ratio was observed in response to gp120 with a more potent effect at apical than basolateral side (Fig. 8A). However, gp120 did not induce caspase-3 activation (Fig. 8B).

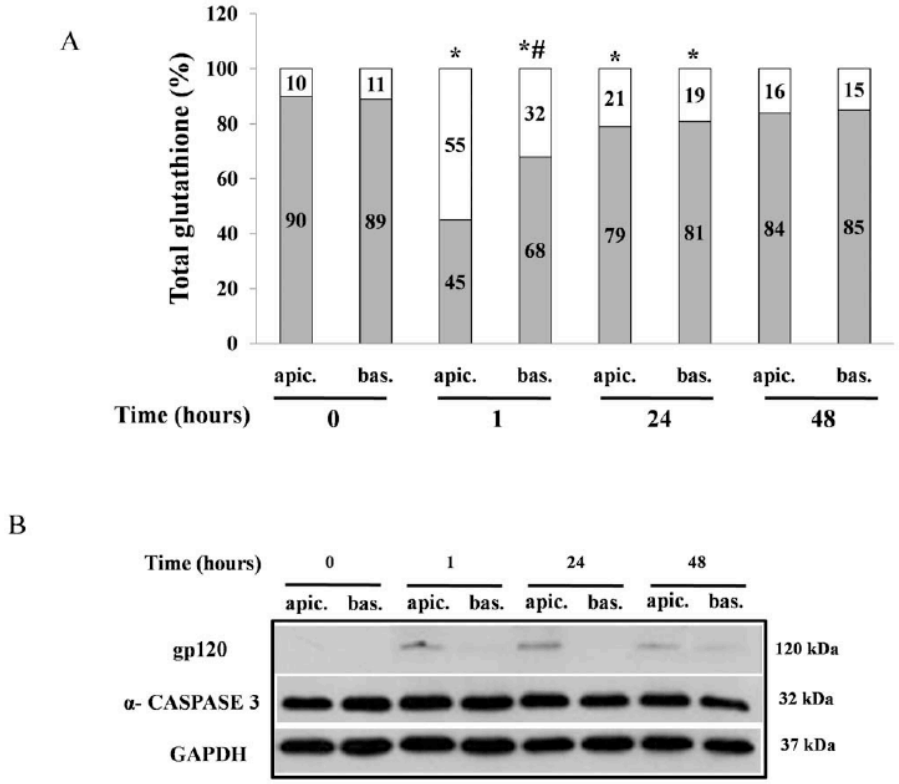

Figure 8. Effects by gp120 on oxidative stress and apoptosis in Caco-2 cell monolayers. gp120 (0.8 nM) was added to apical or basolateral side of Caco-2 cell monolayers and GSH/GSSG ratio (A) and caspase-3 activation (B) were evaluated after 1, 24 and 48 hours. *p<0,05 vs control; #p<0,05 vs apical gp120 stimulation at the same time. After gp120 stimulation cells were collected and western blots were performed with anti-gp120 polyclonal antibody (upper panel) and anti-caspase-3 monoclonal antibody (middle panel). Normalization of western blot was performed with GAPDH in all experiments (lower panel).

Tat-induced oxidative stress and apoptosis are strongly inhibited by pretreatment with antioxidants

Our findings demonstrate that Tat induces oxidative stress and apoptosis in human enterocytes. These two events may be causally related in several pathogenic conditions [36]. We therefore used the antioxidant NAC to determine the relationship between oxidative stress and apoptosis induced by Tat at intestinal level. Our results demonstrate that oxidative stress is completely prevented by pretreatment with NAC. In fact, NAC prevented the Tat-induced ROS increase (Fig. 9A) and preserved the GSH/ GSSG ratio (Fig. 9B).

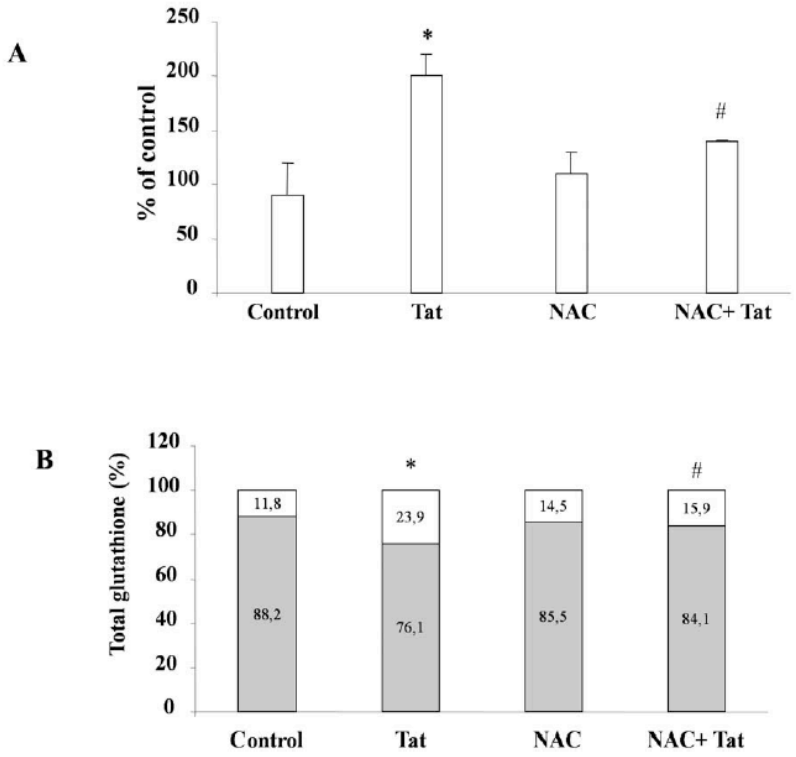

Figure 9. Effect of NAC on the Tat-induced oxidative stress in Caco-2 cells. Intracellular ROS levels, determined by fluorometric method, after exposure of Tat with or without pretreatment with NAC (A). Data were represented as percent of controls. Effect of NAC on Tat-induced GSH/ GSSG imbalance (B). Data are represented as percent of GSH (grey) and GSSG (white) vs total glutathione. *p<0.05 vs control; #p<0.05 vs Tat. Data are representative of 3 separate experiments.

To verify that the redox imbalance induced by Tat was the major cause of cell apoptosis, we performed experiments under a condition of oxidative stress prevention. Pretreatment for 24 hours with NAC completely prevented caspase-3 activity induced by Tat (Fig. 10A), suggesting that the redox imbalance caused apoptosis in our experimental model. We also evaluated caspase-3 and PARP cleavage by western blot and found that NAC prevented activation of caspase-3 signaling (Fig. 10B). Similar results were found in HT-29 cell line. These results support the hypothesis that oxidative stress induced by Tat induces programmed cell death.

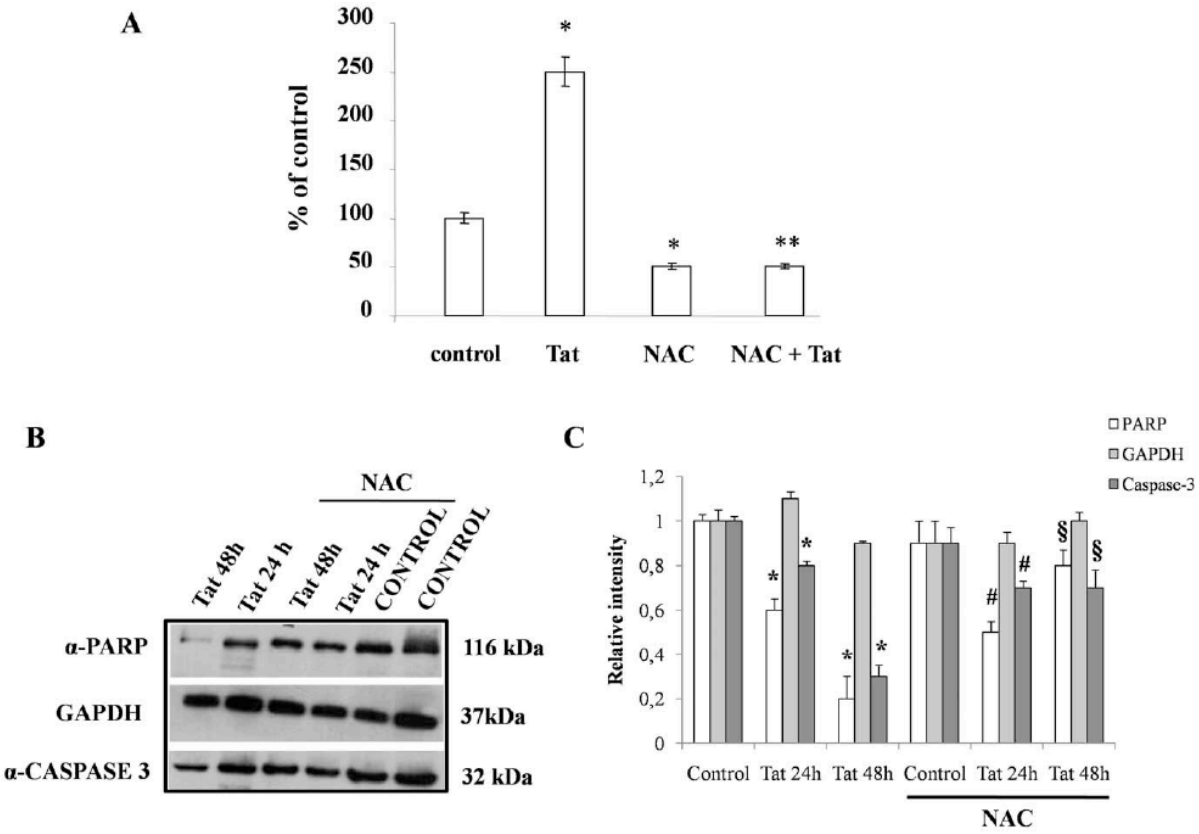

Figure 10. Effect of NAC on the Tat-induced apoptosis in Caco-2 cells. Caspase-3 activity (A) and full-length protein (B lower panel) were evaluated after exposure of Tat with or without pretreatment with NAC. Data were represented as percent of controls. *p<0,05 vs control; **p<0,05 vs Tat. Cleaved PARP was evaluated in the same western blot (B upper panel). Normalization of western blot was performed with GAPDH in all experiments (B middle panel). Data are representative of 3 separate experiments. (C) Densitometric acquisitions are shown from three separate experiments. *p<0,05 vs control; #p<0,05 vs control with NAC; §p<0,05 vs Tat at the same time without NAC.

Intestinal oxidative stress and apoptosis are related and prevented by the antioxidant NAC in human intestinal specimens

To determine whether the findings observed in Caco-2 cells were reproduced in human intestine, we evaluated apoptosis and redox intracellular homeostasis in duodenal biopsies from children (Fig. 11A). Western blot analysis for caspase-3 was performed on human intestinal specimens obtained from 4 HIV- negative patients. Similar to the findings we obtained in in-vitro cell models, exposure of human duodenal mucosa to Tat resulted in caspase-3 activation, as shown by the increase of the proteolytic 17-kDa fragment from caspase-3. Similar to the results we obtained in Caco-2 cells, this process was prevented by NAC (Fig. 11B).

Duodenal biopsies were obtained also from a single HIV- positive child to evaluate apoptosis and redox mechanisms in an ex-vivo model. Caspase-3 activation was determined in basal conditions and in the presence of NAC. Caspase-3 activation in specimens from the child was prevented by NAC (Fig. 11C).

Finally, we measured the GSH/GSSG ratio in intestinal biopsies. The ratio was decreased upon Tat exposure and the imbalance was prevented by NAC pretreatment in intestinal mucosa of HIV-negative patients (Fig. 11D). Interestingly, the baseline GSH/GSSG ratio in the duodenal tissue of an HIV-positive child (Fig. 11E) was similar to that observed in HIV- negative mucosa treated with Tat (Fig. 11D). However, it decreased after treatment with NAC and became similar to HIV-negative controls. These data strongly suggest that intestinal glutathione modifications are directly related to apoptosis in HIV- patients and that Tat, which was prevented by antioxidant treatment in human intestine, plays a key role in this condition.

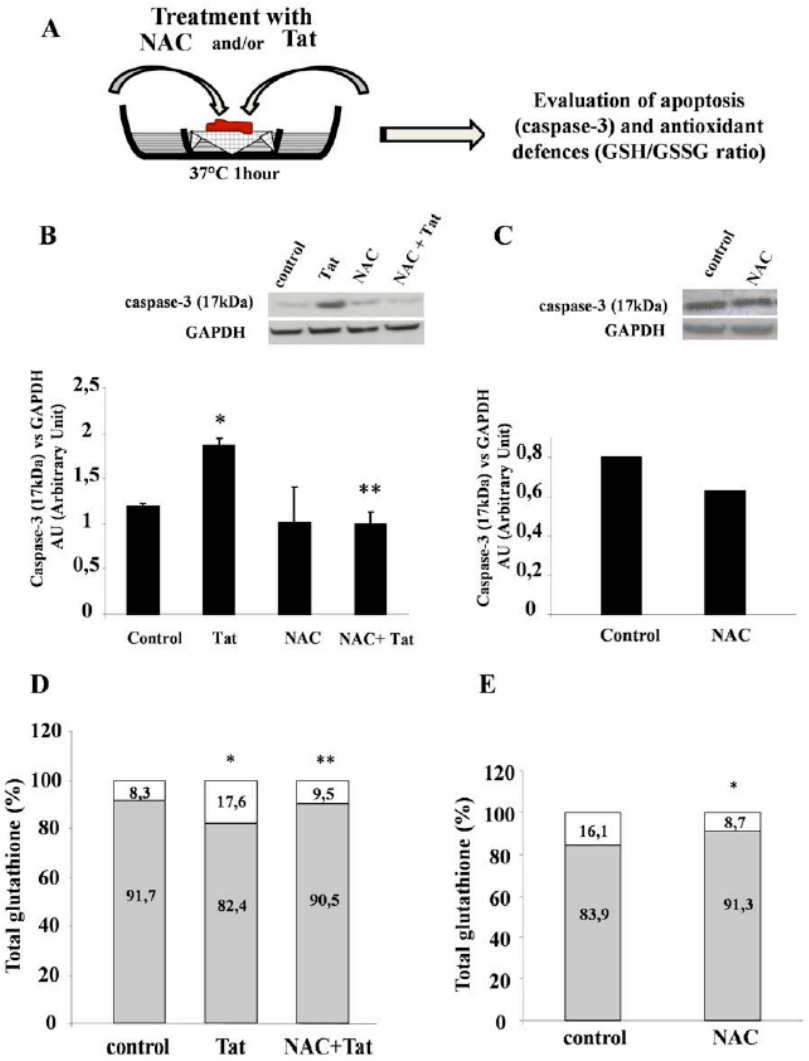

Figure 11. NAC prevented apoptosis and oxidative stress induced by Tat in human intestinal mucosa. Duodenal mucosal specimens were exposed to Tat alone or combined with NAC in an *ex-vivo* organ culture model (A). Panels B and C, Western blot on whole cell lysate was performed using anti-caspase-3 mouse monoclonal antibody (upper panel) and anti-GAPDH mouse monoclonal antibody in 4 HIV-negative children (B) and in an HIV-positive child (C). Densitometric acquisition of caspase-3 protein was normalized for GAPDH expression. Panels D and E, The GSH (grey)/GSSG (white) ratio was evaluated as described under "Methods" in intestinal mucosa of 4 HIV-negative children (D) and in an HIV-positive child (E). *$p<0.05$ vs control; **$p<0.05$ vs Tat. Results are expressed as the means SEM.

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Vitamin D restores autophagic pathway and reduces cytotoxic effect of Rotavirus infection in human enterocytes

Article in writing

INTRODUCTION

Rotavirus (RV) is one of the leading causes of diarrhea in children and one of the major cause of infantile mortality worldwide. Before the introduction of the RV vaccination, 700,000 visits per year were estimated because of RV-associated gastroenteritis (RVGE) in children younger than 5 years in Europe[1]. Scientific authorities recently stressed the need for research efforts to identify possible therapeutic targets and to investigate environmental and host factors, such as immune function, that may affect outcomes in children with infectious diarrhea[2]. RVGE pathogenesis is not completely understood. Its enterotoxicity is retained to be the result of the impaired uptake of fluids and nutrients by the enterocytes, the proliferation of secretory crypt cells, and the active enterocyte chloride secretion driven by the non-structural protein NSP4[1,3]. As for the RV immunity, protective RV response is considered to be multifactorial, but reliable humoral correlates have not been identified, as well as innate immunity has been relatively unexplored[4]. Recent evidence suggests that autophagy is involved in the RV pathogenesis. Autophagy was historically identified as a homeostatic pathway that delivers long-lived proteins and entire organelles for lysosomal degradation[5]. This process has been increasingly studied and it is now recognized to be part of multiple biological processes including innate immune response to viruses[6]. On the other hand, there is evidence that several viruses have evolved virulence factors capable to subvert the autophagy to their own advantage[7]. Rotavirus induces the formation of autophagic vacuoles through the NSP4-driven Ca2+ influx in the endoplasmic reticulum and Ca2+/calmodulin-

dependent kinase kinase-β activation, but impairs the autophagosome delivery to the lysosomes, thus creating sites of efficient viral replication[8].

The active form of vitamin D, 1,25(OH)2-dihydroxivitamin D3 (1,25D3), is involved in a variety of biological effects, including immune response, and its receptor is highly expressed in metabolic tissues such as intestine. Several studies have shown that 1,25D3 induces autophagy through different mechanisms. It increases cytosolic Ca2+ thus inhibiting mTOR, which turns off autophagy[9], and decreases the inhibition of Bcl-2 on Beclin-1, thus promoting autophagy, in breast cancer cells, where it finally exerts proapoptotic effect. 1,25D3 upregulates Beclin-1 in myeloid leukemia cells, in turn promoting differentiation and apoptosis[10]. In macrophages, 1,25D3 recruits cathelicidin LL-37 into autophagosomes in a process that involves Ca2+/calmodulin-dependent kinase kinase-β and AMP-activated kinase, which in turn allows maturation of the autophagosomes and the intracellular killing of mycobacteria[11].

The health promoting effects of 1,25D3 are even more evident if we consider that vitamin D deficiency is associated to higher risk of diarrhea, respiratory infections, severe tuberculosis disease[12-14].

The aim of our study was to explore the effect of 1,25D3 on RV-infected human enterocytes, namely on the restore of the autophagy and on viral replication, as well as on RV-related cytotoxic and enterotoxic effects.

MATERIALS AND METHODS

Cell and cultures

Caco-2 cells were used as small intestinal cell model[15]. At 15 days post-confluence, cells exhibit a well-differentiated brush border on the apical surface and tight junctions with

typical small- intestinal microvillus hydrolases and nutrient transporters. Caco-2 cells were grown in Dulbecco's modified Eagle minimum essential medium (DMEM; Gibco, USA) with a high glucose concentration (4.5 g/L) at 37uC in a 5% CO2 atmosphere. The medium was supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 1% non-essential amino acids, penicillin (50 mU/mL) and streptomycin (50 mg/mL), and changed daily. To verify some data obtained in Caco-2 cells, we used HT-29 as a supplementary intestinal cell model. HT-29 were grown in RPMI 1640 cell culture medium supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 100 mU/ml penicillin and 100 mg/ml streptomycin. Viability was measured through a Trypan Blue assay.

Polimorphonucleates were extracted from peripheral blood through Ficoll at 2000 rpm for 45'min at 4°C. Red blood cells were separated with dextran 6% and hypotonic lysis with NaCl 0.2% e 1.6%.

Rotavirus infection and modulation

The simian rotavirus strain SA11 is well characterized and is able to replicate to high titers in Caco-2 cells[15,16].Virus activation and cell infections were performed in differentiated Caco-2 cells as reported elsewhere[17]. Briefly, the virus was activated with 20 mg/mL trypsin for 30 min at 37°C. Confluent monolayers of Caco-2 cells were washed twice and incubated overnight in fetal calf serum–free medium before virus infection. Viral suspension was added to the apical side of the cell monolayer. After 60 min of incubation at 37°C, the cells were rinsed 3 times and incubated in fetal calf serum–free medium for the established times after infection. The time after infection was started after removal of the excess viral particles. 1,25-dihydroxi-vitamin D3 was purchased by Sigma and diluted at the indicated concentrations starting from a stock of 100 uM in ethanol.

Transepithelial resistance measurements

Transepithelial resistance of cell monolayers grown on filters was measured using a Millicel-ERS resistance monitoring apparatus (Milli- pore). The net resistance was calculated by subtracting the background from the actual value and multiplying the value obtained by the area of the filter (4.9 cm2). The resistance was expressed in ohms/cm2. Transepithelial resistance was measured at 1 h intervals for the first 6 h after infection, then at 6 h intervals for the first 24 h, and subsequently every 12 h for 3 days.

Immunoblotting

Total cells lysates were obtained by homogenization of cell pellets in cold lysis buffer (20 mM Tris, pH 7.5 containing 300 mM sucrose, 60 mM KC1, 15 mM NaC1, 5% (v/v) glycerol, 2 mM EDTA, 1% (v/v) Triton X-100, 1 mM PMSF, 2 mg/ml aprotinin, 2 mg/ml leupeptin and 0.2% (w/v) deoxycholate) for 1 min at 4uC and further sonication for additional 30 sec at 4uC. Equal amounts of protein were subjected to 10% (v/v) SDS-PAGE and transferred to a PVDF membrane (Millipore). The membrane was blocked with 5% (w/v) skim milk and incubated with primary antibody, followed by incubation with an HRP-conjugated secondary antibody. Proteins were visualized with an ECL detection system (GE-Healthcare). The following antibodies were used for Western blot analysis: Sono stati utilizzati i seguenti anticorpi primari: mouse anti-tubulin (Sigma Aldrich, 1:2000), mouse anti-Caspase-3 full-lenght (Santa Cruz Biotechnology, 1:1000), rabbit anti-LC3 I-II (Novus, 1:700), rabbit anti-cleaved Caspase-3 (abcam, 1:700), mouse anti LL-37 (Hycult Biotech, 1:500).

To study autophagy pathway, the endosomal acidification inhibitor Bafilomycin-1a (BAFA, Sigma) was used according to manifacturer at 100 nM.

Immunofluorescence

One step of fixation and permeabilization was performed with 4% paraformaldehyde and 0.1% Triton X-100 for 30 min at +4uC. After three washes in PBS, cells were incubated with primary antibodies at 4°C overnight and sunsequently with FITC/TRITC conjugated secondary antibodies. In selected experiments, Lysotracker (Invitrogen) was used for lysosome staining at 60 nM in DMSO. The monolayers were examined using a Nikon Eclipse 80i epifluorescent microscope. The images were analyzed using NIS Elements D imaging software. A confocal microscope was used for LC3 staining (LSM 510 Zeiss).

Statistical analysis

We used GraphPad Prism Software (San Diego, CA) to evaluate the two-tailed unpaired Student t test and a 2-tailed paired Student t test to evaluate statistical significance. An alpha value of 0.05 was set for statistical significance. p-Values for each analysis are indicated in figure legends.

RESULTS

Autophagy is impaired in RV-infected human enterocytes.

Autophagosome formation was evaluated through immunoblot and with immunofluorescence with LC3-I and LC3-II staining. As judged by the LC3-II staining at immunoblotting, Autophagosome formation is increased in RV infected cells and in starved (FBS-free medium) cells, with regard to control cells (Fig 1). Similarly, the proportion of cells with > 7 LC3 dots per cells were higher in RV-infected cells at 8 hours post infection with regards to non infected cells. (Figure 2).

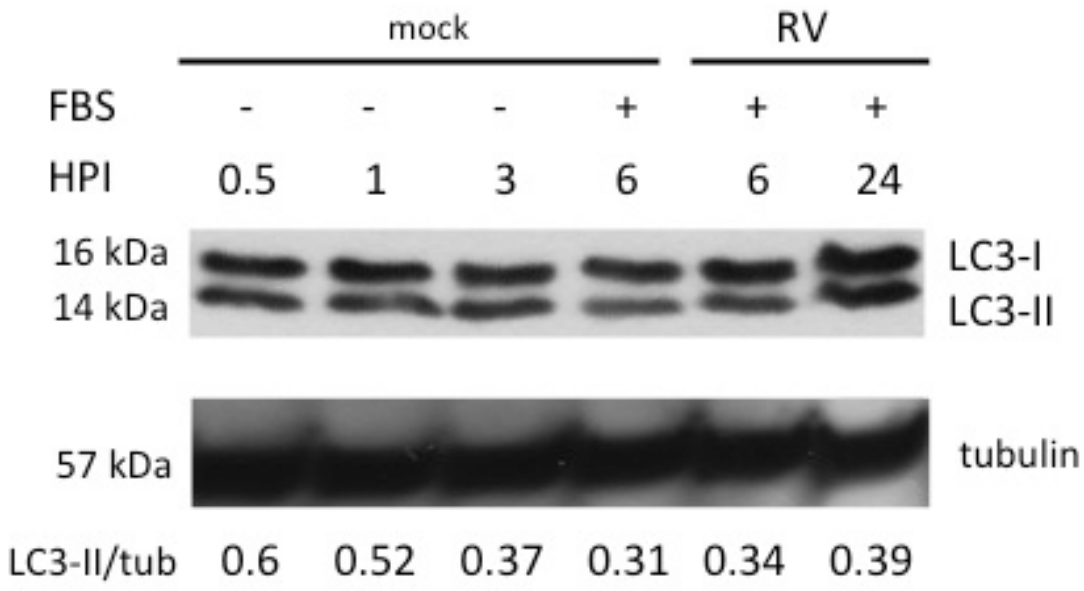

Figure 1- LC3-II expression at immunoblotting on Caco-2 cells infected by RV.
FBS: Fetal bovune serum; HPI: Hours post-infection. Mock: non infected cells. RV: cells infected with RV 25 PFU. FBS-free medium was used as positive control (starvation).

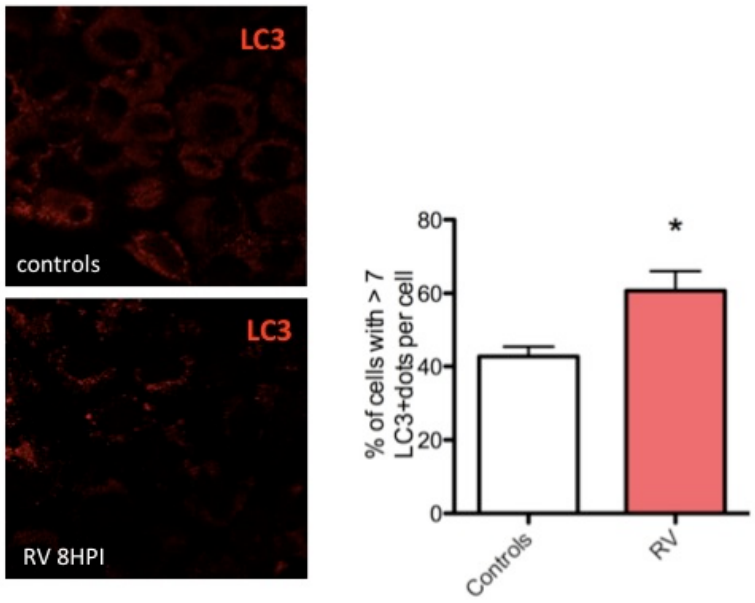

Figure 2: LC3 immunofluorescence in RV infected cells.
HPI: hours post infection; *p<.05.

However, autophagosome (LC3) and lysosomal (LAMP-2) markers did not colocalize at immunofluorescence, suggesting impairment of autophagic pathway (Figure 3). Similarly,

LC3-II did not increase in RV-infected cells treated with Bafylomicin, suggesting a blockage of the autophagosome-lysosome fusion (Figure 4).

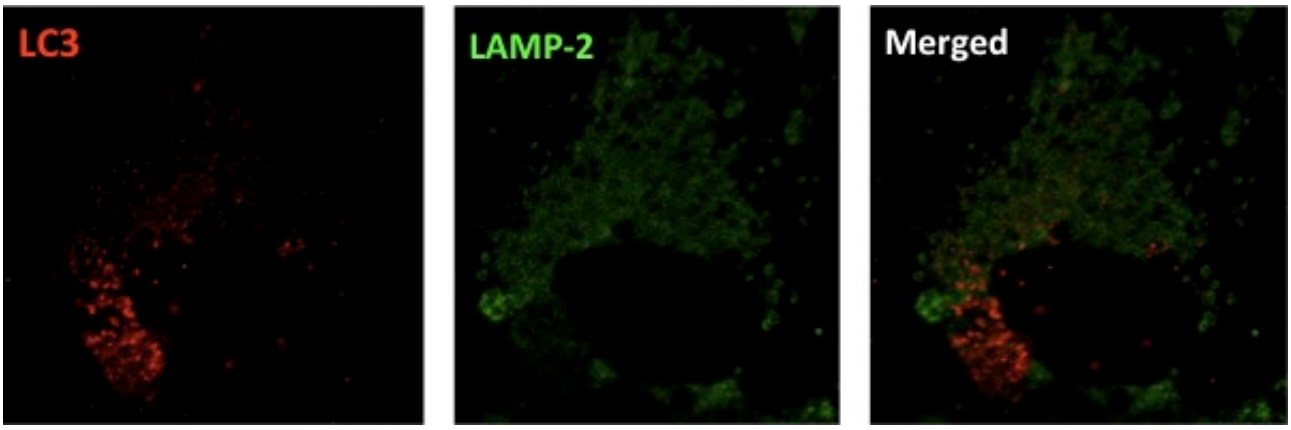

Figure 3: Confocal microscopy of Caco-2 cells infected with RV 25 PFU 8 hours post infection.

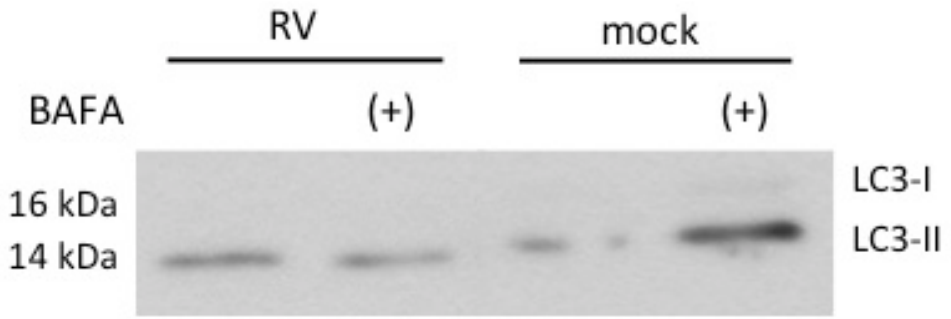

Figure 4: LC3-II expression at immunoblotting in RV-infected Caco-2 cells and in controls. BAFA: cells treated with Bafylomicin A1 100 nM; Mock: control cells.

1,25D3 restores autophagosome maturation

In 1,25 pretreated Caco-2 cells exposed to Bafylomicin A1 the LC3-II was increased with regard to the same cells not exposed to the lysosomal acidification inhibitor, indicating that 1,25 vitamin D restores competent autophagy during RV infection (Figure 5). The restore of the autophagosome-lysosome maturation was also confirmed by colocalization experiments with LC3-II and LAMP-2 immunofluorescence (Figure 6).

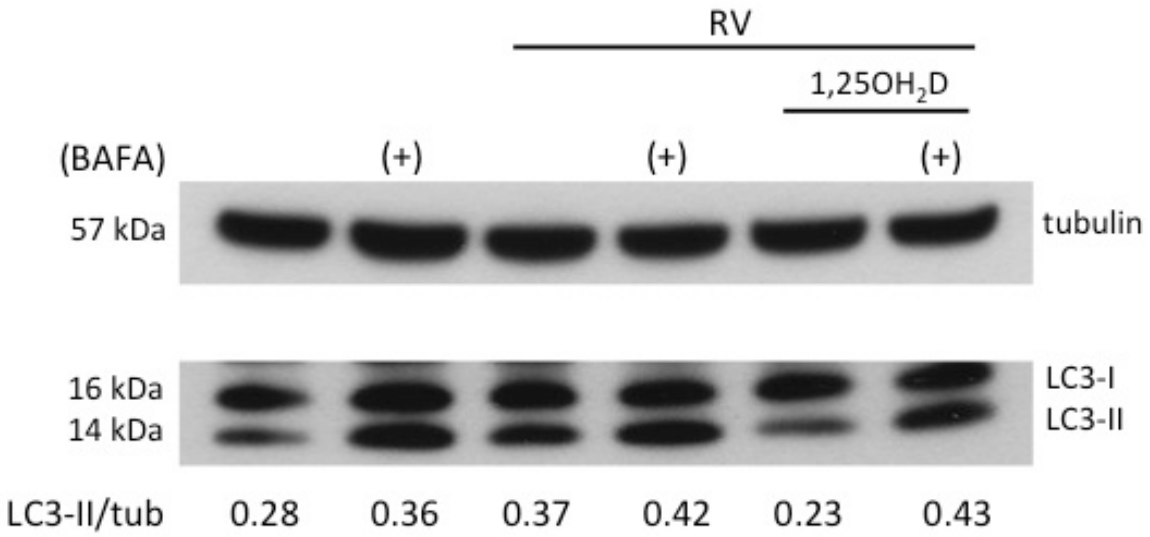

Figure 5: LC3-II expression at immunoblotting in RV-infected Caco-2 cells and in controls. 1,25OH2D: cells pretreated with 1,25D3; BAFA: cells treated with Bafylomicin A1 100 nM; Mock: control cells.

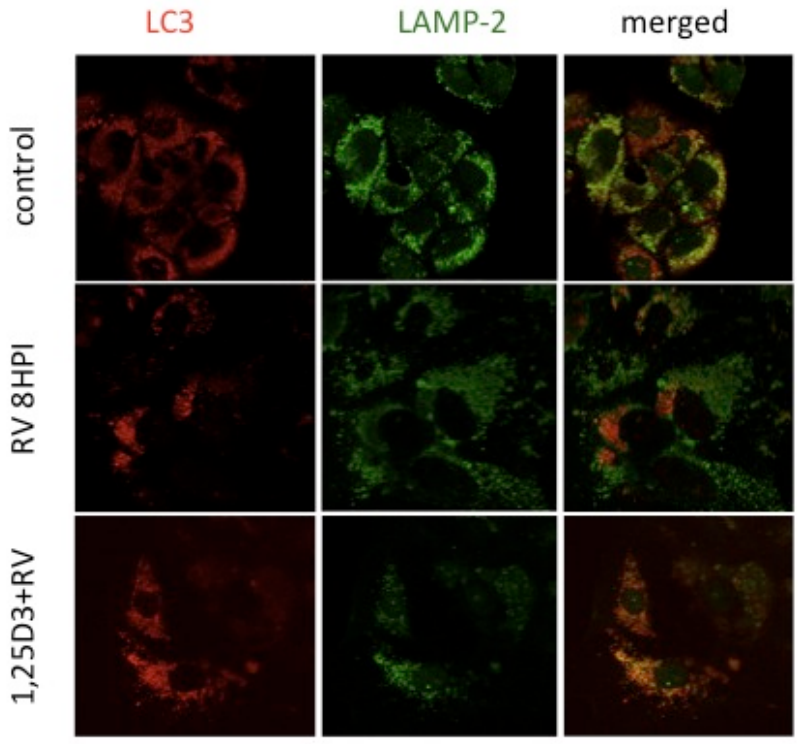

Figure 6: Confocal microscopy of Caco-2 cells. RV: cells infected with RV 25 PFU 8 hours post infection. 1,25D3: cells pretreated with 1,25OH2D3.

LL-37 is upregulated in RV-infected cells

The human cathelicidin LL-37 is upregulated in RV-infected cells as judged by immunofluorescence (Figure 7) and through ELISA assay for LL.37 in cell lysates (Figure 8). Both experiments also show that 1,25D3 increase the RV-induced LL-37 upregulation. These data were confirmed also on cell supernatants (data not shown).

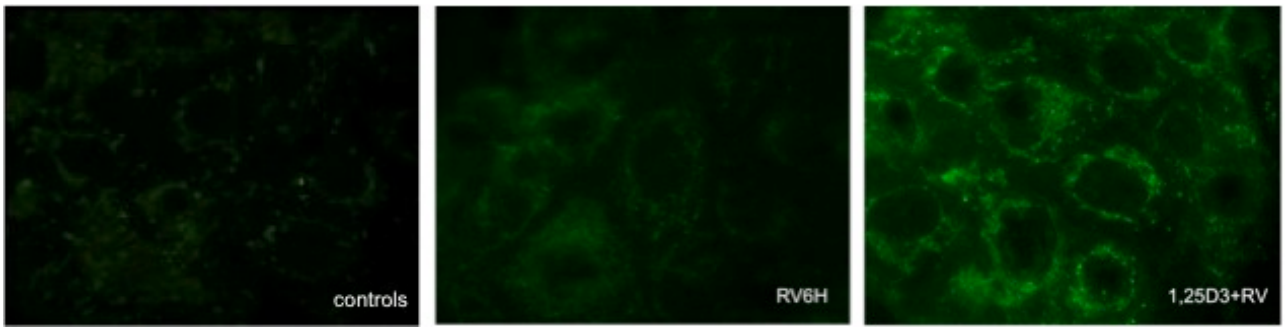

Figure 7: immunofluorescence with anti-LL-37 antibodies in controls, RV-infected and RV-infected 1,25D3 pretreated Caco-2 cells.

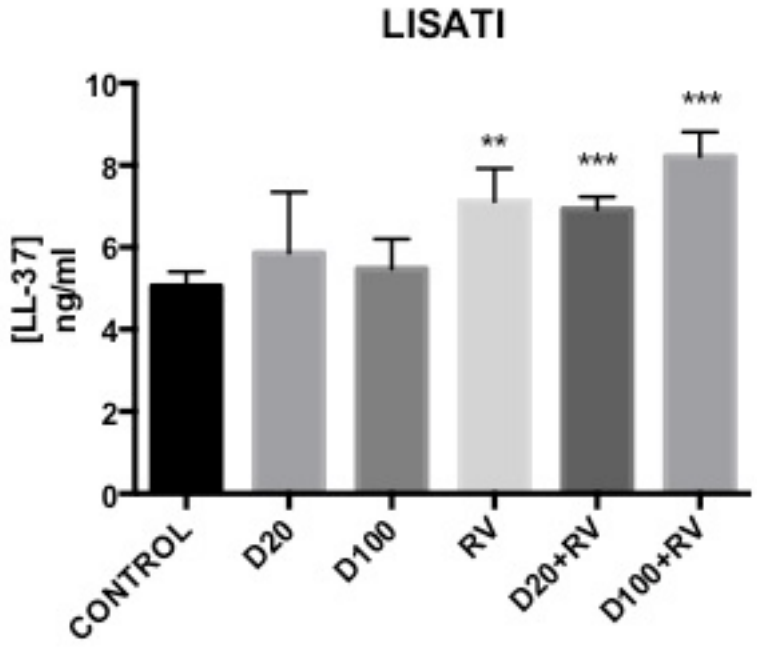

Figure 8: LL-37 ELISA assay on Caco-2 cell lysates. D20: 1,25D3 20 nM; D100: 1,25D3 100 nM; RV: RV-infected cells at 8 hours post infection.

1,25D3 reduce the RV-induced cytotoxic effect

Pretreatment with 1,25D3 reduces the cytotoxic effect of RV infection in Caco-2 cells as assessed through transepithelial resistance assay. TER values of RV infected cells are significantly higher in 1,25D3 pretreated cells with respect to non pretreated cells (Figure 9). Finally, apoptosis is reduced in 1,25D3-pretreated cells at 24 hpurs post infection when compared to non pretreated cells, thus indicating that activated vitamin D reduces epithelial apoptosis and permeability (Figure 10)

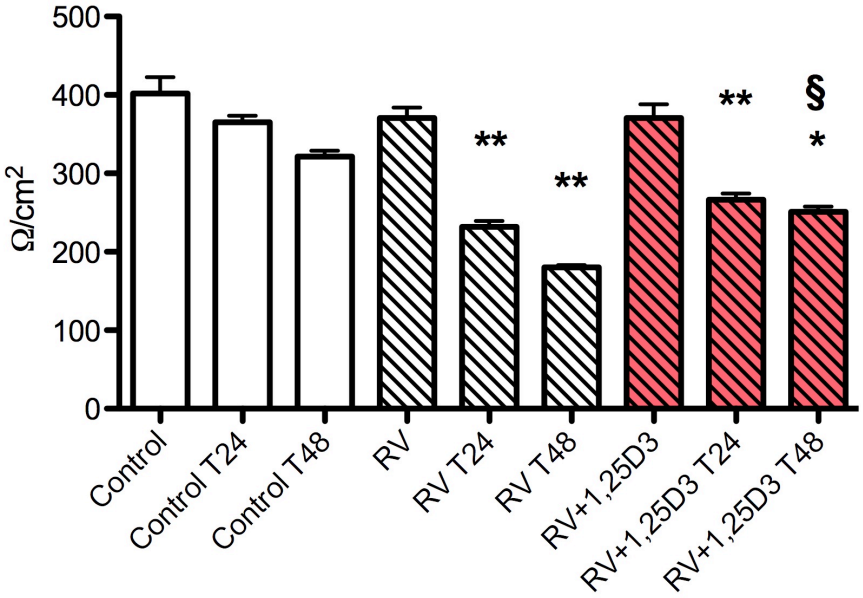

Figura 9: Transepithelial Electric Resistance (TER) at baseline and 24-48 hours post-infection in RV-infected Caco-2 cells with and without 1,25$(OH)_2$D 100 nM. *p < .0001 e **p < .001 vs controls; §p < .0001 versus RV.

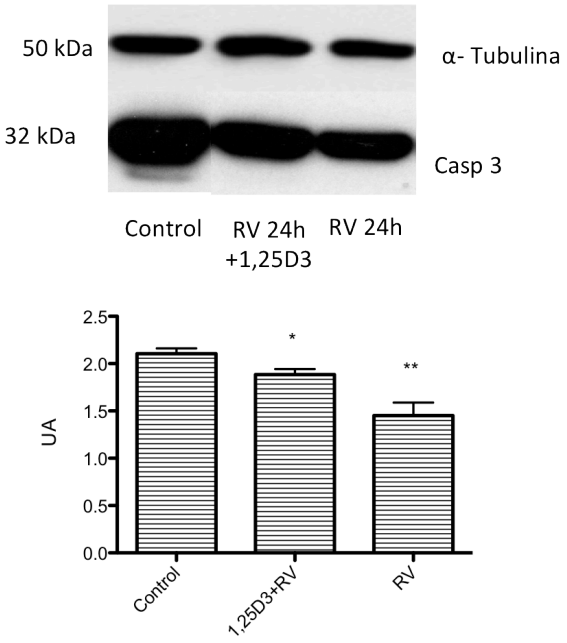

Figura 10: Caspase 3 expression in RV-infected Caco-2 cells after RV 24h post-infection: RV 24h + 1,25D3: cells pretreated with 1,25D3 100nM. *p<.05; **p<.005 vs controls.

DISCUSSION

RVGE pathogenesis is not completely understood. Diarrhea is due to diverse mechanisms: the most recognized mechanism can be defined “enterotoxic”, which is NSP4-dependent and ultimately consists of a rapid and transient chloride secretion. The delayed effects are less clear.

It is accepted, by the way, that RV induce apoptosis, in a time-dependent manner, and that active replication is needed for that effect.

Our model provides an insight in the cell biology of the RV-infected human enterocyte. According to the newest evidence, autophagy could be “highjacked” by RV, that may be able to replicate in inactive autophagosomes. The autophagy impairment could ultimately lead to cell death in different pathways. We provided the first evidence that the active form of vitamin D, 1,25D3, can prevent autophagy blockage thus protecting the enterocyte from cytotoxic effects. In this perspective, vitamin D could be a candidate adjunctive treatment of RVGE.

1,25D3 also increase the LL-37 upregulation that occurs during the RV infection. Based on our data, it is impossible to say if 1,25D3 effect are mediated by LL-37, such as in other cell models, or they just occur along wityh an upregulation of LL-37.

Many other mechanisms are supposed to be involved in RVGE vivo, such as the immune system cross-talk and LL-37 production, as well as a possible role of vitamin D in epithelial differentiation or the important role of the enteric nervous system. However, the resulting effect of any candidate antidiarrheal drug should be better evaluated in vivo.

Moreover, we only assessed the effect of pretreating enterocytes with 1,25D3, but further experiments with 1,25D3 used after RV infection are warranted.

Even with all these limitations, our observations make 1,25 a promising and feasible treatment for acute diarrhea of the children, in adjunct to oral rehydration and probiotic administration.

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Conclusions

Health models can be very different, changing with disease, setting, population, and expected outcomes. Improving the pattern of care can be achieved with a wide spectrum of strategies. All these strategies share few important points: a correct analysis of the practice and of the needs, a reliable measure of the performances, continuous re-thinking of the ongoing processes. Health systems are dealing with a lack of economic resources: in this scenario, it is even more important that health processes be smart and prone to change, to realize a proper learning organization and a real clinical governance.

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Recovery of graft steatosis and protein-losing enteropathy after biliary diversion in a PFIC 1 liver transplanted child

Nicastro E, Stephenne X, Smets F, Fusaro F, de Magnée C, Reding R, Sokal EM. Recovery of graft steatosis and protein-losing enteropathy after biliary diversion in a PFIC 1 liver transplanted child.

Abstract: PFIC 1 is a genetic disorder characterized by hepatic and gastrointestinal disease, often requiring LT during childhood. Extrahepatic symptoms, such as diarrhea and malabsorption, do not improve or may be aggravated after LT, as graft steatosis or steatohepatitis as consequences of the interaction between transplanted liver and native bowel. We describe a patient with PFIC 1 who presented with cholestasis in infancy, who developed intractable pruritus and liver fibrosis. The child underwent living donor LT at 3.6 yr of age, and he early developed severe refractory diarrhea, secondary malabsorption with protein-losing enteropathy, and an early fatty liver disease trough graft steatohepatitis. As the response to cholestyramine was unsatisfactory, we decided to perform an EBD by using the jejunal loop used for the cholangiojejunostomy. Diarrhea resolved rapidly after surgery. He remained well after six months following biliary diversion, with normal stool output and no protein loss. We documented a dramatic improvement of graft steatosis at histology as well as normalization of liver function test. EBD can be considered a valuable treatment option to avoid organ disfunction and loss in PFIC 1 transplanted patients who develop graft steatohepatitis.

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Key words: progressive familial intrahepatic cholestasis type 1 – liver transplantation – diarrhea – protein-losing enteropathy – graft steatohepatitis – external biliary diversion

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PFIC constitutes a heterogeneous group of autosomal recessive disorders caused by mutations in genes involved in bile formation leading to cholestastis of hepatocellular origin (1). PFIC 1, previously called Byler disease, is caused by mutations in *ATP8B1* gene, encoding the ''flippase'' FIC1 expressed on the canalicular pole of the hepatocyte responsible for the enrichment of phosphatidylserine and phosphatidylethanolamine on the inner leaflet of the plasma membrane. FIC1 is also expressed on cholangiocytes and enterocytes, where it is likely to be involved in intestinal bile acid reabsorption and in enterohepatic bile acid circulation. PFIC 1 patients usually present with cholestasis characterized by pruritus and jaundice, often accompanied by intractable diarrhea and growth retardation, and they invariably progress to liver cirrhosis before adulthood; in some patients, gastrointestinal symptoms predominate (2–4). Patients with end stage liver disease, severe pruritus or severe growth retardation are candidates for LT, whereas either PEBD or ileal bypass are considered in cases without cirrhosis (5). It is well known that the liver allograft can be affected by severe steatosis. A variable percentage of patients with post-transplant steatosis progress to steatohepatitis and cirrhosis with possible need of retransplantation (6, 7). Another recognized feature in transplant course of patients with PFIC 1 is the onset or the exacerbation of diarrhea. Interestingly, post-transplant steatosis

Abbreviations: aPTT, activated partial thromboplastin time; BSEP, bile salt export pump; CB, conjugated bilirubin; EBD, external biliary diversion; LT, liver transplantation; NSE, neuron-specific enolase; PEBD, partial external biliary diversion; PFIC 1, progressive familial intrahepatic cholestasis type 1; PT, prothrombin time; TB, total bilirubin; UDCA, ursodeoxycholic acid.

has been documented primarily in patients with diarrhea, and it has been hypothesized that the fatty liver disease spectrum affecting the allograft could be the result of the interaction between the native bowel and the graft liver by diverse mechanisms. Indeed, in a patient with post-transplant diarrhea, symptoms disappeared during a transitional biliary diversion performed for a bile leakage (6).

We describe for the first time the successful treatment of post-transplant refractory diarrhea and secondary protein-losing enteropathy, along with histologically documented graft recovery from post-transplant steatohepatitis, with an EBD in a child with PFIC 1.

Case report

A male infant was born at term to healthy parents, with no familial history of liver disease, and a birth weight of 3660 g. At five months of age, he developed jaundice with dark urine. Growth and development were normal on breast feeding. He had mild hepatomegaly and otherwise normal clinical examination. Liver function tests showed hyperbilirubinemia (TB and CB 5.7 and 1.5 mg/dL, respectively) and mild elevation of aminotransferases (AST 85 IU/L; ALT 58 IU/L); γGT was in the normal range; hepatic synthetic function was normal. Serology for infectious hepatitis was negative. Alpha-1 antitrypsin deficiency and cystic fibrosis were ruled out. Serum total bile salts reached 147 μM (NV < 10). Ophthalmologic examination and spine radiography were unremarkable. Liver ultrasound revealed normal texture and dimension with no focal lesions. Normal gallbladder and biliary tree were shown at cholangio-MRI. Histology showed features of intrahepatic cholestasis with no ductular proliferation. A diagnosis of "low-γGT PFIC" (PFIC 1 or 2) was established and the patient was given UDCA at a dose of 20 mg/kg/day.

The child was referred to our center at the age of 42 months, with intractable pruritus despite UDCA and rifampicin; he was otherwise in good general condition. He had generalized scratch lesions, with no hepatomegaly and no sign of portal hypertension. Blood test showed mild hyperbilirubinemia (TB/CB 3.1/1.8 mg/dL) with normal hepatobiliary enzymes, as well as normal serum total proteins, albumin, and coagulation tests. Liver Doppler ultrasound showed heterogeneous parenchymal structure suggesting fibrosis, with no signs of portal hypertension. Liver histology confirmed portal fibrosis, with mild ductular proliferation, and marked cholestasis.

Living-related donor LT (maternal donor) was performed at the age of 43 months (3.6 yr), with tacrolimus, daclizumab, and mycophenolic acid immunosuppression. Immunohistochemistry showed normal BSEP canalicular staining (courtesy Prof. Tania Roskams). Molecular analysis for *ATP8B1* documented double heterozygosis for the missense mutations p.D554N (c.1660 G > A, exon 16) and p.D734A (c.2199 A > C, exon 19), the latter being novel, according to the diagnosis of PFIC 1 (Dr. Christiane Baussan).

Following LT, pruritus disappeared and serum bilirubin concentration normalized. However, diarrhea appeared progressively within one month following LT, with 6–7 liquid stools/day, and limited improvement with fasting. Repeated stool cultures and parasite examination remained negative, and there was no response to bowel decontamination using oral administration of metronidazole and paromomycin. Although raised values of vasoactive intestinal peptide (VIP) (297 pg/mL, NV 15–65) and NSE (27.2 μg/L, NV < 15) were observed, the absence of abnormal uptake at In^{111}-penteotride and I^{123}-MIBG scintigraphy ruled out a neuroendocrine tumor as the cause of secretory diarrhea. Urinary catecholamines were normal. Abdominal ultrasound examining the bowel wall was unremarkable. Liver ultrasound showed progressive hyperechogenicity of the liver parenchyma. Diarrhea was proposed to be secondary to restored bile flow through the native bowel, and treatment with cholestyramine was administered at a dose of 2 g four times per day. UDCA and mycophenolic acid were discontinued. This led to improvement of the diarrhea (less liquid) and decreased stool output (3–4 stools/day). Liver biopsies at two and three months after LT showed severe macrovacuolar steatosis with mild fibrotic enlargement of the portal spaces (Fig. 1a).

The child returned to his home country on cholestyramine 8 g/day in four doses, with an output of four stools/day, and tacrolimus monotherapy as immunosuppression. Nine months after LT, the child lost weight and developed hypoalbuminemia requiring substitutive treatment and frequent admissions to a university hospital in his home country.

He was referred again to our department two yr after LT, with worsening diarrhea and severe hypoalbuminemia (1.4 g/dL). Serum total protein and immunoglobulin levels were also diminished, along with elevation of the hepatobiliary enzymes (ALT 201 IU/L; γGT 94 IU/L). There was no urinary protein excretion, with

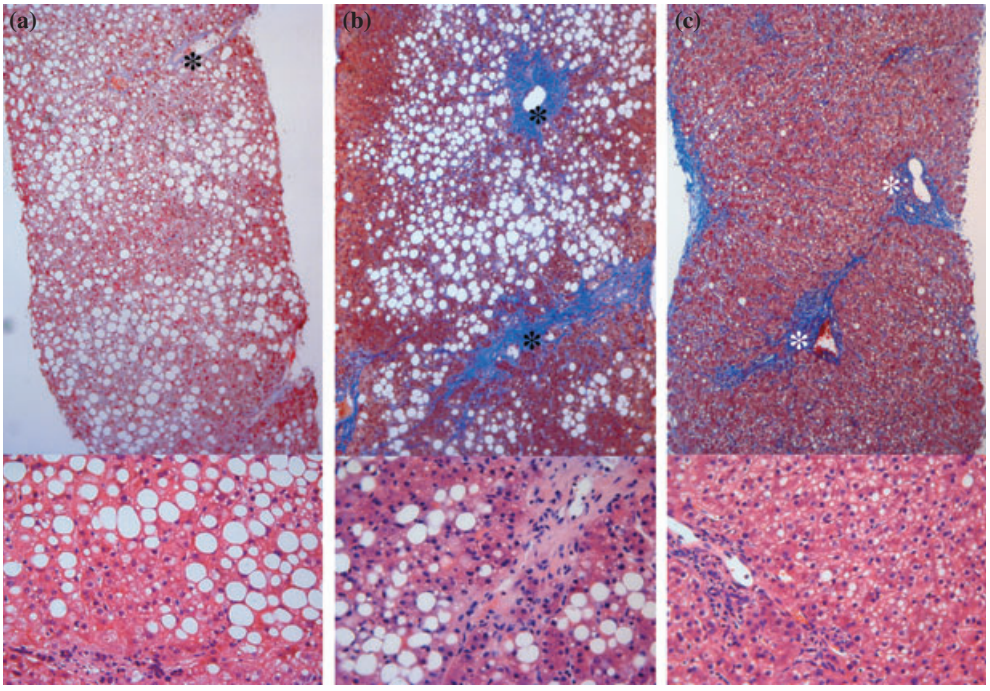

Fig. 1. Liver biopsies of the patient three months (a) and 24 months (b) after LT, and six months after EBD (30 months after LT; c). Both upper (trichrome stains, original magnification ×200) and lower pictures (hematoxylin and eosin, original magnification ×1000) show the diffuse macrovacuolar steatosis and the worsening fibrosis originating from the portal tracts (*). Six months after EBD, a dramatic improvement of the steatosis can be observed (c).

normal renal function and kidney morphology on ultrasound. Echocardiography was unremarkable. Stool cultures were negative for bacteria, parasites, and amoeba. Upper endoscopic examination was normal, with normal duodenal and gastric biopsies, and negative tests for *Giardia* spp. and *Helicobacter pylori*. Fecal *Clostridium difficile* enterotoxin A was negative on immunoassay. Fecal pancreatic elastase-1 was normal. In^{111}-transferrin scintigraphy detected diffuse activity in the small intestine that reached the colon one h post-injection, and the protein loss calculated as a fraction of the whole body retention after 72 h was 108 mL plasma equivalent/day. This confirmed the diagnosis of protein-losing enteropathy, which was attributed to the restored bile flow in the native PFIC 1 small bowel. Liver histology was characterized by fibrous septa of centrilobular origin, associated with the severe macrovacuolar steatosis previously observed (Fig. 1b).

An EBD of the Roux-en-Y loop was carried out 28 months after LT. Briefly, the distal part of the Roux loop was disconnected from the bowel tract and open as a terminal jejunostomy in the right hypochondrium (Fig. 2). The intestinal biopsies performed intraoperatively resulted normal.

Stool frequency normalized within one wk after EBD. Serum albumin and total protein concentrations, as well as immunoglobulin levels returned to normal. Intestinal protein loss assessed at In^{111}-transferrin scintigraphy was reduced by 60% two months later and was

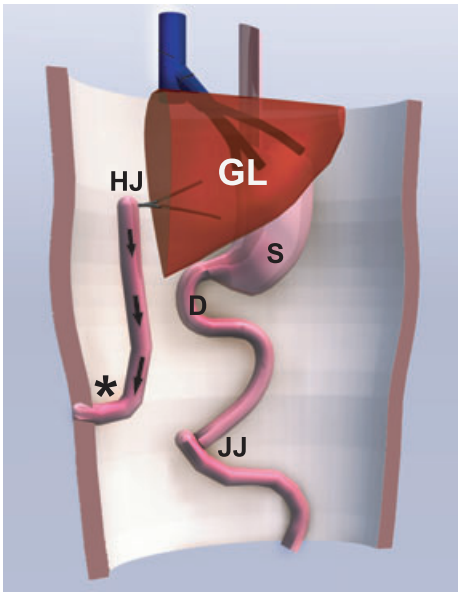

Fig. 2. Diagram of the surgical procedure of the EBD. The distal part of the Roux-en-Y loop was disconnected from the bowel tract and a terminal jejunostomy (*) was open in the right hypochondrium, allowing the bile flow (arrows) to be diverted externally. GL, grafted liver; HJ, hepatico-jejunostomy; S, stomach; D, duodenum; JJ, jejuno-jejunostomy.

absent after six months on both planar abdominal imaging and quantitative assessment. The child remained well on liposoluble vitamin supplementation, and the post-surgical course was uneventful at six months post-EBD. The average bile output was 200 mL/day. The clinical course and biochemistry results are summarized in Table 1. Liver biopsy after six months documented a dramatic improvement of steatosis (Fig. 1c).

A signed written informed consent, approved by the local Ethical Committee (Cliniques Universitaires Saint Luc, Brussels, Belgium), was provided for the patient.

Discussion

PEBD was introduced in 1998, for the treatment for PFIC 1, with the purpose of interrupting the enterohepatic circulation of bile salts by decreasing the bile acid pool. The procedure was shown to diminish serum total bile salts and improve itching, growth, liver function tests, and lipid metabolism in patients without cirrhosis (5, 8). Patients who had undergone PEBD also showed reversal of histologic markers of cholestasis and inflammation (9, 10). However, the long-term outcome of this procedure remains to be demonstrated (11). LT warrants long-term efficacy and it remains the only therapeutic option in patients with advanced liver disease. The potential to receive a liver graft in younger patients because of the option of living donation and split liver technique, as well as an *a priori* poor acceptance of the ostomy are further reasons for some centers to choose LT as the primary option in PFIC patients.

In the described patient, we performed a LT because of the presence of early hepatic fibrosis and of intractable pruritus. However, the occurrence of diarrhea has been reported as a specific complication in such patients. After LT, intestinal FIC1 (the *ATP8B1* gene product) defect is aggravated by the restored bile flow from a normal liver. This leads to the development of refractory diarrhea (12–14). The second concern in the post-transplant course of PFIC 1 patients is the occurrence of allograft steatohepatitis (6, 13, 15, 16). In one of the largest series, steatosis occurred in 8/11 transplanted patients, all having post-LT refractory diarrhea, and seven progressed to steatohepatitis (6). Possible causes of fatty change in transplanted livers are refractory diarrhea and malnutrition secondary to the latter and/or to pancreatitis. Bile adsorptive resin therapy has been shown to improve diarrhea and consequently steatosis, but long-term results of this medical treatment remain unsatisfactory (6, 12, 14).

To date, biliary diversion has not been considered as a cure for these complications after LT, unlike its use in non-transplanted patients. Usui et al. reported a single patient who experienced intractable diarrhea and graft failure because of rejection, in whom EBD was performed at the moment of the retransplantation, with subsequent good outcome (7).

After LT, our patient experienced malnutrition secondary to refractory diarrhea with protein-losing enteropathy, in the absence of pancreatic exocrine insufficiency. This condition rapidly led to fatty degeneration of the transplanted liver with steatohepatitis and worsening fibrosis, severe weight loss and growth retardation and

Table 1. Clinical course and laboratory tests of the patient with PFIC 1 after LT, before and after EBD

	Admission							
Age (months)	42	43	44	46	63	68	71	76
Time post-LT (months)	–	+1	+2	+4	+21	+26	+29	+34
Time post-EBD (months)	–	–	–	–	–	–	+1	+6
ALT, IU/L (NV 14–63)	22	68	156	36	201	102	15	31
GGT, IU/L (NV 7–50)	23	49	35	30	94	103	74	47
TB, mg/dL (NV 0.3–1.2)	3.1	0.4	0.3	0.5	0.5	0.4	0.4	0.7
CB, mg/dL (NV 0–0.2)	1.8	0.1	0.1	0.1	0.1	0.1	0.1	–
aPTT, s (NV 20–33)	27	26	31	24	–	24	26	
PT, s (NV 9–14)	11.1	12.3	12.6	28	–	11.7	12.5	11.6
PT INR (NV 0.8–1.3)	1.03	1.15	1.18	1.15	–	1.08	1.16	1.09
Alb, g/dL (NV 3.4–5.2)	3.7	3.5	2.72	3.5	2.1	1.4	3.11	3.7
TP, g/dL (NV 6.1–7.9)	7.8	6.10	5.10	6.2	4.4	3.6	5.73	6.66
Ig, g/dL (NV 0.5–1)	1	0.9	0.89	1.02	–	0.68	1.01	1.36
Stools/day	1–2	6–7	6–7	4	6–7	6–7	1–2	1–2
Weight/height centile	56	55	–	91	–	21	25	85

ALT, alanine aminotransferase; GGT, gamma-glutamyltranspeptidase; TB, total bilirubin; CB, conjugated bilirubin; aPTT, activated partial thromboplastin time; PT, prothrombin time; Alb, albumin; TP, total proteins; Ig, total immunoglobulins.

generalized edema because of hypoproteinemia. Considering the partial response to bile adsorptive resin therapy, we decided to perform an EBD to improve the diarrhea and nutritional state and to prevent graft failure. This approach is novel, and it proved effective in terms of clinical, histological, and biochemical outcome.

Protein-losing enteropathy, although previously reported in one patient with PFIC 1 following LT, has not been characterized in these patients (6). Intestinal protein loss may be due to causes related to increased interstitial pressure (such as intestinal lymphangectasia, congestive heart failure, and portal hypertension) or to alterations of gastrointestinal mucosa. The rapid good clinical outcome, documented by scintigraphy, suggested that the small bowel defect in bile acid reabsorption, and not fibrosis or portal hypertension, was indeed the cause of both malabsorption and non-erosive mucosal damage finally leading to protein loss. In this perspective, all efforts should be performed to characterize the specific molecular defect among familial cholestasis, and in case of FIC1 mutations, PEBD should be further considered as first treatment option if possible.

A novel *ATP8B1* mutation was found in this patient; an adenine-to-cytosine substitution in position 1660, leading to a missense aspartate-to-alanine (p.D734A). This means, in terms of protein structure, a substitution of a hydrophilic with a hydrophobic residue in the region encoding the largest and catalytically active cytosolic domain, carrying the phosphorylation site and an ATP-binding site. The other mutation carried is a missense aspartate-to-asparagine p.D554N, in the same region. The presence of both mutations in such a key region of *ATP8B1* gene could be related to the dramatic gastrointestinal disease after LT with graft dysfunction. Mutations in such a region have been described previously to be associated with post-transplant steatosis (6).

We conclude that EBD proved to be safe and effective for the treatment of a PFIC 1 patient who developed severe diarrhea, protein-losing enteropathy, and graft steatofibrosis following LT. In this patient, this intervention restored normal graft function and allowed a better quality of life. Internal ileal bypass will be considered as a safer long-term solution.

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We thank Prof. Tania Roskams (Department of Morphology and Molecular Pathology, University Hospital Leuven, Leuven, Belgium) who performed the immunohistochemistry for BSEP and Dr. Christiane Baussan (Biochemistry Unit, Hôpital Bicêtre, Paris, France) for molecular analysis of *ATP8B1*. We are grateful to Roberto Pagnano (http://www.nagalleria.com) for the graphic assistance in the production of the diagram of the surgical procedure.

Author contributions

EN: drafting article, data collection, critical revision of article, data analysis/interpretation; XS: concept/design, critical revision of article, approval of article; FS: concept/design, critical revision of article, approval of article; FF: concept/design, critical revision of article, approval of article; CdM: concept/design, critical revision of article, approval of article; RR: concept/design, data analysis/interpretation, critical revision of article, approval of article; EMS: concept/design, data analysis/interpretation, critical revision of article, approval of article.

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Risk of Perinatal HIV Infection in Infants Born in Italy to Immigrant Mothers

To the Editor—Increasing numbers of human immunodeficiency virus (HIV)–infected children immigrating from countries where HIV infection is endemic have been reported in Europe [1–3]. However, information regarding mother-to-child transmission (MTCT) in infants born in Western countries to immigrant mothers is lacking. We

describe characteristics of infants born to HIV-infected mothers in Italy, focusing on potential inequalities between infants born to native and immigrant women, considered as those originating from countries where HIV infection is endemic [4].

Data from 4470 children born in Italy to HIV-infected mothers during 1996–2010 and enrolled in the Italian Register for HIV infection in children from birth, with an ascertained infectious status, were analyzed. Details regarding data collection and status definitions are described elsewhere [5]. Factors potentially associated with MTCT of HIV infection were explored by logistic regression analysis. Differences among proportions were evaluated using the χ^2 test with Mantel-Haenzel correction.

Crude total rate of MTCT significantly decreased from 5.1% (56 of 1049 cases; 95% confidence interval [CI], 3.98%–6.70%) during 1996–1999 to 2.2% (46 of 2016 cases; 95% CI,1.63%–2.93%) during 2000–2005 and 1.0% (13 of 1298 cases; 95% CI, 0.46%–1.55%) during 2005–2010 ($P < .0001$). The numbers of children born in Italy to immigrant mothers increased from 110 (10.0%) of 1105 during 1996–1999 to 522 (25.5%) of 2049 during 2000–2005 and 561 (43.3%) of 1295 during 2005–2010 ($P < .0001$); 799 (67.0%) of 1193 immigrant mothers originated from sub-Saharan Africa. The rate of MTCT was 2.8% (95% CI, 1.91%–3.79%) among infants born to immigrant mothers and 1.8% (95% CI, 0.89%–2.68%) among infants born to native mothers. In multivariable analysis, being born to an immigrant mother was associated with increased risk of MTCT (adjusted odds ratio, 2.269; 95% CI, 1.115–4.619; $P = .024$) (Table 1). Immigrant mothers less frequently had received combined antiretroviral therapy with ≥3 drugs (679 [56.9%] of 1193 vs 609 of [71.2%] of 855; $P < .0001$) and had less frequently undetectable viral load at delivery, compared with native mothers (531 [44.5%] of 1193 vs 529 [61.9%] of 855; $P < .0001$). A higher proportion of immigrant mothers breastfed their infants, compared with native mothers (1.6% vs 0.3%; $P = .007$). No difference was observed with respect to the proportion of elective caesarean delivery, incidence of premature deliveries, and receipt of intrapartum or postnatal prophylaxis.

Considering data from all the 1478 children with perinatal HIV infection and enrolled in the Italian Register for HIV Infection in Children from 1985, followed up or not since birth, the proportion of HIV-infected children born to immigrant mothers significantly increased over time from 132 (9.9%) of 1328 before 2000 to 79 (54.5%) of 159 during or after 2000 ($P < .0001$). This is consistent with similar findings reported in US [6], French [1], Spanish [2], UK, and Irish [3] pediatric cohorts. However, in our dataset, 115 (54.4%) of 211 infected children born to an immigrant mother were born in Italy, in contrast to what has been observed in other countries, such as the United Kingdom, where the large majority of these children were born abroad [3]. One limit of our study is lack of detailed information regarding time of HIV infection diagnosis in the mothers, time spent in Italy, compliance, and resistance profile. Data regarding mothers' origins and viral loads at delivery were missing in many cases. However, our findings are indicators for social alarm in Italy and shed light on possible barriers to accessing health care facilities, to receiving appropriate counseling and prophylaxis measures, and to existence of possible cultural barriers [7]. Comorbidities may also be more frequent in immigrant mothers [7]. Public health interventions are needed to minimize these missed opportunities for the prevention of HIV infection.

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Table 1. Factors Associated With Mother-to-Child Transmission (MTCT) of HIV Infection in 4470 Infants Born in Italy to HIV-Infected Mothers

	Proportion (%) of infants with HIV infection	aOR (95% CI)[a]	*P*
Birth calendar year (1996–2010)			
Per year		0.950 (0.870–1.038)	.256
Sex			
Female	70/2131 (3.3)	Reference	
Male	45/2237 (1.9)	0.533 (0.354–0.801)	.002
Prematurity (<37 weeks)			
No	101/4473 (2.4)	Reference	
Yes	6/118 (5.1)	0.438 (0.190–1.011)	.053
Unknown	8/119 (6.7)	0.747 (0.222–2.516)	.638
Born to an immigrant mother in Italy			
No	15/855 (1.8)	Reference	
Yes	34/1193 (2.8)	2.269 (1.115–4.619)	.024
Unknown	66/2423 (2.7)	1.782 (1.032–3.079)	.038
Maternal viral load at delivery (RNA copies/mL)			
≥400	22/613 (3.6)	Reference	
<400	2/1586 (0.1)	0.058 (0.013–0.254)	<.0001
Unknown	91/2271 (4.0)	0.729 (0.431–1.234)	.239
Maternal therapy during pregnancy (number of drugs)			
0	66/626 (10.5)		
1	29/844 (3.4)	0.389 (0.232–0.652)	<.0001
2	5/724 (0.7)	0.111 (0.043–0.285)	<.0001
≥3	13/2117 (0.6)	0.155 (0.080–0.302)	<.0001
Unknown	2/159 (1.25)	0.214 (0.050–0.911)	.037
Intrapartum ZDV			
No	69/1187 (5.8)	Reference	
Yes	44/3255 (1.4)	0.920 (0.532–1.591)	.765
Unknown	2/28 (7.1)	0.854 (0.101–7.210)	.884
Elective caesarean delivery			
No	63/711 (8.9)	Reference	
Yes	52/3698 (1.4)	0.364 (0.237–0.560)	<.0001
Unknown	0/61 (0)	0.000	.997
Neonatal prophylaxis			
No	41/632 (6.5)	Reference	
Yes	73/3829 (1.9)	0.144 (0.003–6.050)	.310
Unknown	1/9 (11.1)	0.203 (0.005–8.880)	.408
Breastfeeding			
No	102/4362 (2.3)	Reference	
Yes	12/36 (33.3)	3.816 (1.656–8.794)	.002
Unknown	1/72 (1.4)	1.565 (0.197–12.421)	.672
Injection drug user mother			
No	76/3182 (2.4)	Reference	
Yes	38/1260 (3.0)	1.008 (0.627–1.619)	.974
Unknown	1/28 (3.6)	0.583 (0.074–4.658)	.613

NOTE. aOR, adjusted odds ratio; CI, confidence interval; ZDV, zidovudine.

[a] OR are adjusted for all other variables included in the table and calculated by logistic regression analysis.

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The HIV-1 Transactivator Factor (Tat) Induces Enterocyte Apoptosis through a Redox-Mediated Mechanism

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Abstract

The intestinal mucosa is an important target of human immunodeficiency virus (HIV) infection. HIV virus induces CD4+ T cell loss and epithelial damage which results in increased intestinal permeability. The mechanisms involved in nutrient malabsorption and alterations of intestinal mucosal architecture are unknown. We previously demonstrated that HIV-1 transactivator factor (Tat) induces an enterotoxic effect on intestinal epithelial cells that could be responsible for HIV-associated diarrhea. Since oxidative stress is implicated in the pathogenesis and morbidity of HIV infection, we evaluated whether Tat induces apoptosis of human enterocytes through oxidative stress, and whether the antioxidant N-acetylcysteine (NAC) could prevent it. Caco-2 and HT29 cells or human intestinal mucosa specimens were exposed to Tat alone or combined with NAC. In an *in-vitro* cell model, Tat increased the generation of reactive oxygen species and decreased antioxidant defenses as judged by a reduction in catalase activity and a reduced (GSH)/oxidized (GSSG) glutathione ratio. Tat also induced cytochrome c release from mitochondria to cytosol, and caspase-3 activation. Rectal dialysis samples from HIV-infected patients were positive for the oxidative stress marker 8-hydroxy-2′-deoxyguanosine. GSH/GSSG imbalance and apoptosis occurred in jejunal specimens from HIV-positive patients at baseline and from HIV-negative specimens exposed to Tat. Experiments with neutralizing anti-Tat antibodies showed that these effects were direct and specific. Pre-treatment with NAC prevented Tat-induced apoptosis and restored the glutathione balance in both the *in-vitro* and the *ex-vivo* model. These findings indicate that oxidative stress is one of the mechanism involved in HIV-intestinal disease.

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Introduction

The intestinal mucosa is a functional barrier against pathogens being both a physical obstacle with columnar cells linked together by tight junctions, and the site of mucosal immunological cells. HIV infection is mainly initiated on the intestinal mucosal surface through heterosexual or homosexual transmission [1,2] and HIV acutely induces infiltration of the gut mucosa thereby resulting in the release of activated effector memory CD4+ and CD8+ T cells, damage to the intestinal barrier and increased epithelial apoptosis [3]. Clinical data support a relationship between chronic HIV infection and intestinal dysfunction including increased permeability, altered nutrient absorption, diarrhea and reduction of the absorptive surface [4–10]. Acquired immunodeficiency syndrome (AIDS) enteropathy is an idiopathic, pathogen-negative diarrhea and is associated with an increase in inflammation [11], mucosal immune activation, villous atrophy and crypt hyperplasia that may be observed in all stages of HIV disease even in the absence of HIV virus [12]. The detection of viral proteins and/or nucleic acids in enterocytes and in goblet cells indicated that HIV virus plays a direct pathogenic role at intestinal level [13,14]. Kotler et al. detected HIV DNA, RNA and protein antigens in lamina propria mononuclear cells and epithelial cells of gastrointestinal tract from HIV patients [14].

However, several effects induced by HIV are not mediated by lytic propagation of viral particles, but rather by viral factors that are released by infected cells [15]. We previously demonstrated that the viral protein Tat induces ion secretion in Caco-2 cells and in human colonic mucosa, and inhibits intestinal cell proliferation. Tat-induced ion secretion is associated with an increase in intracellular Ca^{2+} as a result of extracellular Ca^{2+} entrance and mobilization of intracellular stores [16]. A similar effect is induced by Tat in neurons [17]. In addition, Tat causes an imbalance in reactive oxygen species (ROS) generation in neurons, which is neutralized by antioxidants, thereby implicating perturbation of the intracellular redox status in the pathogenesis of HIV-induced cell damage [18].

Oxidative stress is implicated in the pathogenesis and morbidity of HIV infection [19,20]. An increase of ROS and an alteration of antioxidant defenses have been reported in HIV-infected patients [21] associated with decreased levels of antioxidants [22]. The mechanisms involved in HIV-induced oxidative stress are unknown, but HIV-1 proteins gp120 and Tat have been implicated in this process [23] because both induce oxidative stress and cause apoptosis in brain endothelial cells [23].

Antioxidant defenses are also impaired in HIV-infected patients and, in particular, glutathione metabolism is altered [24]. Reduced

glutathione (GSH) is the main intracellular thiol molecule responsible for ROS scavenging and for the maintenance of oxidative balance. It is also involved in the protection of DNA and nuclear proteins from oxidative damage. Intracellular GSH depletion triggers ROS production thereby inducing an arrest in the intestinal cell cycle [25]. GSH levels are depleted in plasma, in epithelial lining fluid of the lower respiratory tract, in peripheral blood mononuclear cells and in monocytes in HIV-infected patients [26]. Antioxidant deficiency leads to severe degeneration of intestinal epithelial cells, and even a mild intracellular redox imbalance inhibits enterocyte proliferation [27].

Interestingly, GSH levels progressively decrease as the HIV-1 viral load increases [28]. A fall in GSH during HIV infection may result from reduced GSH synthesis or increased catabolism. Tat blocks transcription of manganese superoxide dismutase, an enzyme that prevents oxidative stress, and decreases the activity of glucose-6-phosphate dehydrogenase, a key enzyme in pathways that maintain GSH in its reduced state [29]. Moreover, Tat induced oxidative stress in an immortalized endothelial cell line from rat brain capillaries [30].

In this scenario, we hypothesized that the enteropathogenic effects induced by Tat are associated with an imbalance of the redox state in the intestine.

Results

HIV-1 Tat induces intestinal epithelial oxidative stress thereby increasing reactive oxygen species and impairing antioxidant defenses

To evaluate whether an altered redox state could be responsible for the effects induced by Tat, we measured the intracellular levels of ROS and of two main intracellular antioxidant defense systems, catalase and glutathione, in intestinal epithelial cells. Fluorescence microscopy showed that ROS levels were increased in Caco-2 cells exposed to 0.5 nM of Tat for 1 hour as judged by the fluorescence green signal produced by the interaction between dichlorodihydrofluorescein diacetate (DCFH-DA) and ROS (Fig. 1A). ROS production was also increased in HT-29 cells stimulated with HIV-Tat under the same conditions (Fig. 1B). As a positive control, cells were treated with H_2O_2, and cells treated with the same volume of media without Tat protein served as negative control. Increasing Tat concentrations (0.05–1 nM) were added to Caco-2 cells. DCFH-DA was used for ROS quantification and measured 15 min after Tat stimulation. Exposure to Tat protein resulted in a dose-dependent increase of ROS (Fig. 2A). Since ROS generation is usually rapid after a toxic stimulus, we performed time-course experiments in Caco-2 cells exposed to Tat for 15, 30, 60 and 120 min (Fig. 2B). A ROS increase was evident as early as 15 min after exposure to Tat; levels returned to control values after two hours. This suggests that the antioxidant defenses may be activated to counteract oxidative stress.

We carried out neutralization experiments to ascertain whether ROS generation is a specific effect induced by Tat. As shown in Fig. 2C and D, anti-Tat polyclonal antibodies inhibited the increase of ROS intracellular levels in Caco-2 and HT-29 cells. To see whether Tat enters into the cells, we stimulated Caco-2 cells from the same culture with Tat at 1, 24, 48 and 72 hrs and, in parallel, we evaluated ROS concentrations and Tat intracellular levels (Fig. S1). In these conditions, Tat was detected inside the cells 1 hour after stimulation and until at least 72 hours post-exposure (Fig. S1C).

We next investigated whether Tat-induced ROS generation was associated with a decrease of antioxidant defenses by measuring

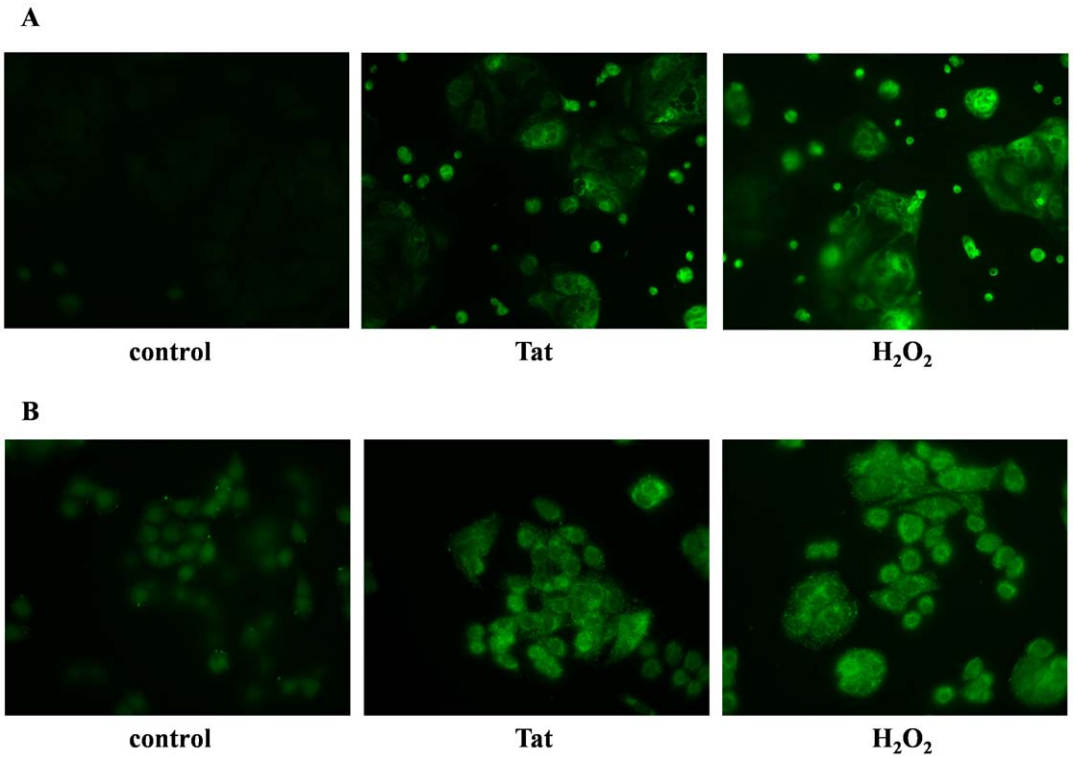

Figure 1. Influence of HIV-Tat protein on ROS generation in Caco-2 cells (A) and HT29 cells (B). Immunofluorescent staining of ROS by DCFH-DA after Tat exposure were compared to H_2O_2- and untreated cells (control). Representative staining is shown at 1 hour post-exposure. Magnification: 200×. Data are representative of 3 separate experiments with 3–4 replicates for each experimental condition.
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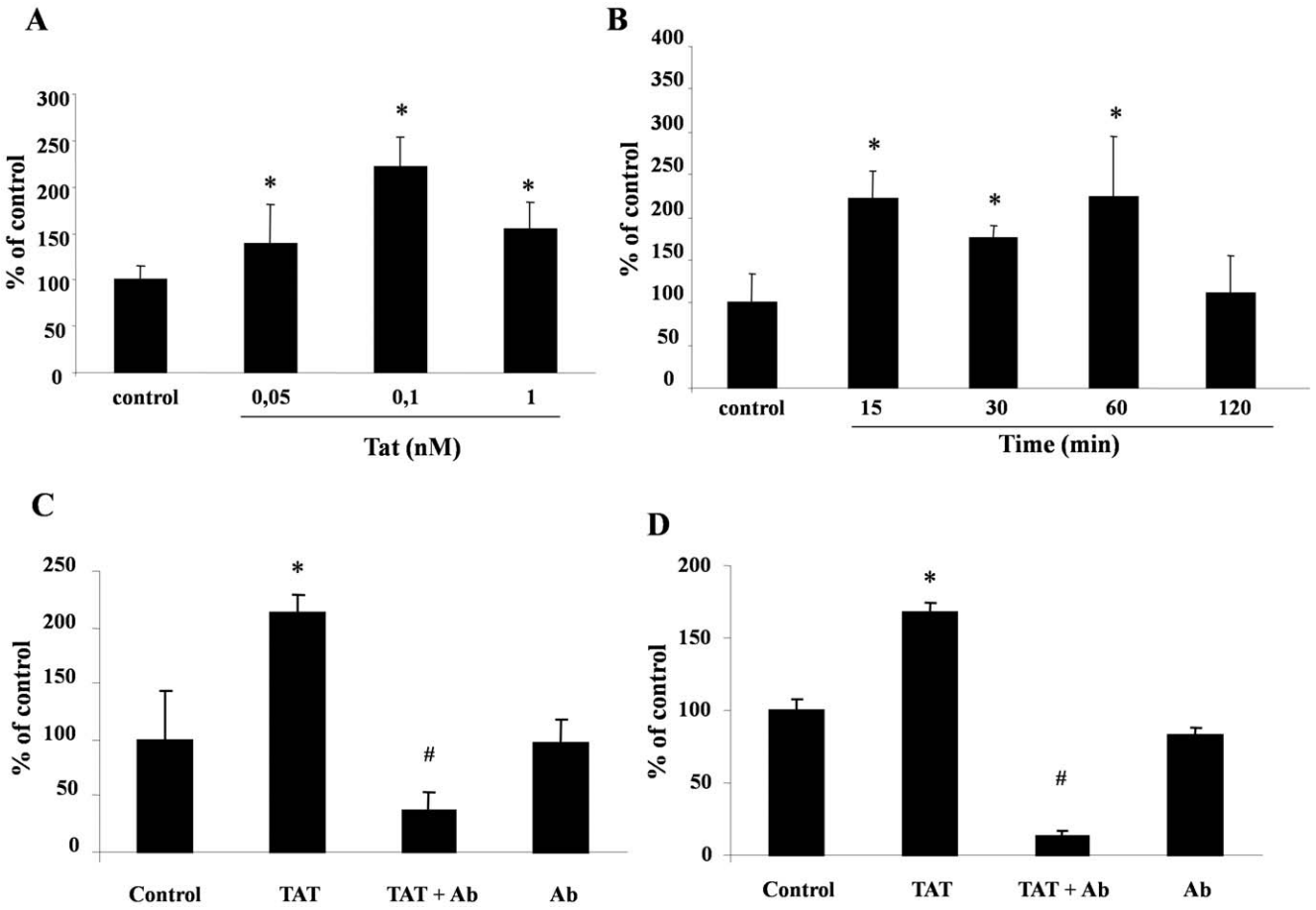

Figure 2. Tat-induced ROS generation is dose- and time-dependent. Caco-2 cells were exposed to different concentrations of Tat for 1 hour (A) and to 0.1 nM for 10, 30, 60 and 120 min (B), and ROS intracellular levels were evaluated by the DCFH-DA fluorometric method. To evaluate the specificity of the effect, Caco-2 cells (C) and HT-29 (D) were incubated with Tat with or without the anti-Tat polyclonal antibody. Data are representative of 3 separate experiments. *p<0,05 vs control; #p<0,05 vs Tat.
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the levels of glutathione, one of the major intracellular ROS scavengers. Glutathione is an important cellular antioxidant, and it plays a major role in protecting cells against oxidative stress. In fact, the intracellular balance between the reduced (GSH) and oxidated (GSSG) glutathione forms in healthy conditions was reported to show a predominantly reducing state being GSH about 80–90% and GSSG 10–20% [31]. We found that the GSH/GSSG ratio was lower in Caco-2 cells exposed to Tat than in controls and that the effect was dose- and time-dependent (Fig. 3A and B). The reduction of GSH was significant between 0.1 and 1 nM of Tat (Fig. 3A) and was already evident at 1 hour post-exposure. GSH remained low at 24 hours and returned to basal level at 48–72 hours (Fig. 3B). Also in this case, anti-Tat polyclonal antibodies completely inhibited the Tat-induced GSH/GSSG imbalance in Caco-2 (Fig. 3C) and in HT-29 cells (Fig. S2). The decreased activity of the enzyme catalase, another component of the antioxidant defense, after 1 hour of exposure to Tat (Fig. 3D) indicates that antioxidant defense is a target of Tat.

Intestinal oxidative stress is increased in HIV-infected children

In nuclear and mitochondrial DNA, 8-hydroxy-2′-deoxyguanosine (8-OHdG) is one of the predominant marker of free radical-induced oxidative damage and is therefore widely used as biomarker for oxidative stress in clinical samples [32–34]. To investigate whether HIV-infected patients are affected by intestinal oxidative stress, we measured 8-OHdG concentration in a small amount of rectal dialysis solution from 20 HIV-positive children treated in our tertiary care center for patients with AIDS (Fig. 4A). The characteristics of patients are shown in Table 1. The concentration of 8-OHdG was higher in HIV-infected children than in serum-negative children (Fig. 4B). To investigate whether intestinal oxidative stress was related to virus replication, we divided the HIV-positive patients in two groups related to plasma viral load. We identified 9 children with a viral load higher than 40 copies/ml and 11 children with undetectable HIV RNA (<40 copies/ml). The mean 8-OHdG concentration was higher in children with HIV RNA >40 copies/ml than in children with HIV RNA <40 copies/ml and in serum-negative children (Fig. 4C), which suggests that in children with chronic HIV infection, oxidative damage measured in rectal mucosa may be linked with viral replication. However, 8-OHdG concentrations in urine and serum did not differ between patients and HIV-negative control children (Fig. S3), suggesting that the oxidative stress is localized in rectal mucosa. Finally, a significant correlation was detected between 8-OHdG and viral load ($r = 0.4653$; $p = 0.0009$).

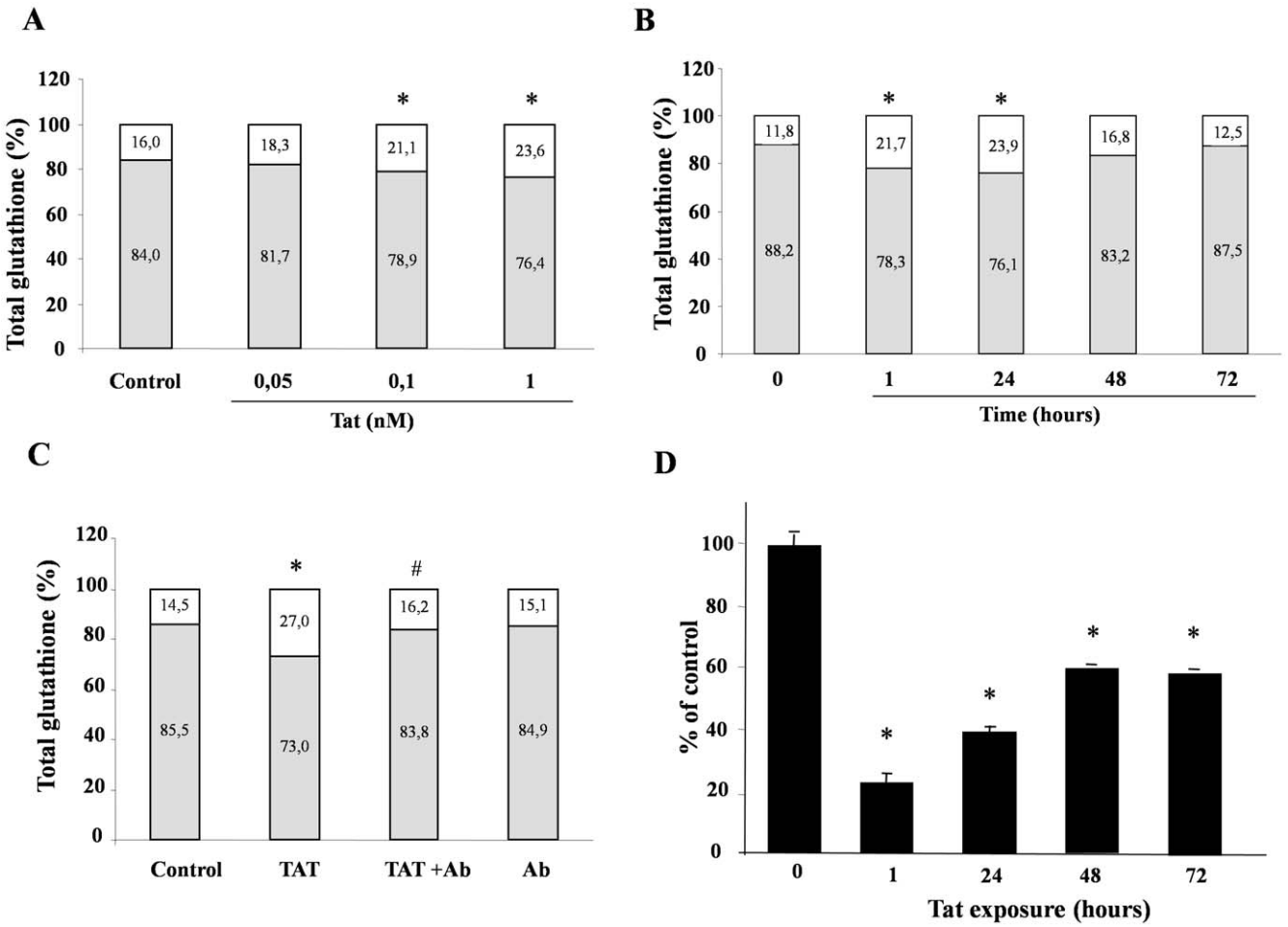

Figure 3. Tat induced alteration of intracellular antioxidant defenses. Caco-2 cells were exposed to different concentrations of Tat for 1 hour (A) and to 0.1 nM for 10, 30, 60 and 120 min (B), and the percent of GSH (grey) and GSSG (white) was evaluated as described under "Methods" in the Method section. To evaluate the specificity of the effect, Caco-2 cells (C) were incubated with Tat with or without the anti-Tat polyclonal antibody. *p<0,05 vs control; #p<0,05 vs Tat. (D) Catalase activity was evaluated in Caco-2 cells after different times of Tat exposure. Data are representative of 3 separate experiments. *p<0,05 vs time 0.
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Activation of caspase-3 and cytochrome c release in the cytosol indicates that Tat induces apoptosis through involvement of the intrinsic pathway

Several viruses induce apoptosis as a strategy to spread the infection or to induce the breakdown of infected cells, thereby favoring viral dissemination. To investigate whether Tat induces intestinal apoptosis, we studied caspase-3 signaling, which is activated by two fundamentally distinct signaling cascades, namely the extrinsic and intrinsic (or mitochondrial) pathways [35]. We found that caspase-3 activity was higher in Caco-2 cells exposed to Tat for 72 hours than in control cells (Fig. 5A). Caspase-3 is a critical effector of apoptosis and is responsible for the proteolytic cleavage of many key proteins including the nuclear enzyme poly (ADP ribose) polymerase (α-PARP). In a western blot time-course experiment, we found that the levels of the full-length forms of caspase-3 protein and α-PARP were reduced after Tat exposure compared with control cells thereby indicating activation of the apoptotic molecular mechanisms (Fig. 5B).

Proapoptotic stimuli, including ROS and calcium overload, are able to activate the intrinsic pathway of apoptosis by inducing mitochondrial membrane permeabilization and the release of cytochrome c in the cytosol. Because we previously found that Tat induced an increase of cytosolic calcium from intracellular stores [16], we used western blot analysis to test the effects of Tat on the release of cytochrome c. Twenty-four hours after Tat exposure, cytochrome c levels in the cytosol of cells were very high and they continued to increase up to 72 hours (Fig. 5C upper panel). Cytochrome c was not detected in the cytosol of control cells. The same filter was reprobed with a monoclonal antibody against cytochrome oxidase subunit IV (COX IV), which is a mitochondrial marker, and then with anti-tubulin antibody, which is a cytosolic marker. The absence of COX IV in the cytosolic samples confirmed the absence of mitochondrial contamination in the cytosolic fraction (Fig. 5C middle panel), and the absence of tubulin in mitochondrial samples indicates that the mitochondrial fractions were free from cytosolic content (Fig. 5C lower panel).

Because oxidative stress disrupts the cytoskeleton, we investigated the intracellular actin architecture. The architecture of actin cytoskeleton was normal in Caco-2 cells, but it became instable and fragmented after Tat exposure. Disruption of the actin cytoskeleton was dependent on the time of Tat exposure (Fig. 6A). To investigate the effects of Tat on microtubules in Caco-2 cells, we performed experiments of co-immunoprecipitation of Tat and tubulin. The protein extracts from Caco-2 cells exposed to Tat

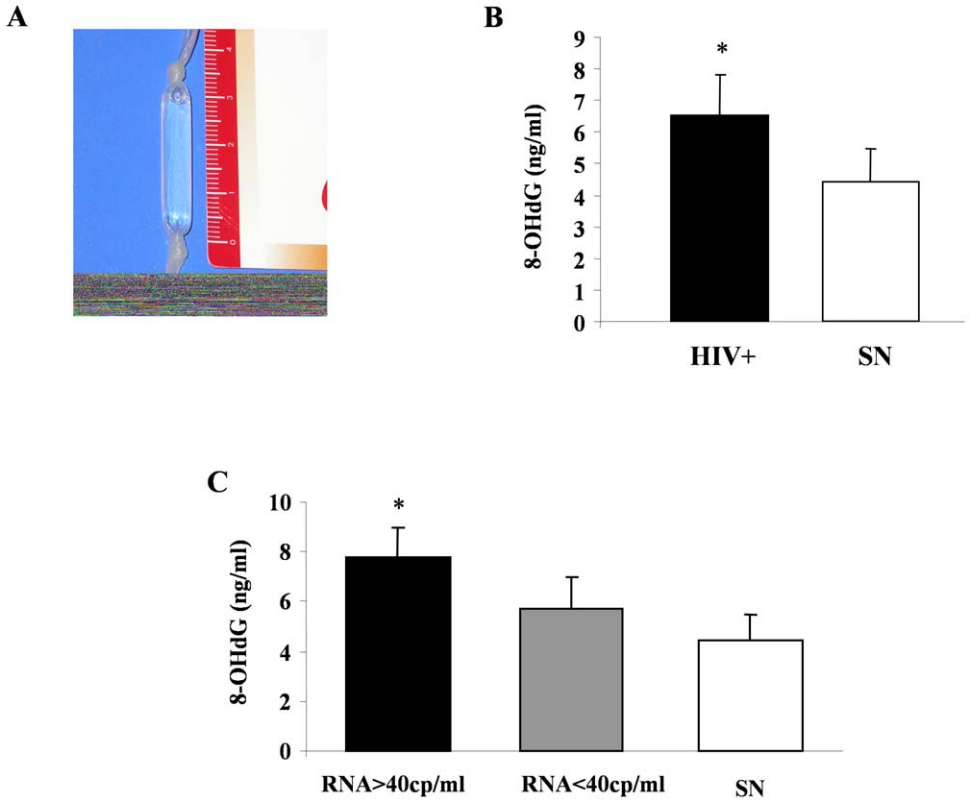

Figure 4. 8-hydroxy-2′-deoxyguanosine (8-OHdG) concentration in rectal dialysis in HIV-negative and positive children. The determinations of rectal 8-OHdG production were performed using a dialysis bag (A) in serum-negative (n = 6, SN) and positive (n = 20, HIV+) children (B). *p<0.05 vs SN. (C) HIV+ children were divided in two groups and compared with controls (n = 6, white): HIV RNA >40cp/ml (n = 9, black), HIV RNA <40cp/ml (n = 11, grey). *p<0.05 vs RNA <40cp/ml and SN groups.
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were subjected to co-immunoprecipitation with an antibody against tubulin or with control IgG, and subsequently analyzed through Western blotting with the anti-Tat polyclonal antibody. Tat coprecipitated with tubulin indicating that it binds to the microtubule cytoskeleton (Fig. 6B).

Table 1. Characteristics of the HIV-positive patients.

Age (months)	
Mean ± standard deviation	134.8±53.3
Range	26–228
Sex	
Male	6
Female	14
CD4+ (cells/ml)	
Mean ± standard deviation	963.85±496.43
Range	176–2027
HIV RNA	
Patients with HIV RNA <40cp/ml	11
Patients with HIV RNA >40cp/ml	9
Mean viral load (cp/ml)± standard deviation	3934.89±6776.77
Range	48–14500

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Polar effects by Tat on the apical and basolateral side of the Caco-2 cell monolayers

To test the hypothesis that Tat induces different effects depending on its addition to the apical or basolateral side of the epithelium, Caco-2 cell monolayers were exposed to Tat 0.5 nM for 1, 24 and 48 hours at apical or basolateral side (Fig. 7). In these conditions Tat reduced the GSH/GSSG ratio at 1–24 hours, with a more potent effect at the basolateral than the apical side (Fig. 7A). In addition, the activation of caspase-3 was observed following basolateral but not apical stimulation (Fig. 7B). The polar effect by Tat is in agreement with our previous data showing chloride secretion induced by basolateral addition of Tat [16].

Effects by gp120 on oxidative stress and apoptosis in Caco-2 cell monolayers

Previous studies investigating the effects of HIV-1 on epithelial barrier function, demonstrate that both Tat and gp120 (the latter being a surface envelope glycoprotein) directly increase permeability of brain endothelial cells through a redox-dependent mechanism [23,30]. In order to investigate the similarities and differences between the effects induced by gp120 and by Tat, we evaluated the effects induced by gp120 on oxidative stress and apoptosis in our experimental system. An imbalance in GSH/GSSG ratio was observed in response to gp120 with a more potent effect at apical than basolateral side (Fig. 8A). However, gp120 did not induce caspase-3 activation (Fig. 8B).

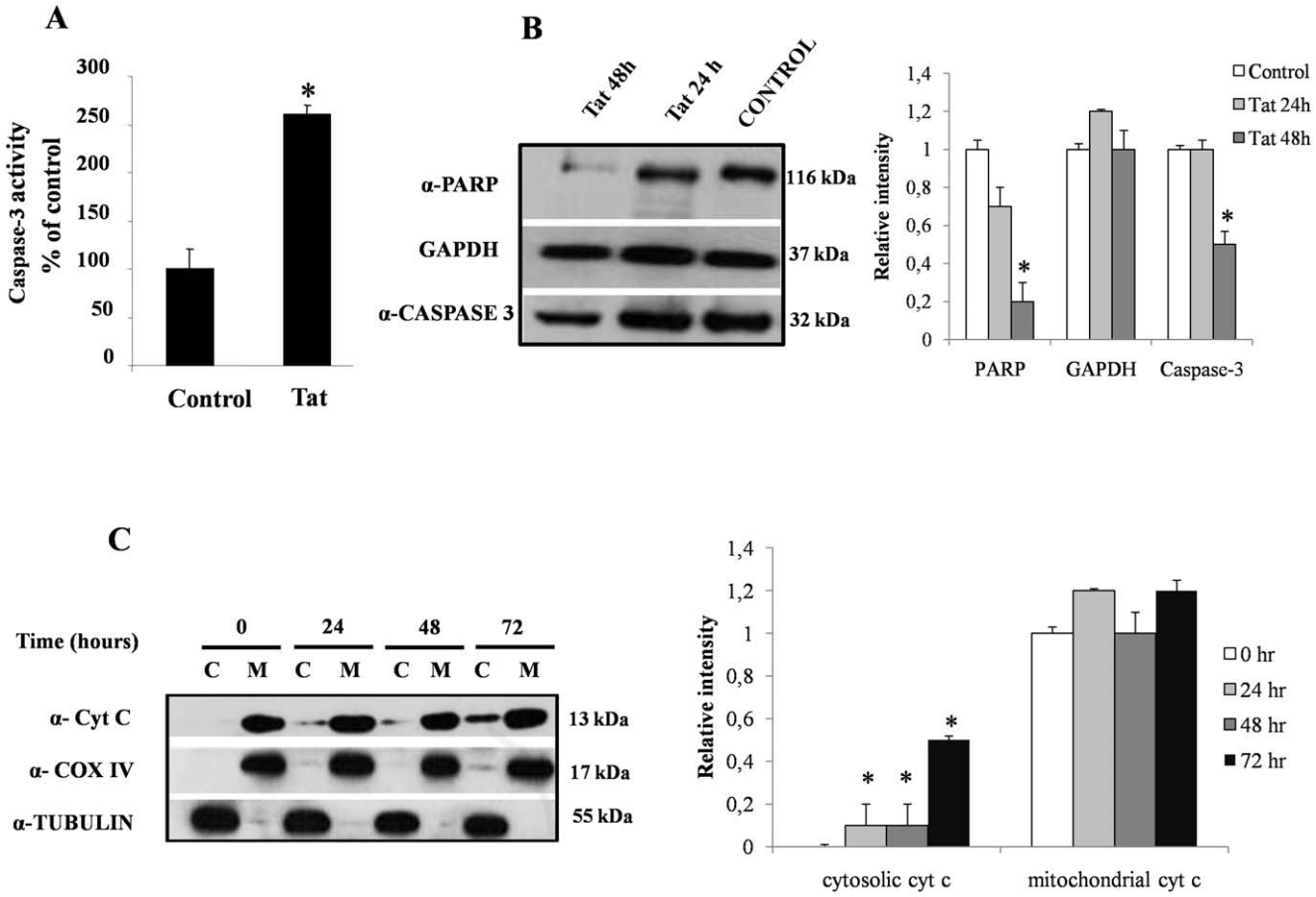

Figure 5. Influence of HIV-Tat protein on apoptosis in Caco-2 cells. Caspase-3 activity (A) and full-length protein (B lower panel) were evaluated in Tat-treated Caco-2 cells. To verify that Tat induced apoptosis, cleaved PARP was evaluated in the same western blot used to evaluate the activation of caspase-3 (B upper panel). Normalization of western blot was performed with GAPDH in all experiments (B middle panel). *$p<0.05$ compared with untreated control cells. Protein extracts from cytosol (C lanes) and mitochondria (M lanes) assayed for cytochrome c by western blot analysis (C); tubulin was used as cytosolic marker, and COX IV as mitochondrial marker. Densitometric acquisitions are shown from three separate experiments. *$p<0,05$ vs time 0.
doi:10.1371/journal.pone.0029436.g005

Tat-induced oxidative stress and apoptosis are strongly inhibited by pretreatment with antioxidants

Our findings demonstrate that Tat induces oxidative stress and apoptosis in human enterocytes. These two events may be causally related in several pathogenic conditions [36]. We therefore used the antioxidant NAC to determine the relationship between oxidative stress and apoptosis induced by Tat at intestinal level. Our results demonstrate that oxidative stress is completely prevented by pretreatment with NAC. In fact, NAC prevented the Tat-induced ROS increase (Fig. 9A) and preserved the GSH/GSSG ratio (Fig. 9B).

To verify that the redox imbalance induced by Tat was the major cause of cell apoptosis, we performed experiments under a condition of oxidative stress prevention. Pretreatment for 24 hours with NAC completely prevented caspase-3 activity induced by Tat (Fig. 10A), suggesting that the redox imbalance caused apoptosis in our experimental model. We also evaluated caspase-3 and PARP cleavage by western blot and found that NAC prevented activation of caspase-3 signaling (Fig. 10B). Similar results were found in HT-29 cell line (Fig. S4). These results support the hypothesis that oxidative stress induced by Tat induces programmed cell death.

Intestinal oxidative stress and apoptosis are related and prevented by the antioxidant NAC in human intestinal specimens

To determine whether the findings observed in Caco-2 cells were reproduced in human intestine, we evaluated apoptosis and redox intracellular homeostasis in duodenal biopsies from children (Fig. 11A). Western blot analysis for caspase-3 was performed on human intestinal specimens obtained from 4 HIV-negative patients. Similar to the findings we obtained in *in-vitro* cell models, exposure of human duodenal mucosa to Tat resulted in caspase-3 activation, as shown by the increase of the proteolytic 17-kDa fragment from caspase-3. Similar to the results we obtained in Caco-2 cells, this process was prevented by NAC (Fig. 11B).

Duodenal biopsies were obtained also from a single HIV-positive child to evaluate apoptosis and redox mechanisms in an *ex-vivo* model. Caspase-3 activation was determined in basal conditions and in the presence of NAC. Caspase-3 activation in specimens from the child was prevented by NAC (Fig. 11C).

Finally, we measured the GSH/GSSG ratio in intestinal biopsies. The ratio was decreased upon Tat exposure and the

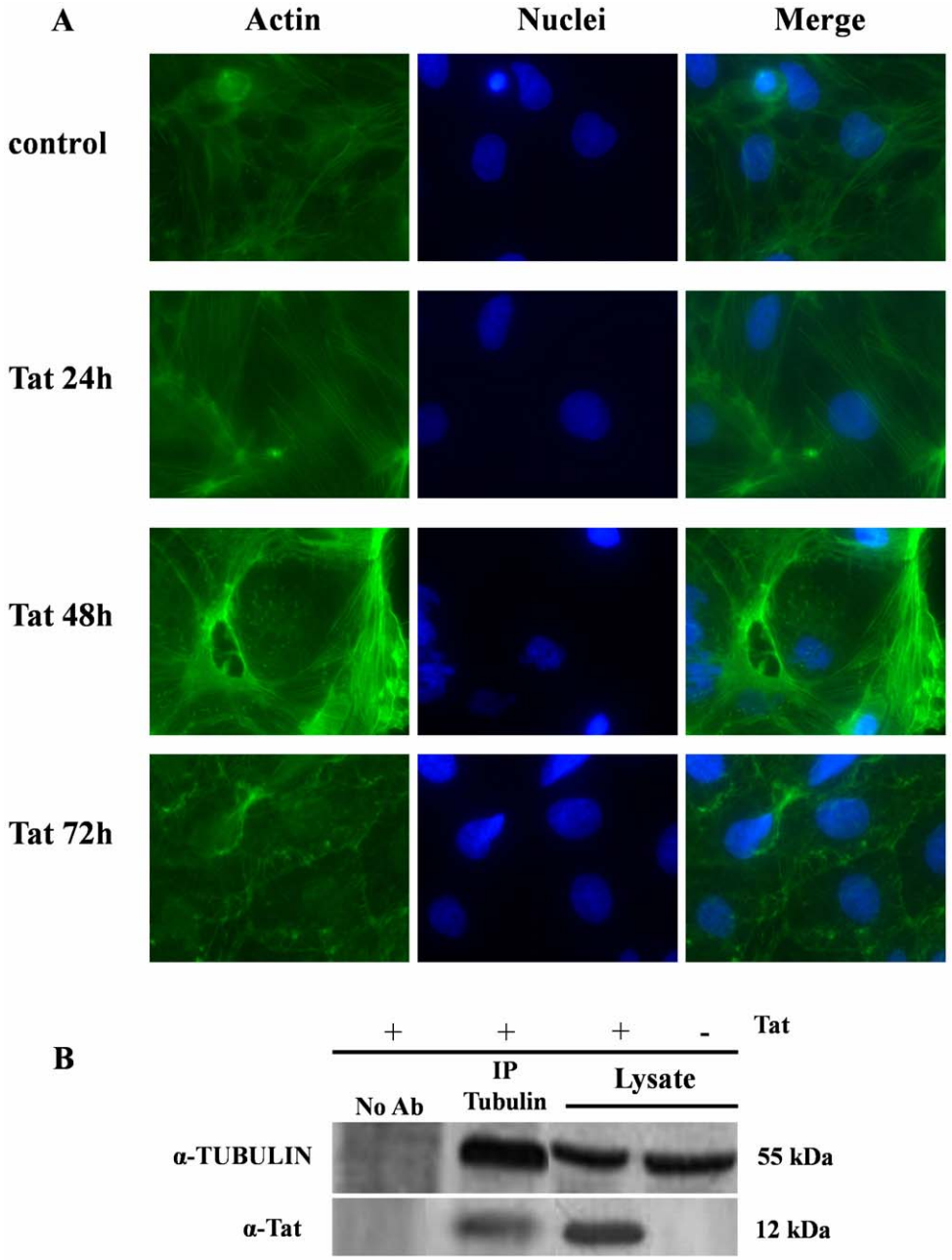

Figure 6. Tat-induced disruption of the actin cytoskeleton. (A) Direct immunofluorescence of actin staining by FITC-conjugated phalloidin (green). The nuclei were stained with Hoerst (blue). Caco-2 cells were exposed to Tat 0.5 nM for 72 hours. Magnification: 1000×. Data are representative of 3 separate experiments with 3–4 replicates for each experimental condition. (B) Cell lysates of Caco-2 cells were stimulated with Tat 0,5 nM for 24 hours and subjected to immunoprecipitation using anti-tubulin antibody or control IgG. Tat protein was detected from immunoprecipitates by Western blotting with anti-Tat antibody.
doi:10.1371/journal.pone.0029436.g006

imbalance was prevented by NAC pretreatment in intestinal mucosa of HIV-negative patients (Fig. 11D). Interestingly, the baseline GSH/GSSG ratio in the duodenal tissue of an HIV-positive child (Fig. 11E) was similar to that observed in HIV-negative mucosa treated with Tat (Fig. 11D). However, it decreased after treatment with NAC and became similar to HIV-negative controls. These data strongly suggest that intestinal glutathione modifications are directly related to apoptosis in HIV-patients and that Tat, which was prevented by antioxidant treatment in human intestine, plays a key role in this condition.

Discussion

Here we demonstrate that HIV-Tat protein directly induces enterocyte apoptosis by a redox-dependent mechanism. Our findings indicate that the increase in ROS intracellular level and impaired antioxidant defenses are associated with epithelial cell apoptosis and actin destruction. The effect was specific for Tat and the timing of the events suggested that Tat induced early oxidative stress followed by apoptosis. The oxidative stress was dose- and time-dependent reaching its peak at a concentration of 0.1 nM.

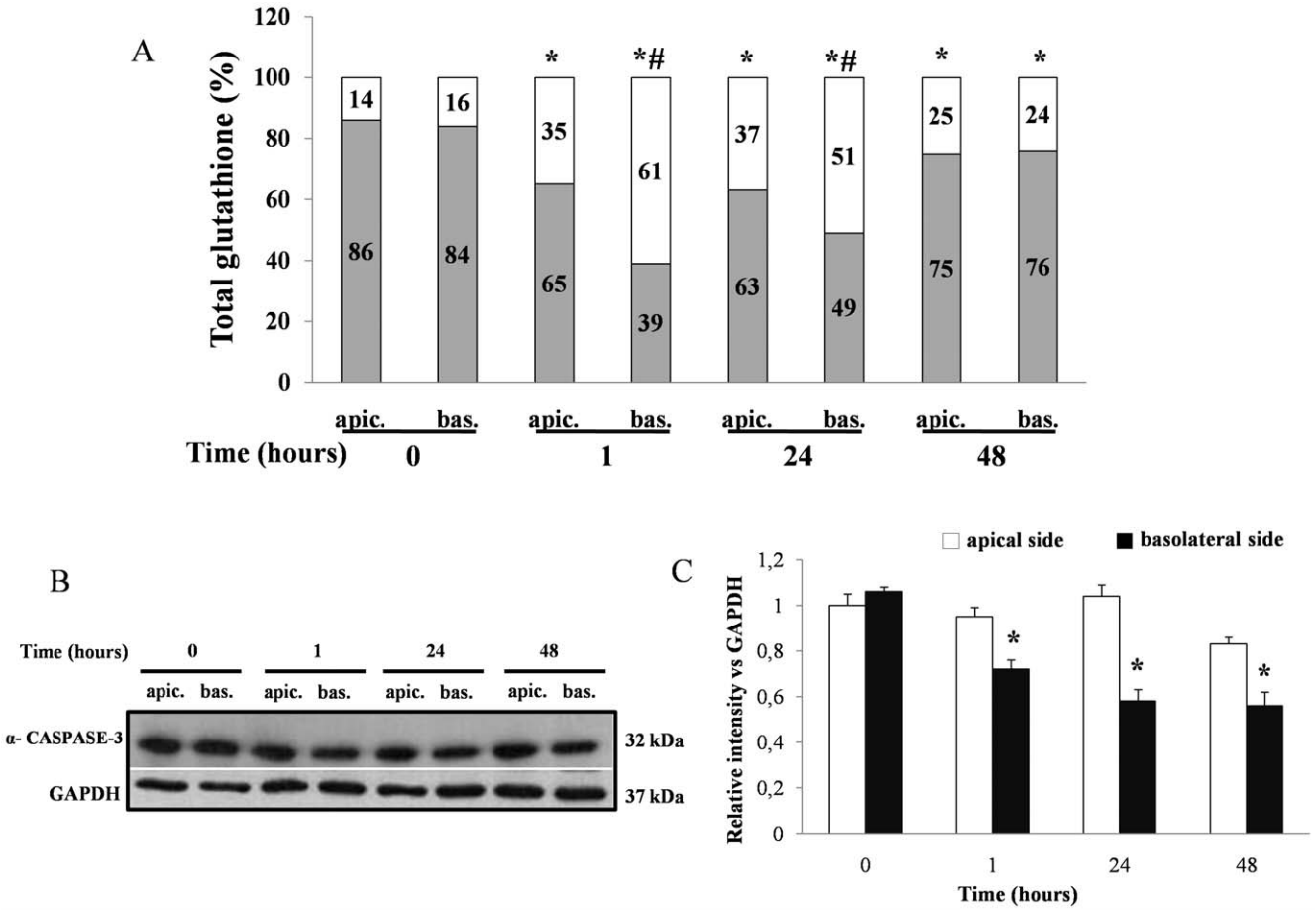

Figure 7. Polar effects by Tat on the apical and basolateral side of the Caco-2 cell monolayers. Tat was added to apical or basolateral side of Caco-2 cell monolayers and GSH (grey)/GSSG (white) ratio (A) and caspase-3 activation (B) were evaluated after 1, 24 and 48 hours. *p<0,05 vs control; #p<0,05 vs apical Tat stimulation at the same time. Normalization of western blot was performed with GAPDH in all experiments. Densitometric acquisitions are shown from three representative separate experiments.
doi:10.1371/journal.pone.0029436.g007

This concentration is similar to that found in sera from HIV patients [37]. We also show that the antioxidant NAC is able to prevent intestinal oxidative stress by maintaining ROS and GSSG intracellular concentrations at a low level. In addition, NAC counteracted intestinal apoptosis, which supports the hypothesis that this event depends on a redox mechanism. Further support to this hypothesis comes from our evaluation of the redox balance and apoptosis in an *ex-vivo* experimental model. Caspase-3 cleaved protein was increased and the GSH/GSSG ratio imbalanced in intestinal biopsies exposed to Tat, which indicates that the apoptotic mechanism was triggered by the impairment of antioxidant defenses. Preincubation with NAC prevented these events with a pattern similar to that observed in cell lines. We had the opportunity to examine an intestinal specimen from an HIV-positive patient. Although we are aware that one HIV-infected patient is insufficient to make any conclusion, we found that apoptosis and the GSH/GSSG ratio in this sample were similar to those in HIV-negative biopsies exposed to Tat. Under these conditions, NAC decreased the levels of activated caspase-3 and re-established a GSH/GSSG ratio similar to those recorded in biopsies of HIV-negative patients. These data demonstrate that a redox-dependent mechanism is involved in the pathophysiology of HIV at intestinal level thereby implicating oxidative stress in HIV enteropathy. It would be interesting to detect Tat in the lamina propria and correlate the viral protein levels with those used in our experimental model. However, several authors detected HIV-1 RNA and viral antigens in epithelial intestinal cells in different stage of infection and in different type of cells [13,14,38]. Therefore it was suggested that the gut mucosa is a reservoir of HIV-1 infection and it is likely to think that viral proteins, including Tat, are present locally.

It has recently been demonstrated that the redox state of HIV-Tat affects its biological activity [39]. In particular, the Tat protein enters the cell and its reduced isoform reaches the cytoplasm and nucleus whereas its oxidized form tends to form multi-aggregates that are less toxic in infected cells. This suggests that the increase in intracellular ROS is not directly induced by Tat but is rather a strategy used by the infected cell to reduce the biological activity of Tat – a process that ultimately leads to programmed cell death.

In clinical studies, the redox balance was severely deranged in HIV-positive patients without highly active antiretroviral therapy (HAART) [40]. These studies showed that HIV-infected individuals are exposed to chronic exogenous oxidative stress that causes perturbations of the antioxidant defense system, including glutathione, thioredoxin, superoxide dismutase, ascorbic acid, glutathione peroxidase, tocopherol and selenium. In addition, hydroperoxidases and elevated levels of malondialdehyde were found in both pediatric and adult patients [41].

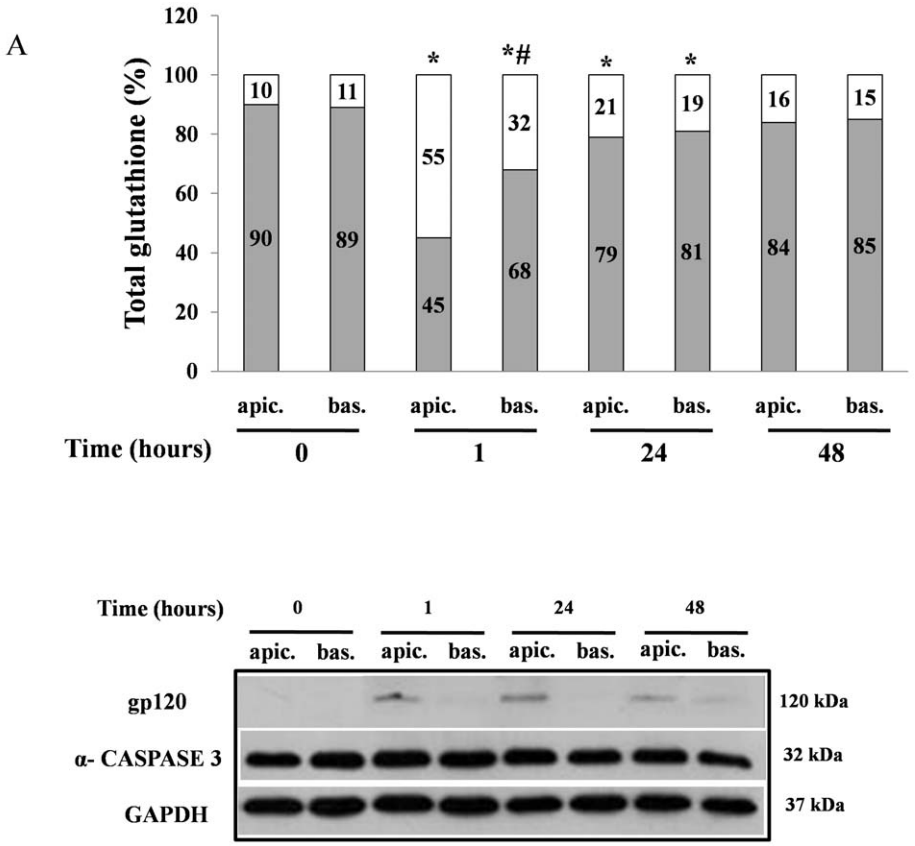

Figure 8. Effects by gp120 on oxidative stress and apoptosis in Caco-2 cell monolayers. gp120 (0.8 nM) was added to apical or basolateral side of Caco-2 cell monolayers and GSH/GSSG ratio (A) and caspase-3 activation (B) were evaluated after 1, 24 and 48 hours. *p<0,05 vs control; #p<0,05 vs apical gp120 stimulation at the same time. After gp120 stimulation cells were collected and western blots were performed with anti-gp120 polyclonal antibody (upper panel) and anti-caspase-3 monoclonal antibody (middle panel). Normalization of western blot was performed with GAPDH in all experiments (lower panel).
doi:10.1371/journal.pone.0029436.g008

We first detected oxidative stress at intestinal level in HIV infection induced directly by Tat in cell models and in human intestinal epithelium. A marker of oxidative stress was also increased in rectal dialysis fluid, but not in serum and urine, of HIV-positive patients seen at our clinical center, which suggests that the stress event is localized at intestinal level. In addition, we found a significant correlation between viral load and concentration of 8-OHdG. We suggest that a specific clinical trial be conducted to evaluate intestinal damage and the therapeutic use of antioxidants.

We previously reported that Tat induces Ca^{2+}-dependent chloride secretion in human intestinal cells [15]. Subsequently, we found that Tat inhibits glucose absorption in Caco-2 cells, and this effect was associated with SGLT-1 missorting [42]. Also α-tubulin staining was drastically decreased in Tat-exposed Caco-2 cells [42], which coincides with the altered actin structure we observed in Tat-treated Caco-2 and HT-29 cells. It was previously reported that Tat affects the microtubule cytoskeleton inducing apoptosis of T cells [43,44]. Similar to what observed in T cells, Tat induces oxidative stress and destroys the cytoskeleton in intestinal cells and this is the result of the direct interaction with the enterocyte. Our findings show a profound disruption of the enterocyte cytoskeleton in HIV infection. Therefore, Tat induces a combined enterotoxic and a cytotoxic effect in human intestine.

The data reported herein were obtained in the same experimental model previously used to evaluate the enteropathogenic effects of Tat [16,42] and show that the redox mechanism is implicated in the enteropathogenic effects of HIV. Tat-induced apoptosis in enterocytes is supported by our finding of the mitochondrial release of cytochrome c in the cytosol, the consequent caspase-3 activation and by increased PARP cleavage. All these effects are well in agreement with our previous result that Tat inhibits enterocyte cell proliferation [16,42].

Attene-Ramos and colleagues demonstrated that the mucosal redox state is linked with intestinal proliferative activity [45]. Proliferating Caco-2 cells are vulnerable to oxidizing agents and a decrease in GSH levels induces transition to an apoptotic phenotype [46]. Moreover, an intracellular redox imbalance results in abnormal expression of genes involved in cell proliferation and growth, cell cycle progression, cytoskeleton structure and cell-to-cell adhesion [47], suggesting that redox alterations impair the enterocyte cycle. We found that Tat significantly induces oxidative stress and apoptosis in human intestinal epithelial cells with a specific sequence of events. Tat induced an early increase of ROS and an imbalance of the GSH/GSSG ratio that resulted in apoptosis. The two events are sequential and linked since an antioxidant prevented apoptotic cell damage.

Previously Nazli et al. demonstrated that gp120, but not Tat, affects intestinal epithelial integrity in a short time evaluation, with a major effect at apical side of intestinal epithelium [15]. Our results indicate that gp120 induces an imbalance in GSH/GSSG

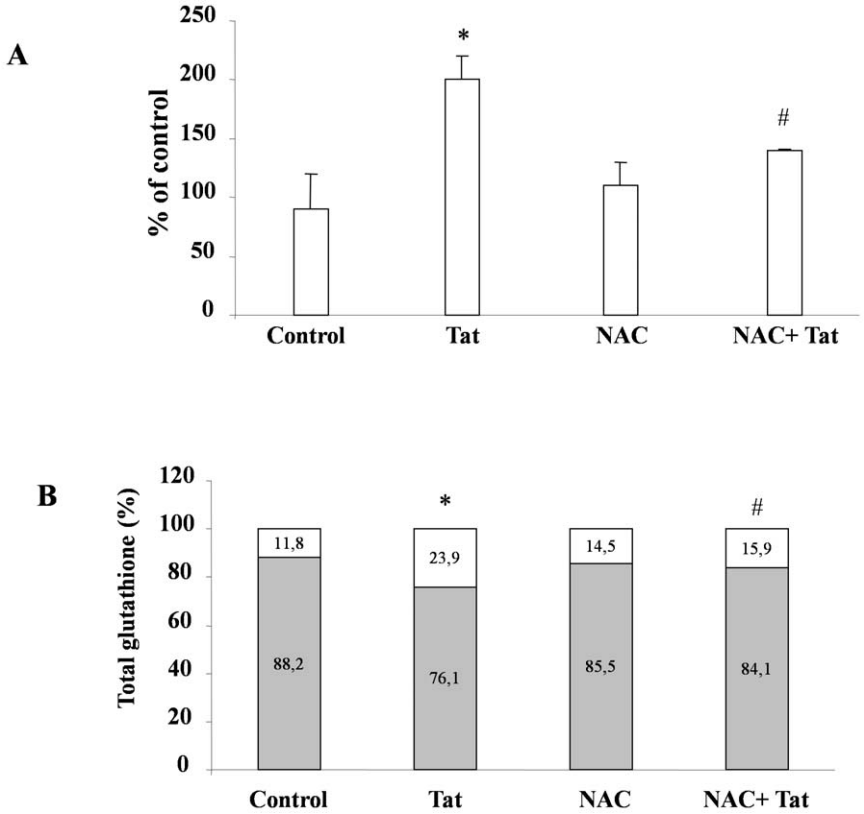

Figure 9. Effect of NAC on the Tat-induced oxidative stress in Caco-2 cells. Intracellular ROS levels, determined by fluorometric method, after exposure of Tat with or without pretreatment with NAC (A). Data were represented as percent of controls. Effect of NAC on Tat-induced GSH/GSSG imbalance (B). Data are represented as percent of GSH (grey) and GSSG (white) vs total glutathione. *$p<0.05$ vs control; #$p<0.05$ vs Tat. Data are representative of 3 separate experiments.
doi:10.1371/journal.pone.0029436.g009

ratio with a more potent effect at apical side after 1 hour of stimulation but it does not induce significant caspase-3 activation suggesting that apoptosis is not induced by this viral protein. According to the different effects induced by Tat and gp120, we hypothesize that the two viral proteins act in an integrated mode with a different timing. gp120 is responsible to damage intestinal epithelium by luminal side in the early phase of infection allowing the virus to pass through the intestinal barrier. Subsequently Tat induces a sustained epithelial damage and oxidative stress which persists in the long term. These results raise the possibility of a novel therapeutic strategy for HIV patients, i.e., restoration of GSH levels. Antioxidants are considered therapeutic agents in oxidative stress-related diseases. Preliminary data indicate that NAC improves immunological functions and GSH levels in HIV patients [48,49]. Our experimental results with NAC support this concept at intestinal level.

In summary, the results of this study indicate that Tat induces oxidative stress by increasing ROS levels and impairing the GSH/GSSG balance in human intestinal epithelial cells suggesting a possible role in the HIV-enteropathy (Fig. 12). Tat also increases apoptosis. There is evidence that the intestinal tract, being a major lymphoid organ, is a source of virus replication [50]. Chun et al. have recently shown, in ileum biopsies from 8 patients, that gut-associated lymphoid tissue remains a major reservoir of HIV with potential residual cryptic replication despite long-term HAART [50]. In addition, HIV detection in gut mucosa is not influenced by levels of plasma viral load or antiretroviral therapy [13]. In this context there is a significant association between CD8+ T-cell response in rectal mucosa and plasma viral load and blood CD4 count in HIV-infected patients [51,52]. The afore-mentioned results are fully in keeping with the concept that the intestine plays a major role in the pathogenesis of HIV infection, even in patients on successful HAART, and that Tat protein could be a major mediator of viral effects. Here we show that HIV-Tat protein plays a key role in inducing intestinal functional alterations including transepithelial secretion, glucose malabsorption and finally an oxidative stress condition with apoptosis leading to a compromised epithelial architecture and pretreatment with the antioxidant NAC prevented redox imbalance and apoptosis.

Methods

Intestinal cell line cultures

Caco-2 cells were used as small intestinal cell model [53]. At 15 days post-confluence, cells exhibit a well-differentiated brush border on the apical surface and tight junctions with typical small-intestinal microvillus hydrolases and nutrient transporters. Caco-2 cells were grown in Dulbecco's modified Eagle minimum essential medium (DMEM; Gibco, USA) with a high glucose concentration (4.5 g/L) at 37°C in a 5% CO_2 atmosphere. The medium was supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 1% non-essential amino acids, penicillin (50 mU/mL) and streptomycin (50 μg/mL), and changed daily. To verify some data obtained in Caco-2 cells, we used HT-29 as a supplementary

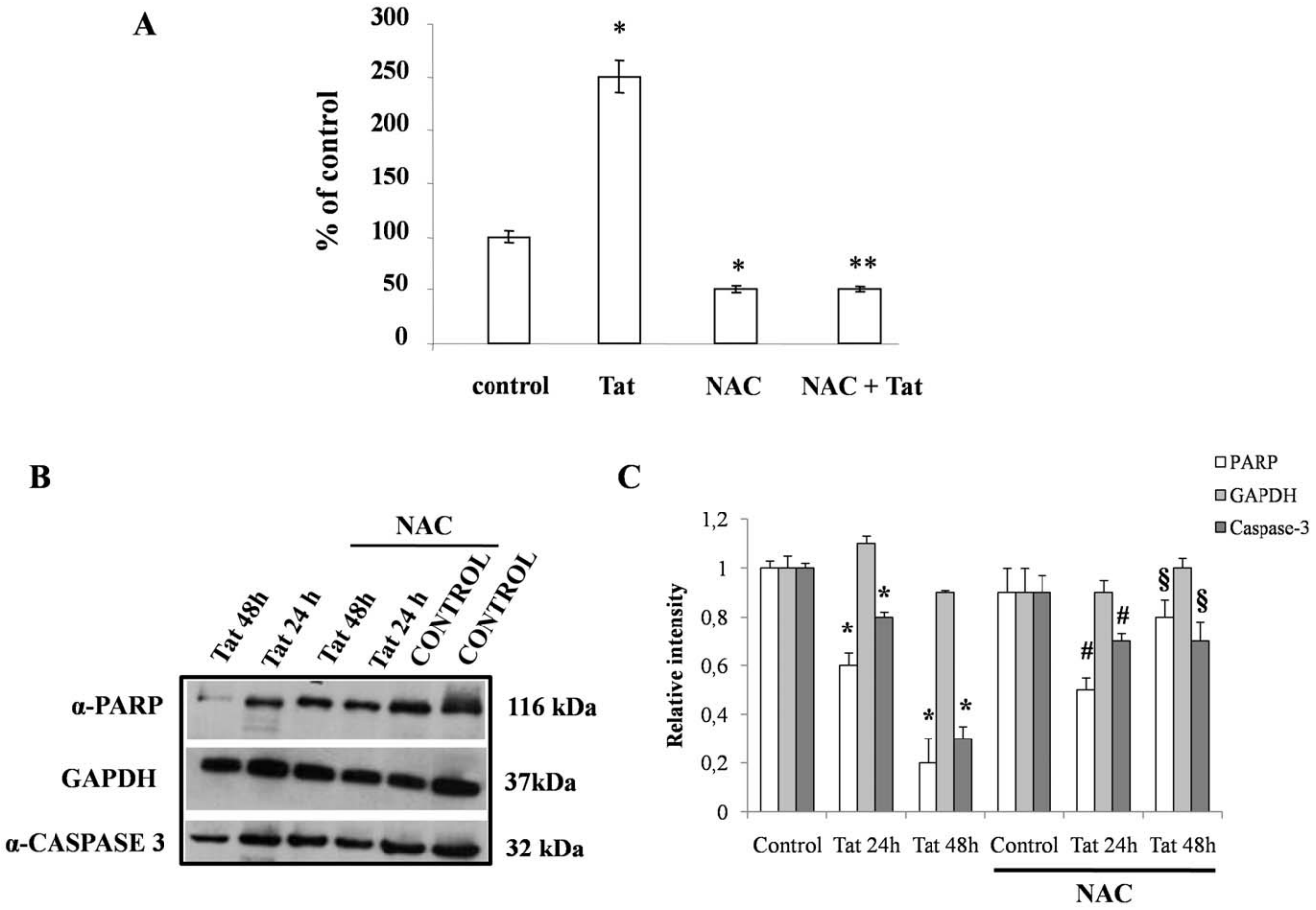

Figure 10. Effect of NAC on the Tat-induced apoptosis in Caco-2 cells. Caspase-3 activity (A) and full-length protein (B lower panel) were evaluated after exposure of Tat with or without pretreatment with NAC. Data were represented as percent of controls. *p<0,05 vs control; **p<0,05 vs Tat. Cleaved PARP was evaluated in the same western blot (B upper panel). Normalization of western blot was performed with GAPDH in all experiments (B middle panel). Data are representative of 3 separate experiments. (C) Densitometric acquisitions are shown from three separate experiments. *p<0,05 vs control; #p<0,05 vs control with NAC; §p<0,05 vs Tat at the same time without NAC.
doi:10.1371/journal.pone.0029436.g010

intestinal cell model. HT-29 were grown in RPMI 1640 cell culture medium supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 100 mU/ml penicillin and 100 μg/ml streptomycin.

To evaluate the polar nature of the effects induced by Tat on Caco-2 cells, monolayers were grown on polycarbonate filter (Corning Incorporated, NY USA) for 15 days post-confluence and then exposed to Tat 0,5 nM for 1, 24 and 48 hours at apical or basolateral side and then GSH/GSSG ratio and caspase-3 activation were evaluated as previously described. Controls were stimulated with the same volume of medium without Tat.

Fluorescence microscopy

Cells were grown on the chambered cover glass for 3 days. Cells were exposed to 20 μM 7′-dichlorofluorescein diacetate (DCFH-DA, D6665; Sigma-Aldrich, St. Louis, MO) for 1 hour at 37°C in the dark. Then, they were treated with Tat (0.5 nM), adding the protein directly to the cell culture, and incubated at 37°C for 1 hour to detect the ROS. Cells were washed in PBS and after mounting, the fluorescence images from multiple fields of view were obtained using a Nikon Eclipse 80i microscopy. The images were analyzed using NIS Elements D imaging software. A positive control was obtained by incubating cells with H_2O_2 10 mM for 5 min.

Reactive oxygen species production

The production of ROS was measured using the DCFH-DA fluorometric method, which is based on the ROS-dependent oxidation of DCFH-DA to DCF. Caco-2 cells were grown in 6-well plates for 15 days post-confluence. Cell monolayers were treated with Tat 0.05, 0.1 and 1 nM for 15 min or with Tat 0.5 n M for 15, 30, 60 and 120 min at 37°C. Medium was removed and cells were washed by PBS. Then, cells were treated with DCFH-DA (20 μM) for 30 min at 37°C in the dark. Intracellular ROS production was measured with a spetrofluorometer (SFM 25; Kontron Instruments, Japan). A positive control was obtained by incubating cells with H_2O_2 10 mM for 5 min. Neutralization experiments were performed incubating Tat 0.5 nM with 30 ng/ml of anti-Tat polyclonal antibodies (Tecnogen, Piana di Monte Verna, Italy) at +4°C overnight with gentle shaking. The preparations were then used to stimulate cells.

Antioxidant defenses evaluation

GSH and GSSG intracellular levels were measured by method described by Allen et al. with few modifications [54]. Briefly, cells were exposed to Tat alone or in combination with NAC and then lysed with Triton X-100. Protein was precipitated with 1%

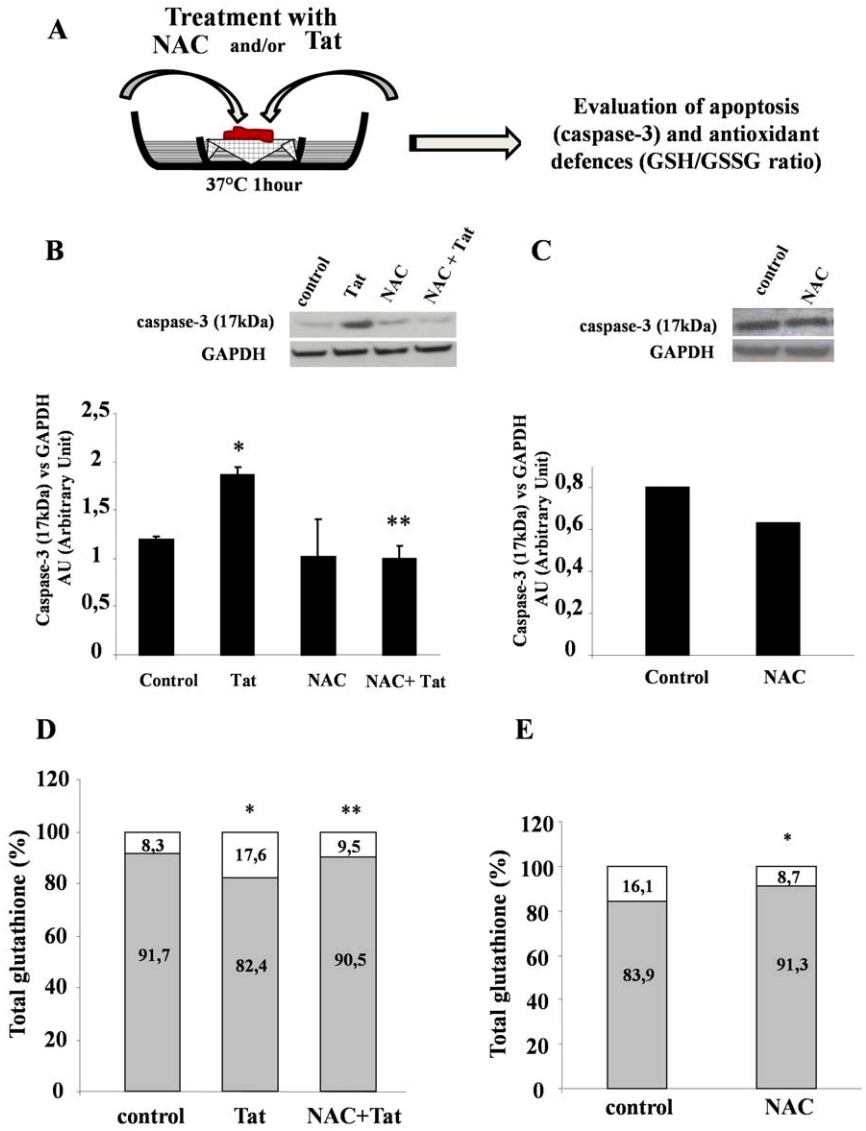

Figure 11. NAC prevented apoptosis and oxidative stress induced by Tat in human intestinal mucosa. Duodenal mucosal specimens were exposed to Tat alone or combined with NAC in an *ex-vivo* organ culture model (A). Panels B and C, Western blot on whole cell lysate was performed using anti-caspase-3 mouse monoclonal antibody (upper panel) and anti-GAPDH mouse monoclonal antibody in 4 HIV-negative children (B) and in an HIV-positive child (C). Densitometric acquisition of caspase-3 protein was normalized for GAPDH expression. Panels D and E, The GSH (grey)/GSSG (white) ratio was evaluated as described under "Methods" in intestinal mucosa of 4 HIV-negative children (D) and in an HIV-positive child (E). *$p<0.05$ vs control; **$p<0.05$ vs Tat. Results are expressed as the means SEM.
doi:10.1371/journal.pone.0029436.g011

sulfosalicylic acid and supernatants used to measure in parallel total and reduced glutathione. Oxidated glutathione (GSSG) was determined by subtracting the reduced form from total glutathione. GSH and GSSG values were normalized for protein content. All assays were performed three times. Catalase activity, a well-established indicator of oxidative stress, was measured with an assay kit from Calbiochem (Gibbstown, NJ) and normalized for protein content. Neutralization experiments were performed incubating Tat 0.5 nM with 30 ng/ml of anti-Tat polyclonal antibodies (Tecnogen, Piana di Monte Verna, Italy) at +4°C overnight with gentle shaking. The preparations were then used to stimulate cells. gp120 (ab69717) and goat polyclonal anti-gp120 antibody (ab21179) were from Abcam plc (Cambridge, UK).

Oxidative stress evaluation in HIV-positive patients

An aliquot of rectal dyalisis, urine and serum samples from 20 patients infected with HIV and 6 control subjects aged 26–228 months were obtained during a routine check-up. All HIV patients had been on HAART for more than 3 years. Immediately after collection, samples were separated into 2 mL aliquots and stored at −80°C until analysis. We used levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG), as biomarker of DNA oxidative stress. 8-OHdG was measured in duplicate using a highly sensitive ELISA kit (Li StarFish Srl, Milan, Italy). The length of dialysis bag is 3 cm and the concentration of oxidative marker was normalized for the exact volume inside the bag. Differences were evaluated by the Mann-Whitney test. The Pearson's correlation coefficient was applied to investigate the correlation between 8-OHdG and viral load.

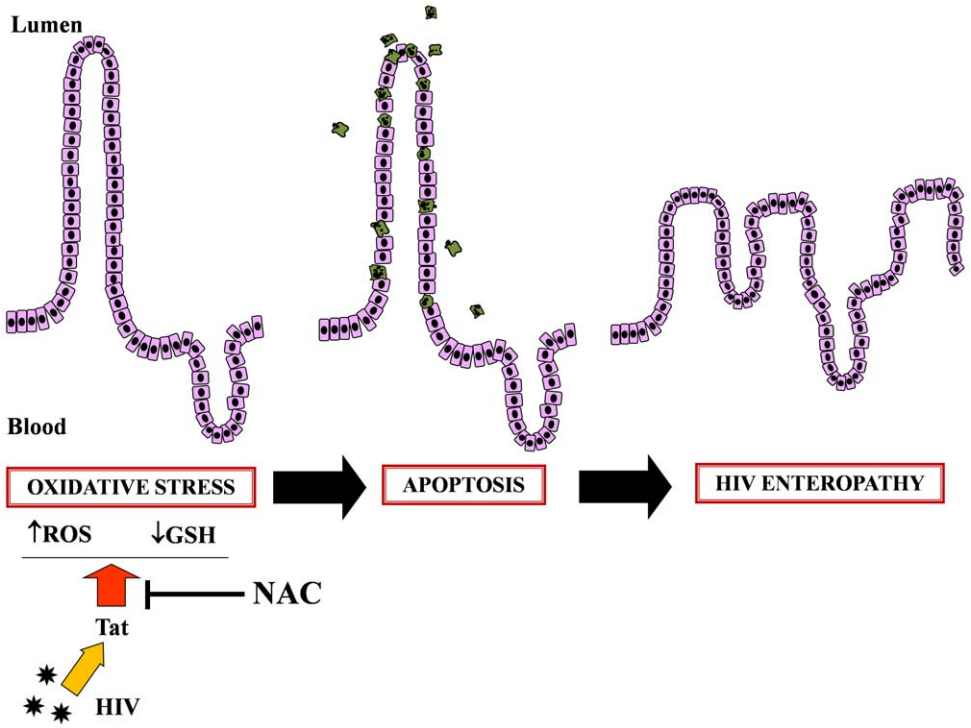

Figure 12. Schematic representation of the mechanism of HIV Tat viral protein-induced oxidative damage to the intestinal mucosa and the protective role of the antioxidant N-acetylcysteine (NAC). Tat induces oxidative stress by increasing the ROS intracellular level and deranging the GSH/GSSG ratio. This leads to programmed cell death (apoptosis) and an increase in epithelial damage. Together with ion secretion [15] and altered glucose transport [39], these steps could represent key mechanisms in HIV enteropathy. Pretreatment with the antioxidant NAC restores the oxidative stress and cell apoptosis, thus protecting intestinal mucosa from gut dysfunction.
doi:10.1371/journal.pone.0029436.g012

Actin staining

One step of fixation and permeabilization was performed with 4% paraformaldehyde and 0.2% Triton X-100 for 30 min at +4°C. After three washes in PBS, the cells were treated with a 50 µg/ml solution of fluorescein isothiocyanate-phalloidin (Sigma-Aldrich, Milan, Italy) in PBS for 40 min. Nuclei were stained with Hoechst 5 µg/ml (Sigma-Aldrich, Milan, Italy) for 5 min at +4°C. The cells were washed three times with PBS and were mounted with Mowiol (Invitrogen S.R.L, San Giuliano Milanese, Italy). The monolayers were examined using a Nikon Eclipse 80i epifluorescent microscope (FITC filter). The images were analyzed using NIS Elements D imaging software.

Caspase-3 activity assay

We used caspase-3 as a marker of apoptosis [55]. An apoptosis assay kit was used to determine caspase-3 activity, according to the manufacturer's instructions (Biovision, Mountain View, CA). Caspase-3 activity was investigated in Caco-2 cells by the release of the chromophore pNA after substrate cleavage. Modifications of caspase-3 activity were determined by comparing the sample optical density (OD) with the control.

Immunoblotting

Total cells lysates were obtained by homogenization of cell pellets in cold lysis buffer (20 mM Tris, pH 7.5 containing 300 mM sucrose, 60 mM KC1, 15 mM NaC1, 5% (v/v) glycerol, 2 mM EDTA, 1% (v/v) Triton X-100, 1 mM PMSF, 2 mg/ml aprotinin, 2 mg/ml leupeptin and 0.2% (w/v) deoxycholate) for 1 min at 4°C and further sonication for additional 30 sec at 4°C. Cytosolic, microsomal and mitochondrial fractions were prepared with the Qproteome Mitochondria Isolation Kit (Qiagen). Equal amounts of protein were subjected to 10% (v/v) SDS-PAGE and transferred to a PVDF membrane (Millipore). The membrane was blocked with 5% (w/v) skim milk and incubated with primary antibody, followed by incubation with an HRP-conjugated secondary antibody. Proteins were visualized with an ECL detection system (GE-Healthcare). The following antibodies were used for Western blot analysis: rabbit polyclonal anti-Tat antibody (Tecnogen, Piana di Monte Verna, Italy), mouse monoclonal anti-caspase3 antibody (full length protein), mouse monoclonal anti-PARP antibody, goat polyclonal cytochrome c antibody, mouse monoclonal anti-COX IV antibody, mouse monoclonal anti-tubulin antibody, mouse monoclonal anti-GAPDH antibody (Santa Cruz Biotechnology). gp120 (ab69717) and goat polyclonal anti-gp120 antibody (ab21179) were from Abcam plc (Cambridge, UK).

Co-immunoprecipitation

A total of 800 µg of protein lysate from the Caco-2 cells exposed to Tat 0,5 nM 24 hrs were precipitated with 1 mg of tubulin antibody. Protein A/G agarose beads (Santa Cruz) were used to collect the immunoprecipitated complexes and the beads were washed with PBST before SDS-PAGE and Western blot analysis with anti-Tat antibody.

Experiments in human small intestinal specimens

Biopsies from the distal part of the duodenum were obtained from 5 children seen at the Department of Pediatrics and undergoing endoscopy (84–192 months of the age) for intestinal disorders. All biopsies were from macroscopically normal areas,

and intestinal histology was subsequently reported to be normal. Tissue samples were transported to the laboratory in culture medium and processed within one hour. Duodenal mucosa specimens were obtained from 4 HIV-negative children and from one HIV-positive child. Specimens were washed and observed by stereomicroscope to exclude tissue necrosis. Organ culture was performed in DMEM with a high glucose concentration (4.5 g/L) supplemented with 0.5% FCS, 1% non-essential amino acids, 2% penicillin (50 mU/mL) and streptomycin (50 mg/mL) and incubated in 5% CO_2/95% air for one hour before treatment. Short-term experiments were run using high Tat concentrations to maximize the cytotoxic effect before spontaneous tissue disruption. Specimens were exposed to Tat alone (0.1 μM) or preincubated with NAC (10 mM for 4 h). Short-term experiments with an higher Tat concentration were performed to maximize the effect before spontaneous tissue disruption. After stimulation, samples were homogenized and lysed in RIPA buffer: 100 mM Tris-HCL pH 7.5, 300 mM NaCl, 2% NP40, 1% Na deoxycholic acid, 0.2% SDS, 100 μg/ml PMSF, 5 μg/mL aprotinin, 1 μg/mL leupeptin, 0.7 μg/ml pepstatin. Whole extracts were centrifuged and protein content was determined by the Bradford assay. GSH/GSSG intracellular levels and caspase-3-cleaved protein were evaluated as described above. For western blot assay we used the mouse monoclonal anti-caspase-3 cleaved protein (Cell Signaling Inc., Danvers, MA). The experiments were undertaken with the understanding and written consent of each child's parents and the study methodologies conformed to the standards set by the Declaration of Helsinki. The study protocol was approved by the Ethics Committee of the School of Medicine, University of Naples Federico II, Italy.

Statistical analysis

We used GraphPad Prism Software (San Diego, CA) to evaluate the two-tailed unpaired Student *t* test and a 2-tailed paired Student *t* test to evaluate statistical significance. An alpha value of 0.05 was set for statistical significance. p-Values for each analysis are indicated in figure legends.

Supporting Information

Figure S1 Fluorescence staining of ROS. ROS intracellular levels were evaluated in Caco-2 cells by DCF fluorimetric method (A) and at fluorescence microscope (B). In parallel, cells from the same culture and in the same conditions were exposed to Tat and a western blot was performed with anti-Tat polyclonal antibody (C). *p<0,05 vs control.
(TIF)

Figure S2 The anti-Tat polyclonal antibody blocks the Tat-induced imbalance of the GSH/GSSG ratio in HT-29 cells. HT-29 cells were incubated with Tat in the presence and absence of the anti-Tat polyclonal antibody. Data are represented as percent of GSH (grey) and GSSG (white) vs total glutathione. Data are representative of 3 separate experiments.*p<0,05 vs control; **p<0,05 vs Tat.
(TIF)

Figure S3 8-OHdG levels in urine and serum in HIV-negative and -positive children. 8-OHdG was used as oxidative stress marker evaluated in urine (A) and serum (B) in serum-negative (SN) and positive (HIV+) children. There were no significant differences between the two groups.
(TIF)

Figure S4 Influence of HIV-Tat protein on apoptosis in HT-29 cells. Caspase-3 activity (A) and full-length protein (B lower panel) were evaluated in Tat-treated HT-29 cells. To verify that Tat induced apoptosis, cleaved PARP was evaluated in the same western blot used to evaluate the activation of caspase-3 (B upper panel). Normalization of western blot was performed with GAPDH in all experiments (B middle panel). Data are representative of 3 separate experiments.
(TIF)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: VB FE AG. Performed the experiments: VB GL. Analyzed the data: VB GL EN. Contributed reagents/materials/analysis tools: EM FE. Wrote the paper: VB FE AG.

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Time- and Segment-related Changes of Postresected Intestine: A 4-dimensional Model of Intestinal Adaptation

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ABSTRACT

Objectives: The aim of the present study was to investigate the segment- and time-related changes in rat short bowel syndrome and construct a 4-dimensional (4D) geometrical model of intestinal adaptation.

Methods: Sprague-Dawley rats were divided into 3 groups: 2-day, 7-day, and 15-day postresection groups in which 75% of the jejunoileum was removed. Histological and morphometrical parameters in the remaining proximal to distal intestinal segments, from the jejunum to the distal colon, were comparatively evaluated in the groups. The data were used to construct a 4D geometric model in which villi were considered as cylinders, and their surface area was expressed as cylinder lateral area.

Results: Major adaptive changes were observed in the ileum consisting of an increase in both the diameter of base and the height of villi. A parallel reduction in their number/mm^2 was observed. The resulting ileal architecture was characterized by a limited number of large villi. An opposite pattern was observed in the jejunum whose postresection structure consisted of an increased number of villi. No changes were observed in the colon. Postresection restructuring was early and faster in the ileum than in the jejunum resulting in an increase in absorptive area of 81.5% and 22.5% in the ileum and jejunum, respectively.

Conclusions: Postresection adaptation is intestinal segment–specific because all of the major changes occur in the ileum rather than in the jejunum. Sparing ileal segments during resection may improve the outcome of patients undergoing extensive intestinal resection. Our 4D model can be used to test interventions aimed at optimizing postresection intestinal adaptation.

Key Words: 4-dimensional model, children, intestinal adaptation, intestinal failure, surgical short bowel

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Short bowel syndrome (SBS) is a clinical condition resulting from massive enterotomy and is the most frequent cause of intestinal failure in children. In children with extensive intestinal resection, parenteral nutrition is required for survival, but this procedure is associated with frequent and severe complications (1). The length of the remnant intestine and the presence of the ileocecal valve predict the chance of acquiring intestinal sufficiency (1,2). After extensive small bowel resection, the remaining intestine undergoes compensatory changes to maintain its absorptive function (3–5) consisting of an increase in small intestinal mucosal thickness, villus length, and crypt depth (6,7). Intestinal crypt cells activate pathways of gene expression of adaptation and development resembling the developing immature intestinal tissues (8). These changes are interpreted as a homeostatic response to increase the remaining digestive-absorptive surface (9). A crucial factor of adaptation is the time of changes. When a large part of the intestine is suddenly removed, a prompt response is needed to ensure transepithelial ion fluxes, restore the barrier against bacterial translocation, and restart motility. Timely changes are essential for survival; however, intestinal adaptation, its mechanisms, and times are still largely unknown (10–12). In addition, the role and adaptive pattern of proximal to distal intestinal segments are also unknown.

Menge et al found that the total mucosal surface of ileal segments already was increased in the proximal but not in the distal remnants at the fourth day postresection (13). We tested the hypothesis that intestinal adaptation in SBS is segment and time specific and adaptive changes take place with a specific structural and temporal pattern in proximal to distal intestinal segments. To this end, we studied the qualitative and quantitative changes of proximal to distal intestinal epithelial architecture, including villus area and volume, at 3 time points after resection, in a model of extensive small bowel resection. Using the morphometric data obtained in the ileum and jejunum, we constructed a 4-dimensional (4D) geometric model of epithelial structure. The model provides an accurate experimental standard for investigations of postresection structural events and can be used to test the effects of nutrients and drugs on adaptation.

METHODS

Experimental protocol: Wistar rats weighing between 210 and 270 g were used. The animals were housed in individual cages, under a 12-hour light-dark regimen and humidity and temperature control. They were divided into 3 groups: resected animals (n = 19), sham-resected control animals that underwent ileal transection with subsequent end-to-end anastomosis (n = 10), and nonoperated control animals (n = 7). The animals were killed by CO_2 inhalation 2, 7, and 15 days after surgery because adaptive intestinal changes reach a plateau 15 days after intestinal resection in rats (14–16).

Surgery was performed on animals anesthetized with diazepam, ketamine, and medetomidine. The length of the small intestine from Treitz ligament to the ileocecal valve was measured in situ, still attached to the mesentery, under constant tension. Then, 75% of the mid-small bowel was resected, leaving the proximal 12.5% corresponding to the remaining jejunum, and the distal 12.5%

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corresponding to the remaining ileum (16,17). A primary end-to-end anastomosis was performed and the length of remaining small intestine ranged from 13 to 20 cm. Animals received 5% glucose solution after surgery, wetted food from the second day after surgery, and then were allowed free access to food. Postoperative analgesia and the antibiotic enrofloxacin were given and animals' weight was recorded daily.

The animals were fed a normal standard diet with no restriction on food or water supply for 1 week before surgery. Before surgery, rats were fasted overnight and weighed. On the first postoperative day, rats had free access to water and rodent diet.

The experimental protocol was approved by the local ethics committee of the Ospedale Cardarelli (no. 1292/09/CB; February 3, 2009). The animals were treated and housed according to national and international regulations governing the use of animals in scientific research.

Tissue sampling: bowel samples were taken 2, 7, and 15 days postoperatively. Six proximal-to-distal intestinal segments were obtained from each animal as follows: antrum (1.5 cm proximal to the pylorus); duodenum (3 cm distal to the pylorus); proximal jejunum (3 cm proximal to the anastomosis in the resected group and 10 cm distal to the ligament of Treitz in the control group); distal ileum (3 cm distal to the anastomosis); right colon (10 cm distal to the cecum); left colon (proximal to the rectum). All of the intestinal segments were weighed, measured, and rinsed with ice-cold saline to remove any luminal contents. Tissue sections measuring 1 cm^2 were cut from each segment along the longitudinal axis and used for histological and morphometric analysis.

Intestinal specimens were fixed in 10% buffered formalin for 24 hours, dehydrated and embedded in paraffin wax using standard techniques. Four-micrometer sections were cut perpendicular to the mucosa, placed on gelatin-coated glass, and stained with hematoxylin and eosin. At least 10 well-oriented sections from each intestinal segment were prepared and evaluated by linear quantitative methods using light microscopy. All of the morphometric and counting procedures were performed by 2 independent, blinded investigators. The following parameters were obtained for each segment: total wall thickness (micrometers), total mucosal thickness (micrometers), and inflammatory cells in the mucosa (number of cells/mm^2 of mucosa and degree of inflammatory infiltrate). The glandular height in the antrum was also measured and is expressed in micrometers.

Villus height (in micrometers), number of villi (villi/mm^2 of mucosal length), crypt depth (micrometers), and villus/crypt ratio were recorded in the duodenum, proximal jejunum, and distal ileum. In the small intestine, only villi and crypts cut throughout their length were measured. The distance from the tip to the base of the villus was taken as villus height. The distance from crypt base to villus-crypt junction was taken as crypt depth. Total mucosal thickness was measured in the proximal and distal jejunum by calculating the distance from the tip of the villus to the muscularis mucosae. Small bowel thickness was determined by calculating the distance from the villus tip to the serosal extremity of longitudinal muscle.

An adaptive response consisting of an increase in mucosal mass may occur through an increase in the number or in the size of intestinal villi or both. We tested the hypothesis that the adaptive response is segment-specific and that the specificity is associated with different mechanisms of adaptation. To address this issue, we applied the geometry of cylinders to villi and calculated epithelial surface expressed as cylinder surface lateral area. The formula to calculate the surface area is the following: surface area $= 2(\pi r^2) + (2\pi r) \times h$, where h is the height of the cylinder and r is half the measure of the base. Then we constructed a 4D geometric model using form-Z, version 6.0 software (Auto·Des·Sys Des·Sys Inc, Columbus, OH) by fitting the villi measures to obtain a spatial representation of adaptive changes in proximal to distal segments. This software is a 3D modeling program combining solids and surface modeling and is used for the first time to construct a biological model. The fourth dimension is time.

For the statistical analysis, results are mean ± standard deviation, with significance determined by analysis of variance test at the $P < 0.05$ level.

RESULTS

General Findings

Two rats died 1 day after surgery. Three animals (1 in the control group and 2 in the 15-day resected group) were excluded from the study because a stricture developed at the anastomotic site. At postmortem analysis, both showed a dilated bowel proximal to the anastomosis. Mean body weight did not differ significantly before and after intestinal resection or between sham-resected and resected rats 2, 7, and 15 days after surgery. Data from nonoperated rats were also identical to the data obtained with the study groups.

Macroscopical Evaluation

The gut reacted to massive resection with a general adaptive response consisting in catch-up growth of the remaining small intestine. Fifteen days after surgery, the small intestine showed an increase in mass of 48.2% ± 7.6% over baseline. The adaptive changes had a well-defined segmental pattern. Figure 1A shows the major macroscopical findings 15 days postsurgery. Changes were clearly evident in the ileum: its weight/cm^2 was almost 2-fold higher than in sham-resected animals. The stomach, the duodenum, and the jejunum underwent minor, not significant modifications, although there was a consistent trend toward an increase in all 3 segments, which could be interpreted as an expression of minimal adaptation. Finally, the proximal and the distal colonic segments were virtually unchanged after small bowel resection 2, 7, and 15 days after surgery compared with sham-resected and control rats. Total bowel thickness showed a similar segmental pattern with the ileum undergoing the most evident changes (Fig. 1B).

Microscopical Evaluation

At microscopic evaluation, intestinal adaptation consisted of an increase in villus height, crypt depth, and mucosal thickness in the jejunum and ileum. The increase in villus height occurred 7 days after surgery in the ileum, whereas villus adaptation ceased 15 days after surgery in the jejunum (Fig. 1C). The crypt adaptive response was a distinct time-dependent process with a peak at day 15 in both ileum and jejunum (Fig. 1C). Two days after surgery, no changes were found in length and crypt depth, in the jejunum, or in the ileum. Overall, the ileum was the major site of intestinal adaptation; the major modifications were observed 15 days after surgery, and consisted of an increase in both villus height and crypt depth. Villus height and crypt depth were significantly increased also in the jejunum, albeit to a lesser extent than in the ileum. There were no substantial changes in the stomach, duodenum, or colon (data not shown).

To evaluate whether the different adaptive responses of intestinal segments were linked to segment-specific restructuring of the intestinal architecture, we analyzed the villus/crypt ratio in all of the intestinal segments. This ratio was conserved in all of the intestinal segments and there was a close overlapping of numerical values between resected and sham-resected animals at 15 days postsurgery (Fig. 2).

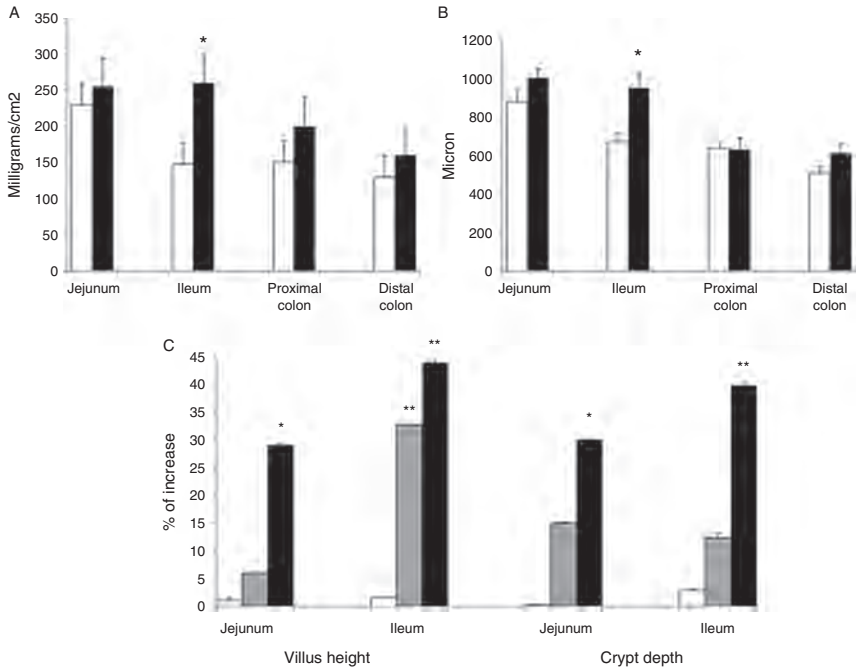

FIGURE 1. A, Total wet weight modifications in different intestinal segments 15 days after extensive small intestinal resection (black bars) compared with sham-resected animals (white bars); $^{*}P < 0.01$ versus sham resected. B, Total bowel thickness in different intestinal segments 15 days after extensive small intestinal resection (black bars) compared with sham-resected animals (white bars); $^{*}P < 0.001$ versus sham resected. C, Increase in villus height and crypt depth in jejunum and ileum 2 (white bar), 7 (grey bar) and 15 days (black bar) after extensive small intestinal resection versus sham-resected animals. $^{*}P < 0.05$ versus sham resected; $^{**}P < 0.01$ versus sham resected.

Light microscopy did not reveal evidence of inflammatory changes in the ileum or jejunum. This indicates that the observed changes were because of a true compensatory increase in epithelial cell mass, rather than to a change in inflammatory mucosal fluid content.

Morphometric Evaluation

The different adaptive pattern in the jejunum and ileum supports the hypothesis that adaptation is the result of distinct mechanisms. To address this issue, we analyzed the segment-specific morphometric changes of the intestinal structure. The number of villi per linear millimeter of mucosa was calculated for each intestinal segment. The area and volume of villi were also measured in all of the segments. Finally, the overall increase of the intestinal surface was calculated for each segment. Minor quantitative and qualitative changes were observed 2 days after surgery, although differences were not significant. Adaptation became progressively more evident at subsequent observations. The number of villi in the ileum was significantly lower in resected animals than in sham-resected animals. In parallel, structural modifications were found in the ileal villus architecture, that is, there was an increase both in the diameter of the villus base and in villus height (Fig. 3). In contrast, the number of villi in the jejunum was similar to that observed in sham-resected animals; however, jejunal villi were longer, whereas the diameter of the villus base remained unchanged (Fig. 3).

We identified a time-related increase in morphometric parameters in the jejunum and ileum (Table 1), which indicates that the time course pattern and the type of architectural restructure were both segment specific. There were no modifications 2 days after surgery, whereas there was a significant increase in villus height and crypt depth at day 7 postsurgery in the ileum and at day 15 postsurgery in the ileum and jejunum segments. In both segments, the area and the volume of the intestinal surface increased, resulting in an increase of surface area, but the bulk of changes occurred in the ileum, which suggests that the latter possesses the highest adaptive plasticity (Fig. 4). Changes were also faster in the ileum than in the jejunum with an increase of >50% of surface area 7 days after surgery in the ileum versus 5% in the jejunum. The ileal segment located distally to the anastomosis was the major site of adaptive changes, which comprised mainly an increase of total bowel thickness, weight, and volume (Figs. 1 and 4).

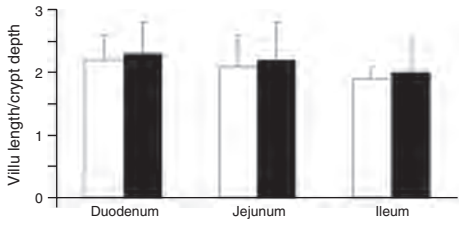

FIGURE 2. Villus/crypt ratio in different small bowel segments 15 days after extensive small intestinal resection (black bars) compared with sham-resected animals (white bars).

Construction of a 4D Model of Segment-specific Intestinal Adaptation

The adaptive changes in jejunum and ileum were associated with a segment-specific restructuring of epithelial architecture. Based on the morphometrical data, a 4D geometric model of adaptation was constructed that provides a view of segment-specific

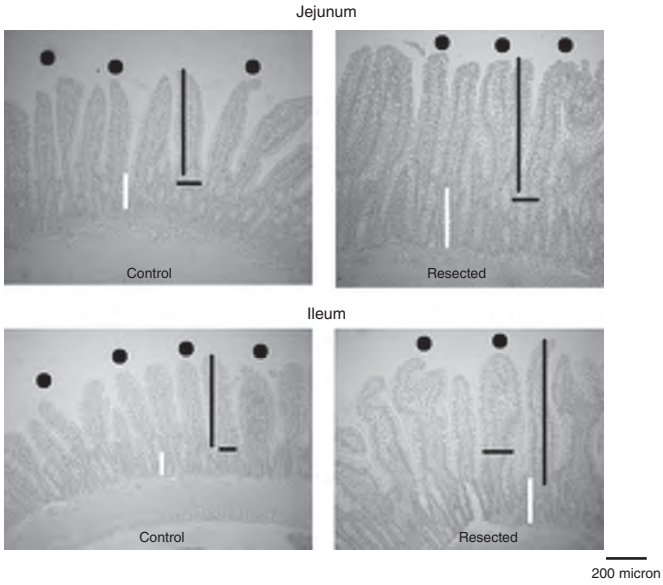

FIGURE 3. Comparative analytical histology of jejunum and ileum 15 days after extensive small intestinal resection compared with sham-resected animals (control). All of the fields are shown at the same magnification (Zeiss, original optical magnification ×200). In the jejunum, villus height is increased (black vertical bars). Base diameter is unchanged (black horizontal bars), and crypt depth is increased (white bars). The number of villi is unchanged (black circles). In the ileum, villus height (black vertical bars) and base diameter are increased (black horizontal bars). Crypt depth is (white bars). The number of villi is decreased (black circles).

changes (Fig. 5). Compared to sham-resected rats, the number of villi/mm^2 of mucosa did not change in the jejunum, whereas it decreased in the ileum. This observation, together with the changes in bowel thickness, wet weight, height, and diameter of villi, strongly suggests that adaptation takes place in the jejunum and ileum with segment-specific events. In the former, the mucosal increase was associated with an increase in villus height, whereas their number and diameter did not change. In contrast, in the ileum, the mucosal increase was the result of a massive increase in villus height and diameter, whereas the villus number decreased (Fig. 5). Villus area and volume were much larger in the distal ileum than in the proximal jejunum; as a result, the nutrient absorptive surface area was strikingly larger in the distal ileum. In contrast, the stomach and colon were not involved in the adaption processes.

DISCUSSION

The total intestinal absorptive surface area is approximately 250 m^2 in adult humans; however, there is a functional and

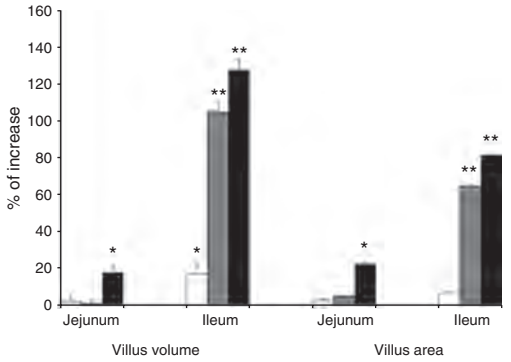

FIGURE 4. Time-course pattern of modifications of villus volume and area in the jejunum and ileum 2 (white bars), 7 (grey bars) and 15 days (black bars) after extensive small intestinal resection versus sham-resected animals. $^{*}P < 0.05$ versus sham resected; $^{**}P < 0.01$ versus sham resected.

structural segmental pattern of the intestine, and distinct intestinal segments have different mechanisms for nutrient absorption and for the transepithelial flux of electrolytes. In SBS, extensive intestinal resection suddenly leads to a dramatic reduction in intestinal surface producing an imbalance of hydroelectrolyte transport and hampered nutrient absorption. Most patients require parenteral nutrition for survival (18,19). In recent years, the anatomic definition of irreversible SBS has changed and, in parallel, the outcome of patients has improved. The longer survival of children with severe SBS provided the opportunity to observe the ability of the remaining intestine to adapt over time (20). Adaptation is affected by the time of refeeding and other clinical variables such as age and nutritional state (21); however, in our rat model, there was a clear segment-specific pattern of adaptation. A major difference in the adaptive responses of jejunum and ileum was the time-related pattern of adaptation. Two days after resection, the mucosal morphology of the rat jejunum and ileum did not differ from that of controls. Adaptation was completed in the ileum within 2 to 7 days, whereas adaptive changes continued in the jejunum at 15 days postsurgery. We do not know whether jejunal changes were completed at 15 days; however, the adaptive response was found to reach a plateau after 2 weeks in similar animal models of short gut (22,23).

The pattern of intestinal adaptation that we observed resembles the intrinsic development of intestine during embryonic development. In human infants, the intestine grows more rapidly during the last trimester of pregnancy, doubling its length by the 40th week or term gestational age (24,25). In contrast, the intestine in rats is immature at birth, but grows rapidly starting from the time of weanling at 18 to 22 days until reaching a plateau at 5 weeks of age (26). In our model, ileal architecture underwent major modifications. A major increase in surface area and volume was

TABLE 1. Comparative morphometric parameters of the adaptive response in jejunum and ileum

	Villus height (mean % ± SD)		Crypt depth (mean % ± SD)		No. villi/mm^2 (mean % ± SD)		Mean absorptive (mean % ± SD)	
	Jejunum	Ileum	Jejunum	Ileum	Jejunum	Ileum	Jejunum	Ileum
2 days	+1 ± 0.5	0 ± 0.02	0 ± 0.1	+2.8 ± 0.2	−1.4 ± 0.7	−8.1 ± 0.9	+2.54 ± 0.8	+6.5 ± 0.5
7 days	+6 ± 0.1	32.7 ± 0.2*	+15 ± 0.2*	+12.3* ± 0.9	+1.3 ± 0.2	−19.5 ± 0.5*	+5.06 ± 0.2	+64.97 ± 0.6*
15 days	+29 ± 0.3*	43.9 ± 0.5*	+30 ± 0.1*	+39.8 ± 0.5*	+5.4 ± 0.3*	−20.7 ± 0.1*	+22.54 ± 0.9*	+81.50 ± 0.7*

Data are expressed as % of increase over baseline.
$^{*}P < 0.05$ versus 2 days.

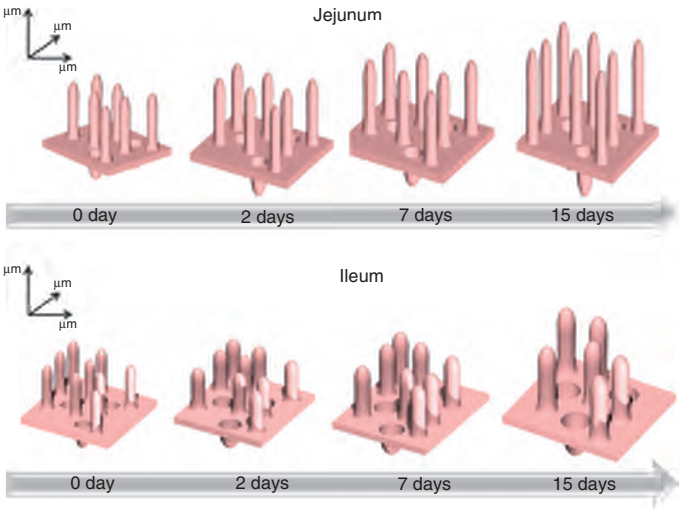

FIGURE 5. Graphic model of the time-related adaptive response observed in the rat short bowel model, showing the mucosal adaptation in the jejunum and ileum. The 4D geometric model was derived using data on villus height and base width and crypt depth as a function of time after bowel resection. It provides a 3D view of the adaptive response after intestinal resection. A differential adaptive response was observed in the jejunum and ileum. In the former, villi height was slightly increased (thin villi pattern). In contrast, in the ileum, the number of villi was decreased, but their volume became large (large villi pattern).

associated with a reduction in villi number. This led to an overall increase in villi surface. From the functional point of view, these changes enhance the adaptive response because the ileum is the main site of absorption of liquids, thereby allowing prompt restoration of transepithelial ion fluxes and of nutrient absorption, which obviously is an advantage in terms of postresection structural/functional intestinal modifications.

In an attempt to restore the intestinal functions, the adaptive response is fully functional in terms of the restructuring because there is an increase in absorptive surface area in a relatively short period.

Both drugs and nutrients have been used to stimulate cell growth and proliferation in SBS, namely, insulin (27), growth hormone and glucagon-like peptide-2 (28,29), glutamine, arginine, zinc, and, more recently, lactoferrin (30–32). The 3D model provides a tool to investigate the type and amount of fuel necessary to optimize absorptive changes and promote intestinal sufficiency. The 3D model was approached by other authors. Liao et al (33) constructed a 3D surface model of different gastrointestinal tracts, from the stomach to the colon. Their macroscopic model was developed to study the geometry and the morphology of the visceral organs to evaluate the visceral distention and curvature during stress events. The 3D modeling approach is a quantitative method that could be used as a useful and analytical tool to study the biochemical properties of intestinal mucosa in different physiological and pathophysiological states.

In conclusion, extensive intestinal resection results in a differential adaptive response in the remaining proximal and distal small intestinal segments. The bulk of changes is observed in the ileum distal to the anastomosis, whereas the adaptive response is less evident in the jejunum and does not involve the colon. The responses follow a different time- and segment-related pattern. Our findings show that the ileum plays a major role in postresection adaptation. This is reflected in the better outcome observed in patients with SBS with preserved rather than removed ileocecal valve (34,35). Sparing even small segments of ileum could result in adaptive changes that may be eventually associated with restoration of full intestinal digestive absorptive functions in children undergoing extensive intestinal resection.

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Bivalirudin in Combination with Heparin to Control Mesenchymal Cell Procoagulant Activity

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Abstract

Islet and hepatocyte transplantation are associated with tissue factor-dependent activation of coagulation which elicits instant blood mediated inflammatory reaction, thereby contributing to a low rate of engraftment. The aim of this study was i) to evaluate the procoagulant activity of human adult liver-derived mesenchymal progenitor cells (hALPCs), ii) to compare it to other mesenchymal cells of extra-hepatic (bone marrow mesenchymal stem cells and skin fibroblasts) or liver origin (liver myofibroblasts), and iii) to determine the ways this activity could be modulated. Using a whole blood coagulation test (thromboelastometry), we demonstrated that all analyzed cell types exhibit procoagulant activity. The hALPCs pronounced procoagulant activity was associated with an increased tissue factor and a decreased tissue factor pathway inhibitor expression as compared with hepatocytes. At therapeutic doses, the procoagulant effect of hALPCs was inhibited by neither antithrombin activators nor direct factor Xa inhibitor or direct thrombin inhibitors individually. However, concomitant administration of an antithrombin activator or direct factor Xa inhibitor and direct thrombin inhibitor proved to be a particularly effective combination for controlling the procoagulant effects of hALPCs both *in vitro* and *in vivo*. The results suggest that this dual antithrombotic therapy should also improve the efficacy of cell transplantation in humans.

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Introduction

Adult mesenchymal stem/progenitor cells are currently under evaluation in several clinical trials. A main concern for clinicians and health authorities is the risk of therapy-induced thrombosis, which has been reported in several patients [1].

We previously showed that hepatocyte transplantation results in clinical benefits to patients with inborn errors of metabolism, for whom this technique may be proposed as an alternative or at least, a bridge to orthotopic liver transplantation [2–7]. However, it was important to increase the degree of cell engraftment to improve the clinical outcome of this procedure. One limitation is the finding that isolated hepatocytes exhibit procoagulant activity (PCA), which was found to be linked to tissue factor (TF) expression. This *in vitro* observation has been found to translate clinically in modifications in coagulation parameters and D-dimer levels in recipients of liver cell transplantation, which is suggestive of infraclinical micro-thrombotic events [8].The TF dependent PCA of hepatocytes has more recently been confirmed by an independent team which reported that all the parameters of the instant blood mediated inflammatory reaction (IBMIR) were documented in a tubing loop model, whole blood coagulation model mimicking blood circulation [9]. Furthermore, the activation of the coagulation cascade was previously shown to be associated with negative clinical outcome following pancreatic islet transplantation. PCA not only led to thrombotic events, but also elicited inflammatory reactions involving the up-regulation of adhesion molecule expression and chemokine production, two critical pathways affecting graft success rate [10–13].

Targeting Cell PCA is thus Essential to Improve Safety and Success of Cell Transplantation

We previously isolated human adult liver-derived mesenchymal progenitor cells (hALPCs) following human liver enzymatic digestion [14]. These cells were able to proliferate, but also differentiate into hepatocyte-like cells both *in vitro* and *in vivo*. Therefore, hALPCs represent an attractive cell source for the treatment of liver-based metabolic diseases.

In this context, the aim of the present study was to determine whether hALPCs display PCA and if hALPCs PCA is related to TF expression. In addition, we compared PCA that of other mesenchymal cells like bone marrow- mesenchymal stem cells, skin fibroblats and liver myofibroblasts. Finally, we investigated how this activity could be modulated by evaluating different antithrombotic strategies targeting clotting factors IIa and Xa.

Results

Procoagulant Activity of hALPCs

The PCA of hALPCs was determined using thromboelastometry in human whole blood and plasma. The clotting time (CT) of hALPCs was shorter than that of hepatocytes when evaluated using the thromboelastogram in both blood and plasma (116.5±33.7 sec [n = 17] *vs.* 285.8±87.0 sec [n = 11] for blood, $p<0.001$) (112.6±18.4 sec [n = 9] *vs.* 363.0±180.1 sec [n = 5] for plasma, $p<0.05$) (Figures 1A and 1B). The control CT without the addition of cells was measured at 644.8±108.8 sec (n = 19) in blood and 781.9±150.5 (n = 9) in plasma. Comparable PCA of hALPCs was observed without adding extrinsic TF (Figure 1C). No PCA was obtained when hALPC culture supernatant was placed in the thromboelastogram instead of cells (Figure S1).

The PCA of hALPCs was also evaluated in the tubing loop model. A decrease in platelet count and increase in D-dimer levels were observed after the incubation of hALPCs with whole blood, with a platelet count decreasing from 295 000/µl to 109 000/µl (Experiment 1) and from 310 000/µl to 134 000/µl (Experiment 2), while D-dimer levels increased from 100 ng/ml to 700 ng/ml (Experiment 1) and from 95 ng/ml to 740 ng/ml (Experiment 2).

Procoagulant Activity of Mesenchymal Cells

The PCA of bone marrow mesenchymal stem cells (279.3±108.3 sec [n = 3], $p<0.01$ as compared to control), skin fibroblasts (121.8±26.53 sec [n = 3], $p<0.01$ as compared to control), and liver myofibroblasts (61.7±7.6 sec [n = 3], $p<0.01$ as compared to control) was evaluated using thromboelastometry on human whole blood. Bone marrow haematopoietic stem cells were used as a control for non-procoagulant cells (590.7±25.3 sec [n = 3]) (Figure 2).

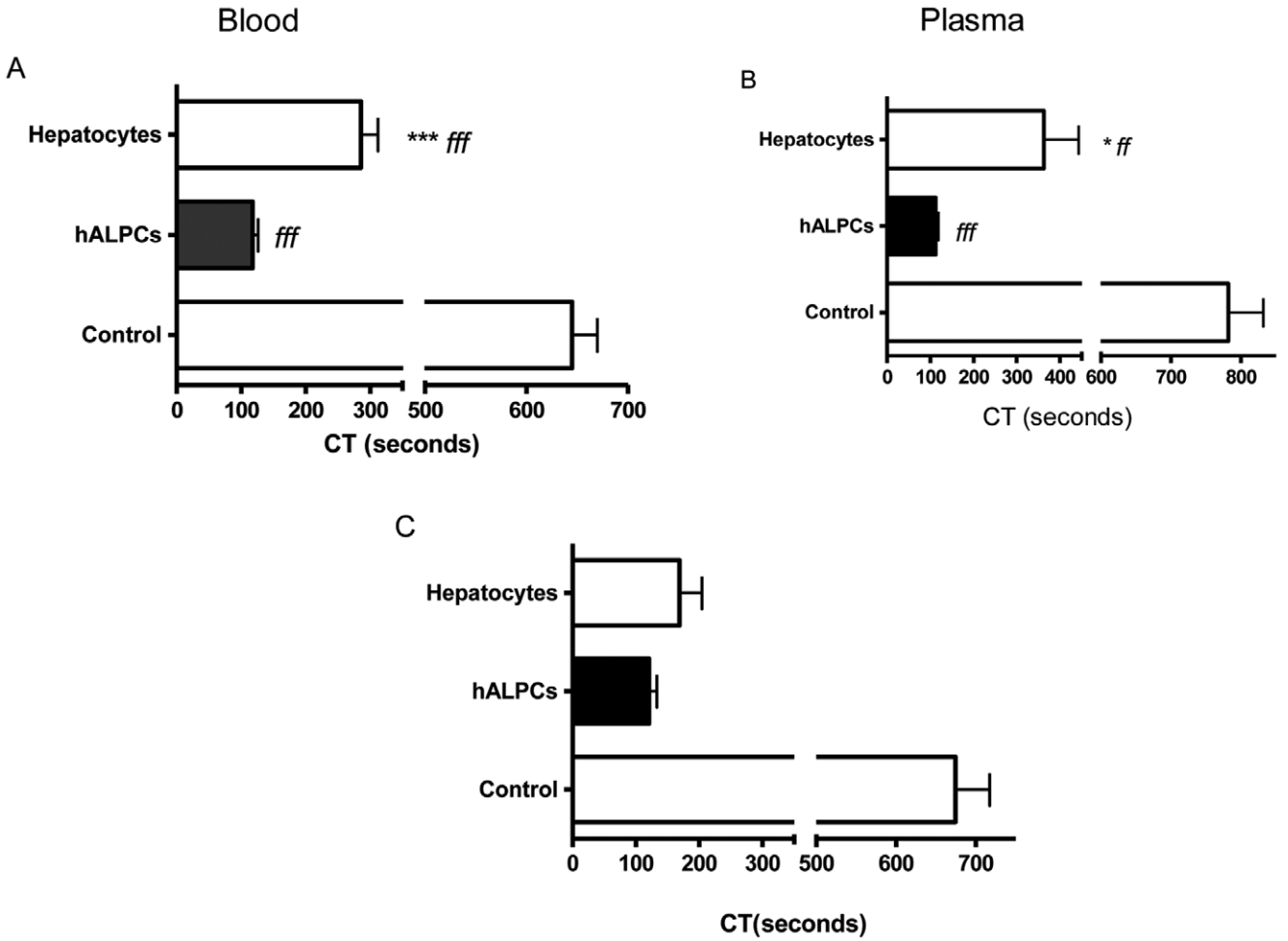

Figure 1.-hALPCs PCA in ROTEM. Figure 1A-hALPCs PCA in ROTEM (Blood). Clotting time (CT) assayed by ROTEM after recalcification, with added tissue factor (ExTem 20 µL) of citrated whole blood (300 µl) in presence or not of cells suspended in human albumin 5%. No coagulation was induced in the absence of recalcification. Hepatocytes (white), human adult liver progenitor cells (hALPCs) (black), control (albumin) (grey). * as compared to hALPCs *f* as compared to control hALPCs *vs.* hepatocytes *vs.* control. ***$p<0.001$ (Kruskal-Wallis test). Figure 1B-hALPCs PCA in ROTEM (Plasma). Clotting time (CT) assayed by ROTEM after recalcification, with added tissue factor (ExTem 20 µL) of plasma (300 µl) obtained from blood incubated in presence or not of cells suspended in human albumin 5%. Hepatocytes (white), human adult liver progenitor cells (hALPCs) (black), control (albumin) (grey). * as compared to hALPCs *f* as compared to control hALPCs *vs.* hepatocytes *vs.* control. ***$p<0.001$ (Kruskal-Wallis test). Figure 1C-hALPCS PCA in ROTEM (no TF addition). Clotting time (CT) essayed by ROTEM after recalcification, without added Tissue Factor (ExTem 20 µL), of citrated whole blood (300 µl) in presence or not of cells suspended in human albumin 5%. No coagulation is induced if absence of recalcification. Hepatocytes (white), hALPCs (black).
doi:10.1371/journal.pone.0042819.g001

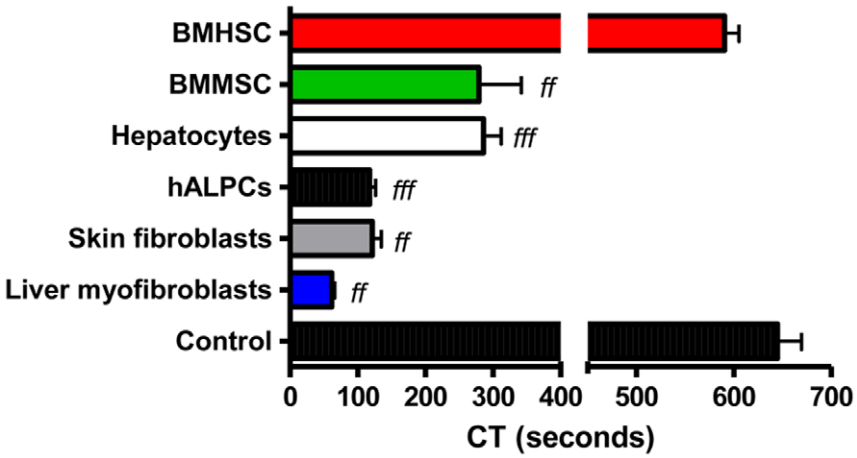

Figure 2. Mesenchymal cells PCA. Clotting time (CT) assayed by ROTEM after recalcification, with added tissue factor (ExTem 20 µL) of citrated whole blood (300 µl) with human adult liver progenitor cells (hALPCs), hepatocytes, skin fibroblasts, bone marrow mesenchymal stem cells (BMMSC), bone marrow haematopoietic stem cells (BMHSC), or liver myofibroblasts suspended in human albumin 5% *f* as compared to control.
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Modulation of Procoagulant Activity of hALPCs

The PCA of hALPCs was first analysed in coagulation factor-deficient plasma. When using factor VII deficient plasma, the physiological cofactor of TF, the PCA of hALPCs was only partially decreased (298.3±42.3 sec [n = 3], $p<0.01$) compared with the PCA in non-deficient plasma (Figure 3). The PCA of hALPCs was not observed in factor II (thrombin) or X deficient plasma, nor in factor V deficient plasma (Figure 3). The PCA of hALPCs was not fully inhibited by unfractionated heparin (225.8±149.8 sec [n = 15], $p<0.001$ as compared to control), low molecular weight heparin (112.3±22.5 sec [n = 3], $p<0.001$ as compared to control), or fondaparinux (209.7±149.7 sec [n = 3], $p<0.001$ as compared to control) (Figure 4A), even when using dosage increases up to five times (Figure S2). No coagulation was observed when heparin was used in the absence of cells.

The direct thrombin inhibitor drugs, hirudin and bivalirudin, allowed for only a partial control of the PCA of hALPCs (256.3±11.8 sec [n = 3] and 377.7±107.2 sec [n = 6], respectively, $p<0.01$ and $p<0.001$ as compared to control respectively) (Figure 4B), even when increasing the dose by two or five times (Figure S3 and S4). Hepatocyte PCA was controlled by unfractionated heparin, low molecular weight heparin, the pentasaccharide fondaparinux (Figure 4C) and the direct thrombin inhibitor drugs, hirudin, and bivalirudin (Figure 4D). A control blood sample in the absence of cells had a CT of 1075.0±107.2 (n = 6) when bivalirudin was added, while no measurable coagulation was observed with hirudin.

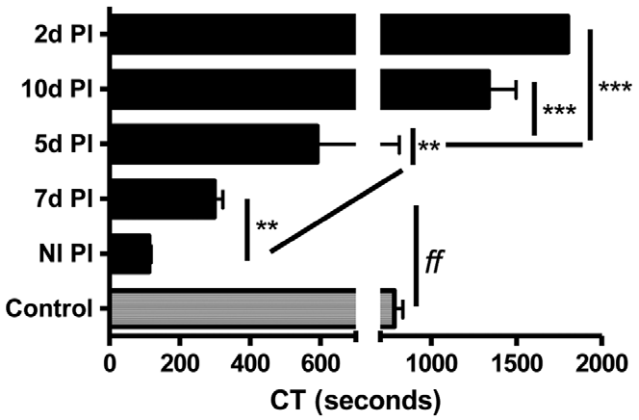

Figure 3. HALPCs PCA in deficient plasma. Clotting time (CT) assayed by ROTEM after recalcification, with added tissue factor (ExTem 20 µL) of plasma (300 µl) deficient in coagulation factors VII, V, X, and II (7d Pl, 5d Pl, 10d Pl, and 2d Pl, respectively) in presence of cells suspended in human albumin 5%. Human adult liver progenitor cells (hALPCs) (black), control (albumin) (grey). * as compared to normal plasma *f* as compared to control.
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Anti-vitamin K drugs (using plasma from patients on long-term anticoagulation when the International Normalized Ratio (INR) was steady between 2 and 3) had no influence on the thromboelastometry, even for controls with the absence of cells (data not shown).

Finally, the concomitant use of bivalirudin with unfractionated heparin (1240.0±338.7 sec [n = 3], $p<0.05$ as compared to bivalirudin alone), enoxaparin (725.0±90.1 sec [n = 3], $p<0.05$ as compared to bivalirudin alone), or fondaparinux (909.0±421.4 sec [n = 3], $p<0.05$ as compared to bivalirudin alone) was shown to be a synergic combination with antithrombin activator and thrombin inhibitor, allowing the PCA of hALPCs to be modulated (Figures 5A and B). However, no complete modulation of the PCA of hALPCs was obtained when combining heparin with enoxaparin or fondaparinux (Figure S5).

We also demonstrated the potential of combining of bivalirudin with a direct anti-thrombotic agent targeting factor-Xa (Rivaroxaban), while the use of rivaroxaban alone was ineffective on hALPCs PCA (Figure S6).

Using analogous experiments, we demonstrated that unfractionated heparin was able to control the PCA of bone marrow mesenchymal cells and skin fibroblasts, but that it remained inactive on liver myofibroblast PCA (Figure S7). In addition, the concomitant use of unfractionated heparin and bivalirudin was shown to modulate the PCA of liver myofibroblasts in contrast with bivalirudin alone (Figure S8).

Comprehension of the PCA of hALPCs

hALPCs express TF and TFPI. TF expression was first documented using immunofluorescence. As shown in Figure 6A–B, we found that all cells expressed TF constitutively (uniform cytoplasmic staining). Flow cytometry analysis of hALPCs

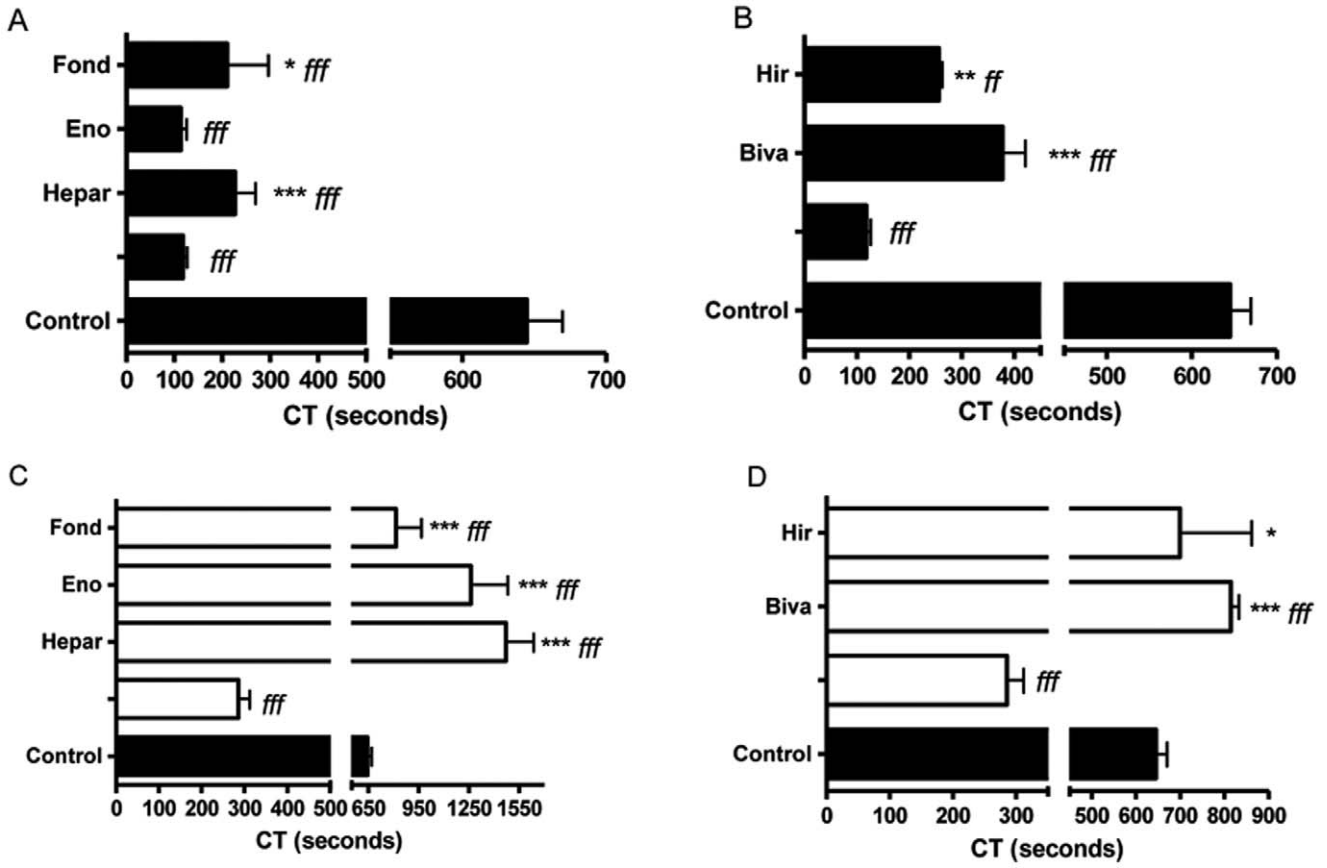

Figure 4. Modulation of hALPCs PCA by anticoagulants. Figure 4A Clotting time (CT) assayed by ROTEM after recalcification, with added tissue factor (ExTem 20 µL) of citrated whole blood (300 µl) in presence or not of human adult liver progenitor cells (hALPCs) suspended in human albumin 5% with heparin (Hepar). In contrast, enoxaparin (Eno) or Fondaparinux (Fond) was extemporaneously added to blood in contact with cells suspended in albumin. hALPCs (black), Control (albumin) (grey). * as compared to hALPCs *f* as compared to control Figure 4B Clotting time (CT) assayed by ROTEM after recalcification, with added tissue factor (ExTem 20 µL) of citrated whole blood (300 µl) in presence or not of human adult liver progenitor cells (hALPCs) suspended in human albumin 5%. Bivalirudin (Biva) or Hirudin (Hir) was extemporaneously added to blood. hALPCs (black), Control (albumin) (grey). * as compared to hALPCs *f* as compared to control Figure 4C Clotting time (CT) assayed by ROTEM after recalcification, with added tissue factor (ExTem 20 µL) of citrated whole blood (300 µl) in presence or not of hepatocytes suspended in human albumin 5% with heparin (Hepar). In contrast, enoxaparin (Eno) or Fondaparinux (Fond) was extemporaneously added to blood in contact with cells suspended in albumin. Hepatocytes (white), Control (albumin) (grey). * as compared to hepatocytes *f* as compared to control Figure 4D Clotting time (CT) assayed by ROTEM after recalcification, with added tissue factor (ExTem 20 µL) of citrated whole blood (300 µl) with or without hepatocytes suspended in human albumin 5%. Bivalirudin (Biva) or Hirudin (Hir) was extemporaneously added to blood. Hepatocytes (white), Control (albumin) (grey). * as compared to hepatocytes *f* as compared to control.
doi:10.1371/journal.pone.0042819.g004

confirmed a positive specific staining for TF (94.9±1.0% for membrane bound form and 93.6±10.2% for cytosolic form as compared to control isotype 24.2±6.1% and 7.6±5.8%, respectively, and unmarked cells 13.2±7.1% and 3.7±4.1%, respectively; n = 3).

The expression of TF and tissue factor pathway inhibitor (TFPI) was assessed at the mRNA level using reverse transcription polymerase chain reaction (RT-PCR) (Figure 6C). Both the membrane form and alternatively spliced variant of TF mRNA were expressed in hALPCs, as was TFPI. In further experiments, we used real-time RT-PCR to quantify TF, alternatively spliced TF (as-TF), and TFPI mRNA levels. As shown in Figure 6D–E–F, the membrane TF was predominantly expressed (n = 3). Furthermore, the expression of TF was higher in hALPCs compared to hepatocytes (n = 3), whereas the expression of TFPI in hepatocytes was higher than in hALPCs (n = 3).

The role of TF in the induction of PCA was determined by the pre-incubation of cells with anti-human TF IgG at a concentration of 0.2 mg/ml. The PCA of hALPCs was partially controlled by blocking TF (324.8±11.4 sec [n = 5], $p<0.01$ compared to without the TF antibody), which was in contrast to hepatocytes, as previously demonstrated (Figure S9) (7).

As shown in Figure 4, only a partial control of the PCA of hALPCs was obtained in factor VII deficient plasma, possibly related to the fact that small amounts of residual factor VII were sufficient to induce coagulation in presence of hALPCs.

HALPCs and heparin. Only minor anti-Xa activity was observed in plasma obtained after the incubation of hALPCs (0.05±0.03 UI/ml) and heparin at a concentration of 10 UI/ml (Figure S10), which correlated with the absence of anticoagulant effect of heparin alone in hALPCs.

Clinical Applications and Anticoagulation Protocol

The anticoagulation protocol was successfully applied, as no thrombotic or haemorrhagic events occurred in the two patients. With bivalirudin use, a substantial increase was observed in

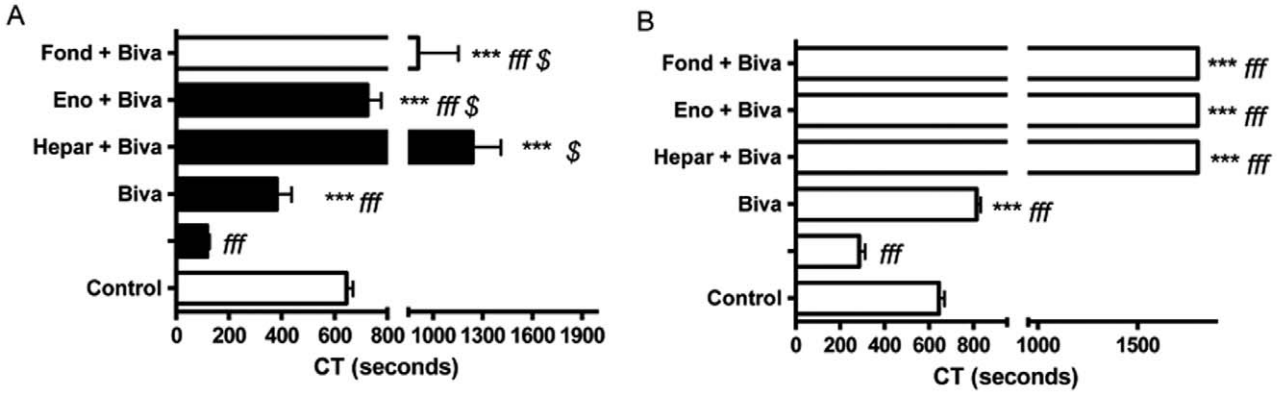

Figure 5. Modulation of hALPCs PCA by combination of anticoagulants. Figure 5A Clotting time (CT) assayed by ROTEM after recalcification, with added tissue factor (ExTem 20 µL) of citrated whole blood (300 µl) in presence or not of human adult liver progenitor cells (hALPCs) suspended in human albumin 5% with heparin (Hepar), enoxaparin (Eno), or fondaparinux (Fond) extemporaneously added to blood. Combination of anticoagulant drugs was obtained when bivalirudin (Biva) was extemporaneously added to blood. hALPCs (black), Control (albumin) (grey). * as compared to hALPCs *f* as compared to control $ as compared to bivalirudin Figure 5B Clotting time (CT) assayed by ROTEM after recalcification, with added tissue factor (ExTem 20 µL) of citrated whole blood (300 µl) in presence or not of hepatocytes suspended in human albumin 5% with heparin (Hepar), enoxaparin (Eno), or fondaparinux (Fond) extemporaneously added to blood. Combination of anticoagulant drugs was obtained when bivalirudin (Biva) was extemporaneously added to blood. Hepatocytes (white), Control (albumin) (grey). * as compared to hepatocytes *f* as compared to control $ as compared to bivalirudin.
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thrombin time (TT), whereas the prothrombin time (PT) remained virtually unchanged. A small increase in D-dimer levels was noted in both patients, reaching 1480 ng/ml for the first patient and 1840 ng/ml for the second. An increase in partial thromboplastin time (PTT) was detected in both patients, which correlated with detectable anti-Xa activity (Figure 7A and B). There was no modification in portal flow using liver Doppler ultrasound during the infusions of both patients.

Discussion

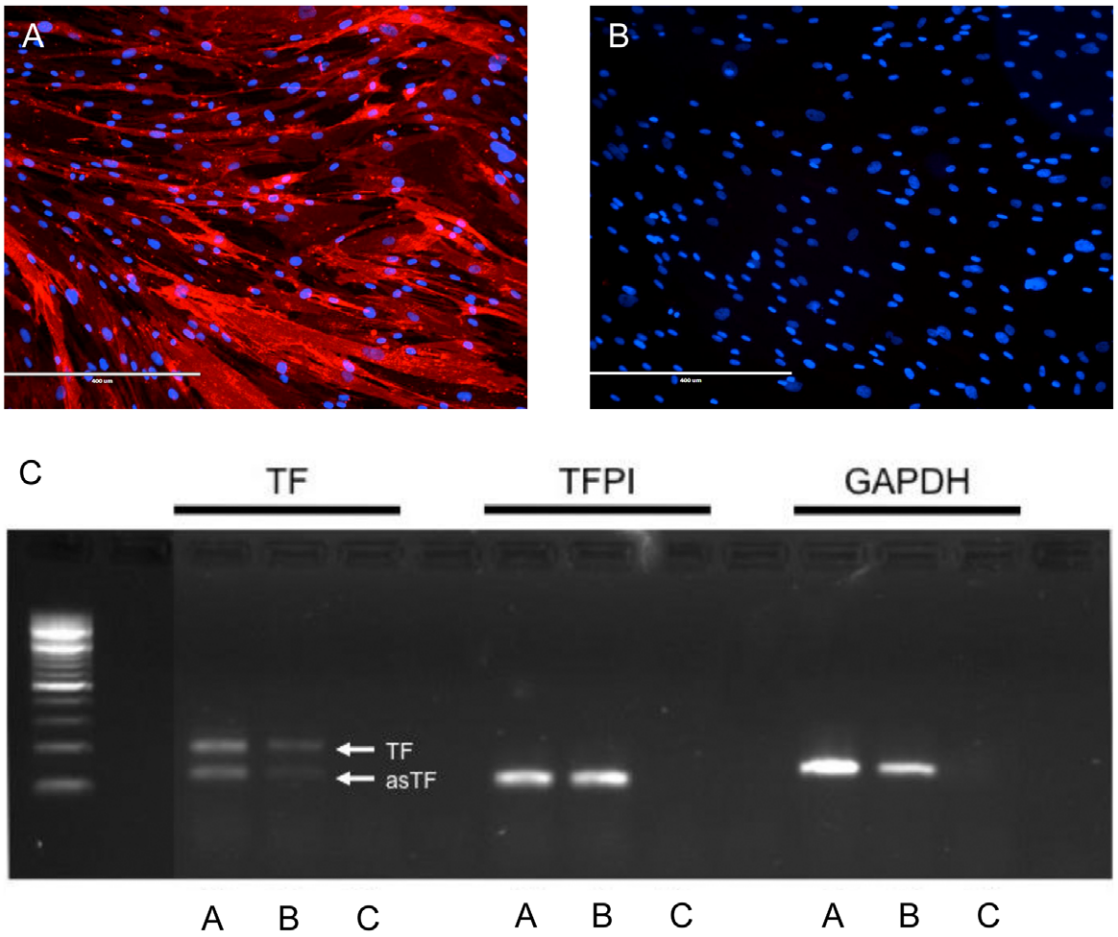
Vein thrombosis at the site of infusion is a potential and major complication in human cell-based therapies. Our present study shows the thrombogenic risk of human mesenchymal cells, as related to the PCA of these cells. We first demonstrated that both hALPCs and bone marrow mesenchymal stem cells exhibited significantly measurable PCA. This was further confirmed for other cells of mesenchymal phenotype like skin fibroblasts and liver myofibroblasts (activated stellate cells). The risk of thrombosis related to the infusion of hALPCs was then shown not to be controlled with antithrombin activator (Heparins, Fondaparinux), direct factor Xa inhibitor (Rivaroxaban) or thrombin inhibitor (Hirudin, Bivalirudin) alone. Instead, an innovative specific anticoagulant protocol acted synergistically to counter this PCA. This dual antithrombotic therapy effectively prevented the risk of thrombosis in human clinical practice.

By revealing the PCA of mesenchymal cells, our study confirmed the previous data from animal studies in which intra-arterial mesenchymal stem cell infusion led to the occlusion of distal vasculature. This result was attributed to the relatively large cell size, and the authors consequently recommended that mesenchymal stem cells be used cautiously when infused via the intravascular route [15]. We demonstrated the procoagulant potential of the cell itself independently of its size.

PCA and subsequent thrombosis events would cause, besides bloodstream modifications, a cell loss and reduction in cell engraftment, thus impairing the final efficacy of cell transplantation [10–13]. We thus investigated in-depth the PCA of hALPCs, as these cells are candidates for curing metabolic diseases in humans [14,16,17]. PCA was determined using thromboelastometry with or without extrinsic TF addition, which is a viscoelastometric method for haemostasis testing in whole blood, measuring the interaction of coagulation factors, inhibitors, and cellular components during the clotting phase and subsequent clot lysis. The rheological conditions of this method mimic the sluggish flow of blood in the veins. The different parameters in thromboelastometry reflect the synergistic activity of the plasma coagulation system, platelet function, and fibrinolysis. A limitation of thromboelastometry is that it only measures part of the process of thrombin generation. It does not take into account the eventual inhibition of thrombin by the natural anticoagulants.

In our study, the PCA of hALPCs was revealed in blood as well as plasma, suggesting that platelet activation did not play a role in this mechanism. The PCA of hALPCs was further confirmed by the tubing loop method. The more pronounced PCA of hALPCs compared to hepatocytes may be explained by the increased TF expression and decreased TFPI expression. As reported in the literature, fibroblasts, vascular smooth muscle cells, and cardiac myocytes, which are all mesenchymal cells, express small amounts of cell-surface TF under physiologic conditions. Indeed, under normal conditions, TF expression is confined to the extravascular sites, separated from the circulating blood by a tissue barrier, which, when disrupted, allows plasma factor VII/VIIA to be exposed to TF and initiate clotting. In this study, TF expression of these extravascular space cells, TF mRNA and antigen, were increased between eight- and ten-fold by serum stimulation in culture [18]. TF expression is also increased by other growth factors, PDGF and EGF, or by non mitogenic agents (bacterial LPS) [19]. In our study, hALPCs were cultured in serum, with cells at passages 4 to 6 being examined, as these may increase TF expression.

We also incubated cells with anti-TF antibody at a concentration inhibiting HUVEC and hepatocytes PCA to ascertain the role of TF in inducing PCA of hALPCs. We only obtained a partial control of hALPCs PCA while using this antibody. This may be related to a non-saturating concentration of the antibody to counteract the increased TF expression by hALPCs. We also only obtained a partial control of hALPCs PCA by using factor VII


A
B
C
TF
TFPI
GAPDH
TF
asTF
A B C A B C A B C
D
ΔΔ Ct
1450
1200
300
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100
0
CAPAN-2
HUVEC
hALPCs
Hepatocytes
E
ΔΔ Ct
800
700
600
200
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0
CAPAN-2
HUVEC
hALPCs
Hepatocytes
F
ΔΔ Ct
14.5
12.0
5.0
2.5
0.0
CAPAN-2
HUVEC
hALPCs
Hepatocytes

Figure 6. TF expression. Figure 6A–B. Tissue factor (TF) expression in hALPCs. Immunofluorescence for TF was performed on human adult liver progenitor cells (hALPCs) placed on coverslips and fixed by paraformaldehyde (magnification 100×) (A). The nuclei were revealed by DAPI (blue staining). As a negative control, immunofluorescence was performed on hALPCs without primary antibody (B). Figure 6C Tissue factor (TF) and tissue factor pathway inhibitor (TFPI) mRNA expression in human adult liver progenitor cells (hALPCs) and hepatocytes evaluated using conventional reverse transcription polymerase chain reaction. TF, alternatively spliced TF (as-TF), TFPI, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (technical control). A) hALPCs, B) Hepatocytes, C) Control Figure 6D–E–F Tissue factor mRNA (TF and as-TF), and tissue factor pathway inhibitor (TFPI) mRNA expression of human adult liver progenitor cells (hALPCs) and hepatocytes evaluated using real-time polymerase chain reaction. Semi-quantitative expression of the mRNA of the TF gene (A), as-TF (B), and the TFPI gene (C) among hALPCs cells and hepatocytes. CAPAN-2 cells and HUVEC are the positive control for TF, as-TF, and TFPI.
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deficient plasma. To interpret these results, we have to consider that factor VII deficient plasma may contain a small amount of factor VII, and that coagulation may have been induced when TF, derived from cells, was exposed to even a small amount of factor VII.

Surprisingly, the PCA of hALPCs and liver myofibroblasts was not inhibited by unfractionated heparin alone, which is not the case for bone marrow mesenchymal stem cells and skin fibroblasts. The anticoagulant effect of unfractionated heparin predominantly acts by binding to and increasing the natural anticoagulant activity of antithrombin and TFPI [20]. Indeed, the release of TFPI into plasma is induced by heparins but is the lowest for unfractionated heparin as compared to low molecular weight heparin [20]. Unfractionated heparin is considered the most important anticoagulant drug used in hepatocyte transplantation, being added to the infusion medium. The absence or low level of anti-Xa activity as measured in plasma in contact with hALPCs and unfractionated heparin (except for high doses) may suggest a particular interaction between cells and heparin.

Heparin is also known to inhibit the proliferation and TF expression of smooth muscle cells in various vascular smooth muscle cell tissue cultures [19,21,22]. The exact mechanism by which heparin inhibits TF expression is still to be elucidated, but it may be triggered after binding of heparin to a receptor [23–25]. To explain the absence of an effect of heparin on the PCA of hALPCs, we may hypothesise that heparin is attached to the binding domain on the cell surface. Heparin is linked to the cell, being the starting point of several reactions aimed at decreasing the proliferation of the cell and TF expression, and thus limiting its availability for a significant blockade of the hALPCs PCA. In addition, hALPCs can also neutralise heparin, but not by specifically using ionic charges. Furthermore, data from Xuereb et al. demonstrated that two downstream pathways could be activated by TF in smooth muscle cells: one PKC dependent, heparin insensitive elicited by LPS, and the other ERK dependent, heparin sensitive elicited by the mitogenic agents but uncoupled to the pathway for proliferative response [19]. Additionally, other antithrombin activators, such as low molecular weight heparin (enoxaparin) or fondaparinux, or direct factor Xa inhibitor did not inhibit the PCA of hALPCs when used alone and even at high concentrations.

Following our observation that PCA was absent when using factor II deficient plasma, we tested the capacity of the direct thrombin inhibitors, hirudin and bivalirudin, in inhibiting PCA in our model. It is also known that direct thrombin inhibitors inhibit clot-bound thrombin more potently than heparins [26]. The PCA of hALPCs was only partially inhibited by these direct thrombin inhibitors. In this context, a recent paper demonstrated that stimulation of human smooth muscle cells with thrombin as well as factor VIIa/factor X led to a significant induction of both TF isoforms on mRNA and protein levels, confirming data from the literature [27], but also to increased TF activity in a chromogenic assay [28]. In contrast to the thrombin-stimulated TF isoforms expression and TF activity, treatment with bivalirudin had no impact on factor VIIa/factor X-induced upregulation of TF and on increased TF activity in smooth muscle cells. These important results showed that increased TF expression and activity differed if obtained after thrombin or factor VIIa/factor X stimulation as bivalirudin has no effect on factor VIIa/factor X TF expression and activity [28]. This is in line with our results that showed that the combination of antithrombotic agents targeting IIa and Xa is needed to control hALPCs PCA, related to increased TF expression. As discussed earlier, two different pathways are probably responsible for increased TF expression, one heparin sensitive and the other heparin insensitive and thus maybe the bivalirudin sensitive one.

Combination of a low molecular weight heparin and recombinant hirudin was found to reduce successfully the thrombus growth in a rabbit jugular vein thrombosis model [29]. Finally, it was also shown experimentally that following hirudin treatment discontinuation, a hypercoagulation rebound occurred, possibly related to the persistence of factor Xa activity in the clot. The authors subsequently demonstrated the complementary effect of DX9065a, a clot-bound factor Xa inhibitor, and r-hirudin, which increased the antithrombotic effect in an in-vitro model for the measurement of clot-bound thrombin [30]. The combination of an antithrombin activator and thrombin inhibitor was used in a recent study on patients with ST-elevation myocardial infarction, resulting in a reduction in mortality or definite thrombosis following primary percutaneous angioplasty [31]. Bivalirudin has a short half-life of 35 to 40 minutes and has a reversible effect, while hirudin has a non-reversible effect and requires novel thrombin synthesis in order to return to normal haemostasis status. Bivalirudin use in children was previously evaluated using an adult dosage (0.75 mg/kg bolus and 1.75 mg/kg/hr infusion), and safely provided the expected anticoagulant effect in a paediatric population undergoing intravascular procedures for congenital heart disease [32]. For clinical application, we chose unfractionated heparin, typically used in hepatocyte transplantation and easily added to the infusion medium, and bivalirudin given its short half-life and available paediatric data.

We successfully tested this anticoagulation protocol in two hALPC-transplanted recipients, without any side effects being observed during or following cell transplantation.

The high increase in D-dimer levels that was observed in previous patients who experienced partial thrombosis was not found in our two patients, confirming the antithrombotic efficacy of this anticoagulation protocol. Furthermore, PCA not only causes local thrombosis and microthrombosis, but also induces local inflammation, which may lead to cell rejection. Thus, the proposed anticoagulation combination may optimise engraftment and repopulation by hALPCs.

To conclude, the original combination of two anticoagulant drugs, an antithrombin activator or a direct factor Xa inhibitor and a thrombin inhibitor, can reduce or even prevent the risk of thrombosis, which is associated with intravascular infusion of cells in humans. This anticoagulation strategy has probably also an interest for islet transplantation where TF expression by islets and

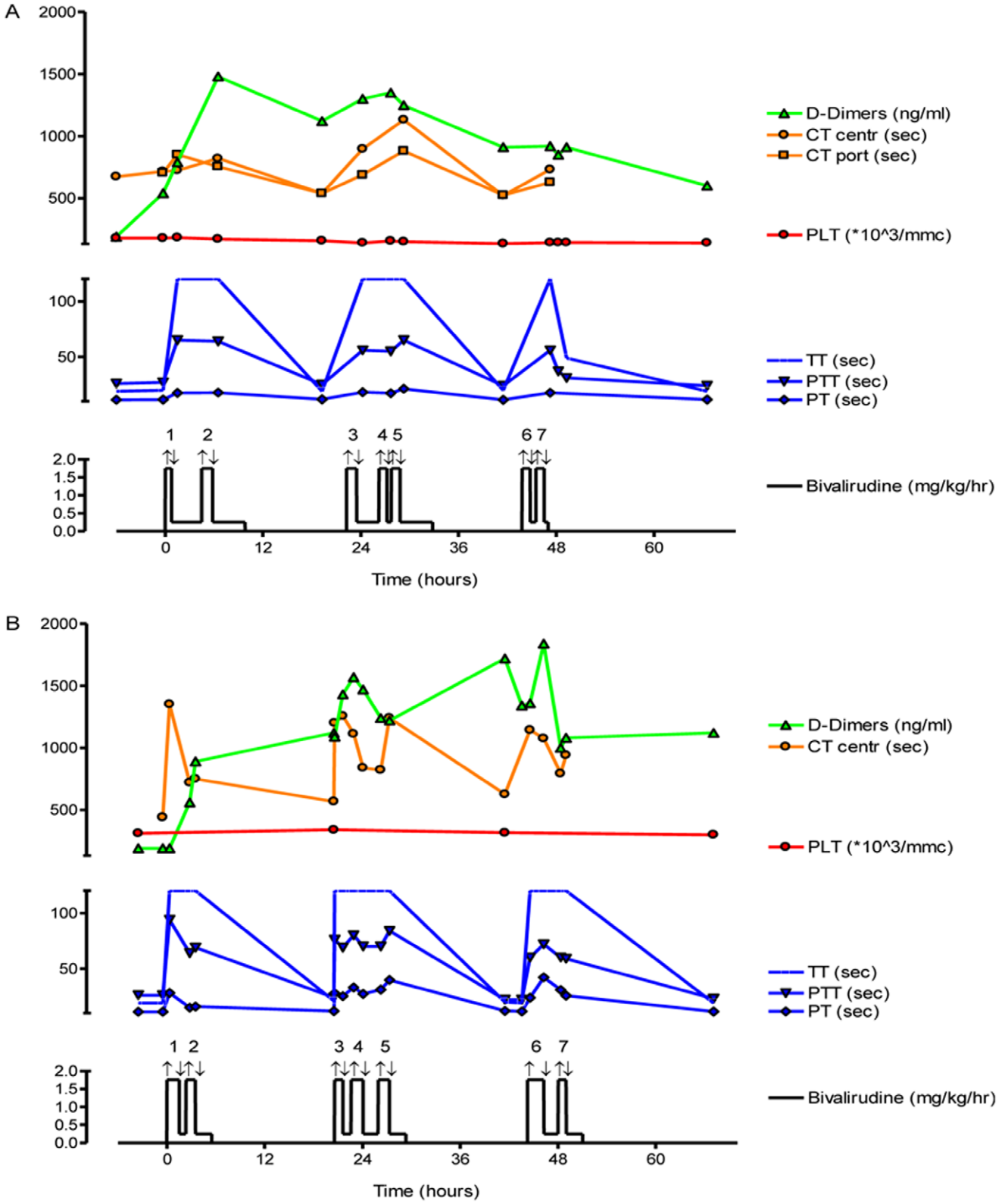

Figure 7. HALPCs infusion and anticoagulation protocol. During cell infusion, patients received bivalirudin (1.75 mg/kg). Between consecutive cell infusions, the dose was decreased to 0.25 mg/kg for 2 to 4 hours. Coagulation tests were repetitively performed before each infusion, 20 min after beginning and at the end, and included the following: thromboelastometry with clotting time (CT) in the portal vein (port) or via the central line (centr); platelets (PLT) (normal values: 150–350 10exp3/µl); D-dimer levels (normal values: <500 ng/ml), thrombin time (TT) (normal values: 15–24 sec); prothrombin time (PT) (normal values: 9–14 sec); partial thromboplastin time (PTT) (normal values: 20–33 sec). A Crigler Najjar patient. B Glycogenosis Type 1a patient.
doi:10.1371/journal.pone.0042819.g007

duct cells plays also a pivotal role in inducing PCA and related instant blood mediated inflammatory reaction. By preventing thrombosis, microthrombosis, and inflammation, this anticoagulation should also optimise the safety and success of cell, pancreatic islet and stem cell transplantation in humans.

Methods

Ethics Statement

The protocol, including all experiments on human samples, as well as the human off-label anticoagulant protocol use and informed consent were approved by the institutional ethics review board (Comission d'éthique biomédicale hospitalo-facultaire, Université Catholique de Louvain, Faculté de Médecine, commission.ethique@md.ucl.ac.be, chaired by J.M. Maloteaux). Written informed consent was obtained from the patient (If applicable) and from the next of kin.

Cell Preparations

hALPCs were obtained from healthy liver donors (n = 6, aged 9 to 44 years) as previously described [14]. We studied freshly trypsinised cells or cells after cryopreservation/thawing at passages 4 to 6, with a viability exceeding 90% at the trypan blue test. Cells were suspended in an albumin solution with or without heparin at a concentration of 10 U/mL (or more when specified). As a control, cryopreserved/thawed human hepatocytes (n = 5, aged 16 to 44 years) were used. Liver isolation and hepatocyte cryopreservation/thawing procedures were previously published in detail [4].

Bone marrow samples were collected by the aspiration of vertebrae or iliac crests of three post-mortem organ donors aged 8 to 67 years. Aspirates were collected into heparinised syringes containing 10% Hanks' balanced salt solution (Invitrogen, Merelbeke, Belgium) and processed within 48 hours according to a previously described protocol [33].

Human fibroblasts were collected from a skin biopsy (medio-anterior side of the forearm) of three volunteers aged 18 to 35 years after obtaining written informed consent as previously described [34].

Human liver non-parenchymal cells were obtained after liver isolation was performed in our tissue bank, involving filtration and two low-speed centrifugations of the cell suspension from three different donors (one neonate liver and two 12-years-old donors). Next, human stellate cells were isolated using Nycodenz gradient centrifugation (Myegaard, Oslo, Norway) according to established protocols and in collaboration with the Department of Cell Biology-VUB (Prof. LA van Grunsven) [35]. Activated myofibroblasts were obtained from the isolated stellate cells.

Blood

Blood was obtained from five male donors aged 29 to 40 years.

Procoagulant Activity of hALPC Suspension

Measurements were performed on a ROTEM® delta analyser (Pentapharm, Munich, Germany). ROTEM® assessed the kinetics and quality of clot formation and clot lysis in real time. Clotting time (CT) was defined as the period of time from the start of analysis until the start of clot formation, normally until 2-mm amplitude was reached. Clot formation time was classified as the period until 20-mm amplitude was reached. The alpha angle was defined as the angle between the centre line and a tangent to the curve through the 2-mm amplitude point, which was at the end of CT. The maximum amplitude of the curve represented the maximum clot firmness, while the maximum of lysis the maximum fibrinolysis detected during the measurement. Our analysis focused on CT.

In short, after a brief rest period, 300 µl of whole blood was pipetted into a cup preheated to 37°C. Suspended cells were subsequently added to the whole blood (five 10exp5 cells if not specified), with 20 µl of trigger reagent containing tissue factor (Innovin, Siemens, Marburg, Germany; final dilution 1:17000/ 0.35 pM) diluted in Owren buffer (Siemens, Marburg, Germany) then being added to the cell-blood mixture followed by the necessary addition of 20 µl of 0.2 M $CaCl_2$. After adding calcium, measurements were initiated automatically. The PCA of cells was also determined without adding Innovin. Thus, in order to ascertain the role of TF in this coagulation model, cells were pre-incubated at room temperature for 10 min with either 0.2 mg/mL anti-human TF IgG1 monoclonal antibody (mAb) (American Diagnostica) or 0.2 mg/mL mouse IgG1 mAb (clone11711.11; RnD Systems, Abingdon, United Kingdom) before extensive washing with albumin 5% followed by the thromboelastometry assay, concentration inhibiting HUVEC and hepatocytes PCA [8,36].

For plasma assays, cells (five 10exp5 cells if not specified) were incubated in 3.8 ml of citrated blood at 37°C for 30 minutes. After incubation, whole blood was centrifuged at 4500 rpm for 10 minutes. Consequently, 300 µl of the obtained plasma was ready for the protocol, pipetted into the cup with the addition or not of Innovin and $CaCl_2$.

For factor-deficient plasma assays, suspended cells (five 10exp5 cells if not specified) were combined with 300 µl of plasma before adding Innovin and $CaCl_2$.

For the modulation of PCA assays, cells were suspended in albumin 5% with or without unfractionated heparin (Heparin Leo®, Leo) at a concentration of 10 UI/ml. The following were then added to blood or plasma: low-molecular-weight heparin, enoxaparin (Clexane®, Aventis Pharma) at a concentration of 1 UI/ml following published data [37], pentasaccharide anti-thrombin activator (Fondaparinux, Arixtra®, GSK) at a concentration of 0.34 mg/l and dose extrapolation of 2.5 mg/kg for adults following published data [38], rivaroxaban (Xarelto®, Bayer Schering) at a concentration of 1 µg/ml following published data [39], hirudin (Refludan®, Celgène Europe Limited) at a concentration of 5.7 µg/ml and dose extrapolation of 0.4 mg/kg, and bivalirudin (Angiox®, The Medicines Company) at a concentration of 10.7 µg/ml and dose extrapolation 0.75 mg/kg. Dose extrapolation was based on circulating blood volume according to weight (70 ml/kg).

If no coagulation was observed after 1800 sec, thromboelastometry was arbitrarily stopped.

Tubing Loop

A whole-blood experiment protocol was adapted from a model previously described [12]. Loops made of polyvinyl chloride tubing (inner diameter 6.3 mm, length 390 mm) and treated with a Corline heparin surface were purchased from Corline (Uppsala, Sweden). Loops were supplemented with cell samples (five 10exp5) suspended in phosphate buffered saline before adding blood. Thereafter, 5 mL of non-anticoagulated blood from healthy volunteers was added to each loop. To generate a blood flow of approximately 45 mL/minute, loop devices were placed on a platform rocker inside a 37°C incubator. Blood samples were collected in tubes containing ethylene diamine tetraacetic acid (4.1 mmol/L final concentration) and citrate (12.9 mmol/L final concentration) before and 30 minutes after the start. Platelets were counted by the XE-2100 automate haematology analyser (Sysmex, Japan), and D-dimer levels were evaluated using the immuno-

turbidimetric assay (Innovance D-Dimer, Siemens, Marburg, Germany) on a CA-7000 system (Sysmex, Japan).

Anti-Xa Activity Measurement

Anti-Xa activity measurement was performed using the Biophen Heparin (LRT) kit adapted on a CA7000 (Siemens, Marburg, Germany). In short, the assay was a chromogenic kinetic method based on the inhibition of a constant amount of factor Xa by the tested heparin (or other anti-Xa substance) in the presence of endogenous antithrombin as well as on the hydrolysis of a factor Xa specific chromogenic substrate by the factor Xa in excess. After a 30-min incubation of the cells suspended in albumin with or without heparin (10 UI/ml, 50 UI/ml, and 100 UI/ml) in the blood, anti-Xa activity was measured in plasma obtained following blood centrifugation.

TF and TFPI Expression of hALPC Suspension

Immunofluorescence studies were performed in order to evaluate the presence of TF. Thus, human adult liver-derived stem cells were placed on coverslips and fixed with paraformaldehyde 4% (Merck, Darmstadt, Germany) for 20 minutes. These cells were then incubated with Triton X-100 (Sigma,Bornem, Belgium) 1% in Tris-base sodium buffer (50 mmol/L Tris-HCl pH 7.4 and 150 mmol/L NaCl) (Organics [VWR], Leuven, Belgium) for 15 minutes and then with 3% milk in Tris-base sodium buffer for 1 hour. The primary antibody, murine mAb anti-TF IgG1 (immunoglobulin [Ig]G1 n4508; American Diagnostica, Andresy, France), was diluted (1/50) in Tris-base sodium and incubated with the cells for 1 hour. The secondary antibody was fluorescein isothiocyanate-conjugated anti-mouse IgG (Sigma). The nuclei were revealed with 4_, 6-diamidino- 2-phenylindole staining (DAPI; Sigma). Negative experimental controls were performed relating to the absence of primary or secondary antibodies. The presence of TF was also confirmed by flow cytometric analysis. In order to detect the membrane-bound form of TF, cells were washed in phosphate buffered saline supplemented with 0.5% bovine serum albumin (FACS buffer) and incubated for 20 minutes at 4°C with the fluorescein isothiocyanate-conjugated IgG1 mAb against TF no. 4508CJ (American Diagnostica) or the corresponding isotype-matched control mAb (BD Biosciences, Erembogedem, Belgium) diluted in FACS buffer containing 10% decomplemented pooled human serum. In order to detect the cytosolic form of TF, cells were incubated with Cytofix/Cytoperm (BD Biosciences) for 20 min at room temperature and washed with Perm/Wash (BD Biosciences).The samples were then incubated for 20 min at room temperature with fluorescein isothiocyanate-conjugated anti-TF mAb or the corresponding isotype-matched control mAb (BD Biosciences) diluted in Perm/Wash. Cell fluorescence was measured using a BD FACS CANTO II flow cytometer and analysed with the BD FACS Diva software.

No anti-TFPI antibody was obtained to evaluate the TFPI expression using immunocytochemistry or flow cytometry analysis.

The two forms of TF and TFPI were analysed by reverse-transcription polymerase chain reaction (RT-PCR). Messenger ribonucleic acid (mRNA) was extracted from 0.5×10^6 cells using the TriPure isolation reagent kit (Roche Applied Science, Brussels, Belgium) according to the manufacturer's instructions. One-step RT-PCR was performed on a Thermocycler instrument (Applied Biosystems, Lennik, Belgium) with primers synthesised at Invitrogen. RT-PCR for TF or glyceraldehyde 3-phosphate dehydrogenase was conducted with the primers detailed in Table 1.

Table 1. TF, TFPI and GAPDH primers.

Primer	Sequence
TF sense primer	5-TGAATGTGACCGTAGAAGATGA-3
TF antisense primer	5-GGAGTTCTCCTTCCAGCTCT-3
as-TF sense primer	5-TCTTCAAGTTCAGGAAAGAAATATTCT-3
as-TF antisense primer	5-CCAGGATGATGACAAGGATGA-3
TFPI sense primer	5-GGAAGAAGATCCTGGAATATCGAGG-3
TFPI antisense primer	5-CTTGGTTGATTGCGGAGTCAGGGAG-3
GAPDH sense primer	5-CGGACTCAACGGATTTGGTCGTAT-3
GAPDH antisense primer	5-AGCCTTCTCCATGGTGGT-3

TF: tissue factor; as-TF: alternatively spliced tissue factor; TFPI: tissue factor pathway inhibitor; GAPD: glyceraldehyde 3-phosphate dehydrogenase.
doi:10.1371/journal.pone.0042819.t001

Products were separated by electrophoresis on 1% agarose gel and visualised with ethidium bromide staining and ultraviolet illumination.

Real-time RT-PCR for TF, as-TF, TFPI, and cyclophilin A was carried out using the StepOnePlus Real-Time PCR system (Applied Biosystems, California, USA) with TaqMan® Gene Expression Assays as listed in Table 2. For TF expression, two assays were used, with one (common TF) amplifying a region present in both membrane and soluble forms (as-TF), and the other (membrane TF) amplifying a region present only in membrane form (standard). The Ct (threshold cycle) parameter was derived for each cDNA sample and primer pair, with Cyclophilin A Ct being subtracted in order to obtain the ΔCt. ΔΔCt was then obtained by subtracting the Ct calibrator gene, with the results expressed as the fold change of the mRNA amount (Figure 6D–E–F). The as-TF expression was calculated as the difference between the ΔΔCt of the common TF and membrane TF. The primers are detailed in Table 2.

CAPAN-2 cell line was used as a TF positive control, while HUVEC cell line as a TFPI positive control.

Infusions of Patients and Anti-coagulation Protocol

A 3-year-old girl, suffering from severe ornithine transcarbamylase deficiency (<1% activity), was the first recipient of hALPCs. The diagnosis was established 12 days after birth and confirmed by DNA analysis, which indicated a *de novo* mutation of exons 6 and 8 on the paternal allele of ornithine transcarbamylase gene.

The girl received from a male donor two separate infusions of 30 million hALPCs per kg of body weight, with a 2-week interval between infusions. In total, the patient received 0.9 billion

Table 2. References for real-time polymerase chain reaction.

Gene	TaqMan® Gene Expression Assays	Amplicon length
as-TF	Hs01076032_m1	69
TF membrane	Hs01076029_m1	85
TFPI	Hs01041344_m1	78
Cyclophilin A	Hs99999904_m1	98

TF: tissue factor; as-TF: alternatively spliced tissue factor; TFPI: tissue factor pathway inhibitor.
doi:10.1371/journal.pone.0042819.t002

Table 3. Coagulation tests.

Coagulation test	
D-dimers level	D-dimer is a fibrin degradation product, a small protein fragment present in the blood after a blood clot is degraded by fibrinolysis. It is so named because it contains two crosslinked D fragments of the fibrinogen protein
Thrombin Time (TT)	The Thrombin Time is a blood test which measures the time it takes for a clot to form in the plasma of a blood sample anticoagulant to which an excess of thrombin has been added. This test is repeated with pooled plasma from normal patients. The difference in time between the test and the 'normal' indicates an abnormality in the conversion of fibrinogen (a soluble protein) to fibrin an insoluble protein.
Prothrombin time (PT) and International Normalized Ratio (INR)	The Prothrombin time and its derived measures of prothrombin ratio and international normalized ratio (INR) are measures of the *extrinsic pathway* of coagulation. They are used to determine the clotting tendency of blood, in the measure of warfarin dosage, liver damage, and vitamin K status. PT measures factors I, II, V, VII, and X.
Partial Thromboplastin Time (PTT)	The Partial Thromboplastin time or activated Partial Thromboplastin Time is a performance indicator measuring the efficacy of both the "intrinsic" (now referred to as the contact activation pathway) and the common coagulation pathways. Apart from detecting abnormalities in blood clotting, it is also used to monitor the treatment effects with heparin, a major anticoagulant. Kaolin Cephalin Clotting Time is a historic name for the activated Partial Thromboplastin Time.

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progenitor cells. The cells were suspended in albumin 5% and unfractionated heparin (10 UI/ml).

The first hALPC infusion was performed under general anaesthesia without any premedication. A transcutaneous catheter was placed in the main portal vein branch under fluoroscopy and ultrasound guidance after injecting one dose of Cefazolin (40 mg/kg). Cell infusion was performed using a syringe of 50 ml at a flow rate of 100 cc/h. Immune suppression was administrated to the patient using tacrolimus (Prograft®, Astellas Pharma) with the monotherapy (0.1 mg/kg) corresponding to 2 mg per day in two divided doses to reach levels of 6–7 ng/ml. Cefazolin (40 mg/kg) was administrated as prophylactic antibiotherapy twice post-infusion with an 8-hour interval between the two doses. The infusion and post-infusion periods were unremarkable, with the child being discharged from hospital on Day 3 post-infusion.

The second hALPC infusion was performed two weeks later. In the interim, a partial thrombosis of the intra-hepatic portal vein branch had occurred and led to stopping the infusion. This adverse event was treated with heparin and coumarinic anticoagulant (5 mg/day).

D-dimer levels were markedly elevated after both courses of cell infusion. This adverse event was without consequence for the patient, but justified a further investigation of the procoagulant effect of progenitor cells.

The second patient was a 24-year-old man with intermediate type I/II Crigler-Najjar syndrome, which did not respond to phenobarbital. The diagnosis was established 1 month after birth and confirmed by DNA analysis, indicating a mutation on the UDP-glucuronosyltransferase 1A1 gene with the presence of the homozygous state for the L443P mutation. The patient received 2.2 billion hALPCs administered in seven infusions over 2 days. Prior to the placement of the portal catheter, the patient was administered premedication, including cefazolin (1 gr). The catheter was under ultrasound control in the portal system. Solumedrol (80 mg) was injected before the infusion. The immunosuppression treatment consisted of tacrolimus (Prograft®, Astellas Pharma), which targeted blood levels of 6–8 ng/ml. A specific coagulation prophylaxis was prescribed, with cells being suspended in albumin 5% and heparin at a concentration of 10 UI/ml. During cell infusion, the subject received bivalirudin (1.75 mg/kg) intraveinously by continuous infusion, while between consecutive cell infusions, he was given bivalirudin (0.25 mg/kg) for 2 to 4 hours depending on the thromboelastometry test. Coagulation tests including thromboelastometry (CT), platelet counts (normal values: 150–350 10exp3/µl), D-dimer levels (normal values: <500 ng/ml), thrombin time (TT, normal values: 15–24 sec), prothrombin time (PT, normal values: 9–14 sec), and partial thromboplastin time (PTT, normal values: 20–33 sec) were repetitively performed before each infusion, 20 min after the beginning, and at the end. A liver Doppler ultra-sound was conducted after each infusion to assess portal flow. The signification of coagulation tests was summarised in table 3.

The third patient was a 17-years-old suffering from glycogenosis type 1a, as documented by genetic analysis (G188R mutation and 380insC insertion) and the absence of glucose-6 phosphatase activity on a liver biopsy. The patient also received antibiotic prophylaxis prior to the placement of the portal catheter as well as steroids before the infusion. The same immunosuppressor regimen as patient 2 was administered. The patient received 3 billion progenitor cells administered in seven infusions over 3 days aimed at controlling recurrent hypoglycemia. The same anticoagulation protocol and coagulation, including liver Doppler ultrasound follow-up, was applied.

Statistics

Mann-Whitney tests were used to assess statistically significant differences ($*P<0.05$, $**P<0.01$, $***P<0.001$). Any significant values were adjusted according to the Bonferroni correction in order to avoid Type 1 errors. The Kruskal-Wallis test was used for one-way ANOVA analysis.

Supporting Information

Figure S1 Supernatant of hALPCs PCA. Clotting time (CT) essayed by ROTEM after recalcification, with added Tissue Factor (ExTem 20 µL), of citrated whole blood (300 µl) in presence of supernatant of hALPCs culture. No coagulation is induced if absence of recalcification.
(DOCM)

Figure S2 Modulation of hALPCs PCA by heparin. A) Clotting time (CT) assayed by ROTEM after recalcification, with added tissue factor (ExTem 20 µL) of citrated whole blood

(300 µl) in presence or not of human adult liver progenitor cells (hALPCs) (Black) suspended in human albumin 5% and with or without heparin (Hepar) at several concentrations (Hepar-10 UI/ml, Hepar 5×−50 UI/ml, and Hepar 10×−100 UI/ml) or not Control (albumin) (grey) *f* as compared to control. B) Clotting time (CT) assayed by ROTEM after recalcification, with added tissue factor (ExTem 20 µL) of citrated whole blood (300 µl) in presence or not of human adult liver progenitor cells (hALPCs) (Black) suspended in human albumin 5% and with or without fondaparinux (Fond) and enoxaparin (Eno) at normal concentrations or increased five times (5×) the normal concentration Control (albumin) (grey) *f* as compared to control Fond *vs.* Fond 5×, non-significant Eno *vs.* Eno 5×, non-significant.
(DOCM)

Figure S3 Modulation of hALPCs PCA by hirudin. Clotting time (CT) assayed by ROTEM after recalcification, with added tissue factor (ExTem 20 µL) of citrated whole blood (300 µl) in presence or not of human adult liver progenitor cells (hALPCs) suspended in human albumin 5%. Increased concentrations of hirudin (Hir) at two (Hir 2x) or five times the normal levels (Hir 5x)) was extemporaneously added to blood. hALPCs (black), Control (albumin) (grey). * as compared to hALPCs *f* as compared to control hALPCs Hir *vs.* hALPCs Hir 2x, n.s.
(DOCM)

Figure S4 Modulation of hALPCs PCA by bivalirudin. Clotting time (CT) assayed by ROTEM after recalcification, with added tissue factor (ExTem 20 µL) of citrated whole blood (300 µl) in presence or not of human adult liver progenitor cells (hALPCs) suspended in human albumin 5%. Increased concentrations of bivalirudin (Biva) two times the normal level (Biva 2x) was extemporaneously added to blood. hALPCs (black), Control (albumin) (grey). * as compared to hALPCs *f* as compared to control.
(DOCM)

Figure S5 Modulation of hALPCs by antithrombin activators in combination. Clotting time (CT) assayed by ROTEM after recalcification, with added tissue factor (ExTem 20 µL) of citrated whole blood (300 µl) in presence or not of human adult liver progenitor cells (hALPCs) suspended in human albumin 5% and with or without heparin (Hepar). Enoxaparin (Eno) or fondaparinux (Fond) was extemporaneously added to blood with cells suspended in heparin or not hALPCs (black), Control (albumin) (grey) *f* as compared to control.
(DOCM)

Figure S6 Modulation of hALPCs by direct inhibition of factor X in combination or not with bivalirudin. Clotting time (CT) assayed by ROTEM after recalcification, with added tissue factor (ExTem 20 µL) of citrated whole blood (300 µl) in presence or not of human adult liver progenitor cells (hALPCs) suspended in human albumin 5% with rivaroxaban. Combination of anticoagulant drugs was obtained when bivalirudin (Biva) was extemporaneously added to blood. hALPCs (black), Control (albumin) (grey). * as compared to hALPCs *f* as compared to control $ as compared to bivalirudin.
(DOCM)

Figure S7 Modulation of mesenchymal cells PCA by heparin. Clotting time (CT) assayed by ROTEM after recalcification, with added tissue factor (ExTem 20 µL) of citrated whole blood (300 µl) with human adult liver progenitor cells (hALPCs), hepatocytes, skin fibroblasts, bone marrow mesenchymal stem cells (BMMSC), bone marrow haematopoietic stem cells (BMHSC), or liver myofibroblasts suspended in human albumin 5% with or without heparin (10 UI/ml) (Hepar) *f* as compared to control.
(DOCM)

Figure S8 Modulation of liver myofibroblats PCA. Clotting time (CT) assayed by ROTEM after recalcification, with added tissue factor (ExTem 20 µL), of citrated whole blood (300 µl) in presence or not of liver myofibroblasts suspended in human albumin 5% with or without heparin (10 UI/ml) (Hepar). A combination of anticoagulant drugs was obtained when bivalirudin (Biva) was extemporaneously added to blood in contact with cells suspended in heparin *f* as compared to control.
(DOCM)

Figure S9 HALPCs PCA and TF blocking antibody Clotting time (CT) assayed by ROTEM after recalcification, with added tissue factor (TF) (ExTem 20 µL) of citrated whole blood (300 µl) in presence or not of cells suspended in human albumin 5% after the incubation of cells with TF antibody (TF+) or not (TF-). Hepatocytes (white), human adult liver progenitor cells (hALPCs) (black), control (albumin) (grey). * as compared to TF- for hALPCs $ as compared to TF- for hepatocytes *f* as compared to control.
(DOCM)

Figure S10 Anti-Xa activity in plasma. After a 30-min incubation of cells suspended in albumin with or without heparin (Hepar) (10 UI/ml, 50 UI/ml, and 100 UI/ml) in blood, anti-Xa activity (UI/ml) was measured in plasma obtained after blood centrifugation Human adult liver progenitor cells (hALPCs) (Black), Hepatocytes (Hep) (White), Control (Grey).
(DOCM)

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Author Contributions

Conceived and designed the experiments: XS SE CH ES. Performed the experiments: XS EN ON CL MN. Analyzed the data: XS ES EN CH SE MN. Contributed reagents/materials/analysis tools: XS EN SE ON CL MN. Wrote the paper: XS ES.

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Functions of intestinal microflora in children

Vittoria Buccigrossi, Emanuele Nicastro, and Alfredo Guarino

Purpose of review
This review discusses the structural composition of intestinal microbiota, the functional relationship between the latter and the host, and the role of abnormal microflora in chronic diseases.

Recent findings
A more complete view of the gut microbiota is being developed following the Human Microbiome Project. The microflora in children is plastic, susceptible to changes in response to diet modifications, antibiotic treatment and other events, providing the opportunity to study its functional role. Increasing evidence highlights the role of nutrition in the age-related development of microflora.
Eubiosis, that is, a normal microflora structure, provides protection against infections, educates the immune system, ensures tolerance to foods, and contributes to nutrient digestion and energy harvest. Changes in microflora, consisting in the overpresence of harmful species or underpresence of commensal species, or dysbiosis produce dysfunctions, such as intestinal inflammation or dysmotility. Moreover abnormal pattern of microflora have been consistently detected in specific diseases.

Summary
A relationship exists between eubiosis and functions and conversely between dysbiosis and dysfunctions or even diseases. Abnormalities in microflora composition may trigger or contribute to specific diseases. This raises the hypothesis to target microflora in order to restore eubiosis through the use of antibiotics, probiotics or nutrients.

Keywords
dysbiosis, enterotype, eubiosis, gut microbiota functions, signature

INTRODUCTION

Intestinal microflora is increasingly considered as a functional human organ and its structure and effects are being clarified as a result of the Human Microbiome Project [1▪▪]. This project aims to produce a reference set of microbial genome sequences and to obtain a preliminary characterization of the human microbiome, to explore the relationship between diseases and changes in the human microbiome, and to develop new technologies and tools for computational analysis. The knowledge of intestinal microbiota composition, how it interacts with the host, and how it causes or contributes to human diseases have been enhanced by culture-independent techniques that allow its phylogenetic investigation and quantification [2▪].

STRUCTURE OF INTESTINAL MICROFLORA AND ITS DETERMINANTS

The entire human population can be classified in three enterotypes on the basis of intestinal microflora. Enterotype defines the quantitatively dominating taxa. Enterotype 1 is dominated by Bacteroides, enterotype 2 by Prevotella and enterotype 3 by Ruminococcus [3▪,4▪▪], and each of the three enterotypes differently affects the host metabolic functions [4▪▪,5]. The enterotypes are associated with protein and animal fat (Bacteroides) or respectively carbohydrates (Prevotella) rich diet and may change as early as within 24 h of initiating a high-fat/low-fiber or low-fat/high-fiber diet [6▪]. However the core structure of enterotypes does not change and is a lifestyle hallmark of individuals. The relationship between diet and the intestinal microflora structure emerged from a comparative evaluation of bacteria in European children, who ate a typical western diet, high in animal protein and fat, and children in Burkina Faso, who were on

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KEY POINTS

- Compared with the complex and resilient adult microbiome, intestinal microflora in children is intrinsically plastic, deeply affected by few variables but less exposed to factors that may change its composition.
- Human microbiota exerts important immune, metabolic, trophic, and protective functions as a result of the symbiosis between the host and intestinal microbes.
- Generally, a reduction in the diversity of gut microflora is associated with intestinal inflammation, dysmotility, and atopy.
- Specific aberrations in microflora composition are microbial 'signatures' of selected diseases, suggesting a specific role of well defined bacterial species in the pathophysiology of those diseases or also their use as biomarkers to predict them or to monitor their course.
- Targeting intestinal microflora may be a novel strategy alone or in combination with traditional treatment for therapy or prevention of chronic diseases.

high-carbohydrate/low animal protein diet [7]. The 'European microbiome' was dominated by Bacteroides enterotype, whereas the 'African microbiome' was dominated by the Prevotella enterotype. The concept that nutrition is a determinant of microbiota composition in children was confirmed in another study [8▪].

Features of a healthy microflora are its richness and evenness. Richness describes the number of bacterial species in a specific ecosystem not taking into account their relative abundance. Evenness indicates the relative abundance of each species in a specific ecosystem. These two definitions are used to describe the microbial diversity in the gastrointestinal tract [9▪].

However, the initial colonization of the intestine is also important for future microflora and functions. At birth, the intestine is sterile, and bacterial colonization begins with amniotic membrane rupture. Bacteria from the mother's intestinal and vaginal sites, and from the outer environment, colonize the neonatal gut within a few hours from birth, and appear in feces shortly thereafter. Vaginally born infants have a greater abundance of Bacteroides and Bifidobacteria compared with infants born by cesarean section [10] and, interestingly, the latter are at higher risk of immunological diseases [11▪]. Other important factors that contribute to build the microbiome composition are antibiotics, hygiene status and functional nutrients. The latter are increasingly used in infancy.

The time of bacterial colonization is also important as shown in animal models [12▪]. In preterm neonates, bacterial colonization is delayed, and the number of colonizing species is limited. An increasing diversity of gut microflora was observed in the first 8 weeks of life in preterm infants, and most infants had staphylococci in their stools as the main species, whereas few infants were colonized with *Bifidobacterium* spp. [13▪▪]. The study also showed a positive relationship between diversity of intestinal microflora and nutrient tolerance and weight gain, supporting a functional relationship between humans and their intestinal microbes.

Nutrition plays a major role at birth and after birth. Breastmilk is important, as it not only provides a range of substrates for bacterial growth [14▪] but it is also a natural bacterial inoculum that affects neonatal colonization [15▪]. Breastfed infants had 2 times the numbers of Bifidobacteria than formula-fed infants, and in the latter, Atopobium and Bacteroides were found in significant counts. Moreover, in formula-fed infants, intestinal microbiota was less complex (or 'diverse') than in breastfed infants [16▪]. Breastfeeding has been associated with a number of beneficial effects in the short and long term and it is likely that microflora contributes many of these effects. The profile of intestinal bifidobacterial population in infants shows the simultaneous co-occurrence of a number of bifidobacterial species [17▪]. Later in life, weaning and the introduction of solid foods are associated with the transition toward an adult-like microbiota [18▪▪] and nutrients again play a key role in determining the final microbiota composition [6▪,7,9▪].

The adult microbiota is relatively stable [19▪▪]. The temporal stability reflects the resilience of microbiome in adults and perturbations, such as antibiotic therapies, have only transient effects on the dominant microbiota. Overall, the adult microbiome is more complex than its infant equivalent, although being stable over time and similar between individuals [20▪▪]. In contrast, infant gut microbiota possesses a relatively simple structure but is rather unstable over time. The microflora in children is intrinsically plastic, affected by few variables and less exposed to factors that may change its composition compared with adults, and this provides an ideal setting to understand the functional roles of gut microbiome.

FUNCTIONS OF GUT MICROBIOTA

Human microbiota exerts important immune, metabolic, trophic, and protective functions that are currently interpreted with a model of symbiosis between the host and intestinal microbes. Many of

the effects by intestinal microbiota are realized with mechanisms that derive from coevolution of bacteria and in the host (Table 1). The commensal microflora inhibits colonization by pathogenic bacteria through a variety of local mechanisms. It also interacts with the immune system at local and systemic level. The immune system in turn protects the host from potential pathogenicity of microbial communities that provide metabolic benefits [21▪▪]. This results in a balanced homeostasis whose histological counterpart is the 'physiological inflammation', defined by the presence of a rich immune cell population within the intestine. A key effect of the innate immunity is to confine bacteria into the intestine preventing them from reaching the systemic immune compartment [22▪,23▪▪]. Vaishnava *et al.* [24▪] demonstrated that RegIIIγ, a secreted antibacterial lectin, is a fundamental immune mechanism that promotes host–bacterial mutualism by regulating the spatial relationships between microbiota and host. Flora abundant in Bacteroides with consequent intestinal inflammation was observed in a murine model deficient in the inflammasome component NLRP6, supporting the concept of the immune-driven dysbiosis [25▪].

While the host immune system controls the development of intestinal microflora, also the opposite is true as the microbiota shapes the immune system [26▪]. Round *et al.* [27▪▪] suggest that the immunologic distinction between pathogens and the commensal microbiota is mediated not solely by direct host mechanisms but also through specialized molecules evolved by symbiotic bacteria that enable commensal colonization. The modulation of mucosal T cells by intestinal bacteria certainly affects systemic immunity as shown by the results obtained with different animal models of microbiota-associated autoimmune diseases [28▪]. Therefore, the intestinal microbiota plays a major role in driving the immune response and vice versa. Microflora is under immune control and this provides the basis of a finely tuned symbiotic relationship.

A similar mutually beneficial relationship exists in terms of energy and nutrition supply, between gut microbiota and the host (Table 1). Bacteria provide the host with energy from indigestible dietary substrates in the form of short-chain fatty acids, whereas the host offers a nutritionally adequate environment to its commensals. A recent clinical trial showed clear associations between gut microbes and nutrient absorption indicating a possible role of microbiota in the regulation of nutrient digestion and energy harvest [29▪▪].

Finally, a novel, fascinating function by microflora is related to neuronal development [30▪]. Microbiota is an active player in the brain gut axis and affects levels of neurotrophins in mice. This translates in behavior control, brain differentiation, and neuronal survival [31▪▪]. However, a recent study showed that the microbial colonization triggers mechanisms that affect neuronal circuits involved in motor control and anxiety behavior [32▪▪].

Table 1. Physiological functions of intestinal microbiota

Functions	Mechanisms/Effects
Protective functions against pathogenic bacteria	Pathogen displacement Nutrient competition Production of antimicrobial factors Activation of local immune response Contribute to the intestinal barrier function
Immune development	IgA production Control of local and general inflammation Tightening of junctions Induction of tolerance to foods
Digestive and metabolic functions	Vitamin production Fermentation of nondigestible carbohydrates Dietary carcinogens metabolism
Neuronal development	Modulation of brain gut axis during neuronal development Motor control and anxiety behavior

IgA, immunoglobulin A.

DYSBIOSIS IN CHILDREN'S DISEASES

In order to understand the functions and dysfunctions on intestinal microflora, an obvious approach is to investigate the composition of microflora in specific diseases. A healthy state of microbiota structure, in which microorganisms with potential health benefits predominate in number over those potentially harmful, is defined 'normobiosis' (or eubiosis). On the contrary, 'dysbiosis' is a condition in which one or more potentially harmful bacterial species are dominant [33]. In many diseases, the diversity of microflora is reduced. However, there are also specific aberrations of microflora in selected childhood diseases. The specific microflora aberrations that have been detected in specific diseases are often defined as 'signature', indicating that microbial aberrations may be a hallmark of that disease (Table 2). An example is provided by celiac disease in which children show a peculiar microbial pattern with abundant Firmicutes [34▪▪]. However, microbial signatures have been detected in several

Table 2. Major changes in the composition of gut microbiome in the intestinal and extraintestinal childhood diseases

Disease	Changes in gut microflora composition
Celiac disease	Lack of bacteria of the phylum Bacteroidetes along with an abundance of Firmicutes
Inflammatory bowel disease	Low concentrations of *Faecalibacterium prausnitzii* and *Bifidobacteria* Increased levels of *Escherichia coli* Reduced diversity of gut microbiota
Inflammatory bowel syndrome	Significantly greater percentage of the class Gammaproteobacteria Presence of unusual Ruminococcus-like microbes
Necrotizing enterocolitis	Predominance of Gammaproteobacteria Reduced diversity of gut microbiota
Atopy	Lower counts of Lactobacilli, Bifidobacteria, and Bacteroides Increased counts of *Clostridium difficile* Reduced diversity of gut microbiota
Obesity	Increased Firmicutes at expenses of the Bacteroidetes group
Cystic fibrosis	Lower species richness Lower counts of lactic acid bacteria, clostridia, Bifidobacterium spp., Veillonella spp., and Bacteroides-Prevotella spp.

chronic diseases such as inflammatory bowel diseases (IBD). Many studies showed that intestinal microbiome profoundly differs between patients with IBD and healthy individuals, and intestinal dysbiosis may contribute to the risk of IBD or its relapses. Schwiertz *et al.* [35] showed that the Firmicutes phylum, and particularly the species *Faecalibacterium prausnitzii*, is less represented in IBD patients than in controls. This effect may not be confined to IBD, as *F. prausnitzii* inversely correlated with the severity of disease in acute appendicitis [36▪]. Interestingly, *F. prausnitzii* has an anti-inflammatory effect and its presence increases with fiber consumption in adults [37▪]. All together, these data suggest that *F. prausnitzii* protects against intestinal inflammation.

In a prospective nationwide cohort study investigating the link between antibiotics and IBD in children, the relative risk of IBD was increased for antibiotic users compared with nonusers [38▪▪]. The association was stronger if only Crohn's disease was considered and for antibiotics given during the early life. In a study in adolescents with IBD, a layered distribution of selected microbiota components was described with a decrease in Bifidobacteria, an increase in Streptococci in Crohn's disease and in Lactobacilli in ulcerative colitis. An increase in mucin degradation by bacteria was also described in ulcerative colitis patients [39▪]. This data support the role of microbiome perturbations in IBD.

Irritable bowel syndrome (IBS), a symptom-based diagnosis defined by Rome III criteria, is associated with dysbiosis and the manipulation of intestinal microbial communities (i.e., probiotics) may effectively alleviate fastidious symptoms. Interestingly, a close relationship between a specific bacterial profile with the severity of symptoms was recently shown in pediatric IBS [40▪▪].

Necrotizing enterocolitis (NEC) is a severe and potentially fatal disease that affects preterm neonates. A decrease in microbiota diversity was observed for all preterm infants, and NEC children showed a further reduction in diversity, with a predominance of Gammaproteobacteria and a reduction of other bacterial species [41]. However, others found an opposite pattern, and in a prospective study, bacterial diversity expressed as band richness was higher in NEC than in controls [42▪▪]. In a different study, microbiota diversity did not differ between infants with NEC and controls in the period before the disease diagnosis, but the authors found that there is a more heterogeneous microbial structure in infants developing NEC [43▪▪]. This observation supports the concept about the identification of a pattern of microbiota at high risk of NEC.

Atopy is also a condition in which microflora has been implicated. Previous studies, summarized in a recent review, showed that atopic infants have lower counts of Lactobacilli, Bifidobacteria, and Bacteroides species, and an increase in *Clostridium difficile* colonization, compared with nonatopic infants [44▪]. Furthermore, prospective studies on microbiota in early life showed that atopy was associated with a reduced ratio of Bifidobacteria to Clostridia and with the presence of *C. difficile* [45▪]. Two recent works showed that reduced microbiome

diversity in early life increases the risk of atopic diseases [46▪], thereby suggesting a link between early microbial colonization and subsequent atopy.

Microflora structure in obesity is a major focus of research, and there is increasing evidence that microflora composition influences the host energy balance and that western diet profoundly changes microbiota in humans [7,47▪]. A high-carbohydrate diet affects the composition of intestinal microflora, which may play an important role in controlling energy metabolism and favor a state of low inflammation [48▪]. Microbial composition differs between obese and lean patients, and this may be related to the extraction and use of energy from food in the intestinal lumen. Changes in gut microbiome are reflected by an abnormal ratio between Firmicutes and Bacteroidetes [49▪]. Interestingly, breastmilk differs from mothers with normal or increased BMI [50▪], and this may be linked with a different microbial imprinting that may become evident in children up to 10 years of age [51▪].

MICROFLORA AS A TARGET FOR THERAPY

The data summarized above indicate that microflora plays a role in triggering – or contributing to – a number of diseases. This raises the hypothesis that modifications of microflora through antibiotics or – conversely – with probiotics or also with functional nutrients (i.e., prebiotics) may have therapeutic effects. Rifaximin was successful in reducing symptoms in a population of adults with IBS without constipation [52▪▪]. Consistent evidence of probiotic efficacy was obtained in children with IBS characterized by abdominal pain [53▪] and constipation [54]. Interestingly, the administration of Lactobacillus casei subsp. Rhamnosus to children at risk of atopy induced a global shift in gut microbial community composition. This resulted in modifications of the relative abundance of a large number of taxa previously associated with either an increased or decreased risk for the development of allergy and atopy [55▪▪]. Overall, the results that are being obtained with probiotics in IBS and other diseases further support the concept that microflora play a role in selected diseases, probably with an age-related pattern [56▪].

However, probiotics should not be regarded as a therapy to be considered in adjunct to the traditional treatment or to be used in minor disorders. They may play an important role in very severe diseases. A general dysbiosis was detected in children with cystic fibrosis (CF) compared with their siblings [57▪]. This may explain the beneficial effects observed on intestinal inflammation and also on respiratory function in children with CF receiving probiotics [58]. More recently, Scanlan *et al.* [59▪▪] showed a reduced richness, evenness and diversity of gut microbiota in CF children in a very small sample-size study. Therefore, dysbiosis may play a major role in CF and the modulation of gut microflora with probiotics may have a positive effect on intestinal and nonintestinal inflammation.

Finally, a very delicate field is the use of probiotics in NEC. Administration of probiotics to preterm infants resulted in the reduction of the incidence of NEC and – even more interestingly – of mortality for all causes in preterm infants [60▪]. The beneficial effects were confirmed in an updated meta-analysis [61]. Such a dramatic outcome and the strength of supporting data, led to the indication to routine administration of probiotics to preterm infants with the aim of preventing the severe complications associated with this condition [62▪]. However, meta-analysis and recommendations are not widely shared. There are probiotic products on the market that cannot be recommended because they have not been studied sufficiently and may be harmful. Because NEC also appears to be a highly heterogeneous and etiologically multifactorial disease, targeting the neonates at highest risk with the lowest potential for harm, rather than routinely prophylaxing all infants appears prudent. Therefore, further data are expected prior to obtaining a conclusive indication in this delicate area [63▪,64▪].

CONCLUSION

Intestinal microflora is considered a fully functional human organ and its structure is the result of early life events such as feeding, illnesses, antibiotic therapies, and environmental exposure. A healthy microbiota, or eubiosis, protects from diseases, whereas an abnormal microflora structure, or dysbiosis, is linked with the risk of diseases.

A reduced diversity of gut microflora is a frequent hallmark of intestinal inflammation. Consistent abnormalities in the microbial structure have been detected in populations of children with specific diseases and have been defined as 'microbiological signatures' of those diseases. The word signature provides a double concept: the specific role of selected bacterial species in causing – or contributing to – specific diseases and the opportunity to recognize the disease (or monitoring its course) by analyzing microflora composition. However, the changes observed in microbial populations raise the option of targeting microflora for therapy. This is being done with increasing success in selected diseases using various strategies, including administration of probiotics.

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None.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Family Group Psychotherapy to Support the Disclosure of HIV Status to Children and Adolescents

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AU1 ▶ **Abstract**

Disclosure of the HIV status to infected children is often delayed due to psychosocial problems in their families. We aimed at improving the quality of life in families of HIV-infected children, thus promoting disclosure of the HIV status to children by parents. Parents of 17 HIV-infected children (4.2–18 years) followed at our Center for pediatric HIV, unaware of their HIV status, were randomly assigned to the intervention group (8 monthly sessions of family group psychotherapy, FGP) or to the control group not receiving psychotherapy. Changes in the Psychological General Well-Being Index (PGWB-I) and in the Short-Form State-Trait Anxiety Inventory (Sf-STAI), as well as the HIV status disclosure to children by parents, were measured. Ten parents were assigned to the FGP group, while 7 parents to the controls. Psychological well-being increased in 70% of the FGP parents and none of the control group ($p = 0.017$), while anxiety decreased in the FGP group but not in controls (60% vs. 0%, $p = 0.03$). HIV disclosure took place for 6/10 children of the intervention group and for 1/7 of controls. Family group psychotherapy had a positive impact on the environment of HIV-infected children, promoting psychological well-being and the disclosure of the HIV status to children.

Introduction

Positive mental health encompasses diverse aspects related to quality of life and general well-being, and can be considered as the achievement of emotional resilience.[1] Resilience is the psychological process developed in response to intense life stressors that facilitates healthy functioning, playing a major role in response to illness and other life adversities.[2] The needs of children living with HIV infection are progressively shifting from those strictly clinical to those related to psychosocial issues.[3] However, this is in contrast to the established model of care of HIV-infected children, which is characterized by a close physician–patient relationship. Such a model is largely the consequence of the social stigma with the need of hiding the disease. However, this often results in delayed disclosure to children and adolescents.[4,5]

Our Unit has been providing care to children, adolescents, and young adults with HIV infection since the onset of AIDS epidemics, in agreement with the guidelines of the National Institutes of Health.[6] In parallel with the evolving aspects of HIV epidemics, we progressively developed a comprehensive approach integrating psychological, social, and biomedical support for the management of HIV. This model provides in- and outpatient care by the medical staff of the reference center, and it also includes home care provided by physicians and nurses working in the hospital.

Using the International Classification of Functioning, Disability and Health (ICF), a specific instrument to evaluate the disabilities and dysfunctions in children, we showed that environmental factors and psychosocial issues had a major negative impact on the quality of life of HIV infected children and their families.[7] Interestingly, the greatest impairment reported by parents was related to the need of hiding the HIV infection status to their children because of social stigma. This was associated with delayed disclosure,[7] which in turn generated a vicious cycle of increased anxiety, ultimately leading to further functional disabilities in HIV-infected children. Therefore, disclosure has a key role in coping with HIV infection. Current recommendations regarding the disclosure of HIV infection to children are based on lessons learned from pediatric oncology.[8] Similar to trends observed in oncology, many parents and care providers of HIV-infected children thought they need to protect children from emotional burdens and social prejudices associated with their disease. With the advent of new therapies

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in the mid 1990's, and the dramatic improvements in the mortality and morbidity of HIV-infected children, changes in disclosure practices began to take place, and the disclosure of HIV infection is now a step of care.[5] Disclosure is not only related to psychosocial aspects and eventually resilience, rather it also affects therapy and its outcome.

In a recent qualitative study, caregivers reported that their children became more adherent to antiretroviral medications following disclosure.[9] In an interview involving 120 families in a resource-limited setting, disclosure was perceived as a step towards self-sufficiency, but also with potential negative social effects.[10] On the contrary, other studies have indicated that children aware of their HIV status may be less likely to adhere.[11] The conflicting relationship between disclosure and adherence may be explained by the age-related patterns of relationship between the child's age, the type of communication, and the support to it. Children and adolescents frequently perceive limited communication before, during, and after disclosure, which is a discrete event rather than a process.[12] Once children are made aware of their HIV status, their care-givers expect them to become able to self-manage treatment, without supervision and reminding.[13] However, we previously showed that adherence was strongly related to caregivers more than any other factor, such as child's age or disease status.[12] Disclosure is therefore an important step that has a broad effect on the HIV infection course and needs to be accompanied by psychological support.

These issues led us to plan a family group psychotherapy (FGP) with the families of children with HIV infection, with the aim of removing barriers that prevent disclosure, and ultimately to increase resilience in the caregivers. The basic concept was to work with small groups of caregivers and to discuss common problems and feelings in order to build competence and self-reliance in families and patients, so that parents and their children could effectively manage their own health.

The intervention was incorporated in our HIV care planning. Specific objectives of the intervention were the following: to provide information for sound management of the disease; to offer an empathetic understanding of the problems and to discuss problems and solutions; and to focus on HIV disclosure in a timely and appropriate manner, acknowledging the need for a close interaction between parents of children, the medical staff and the team of psychologists.

Methods

Children with HIV and their families, seen at our HIV reference center for the management of pediatric HIV infection, were enrolled. Inclusion criteria were the following: age 1–16 years; diagnosis of vertical HIV infection obtained > 1 year before enrollment; unawareness of HIV infection status; therapy according to the criteria reported in the NIH guidelines for the Use of Antiretroviral Agents in Pediatric HIV Infection.[6] To estimate adherence quantitatively, each caregiver was asked how many doses of the total prescribed antiretroviral therapy had been omitted in the previous 4 days, and children were defined as non-adherent if they had taken less than 95% of all prescribed doses of antiretroviral therapy in this period. This method provides a reliable estimate of adherence to antiretroviral therapy in children.[14] Patients and caregivers were randomly assigned to either FGP intervention or control group. Parents decided whether the mother, the father, or the caregiver would participate in the support group. Written informed consent was obtained for every participant.

The intervention was structured through meetings between parents of children and the team of psychologists of the reference center. Eight 2-h group sessions took place once a month. The first and last meetings were longer to allow test administration. The content of the meetings is described in the Appendix. Families in the control group received complete health assistance but no psychological support and they were not aware of the intervention.

The following tests were administered before and after the support group meetings:

- The Psychological General Well-Being Index (PGWB-I)[15] that assesses the psychological and mental health status of an individual through 22 items. The scale consists of six domains: anxiety, depression, sense of positive and well-being, self-control, general health, and vitality. The overall score ranges from 0 to 110 (worst–best states possible). Scores 91–110 are consistent with high level of well-being; scores 71–90 with good level of well-being; scores 51–70 with medium level of well-being; scores 31–50 with low level of well-being; scores 0–30 with very low level of well-being.[16] Changes in psychological well-being index were defined according to these ranges.
- The Short-Form State-Trait Anxiety Inventory (Sf- STAI)[17] is a six-item version of the Spielberger STAI for the assessment of anxiety state.[18] It is a validated instrument for measuring anxiety in adults. It clearly distinguishes between a temporary condition of anxiety in a specific situation and anxiety as a general trait. The results of the scale range from 20 to 80. A score ≥ 60 indicates an elevated level of anxiety; a score between 50–59 indicates a moderate level of anxiety; a score ≤ 49 indicates absence of anxiety.[17] Changes in anxiety were defined according to these ranges. A final self-assessment through rating was obtained after the last meeting.

Both PGWB-I and Sf-STAI have proven reliable in chronic conditions, with a high test/retest coefficient.[19,20]

Results were expressed as number/percent or as mean ± SD. The Student *t*-test, the Wilcoxon matched-pairs signed rank test, and the χ^2 method, or exact Fisher's test when appropriate, were performed to compare the variables and $p < 0.05$ was the cut-off for significance. Data were analyzed with the SPSS package version 20.

Results

A total of 17 parents of as many children (mean age 11.7 ± 3.7 years, range, 4.2–18.0; 7 boys) were enrolled, 13 of them being biological and 4 foster parents. Ten children were single orphans, one was a double orphan and entrusted to a caregiver; 10 children were of Italian origin and 7 were of non-EU origin. Ten caregivers were randomly assigned to support group intervention and the other 7 to controls. The corresponding children populations were age-matched (Table 1). ◀T1 The intervention group had a higher viral load, due to the presence of three patients with > 100 copies/ml. However, all patients had < 1000 viral copies/ml. Of note, viral load was reduced in two children following the intervention (in one patient the viral load was associated with poor compliance, and in another with viral resistance).

Table 1. Baseline Psychological Outcomes and Socioeconomic Features of Patients and Families of Family Group Psychotherapy Group (Intervention) and Control Group

	Intervention (n=*10*)	*Controls* (n=*7*)	p
Sex *n* (%)			
F	5 (50)	5 (71)	0.622
M	5 (50)	2 (29)	
Family *n* (%)			
Natural	7 (70)	6 (86)	0.603
Adoptive	3 (30)	1 (14)	
Age mean±SD	12±4	11±2	0.114
Orphanity *n* (%)			
One	5 (50)	5 (71)	0.784
Both	1 (10)	0 (0)	
None	4 (40)	2 (29)	
Origin *n* (%)			
Italian	5 (50)	5 (71)	0.622
Extra EU	5 (50)	2 (29)	
CG education *n* (%)			
Middle school or less	4 (40)	6 (86)	0.134
High school	5 (50)	1 (14)	
Graduation	1 (10)	0 (0)	
Income *n* (%)			
One	6 (60)	4 (57)	0.59
Both	0 (0)	1 (14)	
None	4 (40)	2 (29)	
Mother transmission *n* (%)			
Sex	3 (30)	5 (72)	0.486
Blood transfusion	2 (20)	0 (0)	
Drug	2 (20)	1 (14)	
Adoptive	3 (30)	1 (14)	
Adherence to HAART *n* (%)			
Adherent	6 (60)	5 (71)	0.627
Non adherent	4 (40)	2 (29)	
CD4 lymphocytes/ μL mean±SD	795±415	604±233	0.124
HIV RNA copies/ mL mean±SD	92±117	20±0.7	0.001

HAART, highly active antiretroviral therapy.

Psychological well-being

Baseline PGWB-I scores differed between FGP and controls (57.4±15 vs. 79.7±7.3, $p=0.04$). The average PGWB-I increased in the intervention group (T0: 57.4±15; T1: 72.5±21, $p=0.22$), and decreased in controls (T0: 79.7±7.3; T1: 48.1±9, $p=0.01$) (Fig 1A). Psychological well-being improved in 70% of the caregivers in the FGP group versus none of the control group; it decreased in 20% of FGP parents versus 71% of the controls ($p=0.01$), and finally remained stable in 10% and 29% of the FGP and controls, respectively (Table 2). An association was observed between parental schooling and changes in parental well-being ($p=0.02$) but not with other social and economic factors (Table 3).

Anxiety

The baseline Sf-STAI scores were not different between FGP and controls. The average Sf-STAI decreased in the intervention group (T0: 51.7±4; T1: 43±4, $p=0.22$), while it increased in controls (T0: 40.6±6; T1: 57.±3, $p=0.01$) (Fig 1B). Anxiety was significantly diminished in 60% of caregivers in the intervention group versus none of the controls ($p=0.03$) (Table 2). Similarly to PGWB-I, changes in anxiety were also associated with parents' education ($p=0.01$) (Table 3).

Disclosure of HIV infection to children and adherence to antiretroviral therapy

Disclosure took place in six of 10 (60%) of the group support caregivers within 12 months following the intervention. Comparatively only 1 of 7 (14%) of controls achieved this step ($p=0.59$). However, disclosure was correlated neither with PGWB-I ($p=0.15$) nor with Sf-STAI ($p=0.45$), nor with any other social, cultural, or economic features (Table 4).

Changes in adherence to HAART of children were not correlated to intervention (Table 2) nor to the disclosure of the HIV status to children by parents.

Assessment of the perceived benefit and efficacy of the intervention

A final self-assessment of the benefits perceived from the intervention was obtained after completing all sessions at the last group meeting: caregivers answered the question 'To what extent did the group help you understanding issues concerning HIV?', and the level was 7.1±2 on a score 0–9 (not helpful–very helpful). A final self-assessment of the empathetic support received was provided by caregivers answering the questions 'How helpful was it to meet other parents of children with HIV?' and 'To what extent did attending the group help you in feeling less isolated with regard to your child's HIV infection?'. On a score 0–9 (not helpful–very helpful), the perceived helpfulness by empathetic support was 9±0 and 9±0, respectively. Only one meeting was missed by two participants.

Discussion

Our results show that an intervention based on group psychotherapy delivered to families of HIV-infected children improves the quality of life, increases their general well-being, and reduces their anxiety state, thereby increasing their resilience. Our observations, even with the limitation of a wide age range of the patients and of the heterogeneity of the type of families, show that the support group effectively breaks the isolation and creates opportunities for carers, by sharing psychological resources and experiences. These results also depend on parents' education, as suggested by the significant association between carers' schooling and their psychological improvement in terms of both general well-being and anxiety. It is well known that the education strongly affects the capacity of living with chronic diseases and that supportive interventions are more effective in high culture settings. This is demonstrated for diseases such as diabetes or chronic obstructive pulmonary disease: positive effects on depression, health-related quality of life, feelings of mastery, and self-efficacy are confined to patients with high education, while those with only a primary education do not benefit from supportive interventions.[21] Our data obtained in a small population of families of low educational level indicate that interventions tailored to those specific

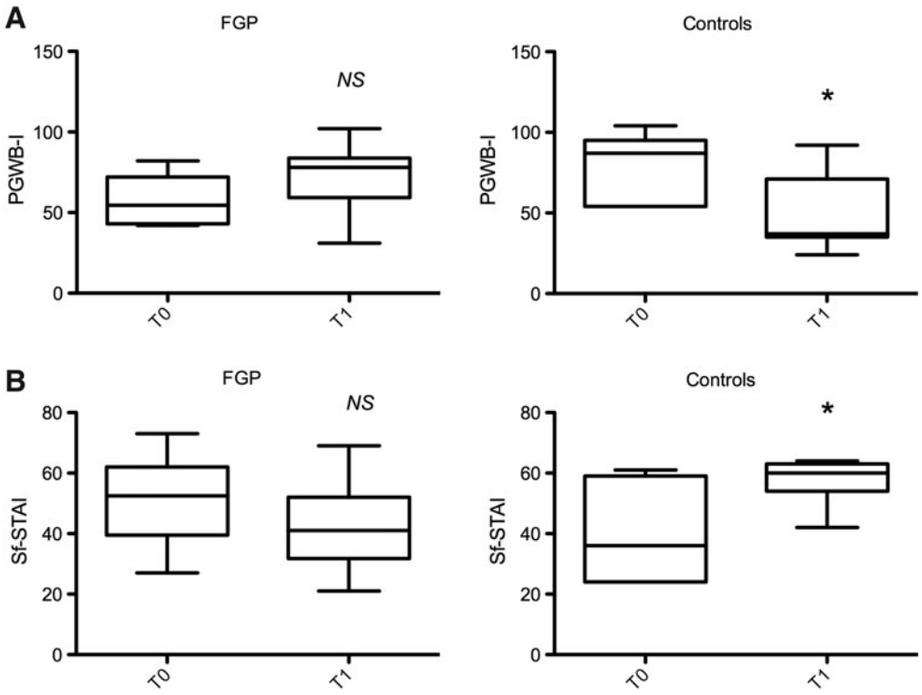

FIG. 1. Psychological general well-being **(A)** and anxiety **(B)** scores before and after family group psychotherapy. FGP, family group psychotherapy; PGWB-I, Psychological General Well-Being Index; Sf-STAI, Short form of State-Trait Anxiety Index; $*p < 0.05$.

Table 2. Changes in Psychological and Clinical Features after the Family Group Psychotherapy

	Intervention (n = *10*)	*Controls* (n = *7*)	p
CG PGWB-I *n* (%)			
Improved	7 (70)	0 (0)	0.017
Stable	1 (10)	2 (29)	
Worsened	2 (20)	5 (71)	
CG Sf-STAI *n* (%)			
Improved	6 (60)	0 (0)	0.037
Stable	2 (20)	2 (29)	
Worsened	2 (20)	5 (71)	
HIV Disclosure *n* (%)			
Yes	6 (60)	1 (14)	0.134
No	4 (40)	6 (86)	
Change in adherence to HAART *n* (%)			
Improved	3 (30)	2 (29)	0.784
Stable	5 (50)	5 (71)	
Worsened	2 (20)	0	
Adherence to HAART T1 n (%)			
Adherent	7 (60)	7 (71)	0.228
Non adherent	3 (40)	0 (29)	
CD4 lymphocytes/ uL T1 mean ± SD	863 ± 379	665 ± 271	0.677
HIV RNA copies/ ml T1 mean ± SD	92 ± 150	20 ± 1	0.06

CG, caregiver; HAART, highly active antiretroviral therapy; PGWB-I, Psychological General Well-Being Index; Sf-STAI, Short form of State-Trait Anxiety Index; T1, after the intervention.

conditions, with simple peer communication are effective and contribute to resilience.

Although there are limited data on psychological support groups in pediatric settings, interventions involving families of children with HIV are generally delivered with the aim of improving patients' care beyond strictly clinical aspects. However, the effects of interventions are rarely measured. We did that and also applied disclosure as a secondary outcome parameter of the intervention. The latter promoted the disclosure of HIV status from carers to children. However, the relationship between psychological support and disclosure process by the caregivers is complex, and this effect could be related to cognitive gain rather than to psychological effect.

Table 3. Change in Psychological General Well-Being and Anxiety of Caregivers After the Intervention According to Parental Schooling

CG education (n)	*Middle or less (10)*	*High (6)*	*Grad (1)*	p
PGWB-I *n* (%)				
Improved	2 (20)	4 (67)	1 (100)	0.021
Stable	1 (10)	2 (33)	0 (0)	
Worsened	7 (70)	0 (0)	0 (0)	
Sf-STAI *n* (%)				
Improved	2 (20)	4 (67)	0 (0)	0.013
Stable	1 (10)	2 (33)	1 (100)	
Worsened	7 (70)	0 (0)	0 (0)	

CG, caregiver; PGWB-I, Psychological General Well-Being Index; Sf-STAI, Short form of State-Trait Anxiety Index.

Table 4. Rate of Disclosure of HIV Status to Children by Families According to Psychological Outcomes of the Intervention

HIV Disclosure (n)	*Yes (7)*	*No (10)*	P
PGWB-I *n* (%)			
Improved	5 (71)	2 (20)	0.156
Stable	0 (0)	3 (30)	
Worsened	2 (29)	5 (50)	
Sf-STAI *n* (%)			
Improved	4 (57)	2 (20)	0.456
Stable	1 (14)	3 (30)	
Worsened	2 (29)	5 (50)	

PGWB-I, Psychological General Well-Being Index; Sf-STAI, Short form of State-Trait Anxiety Index.

Moreover, not all the studies clearly showed an impact of the disclosure on quality of life, as well as the acceptance of disclosure appears to be higher in foster parents.[22,23] We did not find any association between disclosure and type of parents (biological vs. foster), but this might be due to the heterogeneity of our populations. The group therapy fostered the information about HIV infection in carers, enabling them to disclose to their children a troublesome truth. Awareness is a prerequisite to self care and it is a fundamental leading force in the construction of personal future.[24] Current recommendations by the American Academy of Pediatrics support disclosure to children as young as 8 years of age and to all adolescents.[25] From this perspective, the support by the group provided important motivations to self-management and resilience.

Looking at clinical features, our population was largely made of children and adolescents with virologically and immunologically well-controlled disease, reflecting the high standards of clinical care in pediatric HIV. No association was found between either HIV viral load or number of CD4+ lymphocytes and primary or secondary outcomes of our intervention, and only three patients had detectable viral load at baseline.

Unawareness and psychological discomfort could affect adherence and hamper effective management of the disease, especially in adolescents, in whom the lack of responsibility prevents an effective therapeutic alliance. We previously described a changing pattern of adherence over time in HIV-infected children and adolescents, and demonstrated that psychosocial features of caregivers and children play a major role in adherence. Interestingly, children of foster parents had the highest level of adherence and it may be expected that depressed and debilitated parents have limited ability in sustain optimal adherence in their HIV-infected children.[14] However, adherence is the result of a complex interplays of determinants and it is impossible to establish a direct link with psychotherapeutic interventions. In our sample, only 65% of all children showed an optimal adherence to HAART at baseline. We observed that adherence increased over the period of observation. However, this was also the likely consequence of the higher attention paid by physicians and other health care professionals to this outcome during the study. In contrast with the physical well-being, psychological discomfort was high in the families enrolled, and the support group was well accepted and recognized as effective by families. The mutual empathetic support among the participants was perceived as very helpful. These interventions should be integral part of the standard care for HIV-infected children and adolescents. Psychosocial issues in young patients should be carefully considered to ensure clinical success.[26] Management of psychological impairment through a biopsychosocial model of care may reduce anxiety, depression, and social isolation by lowering physical tension, increasing a sense of control and self-efficacy, ultimately increasing resilience in the parents of infected children.[27] The caregivers experienced a sense of relief and expressed positive feelings for being treated in a nonjudgmental way. As previously described, the possibility to freely talk about their own emotions without being censored is a path of growth for HIV-positive subjects.[28]

In conclusion, attention needs to be paid to both psychosocial and biomedical aspects of pediatric AIDS. It is important to involve caregivers and family members who are in close contact with the HIV-infected child. With little effort and easy to perform and sustainable interventions, the bio-psychosocial state of HIV-infected children and their families may be substantially improved.

Appendix: Psychotherapy Sessions

First meeting

A psychologist expert in HIV guided the meeting; a younger psychologist and a social worker were also actively involved. All the group members met in a friendly context. Parents were invited to introduce themselves and their families and share their expectations. The aims of the group support were presented, and the participants were asked to fill the standardized cognitive instruments. Parents reported their fears and feelings of isolation in relation to their and/or their children's illness. Some of the parents reported fear for their own disease. A major issue was related to the disclosure of the HIV status.

Second meeting

An overall increased acknowledgment of the pain and the distress related to the issues raised by parents was recorded and openly discussed. Most interventions focused on the sense of guilt for having vertically transmitted the infection, the fear of illness and death, the uncertainty about the future. Reasons supporting early disclosure to children were gently introduced.

Third meeting

The inner resources of the parents/carer and the family resources were explored under the guide of therapists. The barriers to disclosure were often related to the perceived misconception of the HIV infection in the 'outside world'. The fear of death was strongly rooted in those participants who already had experienced a death for HIV in the family and was contained by an element of life expressed by others. The risk of family break up by a member of the group was counterbalanced by someone else's confidence as well.

Fourth meeting

A conscious sense of guilt arose for the transmission of the infection, in association with the idea of having betrayed their task to be bearers of life rather than death.

Fifth meeting

In this session, the attendees were invited to take advantage from available social services. Particularly, a social worker provided information on local resources, as well as financial resources to support HIV-infected subjects. The physicians of the medical service joined the group, and parents were able to directly and openly ask questions and make comments. The physicians stressed the efficacy of HAART in fostering a full control of the disease, preventing HIV progression, and allowing a full normal life, if optimal adherence to treatment was reached.

Sixth meeting

This session was named 'Not when, rather how' and addressed HIV disclosure, because therapists spoke about the scattered experiences and built a roleplaying to give simple explanations to young children or information about the nature and consequences of illness to older children.

Seventh meeting

In this session, the focus was on consolidation of earlier meetings in order to help the caregiver to find confidence and language competences to talk to the children.

Eighth meeting

In the final session, tests were administered to caregivers in order to obtain a feedback on empathetic support and cognitive advance.

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Author Disclosure Statement

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AU1: Abstract
AU2: Is Disclosure Statement accurate? If not, please amend as needed.

EXPERT OPINION

Clostridium difficile antibodies (WO2013028810): a patent evaluation

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Introduction: Incidence and severity of *Clostridium difficile* infection (CDI) are increasing worldwide. Toxins A (TcdA) and B (TcdB) and host immune response are the major determinates of CD pathogenesis and represent a new, stimulating therapeutic target to control CDI.
Areas covered: The present patent and literature on the pathogenesis and treatment of CD were critically reviewed. The patent was described and put into clinical context, highlighting possible advantages and barriers to use. It consists of a blend of monoclonal antibodies (mAbs) and antigen-binding portions that neutralize TcdA, targeting the enterocyte-binding domain. It demonstrated good efficacy in *in vivo* models and seems promising in clinical practice. However, recent evidence reshaped the central role of TcdA.
Expert opinion: Current treatments are inadequate to control CDI and recurrence. Toxin-targeted mAbs are one of the most promising approaches for CDI, including infection by hypervirulent strains. At-risk subjects and those experiencing recurrence are the ideal targets for this second-line treatment; however CDI epidemiology is fast-changing and mAbs may represent a powerful option also for other patients. The re-evaluation of the pathogenic role of TcdA may potentially limit the use of this product; however, the possible administration in combination with other therapeutic agents may optimize its efficacy.

Keywords: antibiotic-associated diarrhea, *Clostridium difficile*, *Clostridium difficile* infection recurrence, *Clostridium difficile* Toxin A, immunization, monoclonal antibodies

Expert Opin. Ther. Patents [Early Online]

1. Introduction

1.1 *Clostridium difficile* infection

Clostridium difficile (CD) is a sporogenic anaerobic Gram-positive organism that may be responsible for a broad spectrum of diseases in humans, ranging from a self-limiting secretory diarrhea to life-threatening conditions such as pseudomembranous colitis, toxic megacolon, intestinal perforation and septic shock [1].

In the past decade, a dramatic worldwide increase in the incidence of *C. difficile* infection (CDI) has been reported [2]. This relevant increase in CDI occurrence and severity has been related to an injudicious use of antibiotics and other treatment responsible of microflora disruption and to the concomitant emergence of hypervirulent epidemic strains and new risk factors.

Although CD still represents the leading cause of diarrhea in health care settings and at-risk patients (elderly, chronically ill and immune-compromised subjects), it is fast becoming a cause of community-acquired diarrhea in low-risk populations, such as children, healthy adults and pregnant women [3-5].

In addition to the change in epidemiology, the report of unacceptably high failures of traditional first-line treatments (metronidazole and vancomycin) is leading

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Article highlights.

- Current treatments are inadequate to control CDI spreading and recurrence.
- Pathogenesis of CD is strongly related to the action of toxins and host immune response.
- The blockage of toxin A C-terminal domain and consequential inhibition of adhesion to enterocytes may represent a powerful mechanism to neutralize toxin activity.
- Toxin-targeted monoclonal antibodies are a promising therapeutic approaches for the treatment of CDI and its recurrence.
- Considering the recent re-evaluation of toxin A pathogenic role, combination of toxin A-targeted monoclonal antibodies with other treatments (including neutralization of toxin B) may optimize its efficacy.

This box summarizes key points contained in the article.

to a growing concern and the development of new strategies to control CDI and its recurrence [2,6].

1.2 Role of toxins in the pathogenesis of CDI

Although not the only virulence factors, CD toxins A (TcdA) and B (TcdB), play a major role in the pathogenesis of CDI. Both toxins are glycosyltransferases targeting host GTPases, including Rac, Rho and Cdc42, leading to cytoskeleton disruption and apoptosis in enterocytes and immune cells [7]. Moreover, as recently demonstrated, both toxins trigger the inflammasome-mediated IL-1β production in the gut mucosa [8]. Historically, TcdA has been thought to be crucial in the CDI pathogenesis and to have a permissive effect to TcdB-mediated tissue damage, as suggested by toxicity studies in hamster and rabbit models [9,10]. More recently, anti-TcdA IgG titers have been recognized to be protective against the diarrheal disease in CD colonized patients [11]. However, the pathogenic role of the TcdA has been recently reshaped. Hamster mortality models demonstrated that the presence of TcdA, in the absence of TcdB, even at levels higher than in the wild-type strain was not lethal [12]. In human intestine, TcdB has proved to be the main trigger to cell inflammatory response during CDI [13]. The identification of diarrheagenic strains of CD that do not produce TcdA has further reduced the role of this toxin and emphasized the importance of TcdB [14].

1.3 Use of antibodies in human CDI

Immune responses to CD and its toxins are central to the pathophysiology of CDI, and both toxins are highly immunogenic. Moreover, an adequate serum antibody response during the initial episode of CDI seems to be associated with protection against recurrence. In fact, asymptomatic carriers and patients who do not experienced CDI recurrence showed more robust antitoxin immune responses than those with symptomatic and recurrent disease. The risk of recurrent CDI was significantly lower in patients with an enhanced serum antibody response with high titer of IgG against TcdA [11,15] and TcdB [16].

The rationale for immunological approaches, including active vaccination and passive immunotherapy targeting CD toxins, is essentially based on this evidence [11,16,17].

Intravenous immunoglobulins showed variable efficacy as adjunctive treatment for recurrent and severe CDI [18,19]. A growing body of evidence suggests that monoclonal antibodies (mAbs) directed against CD toxins may provide additional benefit to the standard treatment.

2. Chemistry

The object of the present patent is a biologic agent; hence, aspects related to chemistry are limited. The content of major pharmacological and clinical interest is a blend of mouse, chimeric and humanized mAbs and antigen-binding portions that specifically bind CD TcdA. Characteristics of different mAbs are depicted in (**Figure 1**) and the composition of the blend under examination are shown in **Figure 2**. The patent encompasses the methods of production from the murine hybridomas and corresponding mAbs, up to the characterization of the epitope binding.

Briefly, mice were immunized with TcdA C-terminal fragment; different clones of lymphocytes were isolated and fused with immortal myeloma cells to obtain corresponding hybridomas; and all DNA immunoglobulin sequences, except complementarity determining region (CDR), were replaced with human homologue sequences. This technology allows the production of antibodies with low immunogenicity for the *in vivo* use in humans. The patent also provided a pharmacokinetic analysis in rat models and an evaluation of the biological effects (survival to CDI) in mice.

3. Biology and action

These mAbs and antigen-binding portions specifically bind the C-terminal domain of CD TcdA (**Figure 3**). This domain vehicles the binding of TcdA (as well as of TcdB) to the enterocyte apical membrane and is critical to the downstream effects on the host; hence, the blockage through mAbs is a major mechanism to neutralize toxin action [20-22].

In vitro and *in vivo* models demonstrated that mAbs against C-terminal domain are able to neutralize cytotoxicity and hamper bowel fluid secretion and systemic toxicity [23]. Nevertheless, results in humans are still questionable, since the administration of these mAbs to a small group of patients did not demonstrate difference in recurrence, although a trend toward delay in time to relapse was reported [24].

Several other mAbs with different target epitopes of the same domain have been reported in the literature (e.g., 3359, 3358, PCG4), but some recent evidence suggested that a combination of different antibodies binding multiple epitopes may have enhanced neutralizing effects than those achievable with single targeted antibodies [25,26]. The

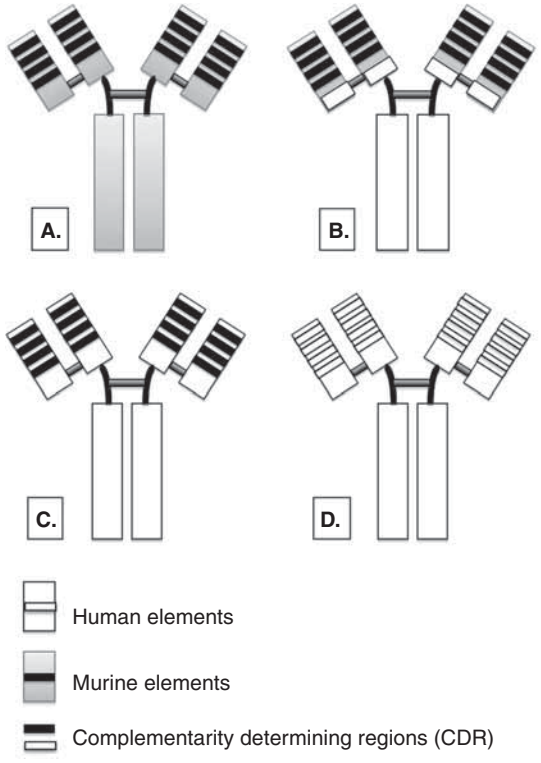


Figure 1. Different types of monoclonal antibodies (mAbs) according to origin and method of production. A, murine mAb; **B**, chimeric mAb; **C**, humanized mAb; **D**, fully human mAb.

neutralizing effect of multiple mAbs may be due to different mechanisms, including steric hindrance of TcdA cell receptors and inhibition of internalization mechanisms. The object of the present patent suits this evidence, including all antibodies isotypes and relative subclasses with monospecific and multispecific activity; that means that each variable fragment is capable of specifically bind a separate antigen or a different epitope on the same antigen.

The patent provides a comparison with the CDA1 mAb, whose efficacy has been demonstrated in a randomized controlled trial with combined anti-TcdA and TcdB antibodies in CDI. The blend of mAbs object of the patent binds an epitope bin which is different from CDA1 but has comparable biological activity in the animal model.

This product may be administered alone or in combination with other therapeutic agents, including a second monoclonal antibody (e.g., anti-TcdB), antibiotics (e.g., vancomycin or metronidazole) or probiotics (e.g., *Saccharomyces boulardii*), through intravenous, subcutaneous, intramuscular or transdermal routes.

4. Expert opinion

The use of toxin-targeted mAbs is one of the most promising new therapeutic approaches for the treatment of CDI and its recurrence. The inhibition of adhesion to enterocytes and the simultaneous blockage or different domain epitopes may represent a powerful mechanism to neutralize TcdA activity, arrest the course of CDI and prevent recurrences.

Administration of monoclonal and policlonal Abs against TcdA alone, not only failed to protect from CDI, but also appears to exacerbate the disease in piglets models [27]. The worldwide prevalence rates of TcdA-negative/TcdB-positive strains varies considerably (0.2 – 3%, with picks as high as 97.9%) [14], and more than one outbreak has been reported [28-30]. This evidence raises doubts on the efficacy of an exclusive anti-TcdA treatment.

Hamster mortality models suggested that combination of anti-TcdA and anti-TcdB mAbs can be more efficacious than the administration of a single mAb [23]. This combined approach has been recently tested by Lowy *et al.* who reported a 72% relative reduction in recurrence rates in patients receiving two fully human neutralizing mAbs against CD toxins, compared to placebo. However, this intervention did not reduce severity of infection, duration of diarrhea or length of hospital stay for the initial episode [31]. However, genetic model of CD deficient in TcdA or TcdB are not conclusive, with a study endorsing the role of TcdB as the only capable of causing disease in hamster [12], and another recognizing a significant role to TcdA [32]. Due to this conflicting evidence, based on studies with different animal models, bacterial strains and antibodies used, the desirable strategy in term of immunopharmacologic intervention for CDI still encompasses a combined anti-TcdA/TcdB approach.

A potential action of anti-TcdA mAbs against hypervirulent CD strains may be hypotizable considering the high level of identity of the cell wall binding domain among different CD strains. However, previous clinical trials assessing the effects of TcdA-targeted mAbs did not reported significant difference in prevalence of epidemic BI/NAP1/027 strain between study population and controls [24].

More recently, downstream toxin-triggered mechanisms have been explored as targets for new treatments, such as empowerment of toxins' nitrosylation (preventing toxin cleavage and release) [33,34] and dampening of the inflammasome-mediated tissue damage with other biologic agents (anti-IL1β) [8].

Subjects who experienced CDI recurrence and patients with underlying chronic conditions, in particular immune deficiencies, are the ideal targets for this treatment. In addition, considering the reported changes in epidemiology, children and otherwise healthy adults may potentially benefit of this approach as a second line treatment.

The potential advantages of the passive immunization with mAbs over other strategies, in adjunct to standard antibiotic treatment, are the immediate effect of prevention and the documented effect on primary CD-related diarrhea and CDI recurrences. High costs may limit applicability of this approach mainly in comparison to other promising emerging therapies such as fecal microbiota transplantation [35].

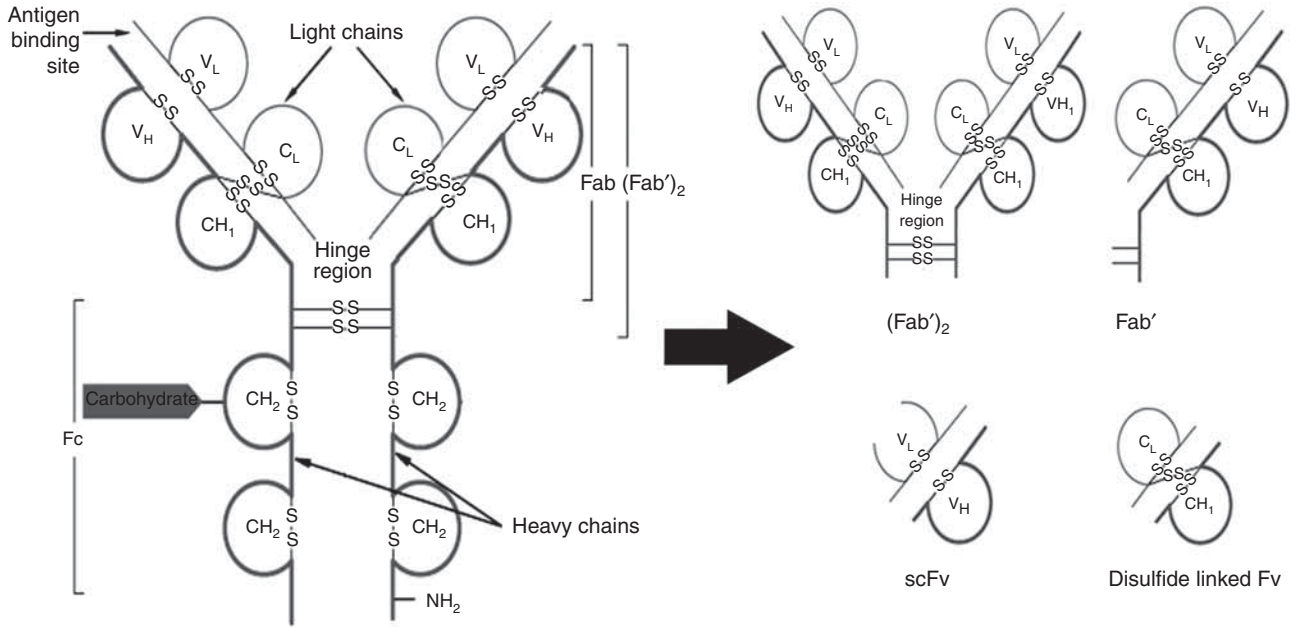
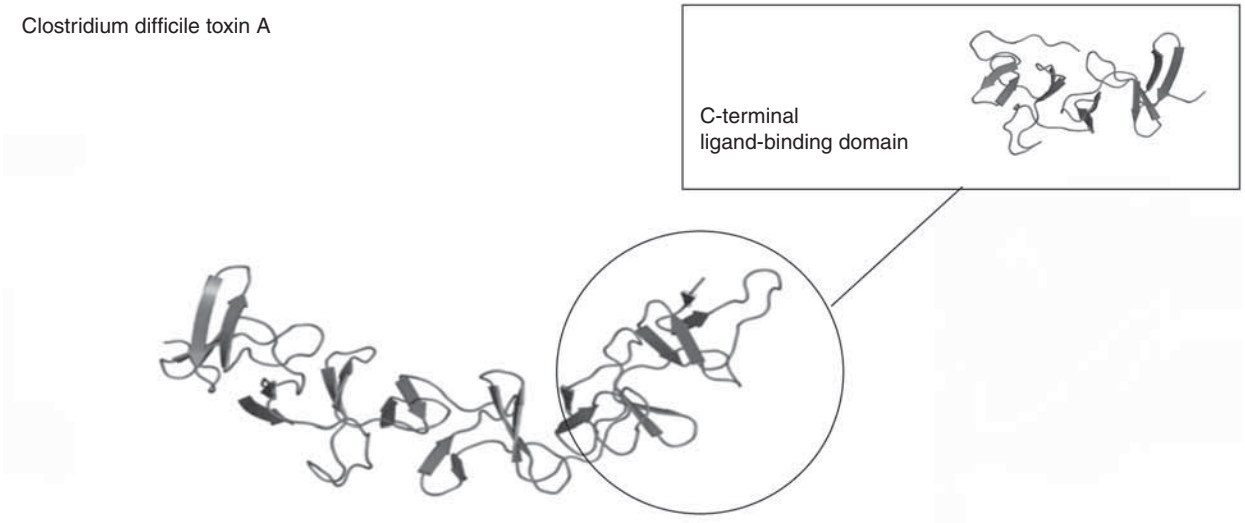


Figure 2. Different components of the blend object of the patent, beyond the whole immunoglobulins, are depicted. Fab′, Fab fragment; F(ab′)2, Fab fragment dimer; scFv, single-chain fragment variable; di-scFv, disulfide-linked fragment variable.



Toxin A sequence

MGSSHHHHHHHHASTGYTSINGKHFYFNTDGIMQIGVFKGPNGFEYFAPANTDANNIEGQAILYQNKFLTLNGKK
YYFGSDSKAVTGLRTIDGKKYYFNTNTAVAVTGWQTINGKKYYFNTNTSIASTGYTIISGKHFYFNTDGIMQIGV
FKGPDGFEYFAPANTDANNIEGQAIRYQNRFLYLHDNIYYFGNNSKAATGWVTIDGNRYYFEPNTAMGANGYKTI
DNKNFYFRNGLPQIGVFKGSNGFEYFAPANTDANNIEGQAIRYQNRFLHLLGKIYYFGNNSKAVTGWQTINGKVY
YFMPDTAMAAAGGLFEIDGVIYFFGVDGVKAP

Figure 3. *Clostridium difficile* Toxin A structure. The C-terminal domain is specifically targeted by the mAbs and antigen-binding portions thereof and naturally binds the enterocyte apical membrane.

Treatments currently available for CDI are inadequate to impede the increased spread and virulence of the infection, avoid recurrence or prevent infection in at-risk populations; future efforts should focus on optimization of immune response to CDI and its toxins.

Although available evidence are promising, before considering mAbs as a routine CDI treatment, several issues should be addressed, including the duration of mAbs biological activity, the impact on the host immune response (self-production of serum Abs) and long-term effect in case of recurrence.

Declaration of interest

The authors state no conflict of interest and have received no payment in preparation of this manuscript.

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