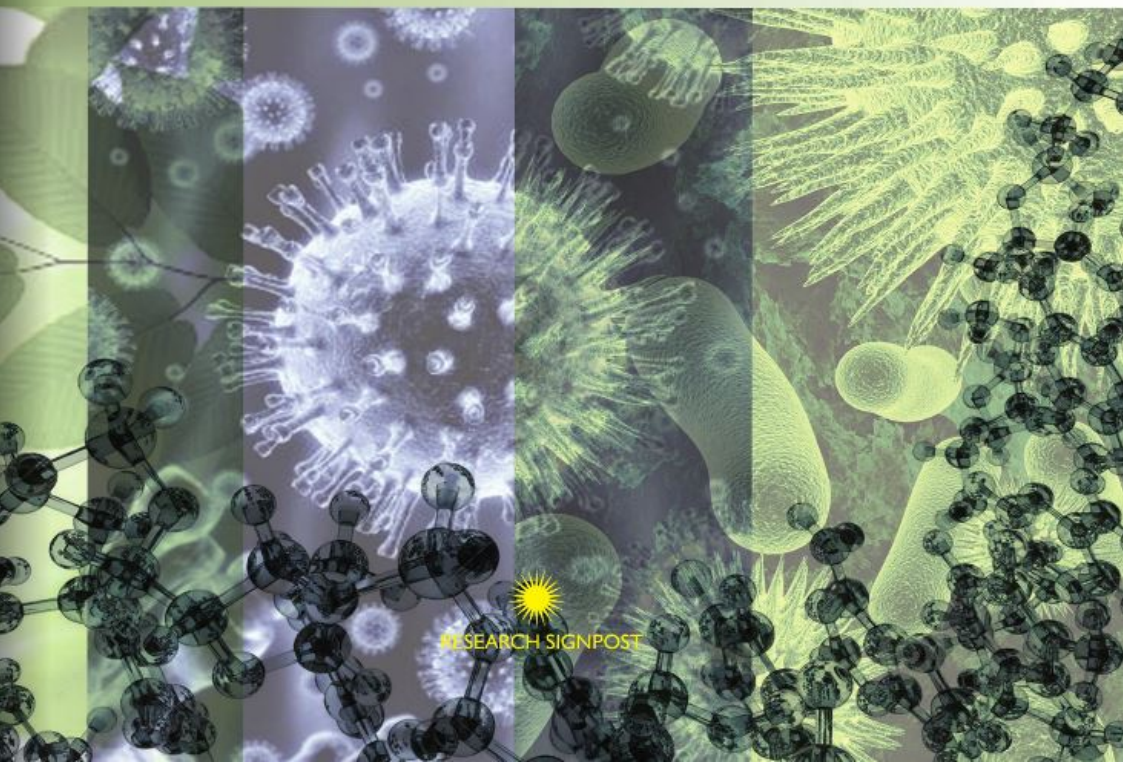
RECENT ADVANCES IN PHARMACEUTICAL SCIENCES

VOL. VI

Editors

Diego Muñoz-Torrero, Àngela Domínguez, Àngels Manresa



Recent Advances in Pharmaceutical Sciences VI

Editors

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Research Signpost, T.C. 37/661 (2), Fort P.O., Trivandrum-695 023
Kerala, India

Published by Research Signpost

Research Signpost
T.C. 37/661(2), Fort P.O.,
Trivandrum-695 023, Kerala, India

E-mail IDs: admin@rsflash.com
signpost99@gmail.com; rsignpost@gmail.com

Websites: http://www.ressign.com
http://www.trnres.com
http://www.signpostejournals.com
http://www.signpostebooks.com

ISBN: 978-81-308-0566-5

Preface

This E-book is the sixth volume of a series that compiles contributions from different areas of the multidisciplinary field of Pharmaceutical Sciences, particularly physical chemistry, food science, toxicology, botany, biochemistry and molecular biology, preventive medicine and public health, pharmacology, physiology, microbiology, and parasitology.

The emergence of drug resistance is a major cause for concern in the pharmacotherapy of infectious diseases and cancer. It is well known that pumping out the drugs from the cell through efflux pumps is one of the mechanisms that account for drug resistance. However, efflux pump mechanisms are not well understood. Chapter 1 sheds light into the role of bilayer lipids that are in the proximity of the efflux pump protein in its folding and function, by addressing the interaction of *Escherichia coli* lactose permease, therein used as a model efflux pump, with the main inner membrane lipid components, using FRET- and atomic force microscopy-based approaches. Polyphenols are a large and heterogeneous class of naturally occurring bioactive compounds, which are mainly present in fruits, vegetables, cereals and their products. Chapter 2 summarizes recent findings about the effects of some polyphenols and polyphenol-rich foods on cardiovascular health, a matter of utmost importance to establish dietary recommendations or even a therapeutic use of polyphenols. Administration of the antiparasitic veterinary drug triclabendazole during the lactating period to animals producing milk for human consumption can result in the appearance of embryotoxic metabolites, particularly triclabendazole sulfoxide, in milk and cheese entering the human food chain. Chapter 3 deals with the assessment of the risk for human embryonic development associated with the daily intake of triclabendazole sulfoxide through maternal milk and cheese consumption during gestation in a rural population of Cajamarca (Peru) where this drug is used in cattle against the endemic fascioliasis.

The arrangement of ribosomal DNA (rDNA) can be useful as a cytogenetic trait for species characterization. Indeed, this topic is attracting a growing interest within the scientific community. Chapter 4 describes the discovery of a new rDNA arrangement in plants of the genus *Artemisia* and its distribution and the diversity of rDNA

arrangements in the family Asteraceae, to which *Artemisia* belongs, and gymnosperms. Also, the resource www.plantrdnadatabase.com with information on type of rDNA arrangement, number and position of rDNA loci in plants is presented. The incidence of type 2 diabetes and one of its major risk factors, namely obesity, are alarmingly increasing worldwide. Insulin resistance is strongly associated with obesity and an early trait in the development of type 2 diabetes. Chapter 5 discusses the physiopathological role of the c-Jun N-terminal kinase (JNK) pathway on the regulation of insulin sensitivity and its modulation as a promising therapeutic approach against insulin resistance and type 2 diabetes. Chapter 6 describes a prospective matched case-control study in 3-59-month old children during the period 2007-2009 to identify potential risk factors of invasive pneumococcal disease, an infection that causes high morbidity and mortality worldwide, and to assess the effectiveness of 7-valent pneumococcal conjugate vaccine. Cathinones are a class of synthetic psychostimulants that can be consumed by adolescents and young adults as recreational drugs, very often in combination with alcoholic drinks. Chapter 7 provides a valuable insight into the effects of the combination of one of such cathinones, namely mephedrone, with ethanol in adolescent mice, paying particular attention to the effects on psychostimulant and conditioning effects, and on neurotoxicity markers. Chapter 8 summarizes some studies on the safety and bioavailability of orally administered maslinic acid, a bioactive pentacyclic triterpene that is present in plants of traditional medicine and in foods of plant origin, and that might be used as a nutraceutical on the basis of its multiple biological activities. The use of agro-industrial residues plays an important role in the sustainable and competitive development of several industrial sectors, as well as in the management of industrial residues. Chapter 9 describes the use of molasses as the basis of a new growth medium that has allowed the optimization of the production by *Bacillus licheniformis* AL 1.1 of lichenysin, a powerful anionic biosurfactant that can prevent and eliminate biofilm formation by pathogenic strains. Infections produced by *Helicobacter pylori* are a major cause of concern, due to their high prevalence and their pathological consequences, i.e. peptic ulceration, gastric adenocarcinoma, and gastric lymphoma. Chapter 10 provides an overview on the usefulness of housekeeping genes for *H. pylori* detection and for the elucidation of the mode of transmission and the relevance of multiple infections. Chapter 11

describes the different species of sand flies in the Balearic Islands (Spain), which can act as vectors of leishmaniosis, as well as the evolution with time of the global density of such sand flies and the prevalence of canine leishmaniosis.

We hope that this new volume turns out to be of interest for the pharmaceutical sciences community, and for researchers of closely related disciplines such as medical, biological, and chemical sciences..

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37/661 (2), Fort P.O.
Trivandrum-695 023
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Recent Advances in Pharmaceutical Sciences VI, 2016: 1-16 ISBN: 978-81-308-0566-5
Editors: Diego Muñoz-Torrero, Àngela Domínguez and Àngels Manresa

1. The lipid-protein interplay in efflux pumps

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Abstract. In this work lactose permease (LacY) of *Escherichia coli* has been taken as model efflux pump to investigate the interactions between the protein and the main lipid components (POPE and POPG) of the inner membrane. Two main approaches have been followed: (i) measuring the fluorescence energy transfer between a single tryptophan mutant of the protein (W151/C154G LacY) and pyrene labeled phospholipids (Pyr-PE and Pyr-PG); and (ii) pulling the protein from the supported lipid bilayers where it is embedded by using the tip of the atomic force microscope (AFM). On one hand, fluorescence measurements at different pHs indicate that LacY present selectivity for PE. On the other hand the observations of the reconstituted protein in lipid bilayers by AFM show a preference of LacY for the fluid phase (L_α) rather than for the gel phase (L_β). To get an estimation of the proportion of each lipid in each phase we have constructed a phase diagram for the system POPE:POPG. The diagram shows that at the temperature of the experiments (24 ºC) there is an almost equimolar proportion of each lipid. The results suggest the existence

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of a boundary region around LacY formed mainly by POPE laterally segregated from a bulk with a random distribution of POPE and POPG. Force spectroscopy allows to establish the force required and the mechanism to unspecifically unfold the protein.

Introduction

Of major interest in health is the emergence of resistance to antibiotic and anticancer agents. Resistance may originate from several mechanisms: drug inactivation, alteration of the specific target, inactivation of the drug or, active efflux of drug mediated by integral membrane proteins commonly known as multidrug transporters. There are two classes of multidrug transporters: the ABC-type dependent on ATP hydrolysis for pumping out the drugs from the cell; and secondary transporters that extrude drugs via mechanisms that are coupled energetically to the electrochemical potential across the membrane. The extraction of drugs through secondary transporters occurs by pumping the substrate out of the cytoplasm whilst H^+ are uptaken. Transmembrane proteins of this kind are known as antiporters, and most of them belong to the major facilitator superfamily (MFS). Some secondary multidrug transporters of this superfamily include NorA from *Staphyloccocus aureus*, TetA from *Escherichia coli* or LmrP from *Lactococcus lactis*, that confer specific resistance to norfloxacin, tetracycline, and daunomycin respectively [1]. Efflux pump mechanisms are not very well understood but experimental evidence suggests that they depend on the affinity of the drug for the lipid bilayer. Among the mechanistic models proposed for drug resistance the drug always reaches the protein from within the bilayer, suggesting an involvement of the phospholipids in the neighborhood of the multidrug transporters in a coupled mechanism that leads to the expulsion of the drug from the cytoplasm. In this work lactose permease (LacY) of *E. coli*, a paradigm for the secondary transporters belonging to the MFS for which we have convenient engineered mutants, will be used to investigate the interactions with the phospholipids of the bilayer where the protein is embedded. LacY consists of twelve transmembrane α-helices, crossing the membrane in a zig-zag fashion that are connected by eleven relatively hydrophilic, periplasmic and cytoplasmic loops, with both amino and carboxyl termini on the cytoplasmic surface (Fig. 1). By combining molecular biology methods with several biochemical and biophysical techniques LacY was one of the first transmembrane protein for which a tertiary structure was proposed. These studies served as the basis for an

early model of the transport mechanism which was confirmed after obtaining the three-dimensional and two-dimensional crystals. These crystals were obtained from a mutant of LacY, with Gly in place of Cys154 (Cys154 → Gly154; C154G), which is arrested in a stable conformation and yields crystals of sufficient quality for high resolution X-ray diffraction studies [2].

In this work we have investigated the interaction between LacY and the main phospholipids of the inner membrane of *E. coli*. We have delineated two different experimental approaches: (i) Förster resonance energy transfer (FRET) has been applied for the investigation of the boundary region of LacY [3]. The FRET strategy consists on measuring the efficiency of the transference of energy between a single-tryptophan mutant of LacY, used as a donor (D), and phospholipid pyrene derivatives as acceptors (A); and (ii) we have exploited the single molecule force spectroscopy (SMFS) mode of the atomic force microscopy (AFM) [4] to unspecifically pull out LacY from a binary mixture that mimics the inner membrane of *E. coli*.

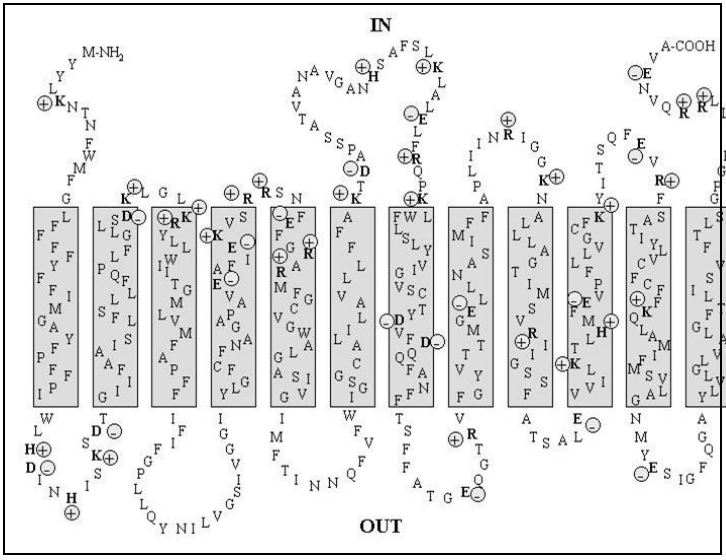

Figure 1. Secondary structure of lactose permease from *Escherichia coli* showing the charged residues (bold). The one-letter amino acid code is used. The putative transmembrane helices are shown in boxes that are connected by hydrophilic loops.

1. Materials and methods

N-Dodecyl-*β*-D-maltoside (DDM) was purchased from Anatrace (Maumee, OH, USA). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phospho ethanolamine (POPE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (sodium salt) (POPG), were purchased from Avanti Polar Lipids (Alabaster, AL, USA). 1-Hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphoglycerol ammonium salt (Pyr-PG) and 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycero-3-phosphoethanolamine ammonium salt (Pyr-PE) were purchased from Invitrogen (Barcelona, Spain). *β*-D-Galactopyranosyl 1-thio-*β*-D-galactopyranoside (TDG), isopropyl-1-thio-ß-D-galactopyranoside (IPTG), and dithiothreitol (DTT) were obtained from Sigma Chemical Co. (St. Louis, MO, USA), and Bio-Beads SM-2 were purchased from Bio-Rad (Hercules, CA, USA). All other common chemicals were ACS grade.

Bacterial strains and protein purification

E. coli BL21(DE3) cells (Novagen, Madison, WI, USA), transformed with plasmid pCS19 encoding single-W151/C154G LacY provided by Dr. H. Ronald Kaback (UCLA, USA), were grown in Luria-Bertani broth at 30 °C containing ampicillin (100 μg/mL) and induced at the appropriate moment with 0.5 mM IPTG. Cells were disrupted and the membrane fraction harvested by ultracentrifugation. Membranes were solubilized by adding DDM and purified by Co (II) affinity chromatography (Talon Superflow^{TM}, Palo Alto, CA, USA). Protein eluted with 150 mM imidazole was subjected to gel filtration chromatography using a Superdex 200 20/30 column (GE-Healthcare, UK), equilibrated with 20 mM Tris-HCl (pH 7.5), 0.008 % DDM. The protein was concentrated by using Vivaspin 20 concentrators (30 kDa cutoff; Vivascience, Germany) and stored on ice. Protein identification was performed by SDS/PGE electrophoresis, and protein quantitation was carried out using a micro-BCA kit (Pierce, Rockford, IL).

Vesicle preparation and protein reconstitution

Chloroform–methanol (2:1, vol/vol) solutions containing appropriate amounts of both labelled and unlabeled phospholipids were dried under a stream of oxygen-free N_2 in a conical tube. The total concentration of phospholipids was calculated as a function of the desired lipid-to-protein ratio (LPR) and protein concentration (1.5 μM). The amount of fluorescent probe was χ =0.015 for all the experiments. The resulting thin film was kept under high vacuum for approximately 3 h to remove organic solvent traces. Multilamellar liposomes (MLVs) were obtained following redispersion of

the film in 20 mM Hepes, 150 mM NaCl buffer, pH 7.40, and applying successive cycles of freezing and thawing below and above the phase transition of the phospholipids, and sonication for 2 min in a bath sonicator. Afterwards, large unilamellar liposomes (LUVs) supplemented with 0.2% of DDM were incubated overnight at room temperature. Liposomes were subsequently mixed with the solubilized protein and incubated at 4 ºC for 30 min with gentle agitation, to obtain a LPR (w/w) of 40. DDM was extracted by addition of polystyrene beads (Bio-Beads SM-2, Bio-Rad).

FRET methodology

Steady state fluorescence measurements were carried out with an SLM-Aminco 8100 (Urbana, IL, USA) spectrofluorometer. The cuvette holder was thermostated with a circulating bath (Haake, Germany), which was used to control temperature within 0.1 ºC. The fluorescence experiments were performed at 25 °C. The excitation and emission bandwidths were 4/4 and 8/8 nm, respectively. For energy transfer measurements single-W151/C154G LacY, the donor, was excited at 295 nm and emission of the pyrene-labeled phospholipids, the acceptor, was monitored at 338 nm. FRET efficiencies (*E*) are calculated according to the equation

$$E = 1 - \frac{I_{\mathrm{DA}}}{I_{\mathrm{D}}} = 1 - \frac{\int_0^\infty i_{DA}(t)dt}{\int_0^\infty i_D(t)dt} \qquad (1)$$

where I_{D} and I_{DA} are the tryptophan emission intensities in the absence or presence of pyrene acceptors, respectively. The reported values of experimental *E* are the averages of triplicate measurements from five separate reconstitutions. In the case of transmembrane proteins, we have to consider the existence of two different populations of phospholipids, those forming the first shell surrounding the protein, confined in the so-called boundary region, and those of the bulk [3]. Assuming these two populations of A molecules, the fluorescence decay of D molecules can be written as

$$i_{DA}(t) = i_D(t)\rho_a(t)\rho_r(t) \qquad (2)$$

where i_D and i_{DA} are the donor fluorescence decays in the absence and presence of acceptor molecules, respectively. Since the number of annular pyrene phospholipids around each protein molecule is expected to follow a binomial population, the annular contribution to the decay can be expressed as

$$\rho_a(t) = \sum_{n=0}^{m} e^{-nk_t t} \binom{m}{n} \mu^n (1-\mu)^{m-n} \tag{3}$$

where m is the number of phospholipid molecules in the first layer surrounding the protein taken as 46 for LacY, μ is defined as the probability of each site in the annular ring being occupied by a labeled pyrene phospholipid and k_t is the rate constant for D-A energy transfer,

$$k_t = \frac{1}{\tau}\left(\frac{R_0}{R}\right)^6 \tag{4}$$

where in turn τ is the donor lifetime and R_0 is the Forster radius (3.0 nm for the Trp/pyrene). On the other hand R, the distance between the D and annular A molecules, can be estimated according to

$$R = \left(w^2 + R_e^2\right)^{1/2} \tag{5}$$

where w is the transverse distance between D and the bilayer centre, and R_e is the exclusion distance along the bilayer plane between the protein axis and the annular lipid molecules. For this system, the value R = 3.2 nm was considered. The geometrical assumptions for FRET measurements are detailed in Fig. 2.

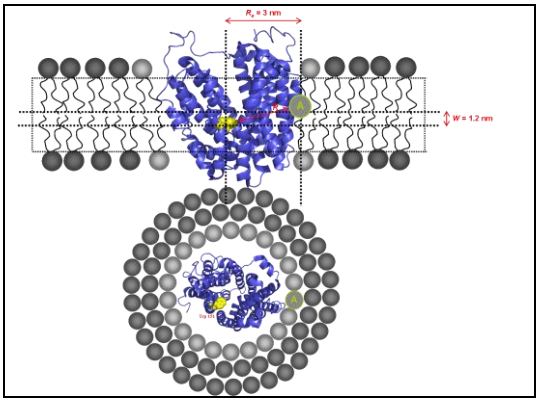

Figure 2. Geometrical assumptions used in FRET modeling. (Up) frontal view showing the location of the donor (Trp151) in yellow and the acceptor (Pyr-PE or Pyr-PG); (Down) sagittal view showing the boundary lipids in light grey where the A are located.

The probability μ can be written as

$$\mu = K_s \frac{n_{pyr}}{n_{pyr} + n_{PL}} = K_s \chi_{pyr} \tag{6}$$

where *n*´s are the mole numbers of the labeled (n_{pyr}) and non-labeled (n_{PL}) phospholipids, χ_{pyr} is the label mole fraction, and K_s is the relative association constant between the labeled and unlabeled phospholipids. Thus, K_s = 1 denotes equal probability of finding acceptors in the annular region and in the bulk, whereas K_s = 0 means no acceptor is in the annular region. Alternatively

$$\mu = \frac{n_{pyr}^{ann}}{n_{pyr}^{ann} + n_{PL}^{ann}} = \chi_{pyr}^{ann} \tag{7}$$

by inserting Eq. 7 into Eq. 6, we obtain a more intuitive meaning of K_s,

$$\chi_{pyr}^{ann} = K_s \chi_{pyr} \tag{8}$$

that is, K_s is the ratio between the acceptor mole fractions in the annular region and in the overall system.

The FRET contribution of acceptors randomly distributed outside the annular region, bulk, can be expressed as

$$\rho_r(t) = \exp\left\{ -4n_2\pi l^2 \int_0^{\frac{1}{\sqrt{l^2+R_e^2}}} \frac{1-\exp\left(-tb^3\alpha^6\right)}{\alpha^3} d\alpha \right\} \tag{9}$$

where n_2 is the acceptor density in each leaflet, l is the distance between the plane of the donors and the plane of acceptors and b being $b = (R_0/l)^2\tau^{-1/3}$.

Supported lipid bilayers and atomic force microscopy

Liposomes or proteoliposomes in Hepes buffer supplemented with 10 mM $CaCl_2$ were deposited onto freshly cleaved mica disks. Samples were incubated at 37 ºC for 2 h in an oven, using a water reservoir to prevent evaporation of the water from the sample. Before imaging, samples were washed with non-calcium-supplemented buffer.

To perform the experiments, it was necessary to drift equilibrate and thermally stabilize the cantilever in the presence of buffer. Images were acquired at 22 ± 0.5 ºC.

Liquid AFM imaging was performed using a Multimode Microscope controlled by Nanoscope V electronics (Bruker, AXS Corporation, Madison, WI). Sample images were acquired in contact mode at scan frequencies of 3 Hz using an optimized feedback parameter and applying minimum vertical force. MSNL-10 V-shaped Si_3N_4 cantilevers (Bruker AFM Probes, Camarillo, CA) with a nominal spring constant of 0.03 $N \cdot m^{-1}$ were used. All images were processed using NanoScope Analysis Software (Bruker AXS Corporation, Santa Barbara, CA).

Force Spectroscopy and protein unfolding

AFM in FS mode was used to obtain nanomechanical magnitudes and to perform protein unfolding [5]. Individual spring constants of the different cantilevers used were determined using the equipartition theorem. Force–distance curves were measured using a constant velocity of 600 $nm \cdot s^{-1}$, with ~1 s of interaction between the AFM tip and the sample surface when protein unfolding was acquired.

The worm-like chain (WLC) model [6,7] was used to fit unfolding events found in the force-distance curves, following the expression

$$F(x) = \frac{k_B T}{4b} \cdot \left((1 - \frac{x}{L})^{-2} - 1 + \frac{4x}{L} \right) \quad (10)$$

where $F(x)$ is the force at a distance x, k_B is the Boltzmann constant, b is the persistence length (0.4 nm) [8], L is the contour length of the unfolded polypeptide chain and T is the temperature. Force peak events of partially unfolded protein were observed in nearly 10% of all force curves, but only experiments with full extension of the protein, that is force curves with force peak patterns with at least 125 nm of extension where used. From these force curves selection only force extensions well-described by the WLC model were accepted for the analysis. All detected unfolding peaks were validated using the root mean square error (RMSE), which is a measure of the difference between the values predicted by the WLC model and the values actually observed. Values with RMSE < 0.015 nN were accepted.

Construction of the phase diagram

A phase diagram for POPE:POPG can be constructed from the excess heat capacity *vs* temperature curves obtained by DSC. Technical details of the experimental procedures have been earlier described [9] .These temperatures are then corrected by the finite width of the transitions of the pure components weighted with their mole fractions. Afterwards, in order to construct a phase diagram of a binary mixture the onset (T_{onset}) and completion (T_{offset}) temperatures observed in the endotherms are plotted against a series of compositions. By connecting the different T_{onset} and T_{offset}, respectively, the solidus and liquidus curves can be defined. A MicroCal MC-2 calorimeter was used for DSC analyses. The data were analysed by the original calorimeter software. The calorimetry accuracy for T_m and for enthalpy changes was ± 0.1 ºC and ± 0.2 kcal mol^{-1}, respectively. Each sample was scanned in triplicate over the temperature range 5-80 °C at a scan rate of 0.44 ºC min^{-1}.

2. Results and discussion

To obtain values of theoretical FRET efficiencies (eq. 1) we have first to solve eq. 3 and 9 and, afterwards inserting the values in eq. 2. As can be seen by inspecting the equations the outcome are μ and K_s. Obviously the accepted outcomes will be those that coincide with the experimental *E*. Details on the development of the model can be found in the literature [10,11] as well as in a review on the applicability to LacY and different levels of lipid-pyrene labeling [3].

Table 1. Experimental efficiencies, probabilities of each site in the boundary region being occupied by a pyrene labelled phospholipid and relative association constant toward LacY.

Labelled lipid (1.5%)	**POPE (98.5%)**			**POPG (98.5%)**		
	E	μ	K_s	*E*	μ	K_s
Pyr-PE	0.79	0.10	6.53	0.66	0.03	2.00
Pyr-PG	0.53	0.00	0.00	0.51	0.00	0.00

In Table 1, the experimental FRET values are listed along with the calculated μ and K_s values. As can be seen μ and K_s could be only calculated for Pyr-PE and never for Pyr-PG. It is worth mentioning that ideally $K_s = 1$ for any probe that mimics the non-labeled phospholipids. Therefore, these $K_s > 1$ may indicate either a boundary region extremely enriched in the label or

that Pyr-PE does not mimic well the unlabeled phospholipid. Since K_s = 0 means no acceptor in the annular region, it becomes clear that Pyr-PG is completely excluded from the boundary. On the other hand, notice that μ and K_s for Pyr-PE in the POPG matrix are compatible with a moderate enrichment of the label in the annular region. All these observations may be likely related with the inverted topology of domains C6 and P7 of LacY (12) when reconstituted in POPG proteoliposomes. Nevertheless, our FRET measurements in POPE and POPG matrices confirm the preference of LacY for PE and its probable predominance in the boundary region.

The requirement of PE for the correct folding and function of LacY has been largely documented [13]. Even a possible specific interaction between PE and some specific amino acid residues of the protein has been postulated [14]. Such interaction could be likely attributed to the ability of PEs to form extensive hydrogen bonds. Hence, variations in the efficiency of FRET should be expected when the pH changes. This phenomena can be observed in Fig. 3, where *E* values at acid, neutral and basic pHs are plotted for POPE:POPG (mol/mol) proteoliposomes doped with Pyr-PE or Pyr-PG (0.1 %).

Actually, whilst POPE is a zwitterion, POPG bears a negative charge at neutral pH. Therefore the decrease of *E* at pH 11 and its increase at pH 3 reported by PyrPE strongly supports the participation of the negative charge of the phosphate group in the interaction with LacY. In agreement, in basic conditions PG would be able to interact and therefore a dramatic increase of

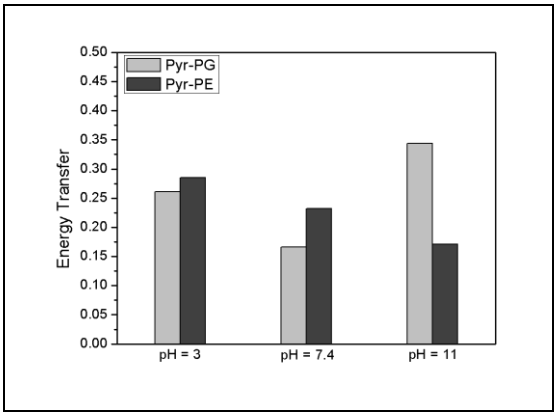

Figure 3. FRET energy transfer at three pH values from single-W151/C154G LacY reconstituted in proteoliposomes of POPE:POPG (3:1, mol/mol) doped with 0.1% of Pyr-PG or Pyr PE.

E values in these conditions is observed. At pH 3, when all groups are protonated, both labels reported a similar FRET efficiency. Interestingly, it has been also reported that PC can support the uphill transport of lactose [15], which emphasizes the fact that the zwitterion character, and the negative charge of the phosphate group are required for LacY proper function.

One interesting property of the POPE:POPG system is that at least in the presence of Ca^{2+} it presents lateral segregation into domains [9]. The presence of divalent cations is required for obtaining the adequate adsorption of the lipids onto the substrate (mica) commonly used to form SLBs. One example of these SLBs is shown in Fig. 4 where the phase separation is clearly observed by the different coloration between the two domains. Noteworthy, in these experiments Ca^{2+} concentration was minimised by swabbing it away after SLB formation. However, the presence of divalent cations between the apical monolayer and the substrate is not excluded.

From the nanomechanical study of these SLBs two magnitudes can be obtained: (i) the yield threshold force (F_y), i.e. the force that the bilayer can withstand before being indented, and (ii) the adhesion force (F_{adh}), i.e. the pull-off force between the tip and the bilayer [16]. Actually, these magnitudes,

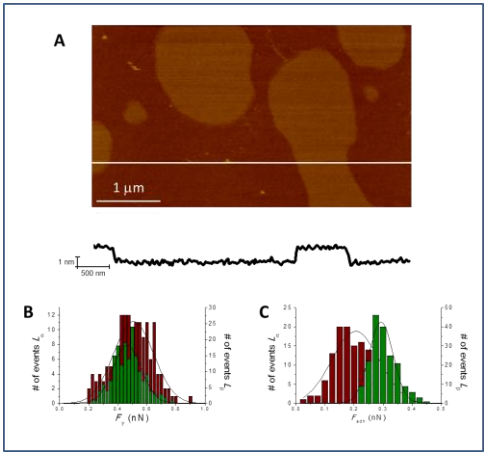

Figure 4. AFM topographic image and height profile analysis of POPE:POPG (3:1, mol/mol) SLB (*Z* scale = 10 nm) (A). Histograms present the distribution of forces of L_α phase (red) and L_β phase (green) for F_y (B) and F_{adh} (C). Fittings to a Gaussian distribution are represented in solid lines. Reprinted from Suárez-Germà *et al.* [5] with permission from Elsevier Science.

particularly F_y, are considered as fingerprints for lipids and phases characterization [16-18]. Distribution of the F_y and F_{adh} values obtained for POPE:POPG are shown in Fig. 4B and 4C, respectively. F_y values ranged from 0.509 ± 0.008 nN to 0.464 ± 0.006 nN and they are characteristic of the gel (L_β) and fluid phase (L_α) of the bilayer, respectively. For F_{adh} we found similar values for both lipid phases, although L_β showed a slightly higher F_{adh} value than L_α (0.292 ± 0.002 nN and 0.205 ± 0.004 nN, respectively).

In order to know the composition of each phase observed, we build a phase diagram of the POPE:POPG system (Fig. 5). A substantial deviation from the ideal mixing behaviour at all the molar fractions is observed. By applying the lever phase rule we can conclude that at 24 ºC, temperature of the AFM observations, we have almost 50% of each phase, which coincides approximately with the distribution of the areas observed in Fig. 4.

Transmembrane proteins tend to insert preferentially into fluid phases. Such is the case of LacY, from which we have already observed this tendency [19]. Thus, when LacY is reconstituted at a LPR (w/w) of 0.5, proteolipid sheets as those shown in Fig. 6A are observed. Similarly, two domains can be distinguished. However, whilst in Fig. 4A, the surfaces were smooth, in Fig. 6A the differences in roughness between both domains is ~ 0.07. The most corrugated domain (higher) contains small entities densely packed that are due to LacY self-segregation into the fluid domains [20].

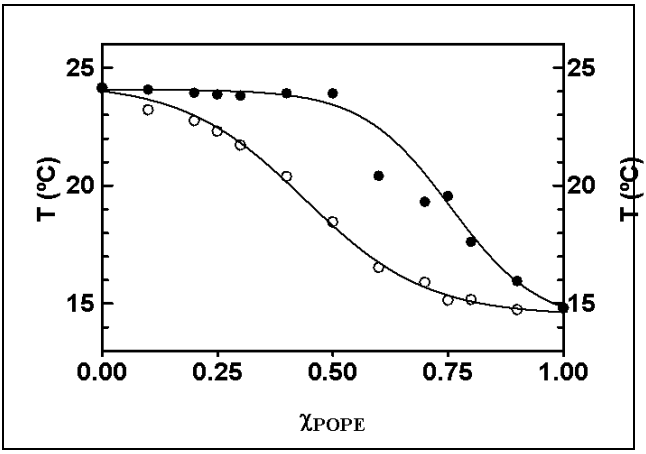

Figure 5. DSC phase diagram for POPE:POPG mixtures obtained from the heat capacity curves (data not shown). Empty circles correspond to the experimental T_{onset} and filled circles to the T_{offset} of the main phase transition.

Interestingly, F_y values for domains with and without LacY were 0.124 ± 0.004 nN and 0.37 ± 0.01 nN, respectively. This indicates a strong perturbation of the system, in both phases, due to the presence of LacY.

The unfolding of LacY from the lipid bilayer where it is embedded by means of force spectroscopy is enhanced in samples where the protein is densely packed as in the high domain in Fig. 6A. The retraction curves of LacY (Fig. 7A), displayed characteristic saw-tooth like pattern of force peaks similar to other secondary transporters characterised by 12 transmembrane α-helix [21,22]. These peaks are characterized by a nonlinear increase in the force as a function of the distance followed by an abrupt drop on force to an ideally zero force value. From the figure we observe some periodicity between the peaks and there are not distinguishable overlapped saw-tooth patterns suggesting that only one protein is pulled out in each event [23]. Indeed, fully extended LacY should present an unfolding curve of about ~170 nm (427 amino acids considering the His-Tag and 0.4 nm per amino acid residue [8]). However the actual representative retracting force-curve in Fig. 7A is shorter. This fact indicates that complete unfolding for a non-specific pulling did not occur most likely due to the strong lipid-protein interactions. For each force-distance curve, several saw-tooth peaks were obtained but only curves displaying at least 6 individual peaks were used. In Fig. 7B, twelve individual superimposed force-distance curves accomplishing

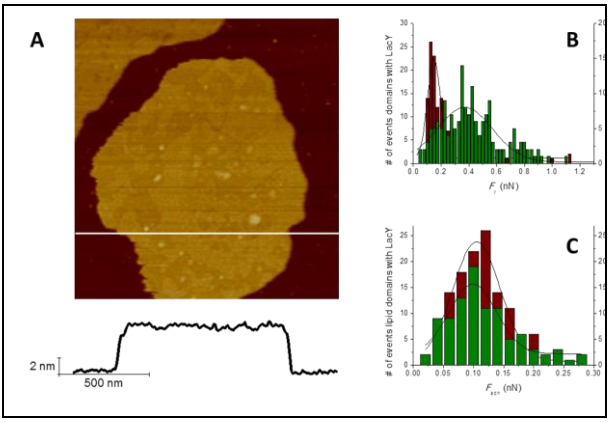

Figure 6. AFM topographic image and height profile analysis of POPE:POPG (3:1, mol/mol) SLB (Z scale = 10 nm) (A). Histograms present the distribution of forces of Lα phase (red) and Lβ phase (green) for F_y (B) and F_{adh} (C). Fittings to a Gaussian distribution are represented in solid lines. Reprinted from Suárez-Germà *et al.* [5] with permission from Elsevier Science.

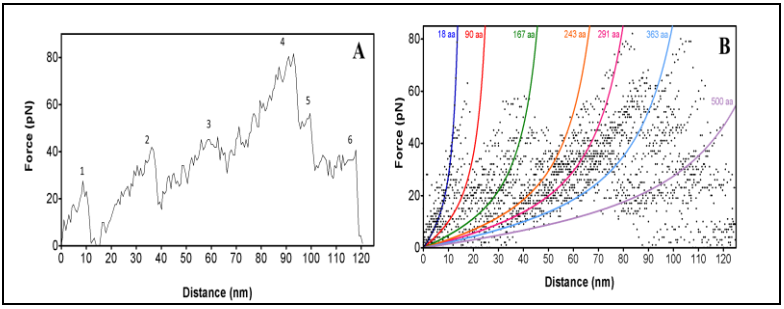

Figure 7. Representative force-distance curve of fully unfolded LacY (A). Superimposed force-distance curves of fully unfolded protein (B). Coloured lines are the WLC curves fitting the mean contour length of individual force peaks while numbering represents the number of amino acids pulled in each event.

the condition demanded are shown. Although there are undefined regions and more data are necessary, the plot becomes significant. There are regions more densely crowded and others with no data at all. To bring some light to the plot we coloured the lines of the WLC curves fitting the mean contour length of individual force peaks. The corresponding number of amino acids pulled out in each event is also shown. We observe that the last curve (purple) represents the extension of 500 amino acids which overcomes the 427 of LacY. This event may result from the complete unfolding of the protein that drags along those phospholipids most likely from the boundary region of LacY.

3. Conclusion

In this work we have observed, by using a bilayer with two phospholipid components that the selectivity of LacY for PE is much higher than for PG. Although PE and PG are both present in almost equimolar proportions in the fluid phase, we do confirm that Pyr-PE is able to get closer to or, alternatively, spend more time next to the LacY. This selectivity is confirmed by FRET measures at different pHs, which suggest the existence of hydrogen bonds between LacY and the phosphatidylethanolamine group. This provides means also for the unfolding patterns obtained when LacY is pulled from the bilayer with the AFM tip. All results indirectly support the hypothesis that bilayer lipids in the neighbourhood of the protein are involved in efflux pump correct folding and function.

Acknowledgements

Thanks to Luis M. Loura and Manuel Prieto for giving us the opportunity to test the FRET model with lactose permease. This work has been supported by the Grant 2014SGR1442 from the Generalitat de Catalunya.

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Research Signpost
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Recent Advances in Pharmaceutical Sciences VI, 2016: 17-36 ISBN: 978-81-308-0566-5
Editors: Diego Muñoz-Torrero, Àngela Domínguez and Àngels Manresa

2. Benefits of polyphenol intake on the cardiovascular risk parameters

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Abstract. Polyphenols are a large and heterogeneous group of compounds widely distributed in fruits, vegetables, cereals and their products such as coffee or wine. These bioactive compounds can ameliorate our health by improving certain risk factors, especially the cardiovascular ones. Thus, many investigations have focused on the effects of some polyphenols and polyphenol-rich foods on cardiovascular and other chronic diseases.

Introduction

Cardiovascular diseases (CVD), including coronary heart disease, cerebrovascular disease, rheumatic heart disease and other disorders of the heart and blood vessels, are the leading cause of mortality and disability in developed countries. According to the World Health Organization (WHO), 31% of all deaths in the world are attributed to CVD, and more than 75% occur in low and middle-income countries [1]. Some risk factors for CVD

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are intrinsic and cannot be modified: sex, age, race and genetic predisposition. However, many of them are related to lifestyle and dietary habits and they are also related to each other: stress, obesity, type-2 Diabetes Mellitus (DM) or insulin resistance, dyslipidemia, and hypertension (Fig.1). In addition to these classic risk factors, there are other non-classic factors including homocysteine, fibrinogen, lipoprotein(a), low-density lipoprotein (LDL) particle size and high-sensitivity C-reactive protein (CRP) [1]. Preventive measures such as smoking cessation, following a healthy diet, being physically active, and regular screenings may help to lower cardiovascular risk [2]. In fact, prevention, even more than treatment, is a priority for the public health agencies, which make huge efforts to raise awareness in the population with the aim of reversing this trend [3].

The Mediterranean Diet (MedDiet), as well as other diets rich in fruits and vegetables such as the Dietary Approaches to Stop Hypertension (DASH) dietary pattern, the US Department of Agriculture (USDA) food pattern, or the American Heart Association (AHA) diet, are an effective way to reduce cardiovascular risk factors due to their high content in vitamins, minerals, fiber, and mono and polyunsaturated fatty acids, and other bioactive compounds [4]. The latter, also known as phytochemicals or phytonutrients, have the capacity to alter biochemical reactions and consequently affect human health [5]. Polyphenols are one of these bioactive compounds. They are a large and heterogeneous group of molecules mainly found in fruits, vegetables, cereals and their products. Up to now, hundreds of different polyphenolic structures have been described in many foods and beverages. Polyphenols have received great attention among nutritionists, consumers and researchers since it has been proved that they have beneficial effects on our health. However, due to the great variety of existing structures, different polyphenol subgroups may differ significantly in stability, bioavailability and physiological functions related to human health. Thus, it is difficult to study their health effects, their bioavailability or their mechanisms of action. For this reason, many studies have focused only in one polyphenol group or even in one polyphenol. Globally, polyphenols seems to improve certain CVDs risk factors such as insulin resistance, inflammation biomarkers, or blood pressure (BP), among others [6]. Additionally, polyphenols have been associated to a decreased risk of certain cancers, neurodegenerative diseases, diabetes and osteoporosis [7].

In this chapter, we summarize recent findings about the effects of polyphenol and polyphenol-rich foods intake on cardiovascular risk factors, especially BP and dyslipidemia.

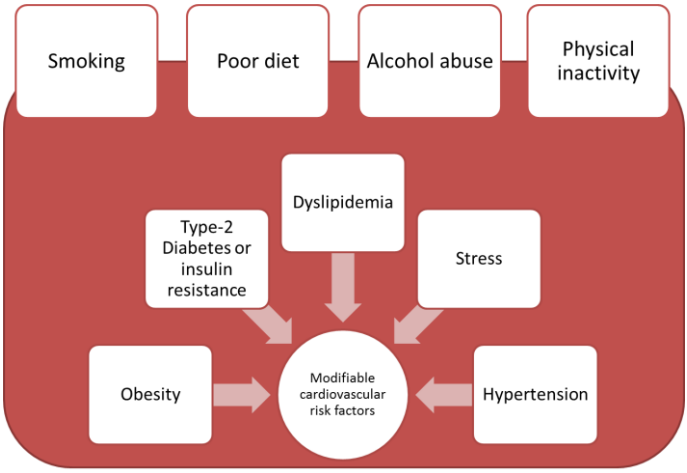


Figure 1. Modifiable cardiovascular risk factors.

1. Polyphenols: General structure and classification

Polyphenols are one of the most numerous and widely distributed groups of natural products in the plant kingdom. Since polyphenols are secondary metabolites of plants, they are naturally found in fruits, vegetables, cereals and beverages, mainly coffee, tea, wine, fruit juices and beer [8]. This group of compounds constitutes the main source of antioxidants in our diet. Total dietary polyphenol intake is estimated to be around 1 g/day, 10 times higher than that of vitamin C and 100 times higher than intakes of vitamin E and carotenoids [9].

More than 8000 phenolic structures are currently known. The structure of polyphenols consists of at least one aromatic ring carrying one or more hydroxyl moiety. The diversity and wide distribution of polyphenols in plants have led to different classifications, for instance, by their source of origin, biological function, or chemical structure [8]. However, the most common way to categorize them by the chemical structure of the aglycones (according to the number of phenol rings they bear and the structural elements that bind these rings). This broadly accepted classification divides polyphenols into two main groups: flavonoids and nonflavonoids [6].

Flavonoids have a C6-C3-C6 structure, consisting on a skeleton of two benzene rings (A and B) connected by a three-carbon chain forming a closed

pyran ring with the benzene A ring. Flavonoids are divided in subgroups according to the oxidation of the central ring and the type of substituents in the heterocyclic ring. These subgroups are flavones, flavonols, flavan-3-ols (and their polymeric forms, proanthocyanidins), flavanones, anthocyanidins, and isoflavones (Fig. 2).

The nonflavonoid group is classified according to the number of carbons they possess, and include phenolic acids, stilbenes, lignans and other polyphenols that are only found in smaller amounts such as simple phenols, hydrolysable tannins, acetophenones, phenilacetic acids, cinnamic acids or seicoiridoids. Phenolic acids comprise hydroxycinnamic acids, hydroxybenzoic acids, hydroxyphenylacetic acids, and hydroxyphenylpropanoic acids [10].

Besides presenting a huge variety of structures, polyphenols are usually attached to various carbohydrates and organic acids, as well as to other polyphenols. In plants, they are usually glycosylated, mainly with glucose or rhamnose and, less frequently, to galactose, arabinose, xylose, glucuronic acid or other sugars (Fig. 3). The number of glycosyl moieties usually varies from one to three, but can be even higher in some cases [11].

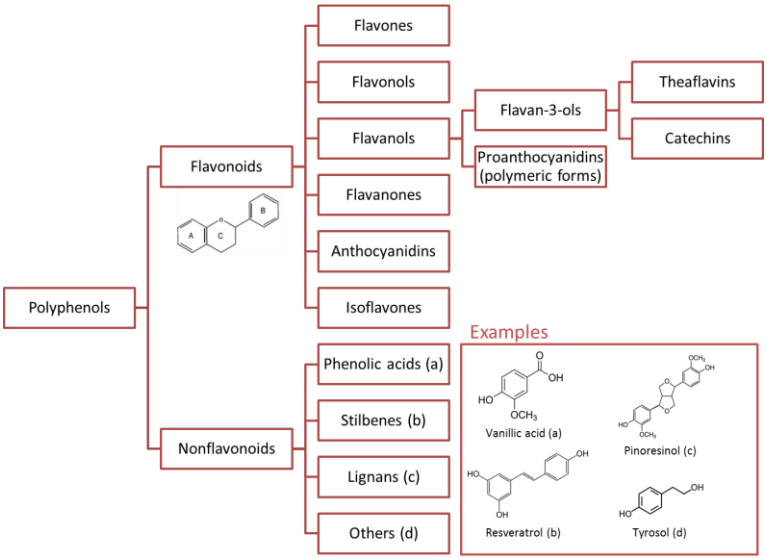
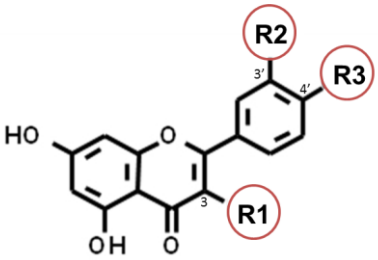


Figure 2. Classification of polyphenols.



	R1	R2	R3
Quercetin	OH	OH	OH
Quercetin-3-glucuronide	O-glucuronid acid	OH	OH
Quercetin-3'-sulfate	OH	OSO_3H	OH
Quercetin-3-rutinoside	Rutinoside	OH	OH
Quercetin-3-glucoside	Glucose	OH	OH
Quercetin-4'-glucoside	OH	OH	Glucose
Isorhamnetin	OH	OCH_3	OH
Isorhamnetin-3-glucuronide	O-glucuronid acid	OCH_3	OH

Figure 3. Example of quercetin metabolites from plants and biological fluids.

2. Distribution of polyphenols in food

As stated above, fruits, beverages and vegetables constitute the main sources of polyphenols. Fruits like apples, grapes, and berries contain up to 200–300 mg polyphenols per 100 g fresh weight, whereas a glass of red wine or a cup of tea or coffee contains about 100 mg polyphenols [12]. Herbs and spices often contain very high level of phenolic compounds but their consumption is limited to low amounts, therefore their contribution to total dietary intake is low [13].

Plant polyphenol composition is highly variable. Some of the compounds, such as quercetin, are ubiquitous, whereas others are more specific from particular foods (e.g., epigallocatechin gallate in green tea, isoflavones in legumes, flavanones in citrus fruit, or phloridzin in apples) [14].

Numerous factors affect the polyphenol content of plants. For example, the phenolic content of the skin of a given fruit is higher than that of the pulp since plants synthesize polyphenols as a mechanism of defense against

external agents. For this reason, fruit and vegetables from organic agriculture have also higher content of polyphenols than those from traditional agriculture [15]. Maturity, genetic factors, environment (sun exposure, rainfall), storage or manipulation will also influence the concentration of polyphenols. The degree of ripening has different effects depending on the polyphenol class: in general, phenolic acid concentrations decrease during ripening, whereas anthocyanin concentrations increase. Finally, the cooking method also have great influence: the phenolic concentration of foods decreases while their bioavailability is higher compared to raw foods [16,17].

Flavonols, one of the flavonoid subgroups, are the most abundant polyphenols in food. They are present in onions, spinach, berries, tea, dark chocolate, vegetables, nuts, and in most of the spices. The richest sources of flavanones are citrus fruits like grapefruit, orange, and lemon. Flavones are notably present in celery, red chicory, artichoke, black olives, and citrus fruits, whereas isoflavones are found in soy foods. Anthocyanins are typical of red dark-colored fruits and their products: berries, cherry, black olives, or red wine. Finally, dark chocolate, berries, nuts, tea and red wine are important sources of flavanols and their polymers, procyanidins [13].

Although flavonoids have traditionally been the most broadly studied group, nonflavonoids also contribute significantly to our polyphenol dietary intake. Phenolic acids, including hydroxycinnamic acids and hydroxybenzoic acids, are widely distributed in foods. The latter is present in higher amounts in chestnut, raspberry, pomegranate juice, and blackberry. Hydroxycinnamic acids are mostly found in coffee, artichoke, prune, chicory, blueberry, black olives, plum, and sweet cherry. Stilbenes and lignans are characteristic of red wine and seeds, respectively [13]. The most known and studied stilbene is *trans*-resveratrol, which is reported to have multiple health effects [18–20]. Finally, olive oil contains several simple phenols such as hydroxytyrosol and tyrosol [21].

Analysis of polyphenols in food is a highly complex process that requires multiple factors to be considered: food matrix, interferences, the variable solubility of the compounds, temperature, extraction time, and concentration levels that varies from traces to milligrams. Low temperatures, organic solvents, lyophilization and working under UV-free light conditions are extensively used methods to prevent the oxidation of polyphenolic compounds [22].

When the main objective is a detailed phenolic profile, mass spectrometry coupled to liquid chromatography is the most common

technique for identification and quantification, although in some cases gas chromatography and capillary electrophoresis can also be used. To quantify total polyphenols or those of a given group, spectrophotometric methods are useful and simpler. The Folin-Ciocalteu (F-C) method has been extensively used to determine total polyphenol content, whereas other more specific reactives are used to determine proanthocyanidins, hydrolyzable tannins, anthocyanidins, and flavan-3-ols [10,22].

3. Bioavailability and metabolism of polyphenols

Bioavailability is the proportion of the nutrient that is digested, absorbed and metabolized so that is available at the site of action [23]. Therefore, it is important to know, not only how much polyphenols we intake, but in which proportion they are available and reach target tissues.

The chemical structure of polyphenols determines their absorption and the structure of the metabolites circulating in the plasma. Given the huge variety of existing phenolic structures, the biological properties of polyphenols greatly differs from one polyphenol to another. Thus, the most abundant polyphenols in our diet are not necessarily those leading to the highest concentrations of active metabolites in our tissues [24]. Absorption of polyphenols also depends on fat intake, food matrix, dose, intestinal transit, and other factors [23]. Recently, emerging findings suggested that microbiota plays a crucial role in the metabolism of polyphenols and, at the same time, polyphenols contribute to the maintenance of gut health by the modulation of the gut microbial balance enhancing the growth of beneficial bacteria while competitively excluding pathogenic bacteria, exerting prebiotic-like effects. Both the formation of bioactive polyphenol-derived metabolites by the microbiota and the modulation of colonic microbiota by polyphenols contribute to host health benefits [25–28]. For instance, equol, enterolactone and enterodiol are produced by the colonic microflora after consumption of soy (or other isoflavones-rich food) and equol have greater phytoestrogenic properties than those of the original isoflavone [29].

Generally, the metabolism of polyphenols takes place as follows (Fig. 5): after the ingestion, some polyphenols, aglycones and anthocyanins, are directly absorbed in the stomach and small intestine. Esters, glycosides and polymers need to be hydrolyzed by intestinal

enzymes or the colonic microflora. Once absorbed, polyphenols undergo some degree of phase I and II metabolism in the enterocytes as methylation, sulfation and/or glucuronidation depending on the nature of the substrate and the dose. Polyphenol metabolites enter to the bloodstream by the portal vein to the liver, where they may be subjected to more conjugations. Then, metabolites travel through the bloodstream again attached to carriers such as albumin until they are excreted. There are two ways of excretion that depends on the molecular weight. The heavier compounds are usually eliminated as bile components. Back in the intestine (enterohepatic circulation), some of them are deconjugated and regenerated by gut microbial enzymes and are reabsorbed. The unabsorbed ones are eliminated via feces. The lighter polyphenols are excreted through the urine via the kidney [24,28].

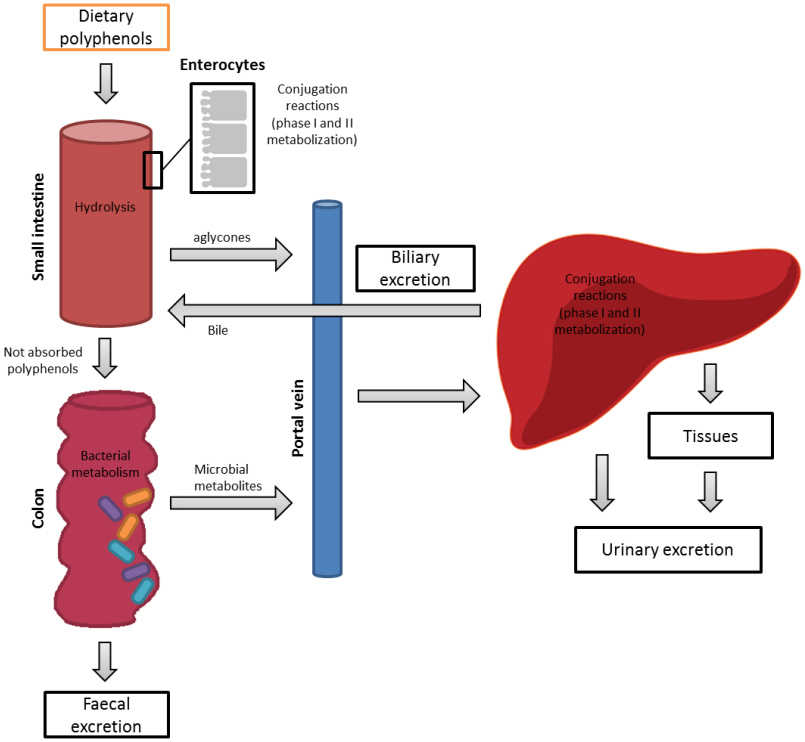


Figure 4. Absorption and metabolism of dietary polyphenols in humans.

4. Polyphenols and health

Numerous epidemiological and human intervention studies have shown that polyphenol intake may protect against cardiovascular and neurodegenerative diseases, some cancers, insulin resistance, and obesity, among others [6,7,30]. However, most of the evidence on the beneficial effects of dietary polyphenols is derived from in vitro and/or animal studies and using concentrations much higher than those achievable through the diet. Thus, translation of these results to humans needs to be tested. Moreover, the generally used compounds for *in vitro* studies are polyphenol aglycones or their sugar conjugates instead of the physiological metabolites [31].

Normal physiological processes such as respiration and metabolic reactions that take place in our body, as well as other exogenous factors, produce reactive oxygen species (ROS). As defense mechanisms, our body has endogenous antioxidants to eliminate the ROS since these molecules can bind to DNA, lipids and proteins, altering their stability and leading to various diseases, such as diabetes, Alzheimer's, Parkinson's, cancer, CVD related diseases and respiratory diseases. Polyphenols can reduce oxidative stress and delay ageing [32–34].

The effect of polyphenols as chemopreventive agents has been extensively demonstrated in animal models but is still very limited in humans and there is only promising data regarding regular consumption of green tea and its main polyphenol epigallocatechin gallate and oral and prostate cancer development [6]. It has been hypothesized that polyphenols may act by blocking the initial stages of the disease by modulating the expression of cytochrome P-450 enzymes involved in the activation of carcinogens and limiting the formation of initiated cells by stimulating DNA repair. In promotion stages, these compounds slow or stop tumor growth by inhibiting the expression of genes involved in tumor proliferation or inducing apoptosis of malignant cells. In progression stages they can inhibit angiogenesis and limit tumor invasion [7,35,36].

The effect of polyphenols in neurodegenerative diseases has usually been performed in animal models and neuronal cells. Polyphenols like resveratrol, proanthocyanidins, epicatechin, catechin and ferulic acid, as well as polyphenols from different foods have shown to reduce β-amyloid peptides in mice, which are involved in the pathogenesis of Alzheimer's disease [37–39]. Studies carried out in humans showed an improvement of memory in old people with mild cognitive problems. These compounds

interfered in the generation and aggregation of β-amyloid peptides [40]. Flavonoid intake was also associated with better cognitive ability in humans [41]. However, it is unclear whether polyphenols directly interact with neural systems because it is unknown if all polyphenols are able to reach the brain [37]. Nowadays, we only know that some metabolites from gallocatechin gallate epicatechin and anthocyanins are able to cross the blood-brain barrier in animals [42,43].

4.1. Cardiovascular effects of phenolic compounds

A large number of epidemiological studies and some human intervention studies have associated the consumption of polyphenols with a decreased risk of CVD. Nowadays, the strongest evidence for the efficacy of polyphenols reducing biomarkers of cardiovascular risk comes from flavan-3-ols-rich foods, especially cocoa and tea [6,44,45]. Other prospective studies associated the intake of anthocyanins and flavanones with a lower risk of CVD and total mortality [46,47].

The protective effect of polyphenols may be explained by the improvements they confer on various risk factors that are much more effective than treating the CVD itself. Indeed, it has been shown that polyphenols improve endothelial function by improving parameters such as LDL cholesterol, platelet aggregation, invasion and proliferation of smooth muscle cells in the arterial wall, nitric oxide (NO) and some markers of inflammation [7,48].

The endothelium is the innermost layer of the blood vessel walls. Endothelial cells, in response to various stimuli, release molecules that are responsible for maintaining normal endothelial function. When the balance is disrupted the homeostatic functions of the endothelium are altered and the process of atherosclerosis, a chronic inflammation of large artery walls, starts. Hypertension, high LDL cholesterol levels or low high-density lipoprotein (HDL) cholesterol levels, diabetes and smoking are risk factors for atherosclerosis [49,50].

Hypertension is a public health issue that affects more than 1 billion people worldwide, causing 7.6 million deaths annually. Hypertension is diagnosed when systolic blood pressure (SBP) is permanently greater than 140 mm Hg and/or diastolic blood pressure (DBP) is greater than 90 mm Hg [51].

Several human intervention studies have related polyphenol-rich foods with a decrease in BP and other related parameters. Results from the most recent studies (from 2010 to 2015) are summarized in Table 1.

Table 1. Human intervention studies on polyphenols and blood pressure.

Type of study	Participants	Age	Administrated substance	Polyphenols †	Dose/day	Length of the study	Biomarker	Changes on BP‡	Reference
Chronic, controlled, cross-sectional	35 healthy men	18-45	Wine grape or grape seed extract (capsules)	Anthocyanins, phenolic acids	6 capsules (800 mg)	14 days	SBP DBP	↔ ↔	[52]
Crossover, randomized, controlled	24 healthy and overweight men	50-65	Orange juice or hesperidin-enriched drink	Hesperidin	500 mL (292 mg)	28 days	DBP	↓(in both intervention groups)	[53]
Crossover, randomized, controlled	24 men with metabolic syndrome	30-70	Grape extract (capsules)	Flavanols, anthocyanins	46 g/day	30 days	SBP FMD NO	↓ ↑ ↔	[54]
Parallel, randomized, controlled	97 overweight men and women	19-55	Algae extract (*Ecklonia cava*)	Fluorotannins	Cans (246 mL and 72 mg extract)	12 weeks	SBP	↓ (with the highest dose)	[55]
Crossover, randomized, controlled	10 healthy men	45-50	Wine, dealcoholized wine and gin	Flavanols, anthocyanins	272 mL wine (733-798 mg EAG/day) or 100 mL gin	20 days	DBP SBP	↓(with wine) ↓(with both wines)	[56]
Crossover, randomized, controlled	51 healthy men and women	30-50	Pomegranate juice	Hydrolizable tannins, anthocyanins	330 mL/d	4 weeks	SBP DBP BP	↓(-3.14 mmHg) ↓(-2.33 mmHg) ↓(-2.60 mmHg)	[57]
Crossover, randomized, controlled	67 men at high cardiovascular risk	55-75	Wine, dealcoholized wine and gin	Flavanols, anthocyanins	272 mL wine (733-798 mg EAG/day) or 100 mL gin	4 weeks	SBP and DBP Plasma NO	↓ ↑ (with dealcoholized wine)	[58]
Parallel, randomized, controlled	84 healthy or mild hypertensives men and women	35-75	Black tea	Catechins	3 cups/day (429 mg)	4 weeks	SBP DBP	↔ ↔	[59]
Crossover, randomized, controlled	49 healthy men	48-68	Quercetin	Quercetin	Capsules (150 mg/day)	8 weeks	SBP postprandial	↓	[60]
Parallel, randomized, controlled.	70 hypertensives (stage 1 or less) men and women	35-75	Grape seed extract	Catechin, proanthocyanidin dimers	Capsules (300 mg/day)	8 weeks	SBP and DBP	↓ (not statistically significant)	[61]
Pilot study	6 healthy men and women	34-68	Boysenberry juice	Proanthocyanidin dimers, epicatechin	180 mL/day (351 mg)	4 weeks	FMD SBP	↑ ↓	[62]
Crossover, randomized	18 healthy men and women	-	Green and black coffee	Chlorogenic acid	40 g (4 cups)	2 weeks	SBP	↓ (after green coffee)	[63]
Parallel, randomized, controlled	15 healthy or mild hypertensive men and women	43.2±12.4	Japanese plum (*Prunus mume*)	Hydroxycinnamic acid derivatives	Capsules (800 mg/day)	12 weeks	BP	↔	[64]
Parallel, randomized, controlled	56 healthy men and women	25-65	Low-calorie cranberry juice	Anthocyanidins, proanthocyanidins	480 mL/day (173 mg)	8 weeks	BP	↓	[65]
Crossover, randomized,	100 healthy men and women	18-65	Orange juice (normal or polyphenol-enriched)	Hesperidin	500 mL/day	12 weeks	SBP DBP	↓ (normal) ↓ (normal)	[66]

SBP, systolic blood pressure; DBP, diastolic blood pressure; BP, blood pressure; AGE: gallic acid equivalents; FMD: flow-mediated dilation.
* Table is organized chronologically.
† Only the top two polyphenols with the highest concentration are listed
‡ ↑increase; ↓ decrease; ↔ no change; unless otherwise stated, % refers to changes from baseline when test substance is given.

A possible mechanism by which polyphenols decrease BP is by enhancing NO production. *In vitro* experiments with isolated arteries showed that polyphenols increased endothelial NO formation and caused NO-mediated endothelium-dependent relaxations [67].

Metabolic syndrome (MS) is a metabolic disorder that consists of a combination of multiple cardiovascular risk factors: obesity, hypertension, dyslipidemia and hyperglycemia. The consumption of polyphenol-rich foods can prevent MS through their protective effect on chronic inflammation linked to obesity, insulin resistance, dyslipidemia, and hypertension [68].

Some *in vitro*, animal and human studies have shown that polyphenols help to control obesity by reducing fat absorption in the intestinal tract, activating thermogenesis and modulating the hormonal response that regulates food intake and satiety [69].

Consumption of cocoa has shown to reduce MS by improving insulin resistance, endothelial function and levels of NO. These effects have been confirmed in multiple literature reviews and meta-analyzes [70]. Similar results were observed with the consumption of green tea [71], olive oil [72] and other polyphenol-rich foods.

Polyphenols could also help to reduce diabetes through different mechanisms. They can inhibit glucose absorption in the small intestine and its reabsorption in the liver, gluconeogenesis, adrenergic stimulation of glucose consumption, or stimulation of insulin release by pancreatic beta cells. For example, polyphenols from cinnamon, resveratrol, isoflavones, and polyphenols from tea, cocoa and grape seeds improve insulin sensitivity, the hormone that regulates plasmatic glucose levels [7,69].

5. Polyphenol intake and its effects in the PREDIMED cohort

In the 60s, the Mediterranean diet (MedDiet) was defined for the first time as the dietary pattern followed by Cretan, Greek and Southern Italian citizens. According to Ancel Keys, this diet was low in saturated fat and high in vegetable oils. The MedDiet has deserved much attention in the past decades due to its numerous beneficial effects in our health. The original definition has evolved to a more extensive one. Nowadays, in general terms, we can consider that guidelines to follow a MedDiet include high intakes of vegetables including leafy green vegetables, fruits, cereals, nuts, legumes and extra virgin olive oil, moderate intakes of fish, meat, dairy products and red wine, and low intakes of eggs and sweets [69].

The PREDIMED study (*PREvención con DIeta MEDiterránea*, ISRCTN35739639) was a prospective, randomized, multicentric and controlled trial aimed to assess the health benefits of a traditional Mediterranean diet (MedDiet) in the primary prevention of cardiovascular diseases [70,71]. The study lasted 9 years, from 2004 to 2013, and

included 7447 elderly participants at high cardiovascular risk. Volunteers were recruited through primary health care centers from 8 different Spanish regions and they were randomized to one of the following nutritional intervention groups: Mediterranean diet supplemented with extra virgin olive oil (MedDiet -EVOO), Mediterranean diet supplemented with nuts (MedDiet -nuts), and a control group which followed a low-fat diet according to the recommendations of the American Heart Association (AHA) [72].

After a median of 4.8 years of follow-up, an external scientific committee advised to finish the study due to the marked differences between the MedDiet groups and the control group. Results revealed that both MedDiet groups had 30% less incidence of CVD events than the control group. Specifically, the adjusted hazard ratio (HR) were 0.70 (95% CI 0.54-0.92) and 0.72 (95% CI 0.54-0.96) when comparing the MedDiet -EVOO and the MedDiet -nuts with the control group [73].

Within the PREDIMED, and after only 3 months of intervention, it was demonstrated that improving a diet towards a MedDiet pattern decreased LDL cholesterol, glucose, BP, and biomarkers of inflammation [70,74]. After one year of intervention, results showed that both MedDiet (MedDiet -VOO and MedDiet -nuts), but not the control diet, were able to revert the metabolic syndrome [75,76] and to increase non enzymatic antioxidant capacity of plasma [77], as well as decrease cellular and circulating inflammatory biomarkers related to atherogenesis [78]. Other investigations were aimed to assess the beneficial effects of the MedDiet on obesity [79,80], cognitive impairment [81], hyperuricemia [82], and type-2 diabetes [83]. MedDiet also delayed the progression of internal carotid intima-media thickness and plaque height [84] and reduced oxidative damage to lipids and DNA in individuals with metabolic syndrome [85]. These results provide further evidence to recommend a MedDiet pattern supplemented with nuts and olive oil to decrease CVDs risk factors, especially when these recommendations are addressed to elderly people at high CVDs risk.

Beyond its main objective, the PREDIMED study has been the nest of other numerous sub-studies involving many exposure variables others than MedDiet and CVD-related outcomes. Polyphenols and polyphenol-rich foods have been one of the studied exposure variables. The concentration of total polyphenols in spot urine samples as a biomarker of total polyphenol intake [86] was negatively associated with BP levels and prevalence of hypertension in a cross-sectional sub-study of 589

participants from the PREDIMED trial [87]. This effect was mediated by the increase of nitric oxide (NO) in plasma [88]. Valls-Pedret *et al*. also found that a high consumption of antioxidant-rich foods such as virgin olive oil, coffee, walnuts or wine, and a high intake of polyphenols were associated with better cognitive function [89]. Total polyphenol intake assessed using yearly food frequency questionnaires and the Phenol-explorer database was associated with cardiovascular mortality and events, and all-cause mortality. From them, lignans, flavanols, and hydroxybenzoic acids were associated with decreased CVDs risk [90] while high intakes of stilbenes and lignans showed a reduced risk of overall mortality [91].

Regarding polyphenol-rich foods, moderate red wine consumption was associated with a lower prevalence of the metabolic syndrome and some of its components: increased waist circumference, low HDL-cholesterol, high BP, and high fasting plasma glucose concentrations [92]. Resveratrol in urine, as a biomarker of wine intake, was also inversely associated with cardiovascular risk factors: blood lipid profiles, fasting blood glucose, and heart rate [93]. Finally, consumption of extra virgin olive oil decreased CVD, atrial fibrillation and carotid intima-media thickness [94–96], while consumption of nuts decreased adiposity in the PREDIMED cohort [97].

7. Conclusion

Polyphenols are bioactive compounds mainly found in fruits, vegetables, cereals and their products. Several studies have demonstrated that polyphenols and polyphenol-rich foods can improve cardiovascular health through different mechanisms. However, some results are contradictory and more mechanisms still need to be elucidated so future research have to substantiate these findings in order to stablish dietary recommendations or to consider the potential polyphenolic compounds as therapeutic agents.

Acknowledgements

This study was supported by CICYT (AGL2013-49083-C3-1-R) from the Spanish Ministry of Economy and Competitiviness, the Generalitat de Catalunya (GC) 2014 SGR 773 and the Instituto de Salud Carlos III, ISCIII (CIBEROBN). CIBEROBN is an initiative of ISCIII, Spain.

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Research Signpost
37/661 (2), Fort P.O.
Trivandrum-695 023
Kerala, India

Recent Advances in Pharmaceutical Sciences VI, 2016: 37-47 ISBN: 978-81-308-0566-5
Editors: Diego Muñoz-Torrero, Àngela Domínguez and Àngels Manresa

3. Risk assessment for human embryonic development of triclabendazole residues in milk and cheese in the diet of a rural population in Cajamarca (Peru): A preliminary approach

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Abstract. Triclabendazole (TCBZ) is a veterinary drug used against *Fasciola hepatica* in cattle. The Cajamarca Valley in Peru is an endemic area of fascioliasis with a high infection rate in animals producing milk for human consumption. The administration of TCBZ during the lactating period can lead to TCBZ derivative residues in milk and cheese entering the human food chain. Milk-derivatives from treated animals have been found

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positive for TCBZ metabolites. One of these metabolites, triclabendazole sulfoxide (TCBZSO), is embryolethal during early developmental stages *in vitro* in mouse and zebrafish. In this study, we have calculated the estimated daily intake (EDI) of TCBZSO due to milk and cheese consumption among a rural population in Cajamarca in order to evaluate the associated risk for human embryonic development. Although the expected maximum plasma concentration of TCBZSO after a worst-case scenario simulation would be below the reported lowest observed adverse effect concentration (LOAEC) for embryolethality *in vitro* (10 µM), several limitations on the available information for exposure, bioavailability and interspecies differences still impede the accomplishment of an accurate risk assessment.

Introduction

Triclabendazole (TCBZ) is a benzimidazolic compound used in veterinary medicine to treat fascioliasis. This parasitic disease, induced by the liver trematode *Fasciola hepatica* (see life cycle in Fig.1 [1]) is endemic in the Cajamarca Valley in Peru, where more than 78% of livestock are infected [2]. Treating the infected animals is a necessity in order to reduce the number of hosts and thus to reduce the disease impact in humans, but also to avoid production losses in cattle industry by means of reductions in animal weight gain, fertility and milk yield [3-5]. TCBZ is the drug of choice to treat fascioliasis in animals because it has high activity against adult and juvenile flukes [6-8] and because it can be administered as a single treatment at the start of the dry-off period, when no milk is being produced for human consumption, and thus avoid TCBZ residues in milk [9].

However, in fascioliasis endemic areas like Cajamarca, TCBZ treatment is administered three to four times a year, even during the period when cows produce milk for human consumption [10]. This use during the lactating period can lead to TCBZ derivative residues entering the human food chain as several studies have shown the presence of TCBZ residues in milk and its transference to dairy products [9].

Taking that into consideration, a maximum residue limit (MRL) of 10 µg/kg of TCBZ in milk has been fixed by the European Union [11], and also an acceptable daily intake (ADI) of TCBZ of 0 to 3 µg/kg body weight (b.w). [12]. However, neither MRL has been established for TCBZ derivatives in milk, nor for TCBZ or TCBZ derivatives in cheese and other dairy products. The presence of these residues in the human food chain acquires special relevance when considering that triclabendazole sulfoxide (TCBZSO), the first metabolite of TCBZ, presents embryotoxic potential

in vitro in mouse and zebrafish (lowest observed adverse effect concentration for lethality; $LOAEC_{lethality}$ = 10 µM; [13]). Therefore, our aim was to evaluate the possible risk for human embryonic development derived from maternal consumption of milk and cheese containing TCBZ derivative residues during gestation.

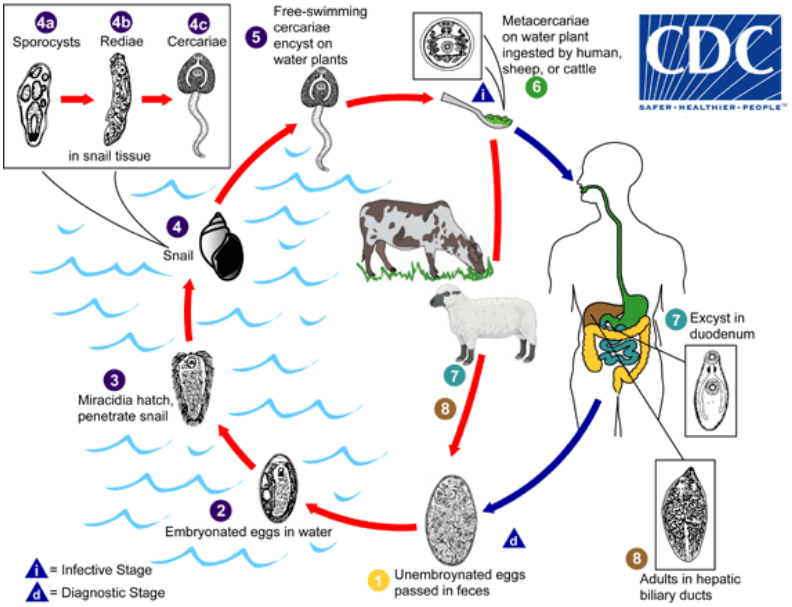

Figure 1. Life cycle of *Fasciola hepatica* and *Fasciola gigantica* [1].
Immature eggs are discharged in the biliary ducts and in the stool. **(1)** Eggs become embryonated in water **(2)**, eggs release miracidia **(3)**, which invade a suitable snail intermediate host **(4)**, including the genera *Galba*, *Fossaria* and *Pseudosuccinea*. In the snail the parasites undergo several developmental stages [sporocysts **(4a)**, rediae **(4b)**, and cercariae **(4c)**]. The cercariae are released from the snail **(5)** and encyst as metacercariae on aquatic vegetation or other surfaces. Mammals acquire the infection by eating vegetation containing metacercariae. Humans can become infected by ingesting metacercariae-containing freshwater plants, especially watercress **(6)**. After ingestion, the metacercariae excyst in the duodenum **(7)** and migrate through the intestinal wall, the peritoneal cavity, and the liver parenchyma into the biliary ducts, where they develop into adults **(8)**. In humans, maturation from metacercariae into adult flukes takes approximately 3 to 4 months. The adult flukes (*F. hepatica:* up to 30 mm by 13 mm; *F. gigantica:* up to 75 mm) reside in the large biliary ducts of the mammalian host. *F. hepatica* parasites can infect various animal species, mostly herbivores (source [1]).

1. Presence of triclabendazole-derivative residues in milk and cheese samples

TCBZ-derivative concentrations in milk and cheese samples were obtained from a study carried out on a dairy farm situated in Cajamarca, Peru [10]. In this study by Imperiale *et al*., seven female Holstein dairy cows between 490 and 630 kg weight were orally treated with a single dose of 12 mg TCBZ/ kg b.w. (Fasinex®, TCBZ 10%, Novartis). Milk samples were collected prior to treatment and at 6, 12, 24, 36, 48, 60, 72, 84, 96, 120 and 144 h post-treatment. Approximately 50 mL of milk were collected after homogenization of the whole milk yield of each cow. The remaining milk production of all experimental animals collected at 12, 24, 36, 48, 60 and 120 h post-treatment was pooled and processed to produce creamy cheese. The extraction procedures to quantify TCBZ and its metabolites in milk (0.5 mL) and cheese (0.5 g) samples were carried out following modifications of previously described methods [10, 14, 15]. Drug concentrations in experimental samples were determined by HPLC with a UV detector using oxibendazole as internal standard (99.2% purity). Both TCBZSO and triclabendazole sulfone ($TCBZSO_2$) metabolites but not TCBZ were detected in milk (up to 36 and 144 h post-treatment, respectively). The sum of total residues (TCBZSO and $TCBZSO_2$) was between 0.6 and 2 µg/mL in pooled milk, and between 1.1 and 20.0 µg/g in cheese.

2. Milk and cheese consumption data

Milk and cheese consumption data concerning the rural population of Cajamarca valley were obtained from two different studies, one from our own group [16] and one from an official Peruvian dietary survey (INEI) [17]. Briefly, in our own study, 36 people from 11 populations located in the rural area of Cajamarca were interviewed for a 24 h dietary recall. To contrast and compare the obtained information, a qualitative food frequency questionnaire was performed by the same interviewer to the same individuals. The official dietary survey of Peru [17] collected information from seven days questionnaires of 1536 households in rural areas and 34698 households in urban areas of Peru. The areas covered in the study included coast, mountains and rainforest.

Milk consumption results reported by these two studies were very different. These differences can be explained by the fact that the INEI

study covered the whole Peruvian population, while the study from Barenys *et al.* [16] was performed exclusively in the Cajamarca valley, which is one of the main milk and dairy producer regions in Peru [18]. Besides, the population interviewed, consisted mainly in teenagers (63% of participants were people between 14 and 17 years old), which are described to consume more milk than the adult population [19].

Ideally, the consumption data should be obtained from women at childbearing age, but this consumption values are not available for the whole Peruvian population [17], and the sample size of this group was too small to drive conclusions in the study by Barenys *et al*. [16]. We have used the values reported in both studies [16, 17] for milk and cheese consumption to further calculate the Estimated Daily Intake (EDI) of TCBZ derivatives.

3. Triclabendazole derivatives estimated daily intake calculation

The results obtained by Imperiale *et al*. [10] for TCBZSO and $TCBZSO_2$ concentrations in milk and cheese and the contribution of milk and cheese to the diet according to our 24 h dietary recall results, and the INEI survey [17] are detailed in Table 1 and were used to calculate the EDI of TCBZ derivatives per individual, expressed in µg of TCBZ derivatives/kg b.w. per day, as follows:

$$EDI = \frac{(\text{max milk consumption} \times \text{max concentr TCBZ derivatives in milk} + \text{max cheese consumption} \times \text{max concentr TCBZ derivatives in cheese})}{\text{median woman body weight}}$$

The median weight value for women population was obtained from Ministerio de Salud del Perú [20]. As detailed in Table 1, the EDI of TCBZSO + TCBZSO2 in a worst-case scenario approach was estimated to be 12.0 µg/kg b.w.

$$EDI = \frac{(180\ \text{mL} \times 2\ \mu\text{g/mL}) + (12\ \text{g} \times 20\ \mu\text{g/g})}{50\ \text{kg b.w.}} = 12\ \mu\text{g TCBZ derivatives/kg b.w.}$$

It is important to remark that there is no study available from the region of Cajamarca indicating which is the real presence of TCBZ derivatives in representative samples in the market and thus, which would be the mean real exposure in the general population. Therefore, the TCBZ derivative values described in Imperiale *et al*., [10], obtained in only one farm directly after the administration of TCBZ to the animals, were taken to simulate a worst-case scenario and to study its associated risk for human embryonic development.

Table 1. Triclabendazole derivatives estimated daily intake calculation.

Median woman body weight [kg]	Milk consumption [mL]		Cheese consumption [g]		TCBZSO+TCBZSO$_2$ concentration in milk [µg/mL]	TCBZSO+TCBZSO$_2$ concentration in cheese [µg/g]	**TCBZSO+TCBZSO$_2$ Estimated Daily Intake [µg/kg b.w.]**
	Perú	Caj	Perú	Caj			**Worst-case scenario**
50[a]	107[b]	180.2[c]	12.0[b]	8.98[d]	2[e]	20[e]	12.0

Perú: data from the 5th quintile of the INEI survey [17], milk consumption includes milk and yoghurt; Caj: data from 24-h dietary recall to Cajamarca population [16]; a: [20]; b: [17] (milk consumption corresponding to milk and yoghurt values); c: calculated from g to mL from [16] using milk density=1.03; d: [16]; e: [10].

4. Plasma concentration-ranges after the estimated daily intake vs LOAEC for embryo lethality *in vitro*

In humans, after ingestion of a therapeutic dose of 10 mg/kg b.w. of TCBZ, the obtained maximum plasma concentration (C_{max}) of TCBZSO is 38.6 µM [21]. At present, there is no characterization of TCBZSO C_{max} reached after direct administration of TCBZSO in humans or in animals, but pharmacokinetic studies with other benzimidazoles have compared the reached concentrations of the sulfoxide metabolite after the administration of the parent compound or after the administration of the sulfoxide metabolite itself [22, 23, 24].

For example, albendazole sulfoxide (ABZSO) produced significantly higher ABZSO C_{max} but not significantly higher area under the curve (AUC) than albendazole (ABZ) in 1-month-old lambs, and significantly higher ABZSO C_{max} and AUC than ABZ in 8-month-old lambs following administration of 5 mg/kg b.w. of each compound [22]. These differences were comparable to those determined previously in goats [22, 23]. Similarly, the plasma concentration of fenbendazole sulfoxide (FBZSO) was much greater when it was directly administered than when fenbendazole (FBZ) was administered in horses, at 10 mg/kg b.w. each. It is remarkable that FBZSO achieved 25.6 or 35 times greater concentrations (expressed as AUC or C_{max}, respectively) when it was directly administered, than after administration of the parent compound [24]. These studies show that when the sulfoxide metabolite is directly administered, the plasma concentration seems to be similar or higher than when the parent compound is administered at the same doses.

Anyhow, a precise characterization of the C_{max} of TCBZSO after TCBZSO administration is still missing and would be essential for a correct risk assessment of TCBZSO effects on human development.

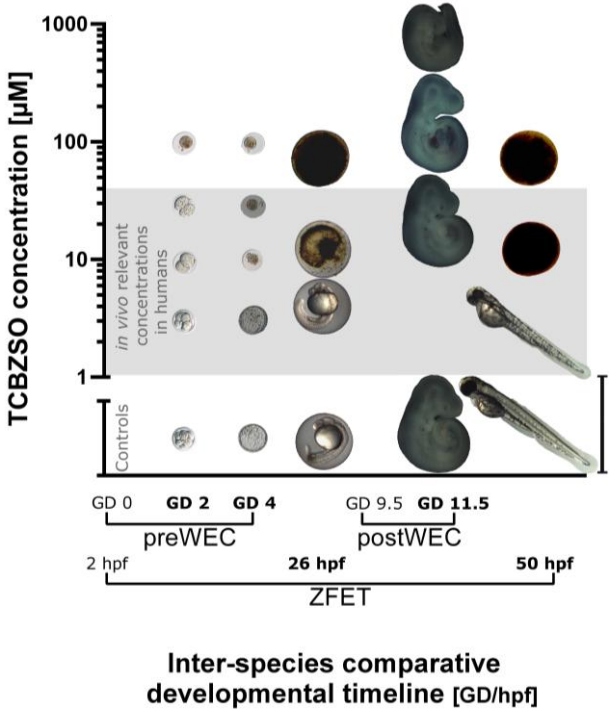

Figure 2. Graphical summary comparing TCBZSO results across species and developing time.

Representative pictures of rodent embryos exposed to increasing concentrations of TCBZSO from GD 0 to GD 4 in the preWEC culture ($LOAEC_{lethality}$= 10 µM) and from GD 9.5 to GD 11.5 in the postWEC culture ($LOAEC_{dysmorphogenesis}$= 666 µM). Zebrafish embryos were exposed to TCBZSO from 2 hpf to 50 hpf, a developmental period comprising the stages covered by both rodent cultures. No dysmorphogenesis were observed (maximum concentration tested= 50 µM), but TCBZSO was embryo lethal during the first 24 h of culture ($LOAEC_{lethality}$= 10 µM). Pictures correspond to the developmental time points marked in bold in the x-axis. Pictures of pre WEC embryos are 10 times magnified respect to pictures of post WEC and zebrafish embryos.

Scale bar: 4 mm for post WEC and zebrafish embryos; and 400 µm for pre WEC embryos.

ZFET: zebrafish embryo test (concentrations= 0, 5, 10 and 50 µM); pre WEC: preimplantation whole embryo culture (concentrations= 0, 3, 10, 30 and 100 µM); postWEC: postimplantation whole embryo culture (concentrations= 0, 27, 267 and 666 µM); GD: gestational day; hpf: hours post-fertilization (ref. [13]).

Taking into account this important limitation, after the administration of the TCBZSO EDI of 12.0 µg/kg b.w., the plasma C_{max} could be calculated following a simplified approach which considers total and instant absorption and no tissue distribution:

$$\text{EDI} = \frac{12.0\ \mu\text{g TCBZSO}}{\text{kg b.w.}} \times \frac{50\ \text{kg b.w.}}{2\ \text{L plasma}} \times \frac{1\ \mu\text{mol TCBZSO}}{376\ \mu\text{g TCBZSO}} = 0.80\ \mu\text{M}$$

Total plasma volume was obtained following the standard weight-based calculation for normal females (0.04L x kg b.w.) [25].

This concentration is still lower than the experimental LOAEC for embryotoxicity observed in *in vitro* developmental toxicity tests in mouse and zebrafish ($LOAEC_{lethality}$= 10 µM; [13]; see Fig 2), but the lack of specific pharmacokinetic data after TCBZSO administration and the possible species differences in pharmacokinetics and embryo toxicity sensitivity, pose a serious obstacle to an accurate risk assessment process.

5. Discussion

At present, no MRL has been established for TCBZSO in milk, in cheese or in other dairy products. Likewise, no ADI has been established for TCBZSO. However, there is extensive scientific evidence that flukicide administration to milk producing animals leads to migration of residues to milk and its derived products as cheese, butter and skim milk powder. It is remarkable that pasteurization or heat treatment during spray drying have no impact in reducing these residues [9].

The present study approaches the subject of risk assessment of TCBZ residues in milk and cheese in the diet of a rural population in Cajamarca (Peru). However, several limitations on the current knowledge about exposure characterization, bioavailability of the compound and interspecies differences in sensitivity impede an accurate accomplishment of the risk assessment process.

Limitations on exposure characterization: in section 3 we have calculated the EDI of total TCBZ derivatives considering a worst-case scenario, as the concentrations used for the calculations are from milk and cheese samples obtained from cows after controlled administration of TCBZ [10]. But in a real situation, not all milk and cheese products consumed by the population would contain TCBZ derivative residues.

Currently, there are no studies detailing the prevalence of milk and cheese samples containing TCBZ derivatives in the Cajamarca market. A study comparable to the one performed by [26] where 27.6% of raw milk samples collected from all farms throughout Southern Greece were found to be positive for the investigated benzimidazoles, would be very valuable to better characterize the real exposure scenario.

Besides, in the EDI calculation, the sum of both metabolites (TCBZSO and $TCBZSO_2$) has been considered, as no concentration values for TCBZSO alone have been detailed. However, the embryotoxic potential of $TCBZSO_2$ has not been investigated yet and it is a shortcoming of the current approach to consider it as if it would be as potent as TCBZSO and work further on with the EDI value obtained from the sum of both metabolites.

Limitations on bioavailability information: as detailed in section 4, there is no specific pharmacokinetic data about TCBZSO bioavailability after TCBZSO administration in humans, neither in animals. A simplified calculation of C_{max} has been used for approximation, but real pharmacokinetic data would help to carry a more accurate risk assessment.

Limitations on information about interspecies differences: Embryolethality ($LOAEC_{lethality}$) from *in vitro* developmental toxicity tests has been established at 10 µM using the mouse preimplantation embryo culture and the zebrafish embryo models [13]. However, there is growing recognition that to predict human safety, it would be advisable to know if there are species differences in sensitivity towards TCBZSO embryo lethal effects [27], by conducting other studies like parallel human and mouse embryonic stem cell tests.

6. Conclusions

In this study we have calculated the EDI of TCBZ derivatives simulating a worst-case scenario. After a TCBZSO EDI, the expected C_{max} would be lower than the experimental LOAEC for embryotoxicity observed in *in vitro* developmental toxicity tests in mouse and in zebrafish. However, our study highlights the current needs of information to carry out an accurate risk assessment for TCBZSO exposure during human development. Specifically, there is a need of:

- A better characterization of TCBZSO presence in commercialized milk and milk-derivatives samples with concrete TCBZSO concentrations.
- A detailed pharmacokinetic profile of TCBZSO concentrations reached after TCBZSO intake.
- More information on human sensitivity towards TCBZSO embryo lethality from human 3D *in vitro* models.

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Research Signpost
37/661 (2), Fort P.O.
Trivandrum-695 023
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Recent Advances in Pharmaceutical Sciences VI, 2016: 49-75 ISBN: 978-81-308-0566-5
Editors: Diego Muñoz-Torrero, Àngela Domínguez and Àngels Manresa

4. Organisation, expression and evolution of rRNA genes in plant genomes

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Abstract. Here we present an overview on the results of eight years of research line devoted to the organisation, expression and evolution of rRNA genes in plant genomes. We describe how we discovered a new rDNA arrangement in genus *Artemisia*, which includes all rRNA genes in a single unit (L-type). This was the first time that such organisation was found in seed plants. We further explored family *Asteraceae* in depth, to which Artemisia belongs, to find that the L-type arrangement may be present in about 25% of its species. Later on we move to gymnosperms to describe the landscape of rRNA arrangements in a representative sample of its diversity. We assess the expression rate of the L-type rRNA in several L-type species, which is comparable to that of species with separated arrangement of rRNA genes (S-type). Finally, we present the resource www.plantrdnadatabase.com which includes information on type of rDNA arrangement, number and position of rDNA loci in plants.

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Introduction

It is broadly accepted that a previous RNA world predated the present one based on DNA, RNA and proteins, probably thanks to the ability of RNA to both store genetic information and to act as an enzyme [1]. However, the mechanism by which RNA turned into the DNA system is still unknown. Nevertheless, several facts, such as the central role of RNA in the translation process and the ability to self-replicate, support this hypothesis. Notably, many RNA molecules, known as ribozymes or RNA enzymes, can catalyse biochemical reactions. Indeed, there are plenty of ribozymes in present-day cells, which could be considered as living fossils. Although ribosomes are composed by RNA and proteins, the catalytic site is formed by RNA only; besides, the deciphering of the 3D structure of ribosome revealed that peptide bond formation (the chemical reaction binding amino acids to form proteins) is catalysed by an adenine residue in the ribosomal RNA [2].

Ribosomal RNAs comprise 80% of the RNA found in a typical cell. The different types of ribosomal RNA (rRNA) account for 60% of the mass of the ribosomes. Ribosomal RNAs are codified by rRNA genes (rDNA), and there can be from 50 to 50,000 copies of rRNA genes per cell. In eukaryotes there are four ribosomal RNA genes. The 18S, 5.8S, and 26S rRNA genes are encoded in a single operon (called 35S rDNA in plants or 45S rDNA in animals and the 5S rRNA gene is encoded outside this operon, by 5S rDNA. Ribosomal DNAs are usually tandemly organised forming long arrays on one or several chromosomal loci. While in prokaryotes all rRNA genes form the same operon and the same RNA polymerase transcribes all of them, in some species of early diverging eukaryote groups e.g. yeast, *Saccharomyces cerevisiae*, although all rRNA genes are organised in a single unit and clustered in tandem, the 18S, 5.8S and 26S rRNA genes are transcribed by RNA polymerase I and the gene for 5S rRNA is transcribed by RNA polymerase III (Pol III). In eukaryotes, the 35S/45S (encoding 18S-5.8S-26S rRNA) and 5S genes are usually arranged separately and they are also transcribed by different polymerases, RNA polymerase I and III, respectively. Typically, in angiosperms, the tandemly arranged 35S rDNA unit is 9–20 kb long occurring in several thousands of copies at one or multiple chromosomal loci [3]. The unit comprises a ~5.0-kb genic region encoding for 18S–5.8S–26S genes and a more variable intergenic spacer (IGS). The 5S rDNA unit is much shorter and is composed of a 120-bp genic region and a 100–1,000-bp spacer [4]. Both the 35S and 5S rRNA genes are

tandemly arranged forming clusters of thousands of regularly spaced units. Figure 1 summarises the possible organisation of rRNA genes in different organisms.

Because of their abundance and sequence conservation, the nuclear genes encoding rRNA have been the subject of much research amongst molecular biologists and cytogeneticists, particularly for inferring evolutionary relationships between species. There are high levels of sequence homogeneity both with coding and non-coding parts of rDNA units within 5S or 35S rDNA loci, a phenomenon known as concerted evolution. Concerted evolution is the process by which the members of a multigene family are homogenised within a species but are different between species.

Independent control of transcription probably enabled physical separation of both loci in chromosomes, an arrangement that is typical for most eukaryotic organisms. Nevertheless, it seems that there are several exceptions to this rule. For example, 5S genes linkage to other repetitive sequences including 35S, histone genes or the trans-spliced leader has been

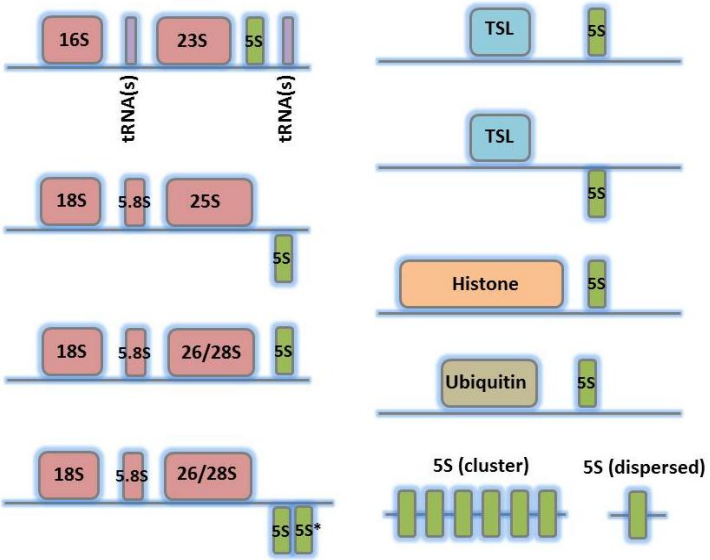

Figure 1. Arrangement of rRNA genes within (or outsides) the tandem repeat units of various multigene families found in different taxonomic groups. TSL=trans-spliced leader : (*) Pseudogene.

demonstrated [5]. These linked arrangements are found among diverse biological taxa including nematodes [6], fungi [7], crustaceans [8], slime moulds [9] or mosses [10, 11], and they are believed to represent transition states between linked (prokaryotic) and unlinked (mostly eukaryotic) arrangements.

1. Organisation of ribosomal RNA genes in *Artemisia*

As pointed previously, the 5S and 35S ribosomal DNA (rDNA), encoding the four major ribosomal RNA species, occur at separate loci in most plants. In some algae, bryophytes and ferns, they are at the same locus (linked arranged) but such molecular organisation had not been described in any angiosperm until our work in genus *Artemisia*. Our research group has been interested for more than 20 years in the evolutionary aspects of this genus [12], one of the most interesting within the family Asteraceae. Genus *Artemisia* is found in the most evolutionary advanced part of the family, belonging to subfamily Asteroideae and tribe Anthemideae. It consists in about 500 species distributed mainly in the Northern hemisphere, with few representatives in the Southern [13]. *Artemisia* species are mostly perennial and landscape dominating in grasslands and semidesert areas of Asia and North America [14]. Certain species (Figure 2) have medical interest, such as *Artemisia annua*, the source of artemisinin, a powerful treatment against malaria. Others are used as a condiment, such as *A. dracunculus*, (tarragon) or as the source of alcoholic beverages such as absinth (*A. absinthium*). Many species are used as forage and some can behave as weeds (*A. vulgaris*). The genus is quite well known from the evolutionary point of view (for a review see [12]) and plenty of karyological and cytogenetic studies have been devoted to the genus. Polyploidy and dysploidy are common in the genus, with ploidy levels up to 16x.

The first molecular cytogenetic study performed in *Artemisia* showed the pattern of ribosomal RNA genes localisation by fluorescent *in situ* hybridisation (FISH), in the *Artemisia campestris* complex [15]. In that study it became evident that both 5S and 35S rDNAs were colocalised, that is, the rDNA probes hybridised in the same chromosomal location, at the end of several submetacentric chromosomes. A further study by [16] deepened in the apparently anomalous organisation of rRNA genes in *Artemisia* and using Southern blot hybridisation, polymerase chain reactions (PCR), fluorescent *in situ* hybridisation, cloning and sequencing the genomic organisation of 5S and 35S rDNA in *Artemisia* was revealed (Figures 3 and 4).

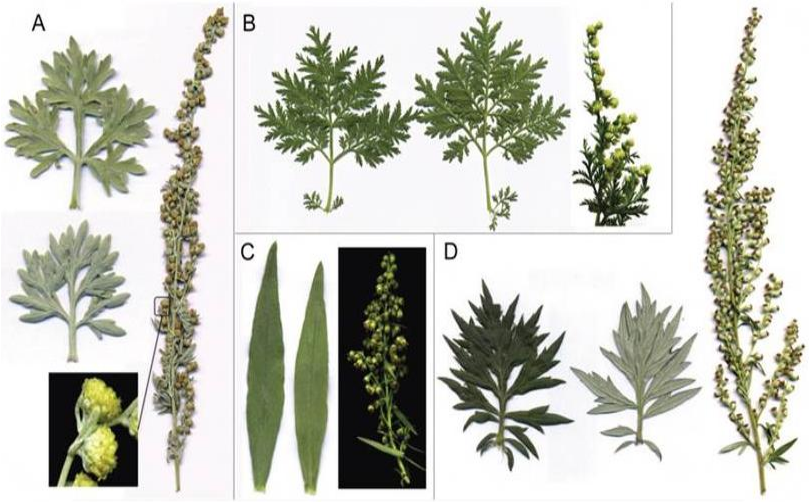

Figure 2. Leaves and synflorescences of four *Artemisia* species. (A) *Artemisia absinthium*. (B) *Artemisia annua*. (C) *Artemisia dracunculus*. (D) *Artemisia vulgaris*. From [12].

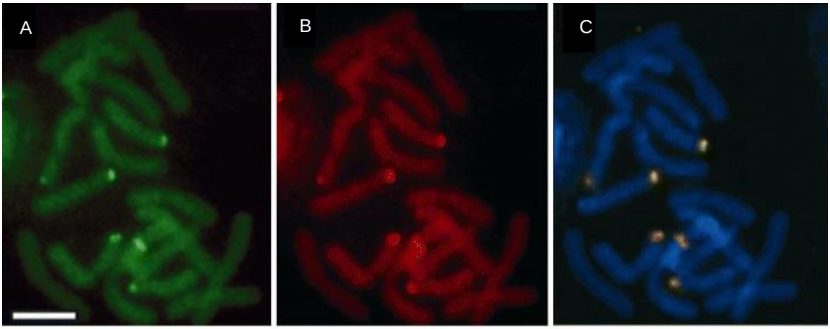

Figure 3. FISH on *Artemisia tridentata* showing the 35S probe (A, green) and the 5S probe (B, red) hybridising at the same loci. C, composite image including DAPI (blue) staining. Scale bar 10 µm. Pictures extracted from [16].

In *Artemisia*, the rRNA genes are all arranged in a single operon containing the 35S genes and the 5S gene in inverted orientation within the large intergenic spacer (IGS) of the 35S rDNA. The 5S gene insertion is typically located next to (about 150 bp) the end of the 26S gene. Also there

is a second less conserved insertion further downstream. The first 5S gene insertion presents all structural features of a functional gene, while the second is most likely a pseudogene since it had a deletion in the internal promoter region (Figure 4). Heterogeneity in unit structure may reflect ongoing homogenisation of variant unit types without fixation for any particular variant.

The study investigated several species within the genus and in all cases the same organisation of rRNA genes was inferred. Besides, closely and not so closely related genera, such as *Ajania* or *Nipponanthemum*, did also present the same arrangement (Figure 5). We hypothesized therefore that the linked arrangement probably evolved before the divergence of *Artemisia* from the rest of Asteraceae (>10 Myrs). Most likely, the activity of transposable elements is involved in this particular arrangement of rRNA genes and, probably, processes of concerted evolution took care of the homogenisation in a given genome (since no unlinked units were detected in *Artemisia*).

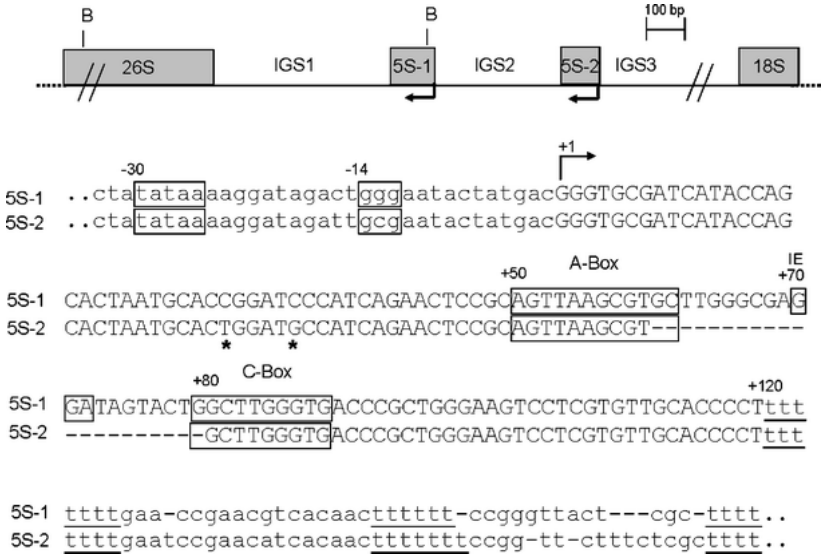

Figure 4. Representation of the 35S intergenic spacer of *Artemisia* containing two 5S copies. While the first one (5S-1) is a complete 5S rRNA gene, the second (5S-2) lacks part of the sequence, including essential regulatory regions, which indicate that it may be a pseudogenised copy. Extracted from [16].

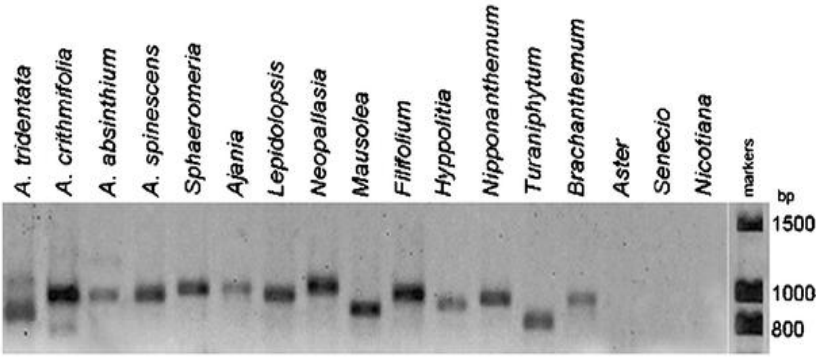

Figure 5. Gel electrophoresis of PCR products obtained after amplification of genomic DNAs with the 5SL-F and 26S-F primers. The sizes of products ranged between 800–1,000 bp. Extracted from [16].

2. Organisation of rRNA genes in family Asteraceae

From the observations established that both rRNA genes have been unified to a single 35S-5S unit in the genus *Artemisia*, we further aimed to reveal the extent, distribution and mechanisms leading to the linked organisation of rDNA in the Asteraceae, the family to which genus *Artemisia* belongs. The Asteraceae or Compositae are the largest angiosperm family with 1,620 genera and 23,600 species [17], and Asteraceae species embrace about 8-10% of all flowering plants. A majority of species of Asteraceae are herbaceous although some trees exist in the family (particularly in the American continent). Asteraceae are easily recognizable by certain morphological characters including fused anthers (syngenesia), a fruit with a single ovule (cypsela), and, in particular, by a specialized inflorescence termed capitulum (Figure 6). The family is widespread and cosmopolitan, growing everywhere but in the Antarctica [17]. Many species are edible and have huge economic interest (sunflowers, lettuces, artichokes, etc.), others are medicinal (calendula, chamomile, etc.) but several of them are also noxious weeds or invasive (wild safflower, cardoon, etc.). Several phylogenetic analyses have been conducted on the family, considered to hold 12 major subfamilies, four of which (Asteroideae, Carduoideae, Cichorioideae and Mutisioideae) comprise 99% of its species diversity [17].

Figure 6. A poster of twelve kinds of capitula of the Asteraceae (not representing their whole diversity), belonging to the two most representative subfamilies: Asteroideae and Cichorioideae: (1) yellow chamomile, *Anthemis tinctoria* (Asteroideae); (2) crown daisy, *Glebionis coronarium* (Asteroideae); (3) *Coleostephus myconis* (Asteroideae); (4) chrysanthemum - *Glebionis* sp. (Asteroideae); (5) sow thistle - *Sonchus oleraceus* (Cichorioideae); (6) chicory - *Cichorium intybus* (Cichoireideae); (7) treasure flower - *Gazania rigens* (Cichorioideae); (8) Mexican sunflower - *Tithonia rotundifolia* (Asteroideae); (9) field marigold - *Calendula arvensis* (Asteroideae); (10) Ox-eye daisy - *Leucanthemum vulgare* (Asteroideae); (11) common hawkweed - *Hieracium lachenalii* (Cichorioideae); (12) Cape daisy - *Osteospermum ecklonis* (Asteroideae). Picture by Dori Alvesgaspar and Tony Wills, licensed under the Creative Commons Attribution-Share Alike 3.0 Unported license.

Although the family is so vast, little had been published about rDNA position, organisation and structure in the whole group. Specific research works centered in certain genera, such as *Tragopogon*, *Centaurea*, *Helianthus* and *Hipochaeris*, among others [18, 19, 20, 21, 22], showed a separate organisation of rRNA genes, in contrast with the situation found in *Artemisia*. Considering the large size of the family, however, we still did not know how frequently an arrangement as the one in *Artemisia* could be found, and this was the purpose of our study. Using molecular (PCR, Southern blot, sequencing, quantitative PCR) and cytogenetic (FISH) methods, we examined ribosomal DNA structure and organisation in selected species (about 200) representing Asteraceae diversity [23].

The linked rDNA arrangement (Figure 7) was found within three large groups in subfamily Asteroideae: tribe Anthemideae (93% of the studied cases), tribe Gnaphalieae (100%) and in the "Heliantheae alliance" (23%). The remaining five tribes of the Asteroideae displayed separated arrangement of rDNA (Figure 8), as did the other groups in the Asteraceae.

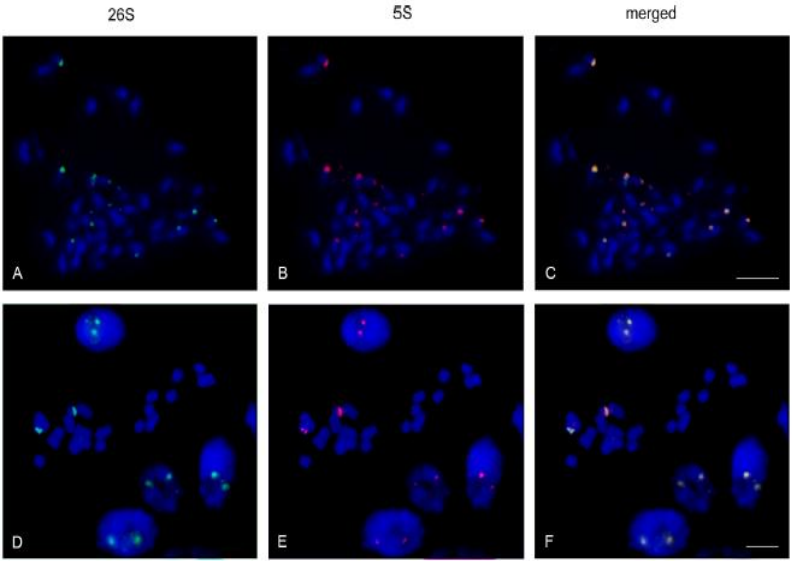

Figure 7. FISH of metaphase chromosomes and interphase nuclei of species that evolved linked arrangement of rRNA genes: (A-C) *Tagetes patula* (2n = 48) and (D-F) *Helichrysum bracteatum* (2n = 22). The 35S (26S probe) and 5S loci are labelled in green and red, respectively. Pictures extracted from [23].

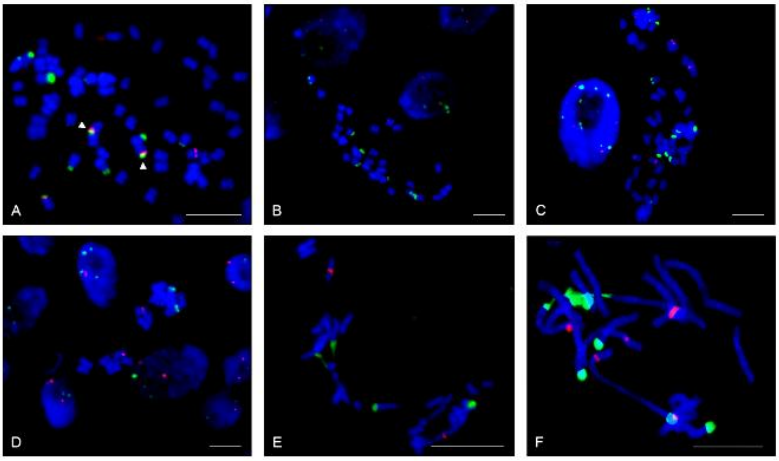

Figure 8. FISH of metaphase chromosomes and interphase nuclei of species that evolved unlinked arrangement of rRNA genes. The individual slides show merged 26S and 5S signals: (A) *Dahlia pinnata* (2n = 64), (B) *Helianthus annuus* (2n =34); (C) *Chrysanthemum zawadskii* (2n = 54), (D) *Aster alpinus* (2n = 18); (E) *Calendula officinalis* (2n = 28); (F) *Tragopogon mirus* (2n = 24). Arrowheads in (A) indicate juxtaposition of 35S and 5S arrays. The 35S (26S probe) and 5S loci are labelled in green and red, respectively. Pictures extracted from [23].

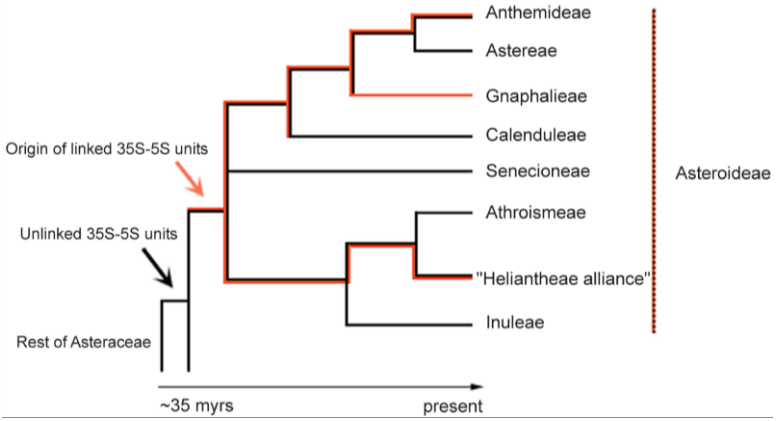

Figure 9. Phylogenetic distribution of rDNA arrangement in the subfamily Asteroideae. Red and black lines indicate evolutionary trajectories of linked and unlinked units, respectively. Extracted from [23].

Therefore, two contrasting modes of rDNA organisation are present in the Asteraceae. According with the distribution of these L-type genera in the phylogeny it seems that the linked arrangement arose independently several times in the largest subfamily, Asteroideae (Figure 9).

Yet, there are differences regarding the tribe; for example, 100% of studied species from Gnaphalieae present the L-type arrangement but conversely, a variable number of species with unlinked rRNA genes were found in Anthemideae (7%) and in the "Heliantheae alliance" (77%). This variability is reminiscent to that of primitive eukaryotes (yeast, other fungi) in which 5S genes are linked either with other rDNAs or other repetitive genes. Indeed, differences in the rDNA organisation are found even between closely related genera such as *Elachanthemum* (S-type) and *Artemisia* (L-type).

Therefore, could rDNA arrangement have a potential use as a phylogenetic tool? Since two closely related genera such as the previously mentioned present contrasting rDNA organisations, the most intuitive answer would be no. Nevertheless, on the basis of the L-type arrangement shared by most Anthemideae, Gnaphalieae and members of the "Heliantheae alliance", perhaps phylogenetic relationships between these three groups should be reassessed, as they could be more closely related than currently considered in Asteraceae phylogenetic treatments [24]. Moreover, the sequence between 26S and 5S genes in L-type species, termed IGS1 (see Figure 4), is not as conserved as the genic regions, displaying significant length and sequence polymorphism. When this sequence is used for a phylogenetic reconstruction, the results are congruent with one of the most used region for inferring phylogenies, the ITS1. For example, between *Artemisia absinthium* and *Artemisia tridentata*, the divergence of ITS1 was around 4%, with 10 substitutions in 250 bp while for IGS1 the divergence was around 7%, with 35 substitutions per 488 bp. Therefore the latter could be used as a suitable marker to infer phylogenetic relationships within the L-type species (the equivalent region in S-type species remains to be tested).

Since the separate organisation of rRNA genes is the most common across eukaryotes, it is likely that this is the most primitive one from which the linked arrangement evolved, although at present this still remains to be proved. According to our results, about 25% of the Asteraceae would present exclusively the linked arrangement; this means that at some point(s) the linked arrays would have overwritten the unlinked ones through concerted evolution. Some hypotheses explaining the mechanisms

of rDNA rearrangements would imply DNA recombination and/or RNA mediated transposition [25].

According to the results in Asteraceae, it is possible that the linked rDNA arrangement is more common than previous assumptions, which considered this organisation as an exceptional finding. Further in-depth studies at the family level are needed, not only to detect similar rDNA arrangements within plant families but also to find the evolutionary advantage, if any, that a linked arrangement of rRNA genes would imply for the organisms. The next step in our quest on rDNA organisation was an in-depth look to gymnosperms' rDNAs, since previous data pointed to certain prevalence of the L-type arrangement in this group.

3. Organisation of rRNA genes in gymnosperms

Gymnosperms were a rather unexplored field for rDNA research at the moment in which our paper dealing with rDNA organisation in these plants was released [26]. Gymnosperms, despite their large diversity in morphology and ecology, are only 14 families and about 1,000 species. This is much reduced in comparison with angiosperms (estimated to be about 352,000 species) or even with certain of its families, like the previously mentioned Asteraceae or the Orchidaceae, both with more than 20,000 species each [26].

There are four orders classically considered in gymnosperms: Pinales, Cycadales, Gnetales and Ginkgoales. The former is the largest and most abundant in terms of species and coverage; it includes about 650 species mostly belonging to the division Pinophyta. Cycadales are the second largest and they currently include three families and 130 species; Gnetales hold three families and 70 species and the latter, Ginkgoales, has a single extant species, *Ginkgo biloba*.

Most molecular cytogenetics research work on gymnosperms has been indeed carried out in family Pinaceae, the most widespread of the whole group. The arrangement of rRNA genes found in most gymnosperms up to date was the separate organisation, with 5S and 35S rRNA genes located in different positions, and usually in different chromosomes. However, the non-Pinaceae gymnosperms are more variable in rDNA organisation. In *Podocarpus* (family Podocarpaceae and still within Pinales, as Pinaceae) FISH revealed colocalisation of 5S and 35S rRNA genes in all loci [27]; similar results were found in certain Cupressaceae, including *Cryptomeria japonica* [28] and in *Ginkgo biloba* [29]. These studies, however, did not

show whether the 35S and 5S were physically linked (as in *Artemisia* and other Asteraceae, see parts 1 and 2 of this article), because rDNA *in situ* hybridisation in metaphase chromosomes does not allow enough resolution to interpret the molecular organisation.

Additionally, PCR screens and sequence analysis revealed a separate organisation of rRNA genes in *Cycas*, *Ginkgo* and *Gnetum* [30]. On the basis of the latter and other studies, it had been proposed that in gymnosperms the 5S and 35S repeats hold the separated configuration typical for vascular plants. Nevertheless, [29] found (using a PCR-based strategy) that in *G. biloba* 5S genes were integrated in the 26S-8S spacer. However, there were also rDNA clones without 5S genes, pointing to some heterogeneity of rDNA arrays. Although *in situ* hybridisation results favoured a dominant linked arrangement, it was unclear which was the proportion of separate units (if any). With this in mind, as well as with the purpose of drawing the global picture of rDNA arrangements in gymnosperms, we conducted an overall study on rDNAs of representatives of the main groups of gymnosperms, with an approach that included PCR, cloning, sequencing and Southern blot hybridisation [26]. Our study provides coverage of basic rDNA organisation for at least one representative of all gymnosperm orders, as well as a 15% increase in coverage of genera and an almost 30% increase in coverage of families of gymnosperms.

3.1. Uniform S-type rDNA arrangement in Pinaceae (Pinales) and Cycadales

Most previous molecular cytogenetic studies had been previously focused on family Pinaceae as it holds economically and ecologically interesting genera such as *Abies*, *Pinus* or *Picea*. Partially colocalising 5S and 35S rDNAs had been observed on chromosomes of *Abies alba* [31], *Picea* [32] and *Pinus* [33] species. In our survey, however, Southern blot did not reveal cohybridisation of 35S (using a 18S probe) and 5S probes in any of these, therefore it is likely that 5S and 35S rDNA signals are one next to the other, but not physically linked. Fluorescent *in situ* hybridisation performed to the species *Cupressus dupreziana* also showed separate arrangement of rRNA genes (Figure 10). Besides, Southern hybridisations showed quite homogeneous signal pattern (Figure 11), possibly the consequence of interloci homogenisation, i.e. efficient concerted evolution acting on these genes.

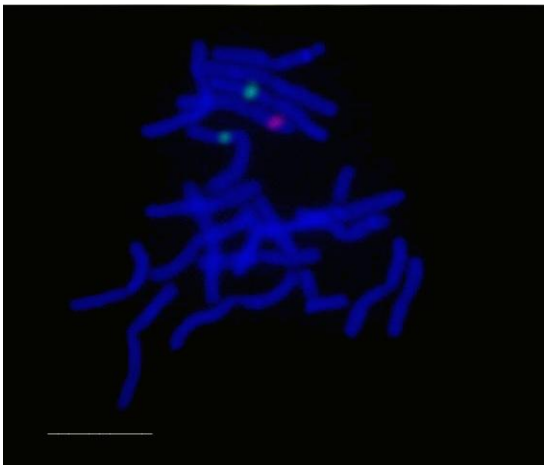

Figure 10. FISH to *Cupressus dupreziana* chromosomes (2n=22). Two 35S sites depicted in green and a single 5S site depicted in red. Scale bar 10 µm.

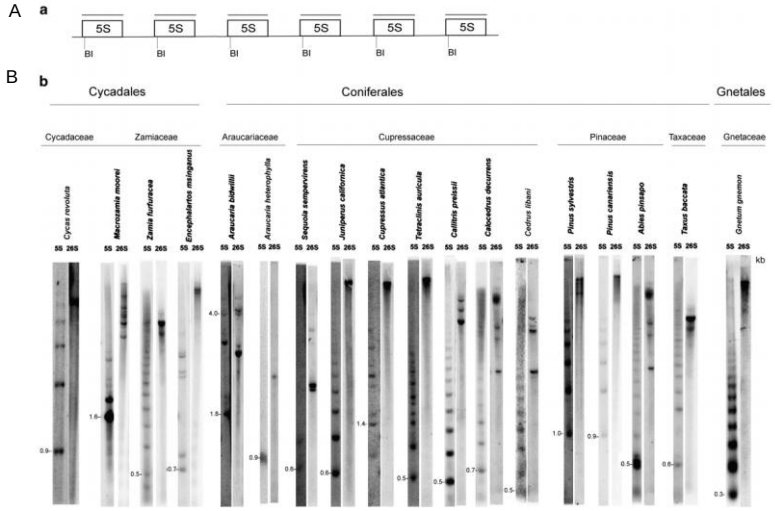

Figure 11. Southern blot hybridisation analysis of species with S-type arrangement of 5S and 35S units. The 5S tandem arrays with conserved *Bam*HI sites and probe hybridisation regions (lines above boxes) are schematically drawn in (A). Genomic DNAs were digested with *Bam*HI and hybridised on blots with the 5S and 26S rDNA probes (B). The sizes of 5S monomeric units are indicated. Extracted from [26].

The representative of Cycadales in this sample also shows a pattern consistent with a separate arrangement of rRNA genes. In this regard, the fact that recent molecular phylogenetic approaches point towards cycadophytes being more ancient than *Ginkgo* [34, 35], with a linked rDNA arrangement, complicates the interpretation on the ancestral condition of rDNA organisation in seed plants.

3.2. Both S- and L-type rDNAs in non-Pinaceae Pinales and in Gnetales.

In this group, we observed certain variations in rDNA organisation. Most members of Araucariaceae, Cupressaceae and Taxaceae showed separation of both 5S and 35S loci (Figure 10), however genera *Podocarpus* and the closely related *Afrocarpus* (Podocarpaceae) show clear linkage of 26S and 5S units (Figure 12).

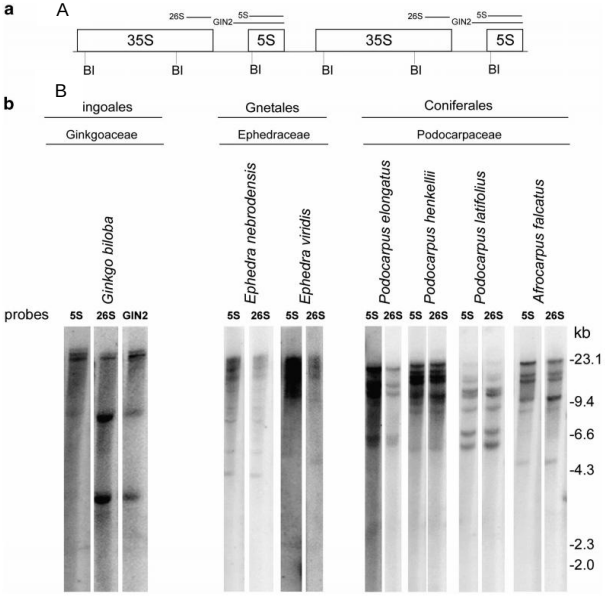

Figure 12. Southern blot hybridisation analysis of species with L-type arrangement of 5S and 35S units. Restriction map showing conserved *Bam*HI sites and regions of probe hybridisation (thin lines above boxes) are schematically drawn in (A). Genomic DNAs were digested with *Bam*HI and hybridised on blots with the 5S, 26S and GIN2 (containing IGS1 from *Ginkgo*) DNA probes (B). Extracted from [26].

The 35S–5S arrays are homogeneous and there is no evidence of separate loci. We estimated, based on Southern blot mapping and cloning, that the 5S insertions are found 6–9 kb downstream from the 26S gene and 42-kb upstream of 18S gene. In *Podocarpus* and *Afrocarpus*, it seems that the direction of transcription would be opposite as the rRNAs are encoded from complementary DNA strands (Figure 13). Also, we found cohybridisation of 26S, 18S and 5S probes in the Ephedraceae (Gnetales) species of our sample (Figure 12), consistent with FISH performed to the chromosomes of *Ephedra distachya* showing overlapping 5S and 35S signals (Figure 14). We also found additional, pseudogene-like copies, of 5S rDNA, together with one apparently functional 5S unit. According to Southern blot mapping and sequencing of *Ephedra* species, their rDNA units are similar to

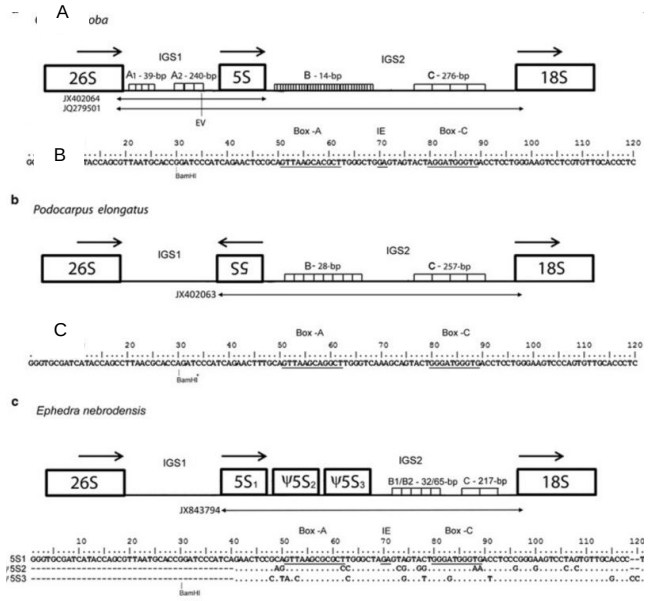

Figure 13. Schematic representation 35S–5S units in *Ginkgo biloba* (A), *Podocarpus elongatus* (B), and *Ephedra nebrodensis* (C). Arrows above boxes indicate direction of transcription. The lines with arrows represent clones carrying 5S insertions. Subrepeated portions of IGS are indicated and labelled as (A–C). The 5S coding region sequences are shown below each graph, with conserved regulatory elements underlined. The positions of *Bam*HI sites are indicated; in *Podocarpus* the site is mutated (asterisk). Extracted from [26].

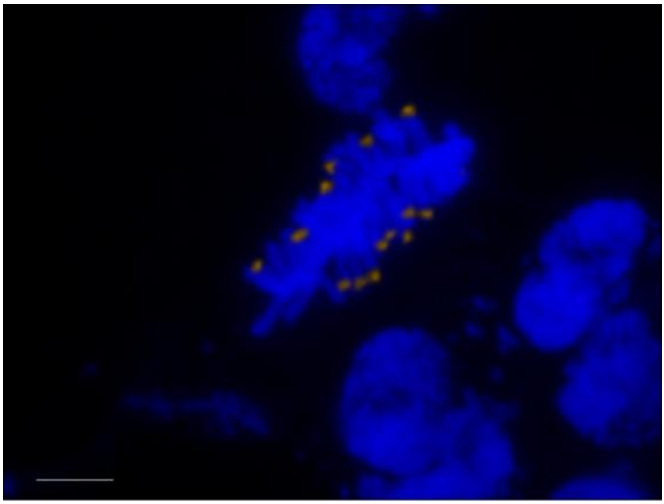

Figure 14. FISH to *Ephedra distachya* chromosomes (2n=28), showing 14 linked (5S-35S) rDNA signals. Scale bar 10 µm.

those of Pinaceae (Pinophyta) in many aspects, both being clearly different from the angiosperm pattern. Duplicated 5S copies had been also detected in other L-type species [26, 30]. However it seems that, again, not all Gnetales would display the L-type arrangement: the sequenced IGS clone from *Gnetum gnemon* appears to lack a 5S insertion (see Southern blot profile of the species in Figure 11, clearly showing separate 5S and 26S hybridization signals), but more research is needed to confirm this point.

3.3. Homogenised L-type rDNAs in Ginkgoales

Fluorescent *in situ* hybridisation showed linkage of 35S and 5S signals on *Ginkgo* chromosomes [28], but the PCR cloning analysis of [30] failed to demonstrate 5S linkage to the large rDNA cluster. In our assays, we also found a 5S insertion in direct orientation located downstream from the 26S gene (Figure 13), so the data support the hypothesis that the predominant organisation of *Ginkgo* rDNAs is L-type (Figure 12). Besides, the different orientation of 5S insertions in *Ginkgo* and *Podocarpus* suggests their independent origins.

The S-type arrangement dominates most angiosperm lineages [30] although in some families the L-type arrangement is quite frequent, as we

have shown. The phylogenetic relationships (Figure 15) indicate that the ribosomal RNA genes have changed their fundamental genomic organisation at least three times during the evolution of gymnosperms, similarly to our findings in Asteraceae.

Sequence analysis of 5S insertions shows interesting differences between angiosperms and gymnosperms. In angiosperms the 5S insertions occur in a non-repetitive part of the IGS quite close to the 26S gene, while in gymnosperms the 5S gene is located distally to the 26S and 18S genes, embedded in a highly repetitive DNA region as in bryophytes [11]. Up to our knowledge, the 5S genes of L-type angiosperms are encoded by the opposite DNA strand to the 35S genes while in gymnosperms both direct and inverse orientations occur. Moreover, retroelement signatures in close proximity of the 5S insertions in the IGS have been found in angiosperms [36], but not in gymnosperms.

Both cycads and *Ginkgo* are considered to be early diverging, living-fossil taxa [37] and it is remarkable that they show contrasting L- and S-type arrangements, respectively. Since both arrangements are also present in modern plants, it is likely that both are evolutionary neutral. However, the overwhelming prevalence of S-type arrangement in seed plants and vertebrates still needs to be explained.

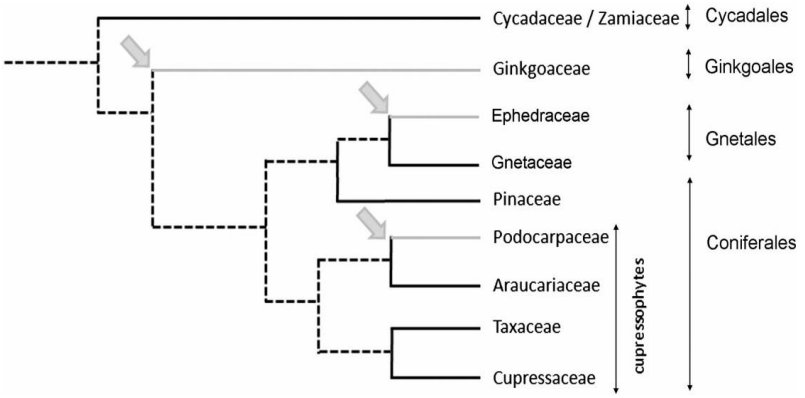

Figure 15. A proposed organisation of 5S–35S genes in gymnosperm phylogeny. Grey lines—L-type arrangement; black lines—S-type arrangement. Grey arrows show putative integration events of 5S genes into the 26S–18S IGS. Dashed lines—putative ancestors having either L- of S-type units. Extracted from [26].

4. Expression of rRNA genes in L-type arrangement

Despite numerous data confirming the existence of the L-type arrangement of rRNA genes in certain plants groups, the activity of such linked genes remained unknown. Therefore our next step was studying the homogeneity and expression of 5 S genes in several species from family Asteraceae, known to contain linked 35 S-5 S units, together with the determination of their methylation status by bisulfite sequencing [38]. Our purpose was to know which proportion (if any) of linked genes is expressed and functional.

For an adequate ribosome synthesis there has to be a stoichiometric ratio of rRNA molecules, but there are certain differences regarding the regulation of the transcription of the individual genes taking part of the ribosomal units. The 35S transcript is produced by RNA polymerase I and it is processed in the nucleolus to become 18S, 5.8S and 26S rRNA molecules. The 5S transcription is performed by RNA polymerase III and requires an internal promoter within the genic region (internal control region, ICR). This promoter consists on the A-box, an internal element (IE) typically two nucleotides, and a C-box (Figure 16). The internal promoter sequence is highly conserved among plants and animals [39]. The regulation of the synthesis of rRNAs is also epigenetically controlled: non-transcribed rRNA genes can be silenced by epigenetic processes involving chromatin modifications including DNA methylation [40, 41].

Using an approach that included RT-PCR, cloning, bisulfite sequencing and FISH to determine the expression, methylation status and chromatin condensation levels we showed that in several representative plant species the linked 5S genes are expressed and dominantly contribute to the cellular 5S rRNA pools [38].

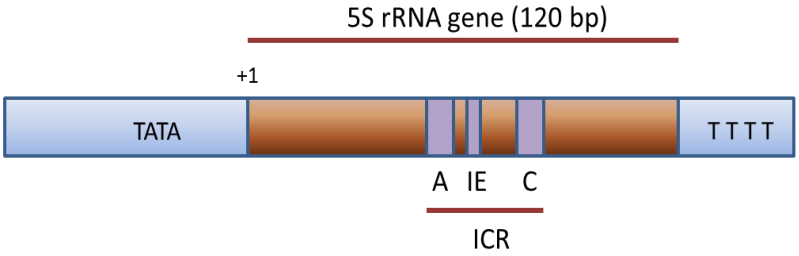

Figure 16. Structure of the 5S rRNA gene, showing the internal control region (ICR), which is subdivided into the A box, the intermediate element (IE), and the C box.

Another question that arose was the presence of minor separate rRNA genes (S-type arrangement) in species which are mostly L-type. Previously, Southern blot had shown almost totally homogenized linked 5S-35S units in several L-type genera of Asteraceae. However, several separate 5S tandems were also recovered in certain species (although in most L-type genomes there were no separate tandems). The interpretation was that these rare separate tandems likely came from loci that did not hybridise with 26S probe on Southern blots, although quantitative estimates by RT-PCR indicate that these are less than 10% in L-type genomes. Indeed, four types of rDNA arrangement were found: 1) completely homogenized L-type arrangement (with no 5S tandems outsides the linked units), as found in genera *Helichrysum*, *Matricaria* or *Tagetes*; 2) L_s-type, with most but not all rDNA units homogenized to the linked arrangement and some separate 5S tandems, as in genera *Artemisia* and *Gnaphalium*; 3) S_L-type, with a dominance of independent 5S tandems but few L-type rDNA units, like in genus *Elachanthemum*; 4) S-type arrangement with 5S independent tandems only, as found in most angiosperms.

4.1. Which 5S is transcribed?

In L_s genomes both L-type and S-type loci encode potentially transcribed genes, although the latter are much less abundant. It is known that only a fraction, sometimes quite reduced, of rRNA genes is transcribed in the cell while the rest is epigenetically inactivated, so it is interesting to set the origin of 5S rRNA transcripts. We suggest that the contribution to the total 5S rRNA pool from the scarce separate 5S tandem arrays is low, if any. Indeed, the linked 5S are being expressed, because:

1) 5S transcripts including IGS1 sequences (beyond the first termination signals) were identified, implying that transcribed 5S sequences actually stem from linked 5S genes,
2) no read-through transcripts were detected from tandemly arranged 5S-5S in L_s-type species,
3) the RNAs derived from linked genes adopted a secondary structure typical of a functional molecule according to the RNA folding simulations performed (Figure 17),
4) linked arrays contained undermethylated and decondensed chromatin fractions, likely corresponding to active genes.

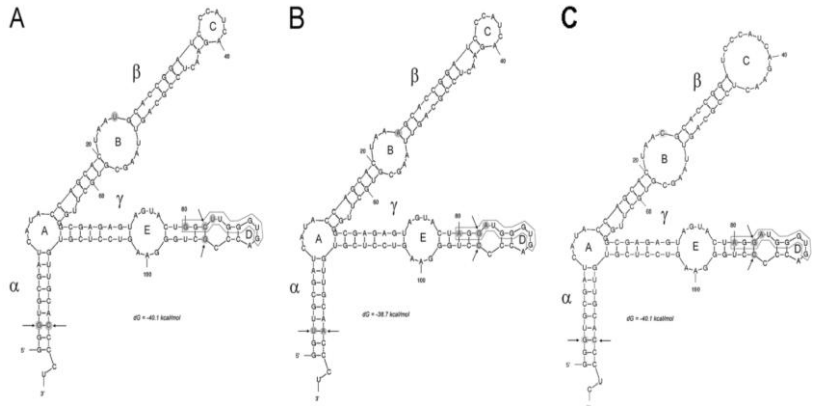

Figure 17. Secondary structure models for 5S rRNA from *Artemisia absinthium* (A), *Tagetes patula* (B) and *Helichrysum bracteatum* (C). Variable nucleotides highlighted by gray shading. Arrows indicate compensatory substitutions. The C-box element is boxed. Structural domains and loops are respectively in Greek and Latin letters. Extracted from [38].

4.2. Divergences in plant C-box consensus sequence

As pointed out previously, 5S rDNA contains regulatory elements (ICR) within the genic region, contrasting to most genes. The sequence and position of these elements (Figure 18) is highly conserved across eukaryotes [42]. However, it seems that certain Anthemideae species have evolved a variant of the C-box (C*) that is different from the angiosperm consensus. Interestingly, mutations in this region are related with reduced transcription rates in *Arabidopsis* [43]. Nevertheless, these changes in C-box do not affect the secondary structure of 5S rDNA which implies that the interactions between rRNA and transcription factor III are not damaged. The C*-box is found in some, but not all, L-type species (and the other way round) so it seems that there is no relationship between the occurrence of the promoter variants and the genomic arrangement of rRNA genes.

4.3. 5S rDNA methylation profiles of L-type and S-type species

The CG, CHG and CHH methylation profiles typically found in plant repetitive DNA [44] are also found in L-type and S-type species. Therefore it

Figure 18. Aligned C-box sequences from different plant and animal species. Shaded letters indicate conserved nucleotides in plants. Cassandra is a non-autonomous retroelement homologous to 5S gene (in this case, from *Rosa rugosa*). Variable nucleotides within the unique C*box are underlined and in bold. Extracted from [38].

seems that the tandem arrangement of 5S is not essential for its methylation, as 5S methylation patterns of L-type species are similar to those of S-type species. We found a significant rate of methylation in non-CG motifs in *Helianthus annuus* (with S-type arrangement) which shows a pericentromeric 5S locus, while much less methylated 5S are detected in *Tagetes patula* or *Artemisia absinthium*, presenting a terminal position of these genes. Previously researchers had found in *Arabidopsis* [45] that the 5S loci close to the centromeres were more methylated than other distally located, so it is likely that the specific location in chromosomes is related with its methylation rate.

4.4. Chromatin condensation and expression of rDNA

RNA polymerase I transcribes the 35S genes and occurs in the nucleolus and RNA polymerase III transcribes the 5S genes and is found in the nucleoplasm. Therefore transcription of both 5S and 35S cannot take place in the same unit at the same time, which may explain why we did not find products of bidirectional 26S-5S transcription. It is possible that the regulation of the transcription takes places at individual chromosome sites. While one chromosome homolog is acting as a NOR, transcribing 35S rRNA genes, the other could be transcribing the 5S rRNA genes. Figure 19 shows a late anaphase/telophase of *Helichrysum bracteatum* in which the nucleoli were assembled on one highly decondensed homolog, while the other was highly condensed and probably not involved in nucleolus assembly. The 35S-5S arrays closely associate with the nucleolus suggest that 5S transcription may occur in close proximity to the nucleolus, possibly at its periphery.

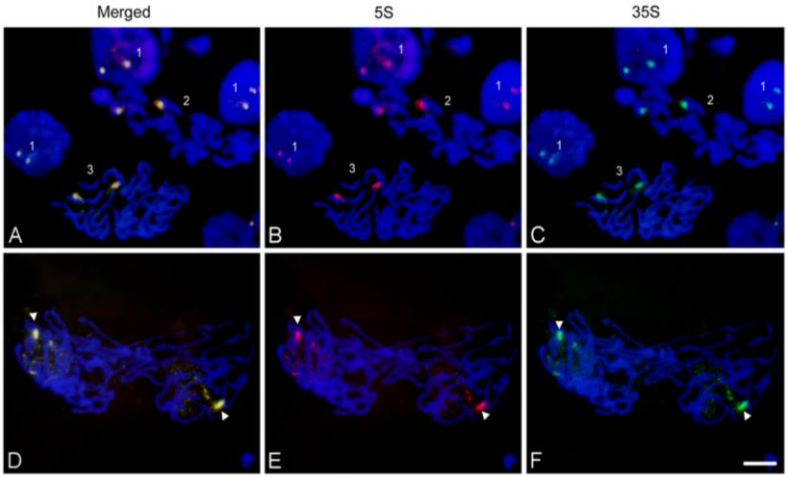

Figure 19. FISH of 5S (red) and 35S (green) rDNA probes to *Helichrysum bracteatum* nuclei. Pictures in the first row (A, B and C) show rDNA signals in interphase (field 1), metaphase chromosomes (field 2), and a prophasic cell (field 3). The second row (D, E and F) shows late anaphase/early telophase. One rDNA homolog is highly condensed (arrowheads) whereas the other is spread throughout the nucleus and decondensed. Pictures extracted from [38].

5. The Plant rDNA database

The idea of creating a database that stores information on number, position and structure of the 5S and 18S-5.8S-26S ribosomal DNA (rDNA) loci

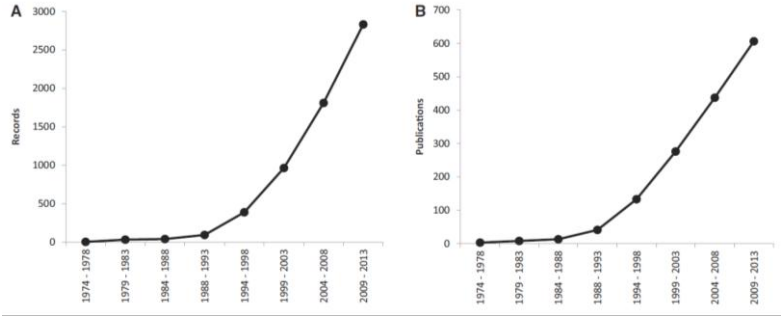

Figure 20. Mean number of records (A) and publications (B) reported per year over eight successive 5-year periods, between 1974 and 2013. Extracted from [49].

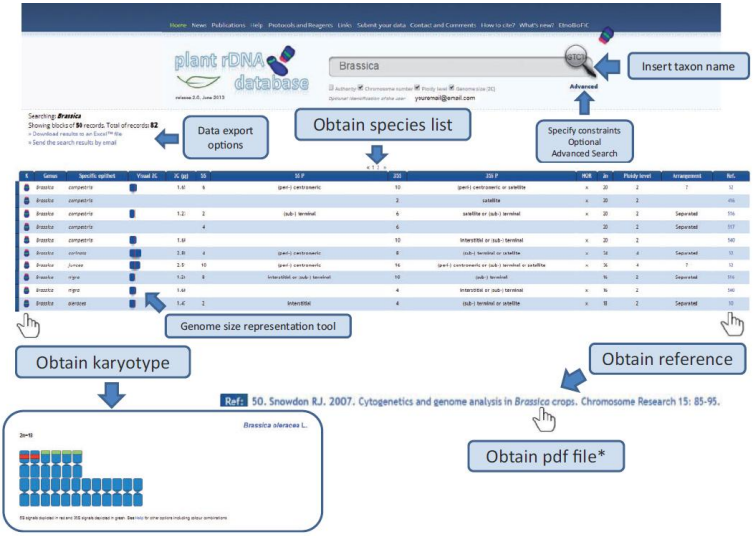

Figure 21. Flowchart showing the possible database outputs and options for a given search. The karyotype appears as a pop-up window, in which the 5S rDNAs are depicted in red and the 35S in green. Extracted from [49].

stemmed from our previous observations of particular rDNA linkages in certain plant groups (see parts 2 and 3 of this article). With the purpose of finding more linked rDNAs in plants, we started a review of any publication containing plant rDNA-FISH data. However, we realised that number, position and structure of rDNAs are useful cytogenetic traits that can help at species characterisation [46, 47], and we detected a growing interest within the scientific community to produce such data (Figure 20). Therefore, in order to store this information, and to make it accessible to a broader scientific community, an online resource, the Plant rDNA database (www.plantrdnadatabase.com) was developed in 2011 [48] and updated in 2013 [49]. The data comes from fluorescent *in situ* hybridisation experiments (FISH) and additional information is also provided, such as ploidy level, mutual arrangement of rRNA genes, genome size and life cycle. The webpage is intuitive and user-friendly, including different search options (Figure 21).

6. Further work

The next steps in our quest on ribosomal DNA will take a deeper look on the evolutionary role of the 5S rRNA gene across the tree of life. In particular, we will explore the evolutionary models of concerted evolution and birth-and-death and we will see how these adjust to globally explain the evolution of this ubiquitous and unquiet sequence. We are also interested in what is the participation of transposable elements in its mobility. Besides, we will continue working on the Plant rDNA database: a new update is planned to be released in 2016 and a global analysis of the data contained is also being prepared.

Acknowledgements

We acknowledge Professor Joan Vallès for his careful reading and corrections performed on the manuscript, as well as all members of our research team, www.etnobiofic.com which have participated at different stages of the project. This work was supported by Ministry of Economy and Competitiveness from the Government of Spain (CGL2010-22234-C02-01,02/BOS and CGL2013-49097-C2-2-P), the Generalitat de Catalunya, Government of Catalonia ("Ajuts a grups de recerca consolidats", 2009SGR0439 and 2014SGR514) and the IRBio (Institut de Recerca de la Biodiversitat, Universitat de Barcelona). SG benefited from a “Ramón y

Cajal" researcher contract of the Ministry of Economy and Competitiveness from the Government of Spain.

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Research Signpost
37/661 (2), Fort P.O.
Trivandrum-695 023
Kerala, India

Recent Advances in Pharmaceutical Sciences VI, 2016: 77-90 ISBN: 978-81-308-0566-5
Editors: Diego Muñoz-Torrero, Àngela Domínguez and Àngels Manresa

5. The role of the c-Jun N-terminal kinase (JNK) pathway in insulin resistance

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Abstract. Obesity is usually associated with a decreased response to insulin, a major metabolic defect known as insulin resistance and an early trait in the development of type 2 diabetes. The c-Jun N-terminal kinase (JNK) pathway has emerged as a central regulator of insulin sensitivity, locally and systemically, thereby, of body's metabolic homeostasis. As the incidence of obesity and type 2 diabetes has alarmingly increased in the last few decades, there is a tremendous necessity to identify novel pharmacological targets to efficiently improve the therapeutic outcome. In this regard, the JNK pathway seems to meet most of the requirements for being an adequate candidate to direct pharmacological intervention.

Introduction

The incidence of type 2 diabetes is alarmingly increasing worldwide specially as a result of the tremendous rise in obesity, a major risk factor for

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the development of this disease. Decreased sensitivity of target tissues to insulin, known as insulin resistance, is an early trait in the development of type 2 diabetes and results in glucose intolerance; afterwards, failure of pancreatic insulin-secreting cells (β-cells) is the major determinant of progression to hyperglycemia, the hallmark of diabetes. Insulin resistance is strongly associated to obesity; its incidence in the population parallels that of obesity; and represents the link of obesity to type 2 diabetes [1].

Insulin resistance is a major metabolic disorder and develops because defective insulin receptor (IR) signaling in insulin-target tissues such as liver, adipose tissue and skeletal muscle, among others. The etiology of insulin resistance has been the focus of intensive research, and it is currently accepted that chronic inflammation is an important cause [2]. In this regard, obesity, which is the most common origin for the development of insulin resistance, is usually associated with a low-grade, chronic inflammatory state that initially affects the adipose tissue but at latter stages spreads out to other tissues because the fat accumulation at these anomalous locations [3]. Other conditions associated to the expansion of the adipose tissue such as the increased production of reactive oxygen species (ROS) or the endoplasmic reticulum (ER) stress also promote insulin resistance either directly or through the enhancement of the inflammatory response [4]. At molecular level, pro-inflammatory cytokines are main players in promoting insulin resistance, as they are activators of signaling pathways, such as the c-Jun N-terminal kinase (JNK) and the inhibitor of nuclear factor κ-B kinase (IKK) pathway, that negatively interfere with the early steps in the IR signaling cascade [5]. In this chapter we are going to focus the attention on the physiopathological role of the JNK pathway on the regulation of insulin sensitivity.

1. The JNK signaling pathway

JNK comprises a group of serine/threonine protein kinases that belongs to the stress-activated protein kinase (SAPK) subfamily of mitogen-activated protein kinases (MAPKs). MAPKs participate in ubiquitous and evolutionary conserved signal transduction pathways in eukaryotes. MAPK pathways enable coordinated responses to a vast array of stimuli regulating different cellular processes such as gene transcription, mRNA stability, protein synthesis, cell proliferation, cell differentiation, or apoptosis. The

MAPKs are the final step of a protein kinase cascade (the MAPK module) composed, in addition to the MAPK, by a MAPK kinase (MAP2K), and a MAP2K kinase (MAP3K), which are sequentially activated through phosphorylation by the upstream kinase. In addition, non-enzymatic scaffold proteins may also participate in these MAPK modules to stabilize physical interactions and/or promote recruitment of the components. MAPK modules enhance efficiency and selectivity in signal transmission. MAPKs are activated by dual phosphorylation on tyrosine and threonine residues within the evolutionary conserved Thr-Xxx-Tyr motif located in their activation loop. MAPKs are proline directed kinases thus; they phosphorylate serine and threonine residues followed by a proline, though engagement of substrates requires additional interactions out of the catalytic site mediated by specific docking sites located apart of the phosphorylation sites. MAPKs are inactivated by dephosphorylation performed by protein phosphatases such as the MAP kinase phosphatase (MKP)/dual specificity phosphatase (DUSP)-1 [6].

JNK was initially identified as the major protein kinase responsible for the phosphorylation of residues at the N-terminal/transactivation domain of c-Jun, a component of the AP-1 complex, triggering its transactivation function (Fig. 1) [7-9]. The JNK signalling pathway is preferentially activated by pro-inflammatory signals, such as tumor necrosis factor (TNF)-α or interleukin (IL)-1β, and stress stimuli, such as ultraviolet (UV), ER stress and ROS. Consistently, JNK pathway is a target of anti-inflammatory drugs such as glucocorticoids, which efficiently repress the activation of the JNK pathway by different mechanisms [10-12].

JNKs are encoded by three genes, *jnk*-1, *jnk*-2, and *jnk*-3, which through alternative splicing mechanisms give rise up to twelve different isoforms. Expression of *jnk*-1 and *jnk*-2 is ubiquitous whereas *jnk*-3 is expressed in a tissue specific manner [13,14]. The MAP2K MKK4 and MKK7 catalyze the phosphorylation, and concomitant activation, of JNK. Whereas MKK4 also phosphorylates the MAPK p38, MKK7 is highly specific for JNK and other substrates have not been identified so far for this protein kinase. Accordingly, MKK4 and MKK7 show nonredundant functions *in vivo*. For instance, MKK4 activates JNK in response to stress stimuli, such as anisomycin or heat-shock, whereas MKK7 is required for JNK activation by pro-inflammatory signals [15,16]. Consistent with the variety of stimuli that activate the JNK pathway, a diverse group of MAP3Ks phosphorylate, and concomitantly activate, MKK4 and/or MKK7 [6].

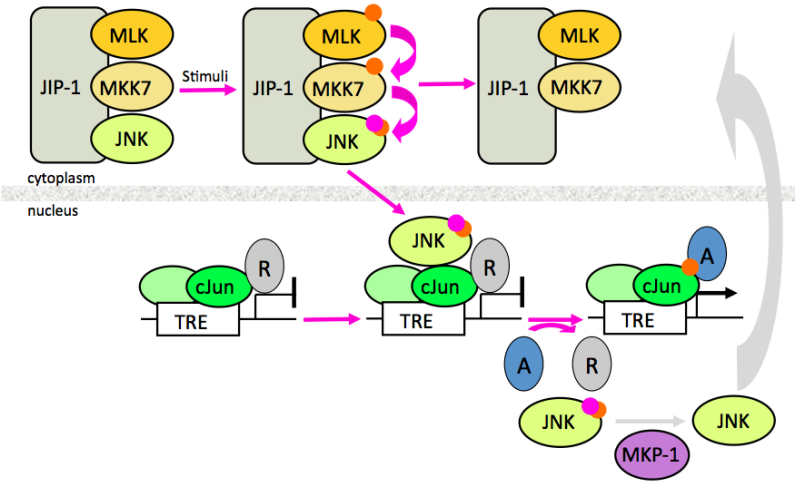

Figure 1. Regulation of the AP-1-dependent gene transcription by the JNK pathway. In non-stimulated conditions, protein kinases (MAP3K: MLK, MAP2K: MKK7, and MAPK: JNK) and scaffolds (JIP-1) of the JNK pathway module are pre-assembled in the cytoplasm but inactive. In the nucleus, AP-1 complexes are associated to their cognate DNA binding elements, known as TPA response elements (TREs), but the interaction of co-repressors (R) with c-Jun, a component of the AP-1 complex, inhibits transcription of the AP-1 target genes. Upon stimulation, the protein kinases in the module are sequentially activated by phosphorylation (phosphotyrosine and phosphoserine/phosphothreonine are indicated in magenta and orange dots, respectively). Activated JNK dissociates from the module and translocates into the nucleus where interacts and phosphorylates c-Jun at specific sites in its transactivation domain. c-Jun phosphorylation induces the dissociation of co-repressors allowing the interaction with co-activators (A), consistently, the AP-1-dependent gene transcription is induced. MKP-1, a nuclear protein phosphatase, inactivates JNK by dephosphorylation, and inactive JNK returns to the cytoplasm.

2. The IR signaling pathway

Insulin is a hormone secreted by the pancreatic β-cells in response to an increase in glycemia. It has very important endocrine functions mostly, but not exclusively, related to carbohydrate and lipid homeostasis. Insulin reduces glycemia by promoting glucose uptake in adipose tissue and skeletal muscle and suppressing hepatic gluconeogenesis. In addition, insulin promotes glycogen, lipid and protein synthesis in target tissues. Insulin signaling is triggered by its binding of the IR, a receptor that belongs to the

tyrosine kinase receptor family and that is composed by two extracellular subunits (α chains) and two transmembrane subunits (β chains). Hormone engagement activates the intrinsic tyrosine kinase activity of the IR resulting in the autophosphorylation on the β subunit cytoplasmic tails. These phosphotyrosine residues are docking sites for the recruitment of proteins from the IR substrate (IRS) family, such as IRS-1 or IRS-2. IRS-1/2 are also phosphorylated on tyrosine residues by the IR resulting in the generation of a docking platform for proteins of the IR signaling, such as the subunit of the phosphatidylinositol-3-kinase (PI3K), p85, or the Grb2-SOS adaptor Shc.

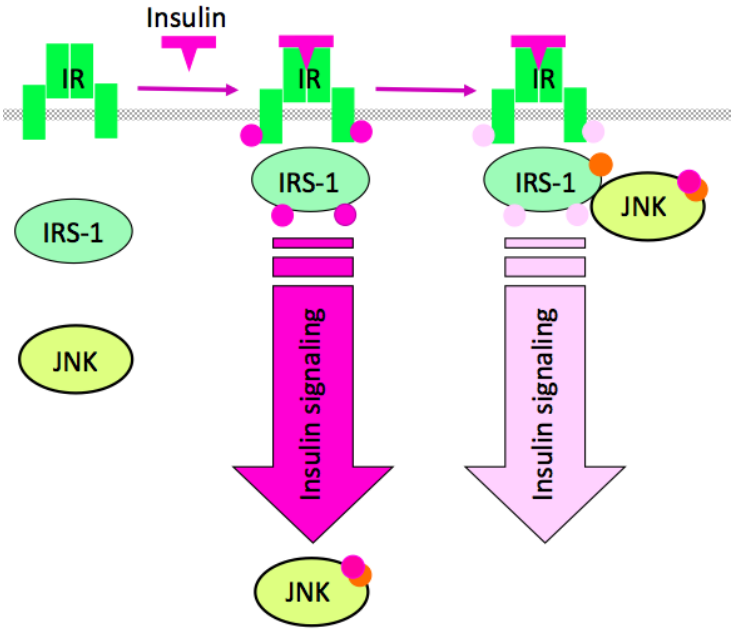

Figure 2. Insulin-induced JNK activation inhibits IR signaling by phosphorylation of IRS-1 on serine-307. Insulin binding to the IR triggers its tyrosine kinase activity eliciting the autophosphorylation on tyrosine residues (magenta dots) of the cytoplasmic tails of the IR β subunits. These phosphotyrosine residues are docking sites for the recruitment of proteins from the IRS family such as IRS-1. Upon interaction, IRS-1 is phosphorylated on tyrosine residues by the IR resulting in the generation of a docking platform for the interaction with a variety of proteins involved in IR signaling. Insulin induces the activation of JNK, which in turn binds to IRS-1 and phosphorylates it on serine-307 (orange dot). IRS-1 phosphorylation on serine-307 disrupts its interaction with the IR ensuing the physiological down-regulation of IR signalling.

IR-activated PI3K promotes AKT activation, which eventually elicits the regulation of glucose metabolism and protein synthesis, and, through the sterol-regulatory element binding protein (SREBP)-1c, the metabolism of lipids. In contrast, insulin mitogenic effects are mostly mediated by the induction of the SOS-Ras-extracellular signal-regulated protein kinase (ERK) signaling pathway (Fig. 2) [17].

IRS-1 and IRS-2 are tightly regulated by phosphorylation on multiple serine/threonine residues mediated by several insulin-stimulated protein kinases, an autologous mechanism that regulates, positively or negatively, insulin sensitivity [18]. Concretely, IRS-1 phosphorylation on serine-307 is induced by insulin and inhibits IRS-1 interaction with the insulin-activated IR, thereby inhibits IR signaling. Serine-307 is targeted by JNK, which is activated in response to insulin and in this manner operates as a negative feedback mechanism to turn off IR signalling in physiological conditions (Fig. 2) [19-21]. Likewise, IRS-2 is also phosphorylated by JNK on serine-488 with similar functional consequences [22].

3. Obesity-induced insulin resistance and the JNK pathway

The relationship between obesity-induced insulin resistance and inflammation originates with the observation of an increased TNF-α gene expression in adipose tissue of obese rodents, which turns into increased levels of this cytokine locally and, eventually, systemically. Moreover, TNF-α neutralization in these animals improved insulin sensitivity [23]. In the following years, many studies have contributed to decipher the cellular and molecular mechanisms underlying the role of inflammation in the promotion and progression of obesity-induced insulin resistance, however most of them are out of the scope of this revision hence they will not be mentioned but are profoundly reviewed in references 2 to 5.

In obesity there is an overexpansion of the adipose tissue that is accomplished by processes of adipocyte hyperplasia and hypertrophy. Hypertrophic adipocytes undergo a series of perturbations such as an increased demand on the synthetic machinery, an accumulation of unfolded proteins in the ER or an excessive production of ROS that generate stress in the ER. To restore organelle functionality a response known as the unfolded protein response (UPR) emanates from the ER. The UPR is a complex response system with different branches but notably one of them, mediated by the inositol-requiring enzyme (IRE)-1, activates JNK through a pathway involving TNF-receptor associated factor (TRAF)-2 [24]. This activation of JNK leads to IRS-1/2 phosphorylation with the concomitant inhibition of IR

signaling. In addition, the JNK pathway-induction of pro-inflammatory gene expression works as a feed-forward mechanism to further increase JNK activity. In this scenario, this chronically activated JNK leads to the promotion of insulin resistance (Fig. 3). In addition, in obesity there is an increase in the systemic level of free fatty acids (FAA), which by binding to the Toll-like receptors activate the JNK pathway and, thereby, foster insulin resistance [25].

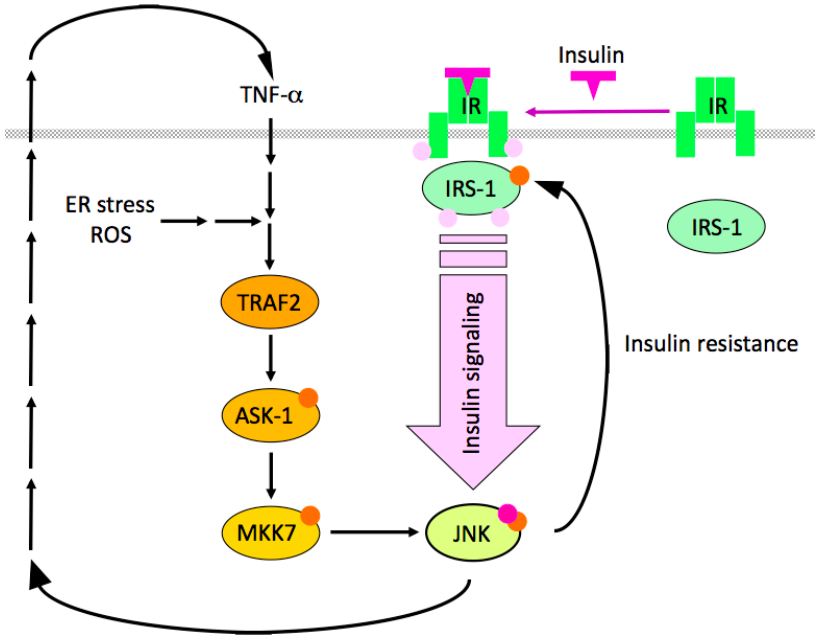

Figure 3. Obesity-induced JNK activation induces insulin resistance through IRS-1 phosphorylation on serine-307. In obesity there are conditions, such as the increased level of pro-inflammatory cytokines (TNF-α) and ROS, or the induction of ER stress, that exacerbate the activity of the JNK pathway by a TRAF-2-dependent induction of phosphorylation, and concomitant activation, of the protein kinases of the JNK module (magenta and orange dots on ASK-1, MKK7 and JNK, respectively). This obesity-induced JNK activity inhibits IR signaling through the phosphorylation of IRS-1 on serine-307 (orange dot). In addition, enhanced expression of pro-inflammatory mediators, such as the TNF-α, mediated by the JNK pathway further contributes to the inhibition of IR signaling mediated by the JNK-dependent IRS-1 phosphorylation. Thereby, in this scenario of chronically exacerbated JNK activity insulin resistance develops.

In accordance with this, JNK activity is augmented in tissues such as liver, adipose tissue, and skeletal muscle of obese humans [26] and rodents [27]. Moreover, a central role of the JNK pathway on obesity-induced insulin resistance is also substantiated by genetic data.

Epidemiologic studies identified the gene encoding the JNK interacting protein (JIP)-1, a scaffold protein of the JNK module, as a candidate for type 2 diabetes [28]. In addition, JIP-1-genetic deficiency in mice prevents obesity-induced activation of JNK in adipose tissue and protects from diet-induced obesity and insulin resistance [29].

Moreover, *jnk*-1-deficient mice are protected from the development of adiposity and show significantly improved insulin sensitivity and enhanced IR signalling in diet- and genetically-induced obesity mouse models [27]. This study demonstrated that JNK-1 isoform has a prominent role in obesity-induced insulin resistance given that *jnk*-2-deficient mice showed a wild type phenotype [27]. However, the fact that *jnk*-2-deficient mice heterozygous for *jnk*-1 are also protected from diet-induced obesity and insulin resistance points to a compensatory role of JNK-1 in the absence of JNK-2 [30].

The pivotal role of JNK in obesity-induced insulin resistance has strongly instigated the pharmacological targeting of the JNK pathway as a therapeutic approach for the treatment of type 2 diabetes. In this regard, treatment of obese mice with a small molecule inhibitor of JNK improved insulin sensitivity [31].

Notably, it has been described that thiazolidinediones (TZDs), a group of synthetic peroxisome proliferator-activated receptor (PPAR)-γ ligands used in clinics because their insulin-sensitizing activity [32], are active in the inhibition of obesity-induced JNK activity in insulin-target tissues such as liver and adipose tissue. Indeed, the lack of TZD antidiabetes action in obese *jnk*-1-deficient mice indicates that JNK inhibition mediates this pharmacological property. Moreover, PPAR-γ is expressed in immune cells, such as macrophages, and in adipocytes; therefore, TZDs may inhibit JNK in both cellular compartments, immune and metabolic, of the adipose tissue, and regardless the nature of the stimuli, a pro-inflammatory cytokine or a stress mediator (Fig. 4) [33].

The contribution of tissue-specific JNK activity on insulin sensitivity has been studied in transgenic mice by tissue specific genetic inactivation of *jnk*-1, or to a lesser extend *jnk*-2, or over-expression of dominant-negative or constitutively-activated forms of JNK or its immediately activating kinase, MKK7.

Adipose-tissue specific *jnk*-1 genetic ablation does not prevent from adiposity but protects from the development of insulin resistance in adipose tissue and liver induced by a high-fat diet. This effect on hepatic insulin sensitivity seems to be mediated by the reduction in the interleukin (IL)-6 release from the adipose tissue [34].

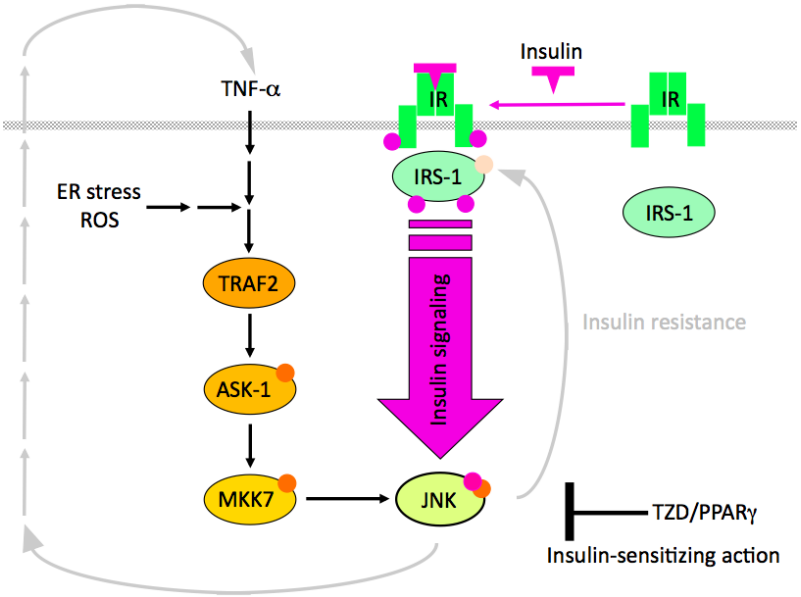

Figure 4. TZD insulin-sensitizing action is mediated by inhibition of the JNK pathway in adipocytes and macrophages. In obesity, the JNK pathway is activated by different stimuli and inhibits IR signaling through IRS-1 phosphorylation on serine-307 (see text and legend of Fig. 3). TZDs inhibit obesity-induced JNK activity, an action that requires their interaction with PPAR-γ, and, in this way, restore IR signaling and, thereby, insulin sensitivity.

In the liver, overall JNK activity inhibition by adenovirus-enforced overexpression of a dominant-negative version of JNK or by simultaneous inactivation of *jnk*-1 and *jnk*-2 protects against insulin resistance in diet-induced obese mice [35,36]. In addition to the negative action of JNK on IR signaling through IRS-1/2 phosphorylation, the inhibitory role of JNK on the PPAR-α/fibroblast growth factor (FGF)21 pathway also contributes to this protective phenotype [36-38]. Nonetheless, there is some

controversy regarding the specific role of each liver JNK isoform on systemic glucose metabolism. On one hand, knock down of *jnk*-1 or *jnk*-2 gene expression in the liver of obese animals resulted in reduced hyperglycemia and hyperinsulinemia but with distinct effects on hepatic steatosis [39,40]. However, single *jnk*-1 genetic ablation specifically in hepatocytes induced glucose intolerance, insulin resistance and hepatic steatosis in lean mice and had no effect on obese animals, indicating that this isoform has a protective function in this particular cell type [41]. This discrepancies may be due to a dual role of JNK-1 in hepatocytes that is, in addition to down-regulate IR signalling, JNK-1 is required for gluconeogenesis, lipogenesis and clearance of circulating insulin [41], in addition to not yet characterized interactions among the distinct cell types in the liver. Finally, activation of the JNK pathway by adenovirus-mediated over-expression of wild type JNK decreased insulin sensitivity and augmented insulinemia due to an increased hepatic glucose production [35].

Regarding skeletal muscle, whereas *jnk*-1-deficiency specifically in this tissue does not protect from diet-induced obesity, it ameliorates systemic insulin resistance by preserving IR signalling locally. This protective effect is not widespread to adipose tissue or liver, in fact, theses mice showed enhanced hypertriglyceridemia, due to a reduction on muscle lipoprotein lipase, macrophage infiltration in adipose tissue and hepatic steatosis [42]. In contrast, JNK over-expression studies in skeletal muscle have rendered contradictory and therefore, require further analysis [43,44].

JNK has also been implicated in promoting insulin resistance in pancreatic β-cells. In particular, FFA administration results in JNK activation with the concomitant inhibition of IR signalling in these cells [45]. Moreover, *in vivo* activation of JNK by over-expression of a constitutively active version of MKK7 in pancreatic β-cells leads to glucose intolerance due to the failure to secrete insulin in response to glycemia. Static insulin secretion assays with isolated pancreatic islets showed that JNK activation blocks the second phase of glucose-induced secretion of insulin, which depends on IR signalling [46]. In addition, JNK might be involved in pancreatic β-cell failure, as it is required, though not sufficient, for pro-inflammatory cytokine-induced apoptosis in this cell type [46,47].

Finally, JNK also regulates glucose metabolism from the central nervous system (CNS). As in peripheral tissues, obesity increases JNK activity in the hypothalamus [48], and CNS-specific *jnk*-1-genetic ablation

protects mice from diet-induced obesity, glucose intolerance and insulin resistance [49,50]. This phenotype is achieved through augmented energy expenditure and locomotor activity [49,50]. Moreover, a similar phenotype was also observed in genetically modified mice lacking JNK-1 and JNK-2 in the adenohypophysis [51]. In contrast, JNK activation in the agouti-related peptide (AgRP)-expressing neurons of the hypothalamus induces obesity as a consequence of hyperphagia [52]. Altogether, these results indicate that at the CNS JNK regulates the activity of the hypothalamic-pituitary-adrenal axis [49-52].

4. Conclusion

The JNK pathway is currently regarded as a promising candidate for the pharmacological treatment of insulin resistance and type 2 diabetes because its pivotal role in the control of insulin sensitivity. For instance, the ability of the insulin-sensitizing drugs TZDs to inhibit the JNK pathway is fundamental for their pharmacological action. Even though in most of the experimental models studied exacerbated activity of the JNK pathway is detrimental for metabolic homeostasis, some examples showed that inhibition of JNK pathway activity in a particular tissue has negative consequences in others. In addition, incipient studies have demonstrated compensatory as well as non-redundant functions between the different JNK isoforms. Therefore, tissue-specific and isoform-specific JNK actions on metabolic control require further investigations for the design of the most advantageous therapeutic strategy.

Acknowledgements

This work was supported by the Plan Nacional I+D grants SAF2013-40832-P and SAF2014-57856-P from the Spanish Ministry of Economy and Competitivity. JL-M and JD-D, and MIA were recipients of fellowships from FPI, and FPU programs from the Spanish Government, respectively. CB was supported by an ADR fellowship from University of Barcelona, Spain.

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Research Signpost
37/661 (2), Fort P.O.
Trivandrum-695 023
Kerala, India

Recent Advances in Pharmaceutical Sciences VI, 2016: 91-110 ISBN: 978-81-308-0566-5
Editors: Diego Muñoz-Torrero, Àngela Domínguez and Àngels Manresa

6. Invasive pneumococcal disease in children: Risk factors and vaccine effectiveness

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Abstract. Invasive pneumococcal disease (IPD) has high morbidity and mortality worldwide. The overall incidence of IPD in Catalonia in 2005-2009 was 16.6 per 100,000 persons-year, 66.4 in children aged < 2 years and 50.7 in children aged 2-4 years. 7-valent pneumococcal conjugate vaccine (PCV7) coverage in Catalonia is intermediate. A prospective matched case-control study in children aged 3-59 months treated at two hospitals in Catalonia during 2007-2009 was performed. Potential risk factors for IPD and PCV7 effectiveness in preventing IPD were investigated. 293 cases and 785 controls were included. Attendance at daycare or school was a risk factor for IPD (aOR 3.07, 95% CI 1.97-4.78) and the effectiveness of PCV7 against vaccine serotypes was 93.7% (95% CI 51.8 -99.2).

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Introduction

Invasive pneumococcal disease (IPD) causes high morbidity and mortality worldwide. The estimated burden of IPD in children aged <5 years is 14.5 million annual episodes, and about 800,000 deaths, mostly in developing countries [1]. In developed countries, children aged <2 years, older adults and people with immune deficiency are at increased risk of IPD [2].

The 7-valent pneumococcal conjugate vaccine (PCV7) including seven of the 90 *Streptococcus pneumoniae* serotypes (4, 6B, 9V, 14, 18C, 19F and 23F) was marketed in 2000 in the US and 2001 in Europe. Studies of PCV7 effectiveness corroborated previous efficacy results [3-5]. The 10-valent pneumococcal conjugate vaccine (PCV10) was marketed at the end of 2009 and the 13-valent conjugate pneumococcal vaccine (PCV13) in 2010. PCV13 replaced PCV7 and contains PCV7 serotypes plus serotypes 1, 3, 5, 6A, 7F and 19A. PCV10 contains the PCV7 serotypes plus serotypes 1, 5, and 7F. The decline in PCV7 serotypes caused a decrease in IPD incidence which, however, then increased slightly due to the emergence of serotype 19A [6].

During 2005-2009 in Catalonia, the overall incidence of IPD was 16.6 cases per 100,000 persons-year. The highest incidence was in children aged <2 years (66.4 cases per 100,000 persons-year), children aged 2-4 years (50.7 cases per 100,000 persons-year), and persons aged ≥ 65 years (35.5 cases per 100,000 persons-year). In this period, PCV7 serotypes accounted for 17.5% of cases, PCV10 serotypes for 48.6%, and PCV13 serotypes for 69.8% [7]. In children aged <2 years, in 2008, PCV7 serotypes accounted for 19.4% of cases, PCV10 serotypes for 41.8% and PCV13 serotypes for 77.6% [8]. PCV7 is not included in the Spanish routine vaccination schedule (except in the Autonomous Community of Madrid during 2006-2012 and in Galicia in January 2012), although it was administered in children aged <5 years with risk medical conditions [9] following international recommendations [10].The Spanish Association of Pediatrics recommended the administration of the vaccine in 2003 [11]. PCV7 vaccination coverage in Catalonia in 2007-2009 was around 50% [12].

In Spain, where there is widespread use of antibiotics [13], an increase in IPD cases caused by multiresistant clones expressing non-vaccine serotypes [14] was detected. This made it necessary to determine the risk factors associated with these cases.

From the public health perspective it is important to determine the factors related to the host (age, genetic factors, risk medical conditions),

socio-demographic factors (daycare, social class) and clinical factors possibly associated with IPD [15-17].

The objectives of this study were to investigate risk factors for IPD and for non-penicillin susceptible strains, to evaluate factors associated with PCV7 vaccination and to investigate the effectiveness of PCV7 against IPD caused by vaccine serotypes in children aged <5 years in Catalonia.

1. Methods

Study design

We made a matched case-control study in 2007-2009 in two pediatric hospitals in Barcelona (Sant Joan de Deu Hospital and Valle Hebron Hospital). A case was defined as a patient aged <5 years attended with clinical signs of infection and microbiological confirmation (culture or detection of *Streptococcus pneumoniae* DNA by PCR) in normally sterile fluid [12].

Serotyping was made by Quellung reaction or PCR [18, 19]. Susceptibility to penicillin was studied by agar dilution (defined according to the 2008 Clinical Laboratory Standards Institute cutoffs) [20]. Controls were patients aged 3-59 months treated by the two hospitals for other reasons than infectious diseases. Three controls per case matched by sex, age (<12 m: +/- 3m; 12-23 m: +/- 6m; 24-59 m: +/-12m), date of hospitalization and underlying disease (when present) were selected. Cases and controls in whom the PCV7 vaccine status was unknown, and cases in whom the serotype could not be determined, were excluded.

The clinical variables studied were age, sex, and medical risk condition. In cases, signs and symptoms, ICU admission, evolution, serotype and penicillin susceptibility were collected. Daycare or school attendance, home exposure to smoking (number of cigarettes/day), number of cohabitants, age of siblings, breastfeeding in the previous month, antibiotics in the previous month, and respiratory infection in the previous month were collected using a single questionnaire. The parents' socioeconomic status according to the British occupation classification was collected [21].

PCV7 vaccination (date and number of doses) was collected through the vaccination card, medical record, or health center record. A vaccinated patient defined as vaccine reception ≥ 15 days before symptom onset (if

any) or hospital admission date (controls) and a fully vaccinated patient as reception of the recommended doses according to age.

The minimum sample size was calculated using Schlesselman's criteria [22]. A prevalence of vaccination of 25% in the healthy population (data before the start of the study in Catalonia) [23] and a PCV7 effectiveness against IPD of 80% was assumed. It was assumed that vaccine serotypes produce 20% of cases. With an alpha error of 0.05 (two-tailed), a beta error of 0.20 and three controls per case and analyzing two age groups its was estimated that 270 cases and 810 controls were required.

The vaccine effectiveness (VE) was calculated in cases with vaccine serotypes, non-vaccine serotypes, and all serotypes in the 24-59 months and 7-23 months age groups. The study was approved by the ethics committee of each hospital.

Statistical analysis

Crude odds ratios (OR) and adjusted odds ratios (aOR) of the associations between study variables and PCV7 vaccination (fully vaccinated and non-vaccinated) were calculated using unconditional multivariate logistic regression for all cases and controls. To study possible risk factors and IPD in cases and controls, multivariate conditional logistic regression was made and independent variables with a significance of $p < 0.2$ included. The vaccination history, daycare or school attendance, and antibiotic treatment were included in the analysis because of their clinical relevance. Unconditional logistic regression was used to calculate the OR and aOR of the association between study variables and non-penicillin susceptible strains in all cases of IPD. VE was calculated using the formula VE = (1- aOR) x 100. All analyses were made using SPSS version 18 (SPSS Inc. USA).

2. Results

Risk factors associated with IPD

We included 293 cases and 785 controls [12]. Over 60% of cases were confirmed by PCR. Pneumonia with empyema was the most frequent clinical form (64.5%), followed by uncomplicated pneumonia (15.7%). Non- PCV7 serotypes accounted for 91.1% of cases, PCV10 serotypes for

39.2% and PCV13 serotypes for 70%. The most common serotypes were 1 (21.2%), 19A (16.0%), 3 (12.6%) and 7F/A (6.8%). Diseases predisposing to IPD were present in 1.4% of cases (n = 4) (immunosuppression in 2 cases, diabetes mellitus in 1 and pulmonary emphysema in 1 case). In all four cases, IPD was caused by non-PCV7 serotypes and had received ≥ 1 PCV7 doses. The case fatality rate was 1% (3 cases, all with meningitis and due to serotypes 6A, 7F/A, and 23F). Of controls, 47.1% were correctly vaccinated with PCV7, while 58.7% had received ≥ 1 doses.

Daycare or school attendance (aOR 3.07, 95% CI 1.97-4.78) and > 4 cohabitants (aOR 2.0, 95% CI 1.37-2. 90) were global risk factors for IPD. Daycare or school attendance were risk factors for IPD in the 12-23 months (aOR 4.89, 95% CI 2.25-10.25) and 24-59 months (aOR 2.82, 95% CI 1.13-7.05) age groups. Other risk factors were > 4 cohabitants in the 24-59 months age group (aOR 2.04, 95% CI 1.27-3.28) and previous respiratory infection in the 12-23 months age group (aOR 1.98, 95% CI 1.02-3.86).

Protective factors against IPD were reception of ≥ 1 doses of PCV7 in the 3-11 months (aOR 0.35, 95% CI 0.14-0.86) and 12-23 months (aOR 0.47, 95% CI 0.24-0.94) age groups, but not in the 24-59 months age group (aOR 1.01, 95% CI 0.67-1.52). Children aged 24-59 months who had received antibiotic treatment in the previous month had greater protection against IPD (aOR 0.51, 95% CI 0.29-0.89). Other variables (having siblings aged <5 years, breastfeeding in the previous month and exposure to smoking in the home were not risk factors for IPD.

Serotype 1 was associated with the 24-59 months age group, pneumonia, pneumonia with empyema, and daycare or school attendance. All IPD cases due to serotype 1 were penicillin susceptible (Table 1). Serotype 19A was associated with age <24 months, pneumonia with empyema, previous respiratory infection and non-penicillin susceptible strains. Serotype 3 was associated with pneumonia and empyema, and reception of ≥1 dose of PCV7. Serotype 7F was not associated with any of the variables studied [24].

Penicillin susceptibility was analyzed in all strains (115): 40 (34.8%) were not susceptible. Children aged 24-59 months had a lower risk of non-penicillin susceptible IPD than those aged 3-23 months. Antibiotic treatment during the previous month and serotype 19A were risk factors for non-penicillin susceptible IPD [12] (Table 2).

Table 1. IPD risk factors associated with serotypes in children aged 3-59 months.

	Serotype 1 (n=62) %	Others (n=231) %	aOR (95%CI)
Age			
3-23 months	**8.1**	**47.6**	**Reference**
24-59 months	**91.9**	**5.4**	**7.70 (2.70-21.98)**
Pneumonia	**100.0**	**74.9**	**---**
Empyema	**75.8**	**61.5**	**2.57 (1.33-4.96)**
Daycare or school attendance	**93.3**	**78.1**	**3.55 (1.21-10.38)**
Non-penicillin susceptible	**0.0**	**100.0**	**----**
	Serotype 19A (n=47) %	**Others (n=246) %**	**aOR (95% CI)**
Age			
3-23 months	**70.2**	**33.3**	**Reference**
24-59 months	**29.8**	**66.7**	**0.19 (0.09-0.41)**
Empyema	**57.4**	**65.9**	**7.80 (2.91-20.86)**
Respiratory infection	**65.1**	**44.6**	**2.26 (1.03-4.94)**
Non-penicillin susceptible	**60.0**	**25.9**	**1.89 (1.13-3.16)**
	Serotype 3 (n=37) %	**Others (n=256) %**	**aOR (95% C I)**
Empyema	**75.7**	**62.9**	**3.01 (1.22-7.43)**
Vaccination status			
Non vaccinated	**24.3**	**50.0**	**Reference**
Vaccinated	**73.0**	**41.4**	**4.87 (2.05-11.59)**

Adjusted odds ratio (95%CI) for conditional logistic regression model, which included vaccination with≥1 dose of PCV7, attending daycare or school and antibiotics prescribed within 30 days before the onset of clinical symptoms.

Table 2. Factors associated with IPD penicillin-nonsusceptible in children aged 3-59 months.

Variables	IPD non susceptible (N = 40)		IPD susceptible (N = 75)		OR (95% CI)	aOR (95% CI)
	N	%	N	%		
Sex (male)	25	62.5	41	54.7	1.38 (0.63-3.03)	1.27 (0.51-3.15)
Age						
3-23 months	3. 4	85.0	33	44.0	Reference	Reference
24-59 months	6	15.0	42	56.0	0.14 (0.05-0.37)	0.14 (0.04-0.44)
≥1 dose PCV7	19	47.5	40	53.3	0.79 (0.37-1.71)	0.74 (0.27-1.51)
Daycare or school attendance	27	71.1	61	82.4	0.52 (0.21-1.31)	0.44 (0.16-1.23)
Antibiotic treatment	10	26.3	5	6.8	4.93 (1.54-15.72)	4.30 (1.09-16.94)
Respiratory infection	22	57.9	36	48.6	1.45 (0.66-3.19)	1.24 (0.48-3.22)
Clinical form						
Meningitis	9	22.5	12	16.0	1.52 (0.58-4.00)	1.21 (0.40-3.69)
Pneumonia	18	45.0	47	62.6	0.49 (0.22-1.06)	0.70 (0.25-1.97)
Bacteremia	11	27.5	11	14.7	2.21 (0.86-5.67)	1.28 (0.45-3.69)
Other clinical	2	5.0	5	6.7	0.74 (0.14-3.98)	0.48 (0.08-2.81)
Serotype						
1	0	0.0	28	37.3	Reference	Reference
19A	18	45.0	12	16.0	4.29 (1.79-10.32)	3.58 (1.28-10.05)
3	1	2.5	5	6.7	0.36 (0.04-3.18)	0.40 (0.04-4.49)
7F/A	0	0	10	13.3	----	----

Adjusted OR (95% CI) for unconditional logistic regression model, which included vaccination with ≥1 dose of PCV7, attending daycare or school and antibiotics prescribed within 30 days before the onset of clinical symptoms.

Factors associated with PCV7

Children attending daycare were more frequently vaccinated than children who did not. Children aged 24-59 months were less frequently vaccinated than children below this age. Children with > 4 cohabitants and those with a lower social class were less frequently vaccinated [12] (Table 3).

Table 3. Factors associated with 7-valent conjugate pneumococcal vaccination in children 3-59 months.

Variables	Fully vaccinated (N = 501)		Non-vaccinated (n = 460)		OR (95% CI)	aOR (95% CI)
	N	%	N	%		
Age						
3-11 months	67	13.4	64	13.9	Reference	Reference
12-23 months	148	29.5	112	24.3	1.26 (0.83-1.92)	0.98 (0.62-1.56)
24-59 months	286	57.1	284	61.7	0.96 (0.66-1.41)	0.54 (0.33-0.88)
Daycare or school attendance	381	76.4	310	68.3	1.50 (1.13-2.00)	1.70 (1.12-2.56)
Breastfeeding	30	6.1	39	8.7	0.68 (0.41-1.11)	0.71 (0.41-1.22)
Exposure smoking						
0 cig/day	285	61.3	247	58.8	Reference	Reference
1-19 cig/day	84	18.1	68	16.2	1.07 (0.74-1.54)	1.31 (0.87-1.97)
≥20 cig/day	99	20.6	105	25.0	0.79 (0.57-1.10)	0.88 (0.61-1.27)
> 4 cohabitants	58	11.8	106	23.5	0.44 (0.31-0.62)	0.58 (0.39-0.86)
Siblings <5 years	135	27.4	126	27.9	0.98 (0.73-1.30)	1.17 (0.83-1.65)
Social class						
I-III	282	68.0	167	46.4	Reference	Reference
IV-V	133	32.0	193	53.6	0.41 (0.30-0.55)	0.46 (0.34-0.62)
Medical risk condition	4	1.0	4	0.87	1.15 (0.31-4.31)	0.99 (0.24-4.02)

Adjusted OR (95% CI) for unconditional logistic regression model.

Vaccine effectiveness

The VE of complete PCV7 vaccination was 93.7% (Table 4). VE was higher in children aged 7-23 months than those aged 24-59 months. In this age group no significant differences were found because the statistical power of the study was low (40%) since few cases caused by vaccine serotypes were found. No protection was found against IPD caused by non-vaccine serotypes or all serotypes [25].

Table 4. Effectiveness of 7-valent pneumococcal vaccine in fully vaccinated children aged 7-59 months.

Serotype	Cases	Controls	Crude vaccination effectiveness	Adjusted vaccination effectiveness
	Vaccinated*/ N (%)	Vaccinated*/ N (%)	(95% CI)	(95% CI)
Vaccine serotypes	4/23 (17.4)	36/61 (59.0)	93.8% (51.9-99.2)	93.7% (51.8-99.2)
7-23 months	1/14 (7.1)	24/40 (60.0)	92.3% (38.1-99.0)	92.5% (39.3-99.1)
24-59 months	3/9 (33.3)	12/21 (57.1)	79.2% (-84.7 to 97.7)	79.4% (-84.0 to 97.7)
Non vaccine serotypes	120/228 (52.6)	315/597 (52.8)	-8.0% (-56.4 to 25.4)	-10.9% (-65.6 to 25.7)
All serotypes	124/251 (49.6)	351/658 (53.3)	16.1% (-13.5 to 37.9)	13.2% (-20.7 to 37.6)

*Incompletely vaccinated children were excluded from the analysis.
Adjusted using conditional logistic regression for attendance at daycare or school (all serotypes and non-vaccine serotypes), cohabitants (all serotypes and non-vaccine serotypes) and age (all serotypes, vaccine serotypes in all age groups and non-vaccine serotypes).

3. Discussion

In children aged <5 years, PCV7 serotypes caused 8.9% of cases of IPD in 2007-2009: 39.2% and 70% of cases were caused by PCV10 serotypes and PCV13 serotypes, respectively. The most common serotypes were all PCV13 serotypes: 1 (21.2%), 19A (16.0%), 3 (12.6%) and 7F/A (6.8%). The differences in the distribution of serotypes in children aged <5 years

compared with a 2005-2009 study in 50 Catalan hospitals [7] may be explained, at least in part, by the diagnosis of 60.8% by PCR in the present study compared with 19.1% in the previous study. In addition, the cases studied were attended in two reference centers and, therefore, the IPD cases may have been more severe: the serotypes involved could also have influenced this distribution. Gutierrez *et al.* [26] found that serotype 19A was predominant in children aged <5 years in the Autonomous Community of Madrid in 2007, after the inclusion of PCV7 in the routine vaccination schedule, whereas in our study it was serotype 1.

The most frequent clinical forms were pneumonia with empyema (64.5%), pneumonia without empyema (15.7%), meningitis (9.6%), non-focal bacteremia (7.5%), and other clinical forms (2.7%). The increase in IPD due to serotype 1 was caused primarily by the emergence of clone 306, which is related to this serotype and has caused various cases of pneumonia with empyema [14]. Other studies have shown an increase in empyema in children, especially after the introduction of PCV7. Calbo *et al.* [27], in Barcelona, found that the rate of pneumonia with empyema increased from 1.7 cases per 100,000 persons-year in the pre-vaccine era (1999-2001) to 8.5 cases in the vaccine era (2002-2004). Obando *et al.* [28] found a 13-fold increase in the incidence in Seville and Malaga and a 6-fold increase in Barcelona, after comparing data from 1998 and 2006. A lower increase in empyema in children was observed in the US [29]. In England, Koshy *et al.* [30] found an increase in empyema before the introduction of PCV7, but comparison of the results before and after the introduction of the vaccine showed the increase was not significant.

We found an association between daycare or school attendance and IPD, as described in other studies in the pre-vaccine [31-34] and vaccine eras [35].This association was observed in children aged 12-59 months, but not in those aged <12 months, possibly due to the small number of cases studied in this age group. In a semi-closed environment (such as a daycare center) there is a greater possibility of contact with colonized children and, therefore, greater exposure to *S. pneumoniae*. Most children attending daycare or school were correctly vaccinated with PCV7 for their age, but had a higher risk of IPD due to PCV7 serotypes than those not attending.

Having siblings aged <5 years was not a risk factor for IPD. A 1986-1989 Finnish case-control study by Takala *et al.* [34] in 248 cases aged <15 years found that having siblings aged <7 years was associated with IPD in children aged ≥ 2 years but not in those aged < 2 years. A 1980-2005 Danish case-control study by Hjuler *et al.* [32] in 1,381 cases aged <5 years

concluded that children aged <5 months with older siblings had higher risk of IPD than those without, while having older siblings was a protective factor for IPD in children 6-23 months, possibly due to natural immunization against IPD. In our study, PCV7 administration could be reducing the carrier status of siblings, as suggested by Givon-Lavi *et al* [32], while daycare attendance could have a greater influence than having siblings aged <5 years.

We found an association between cohabitation with ≥ 4 people and IPD only in children aged 24-59 months. The lack of association in the 3-11 months and 12-23 months age groups may have been due to the low number of cases in these groups.

A US study by Levine *et al.* [31] in the pre-vaccine era found no association between antibiotic treatment in the previous 3 months and IPD caused by strains with an MIC ≥2 mg/mL. In our study, children aged <2 years, serotype 19A and treatment with antibiotics during the previous month were associated with non-penicillin susceptible serotypes, reinforcing the importance of correct antibiotic prescription to prevent the spread of penicillin-resistant clones. We found a negative association for antibiotic treatment in the previous month in children 24-59 months once possible confounders, such as a history of vaccination and daycare or school attendance were controlled for. This may be related to the large number of cases of IPD due to penicillin-sensitive serotype 1 [14].

Viral respiratory infection in the month prior to IPD has been described as a risk factor for developing the disease [37, 38]. Respiratory viruses alter endothelial cells, promoting bacterial adherence [39]. In addition, there is less clearance of *S. pneumoniae* in previously colonized airways [40], which favors the spread of pneumococci in normally sterile areas. We only found this association in children aged 12-23 months. Other factors may be involved, such as the etiologic agent causing the viral infection or the child's immune system [41].

In our study breastfeeding had no protective effect against IPD, as did a study by Pilishvili *et al.* [35] after the introduction of PCV7. However, a study by Levine *et al.* [31] before the introduction of PCV7 found a protective effect of breastfeeding against IPD. This protective effect due to the transfer of antibodies from mother to child may be lower than the protective effect and the herd immunity provided by PCV7.

We found no association between parental smoking and IPD, similar to other studies [34, 42, 43]. However, studies by O'Dempsey *et al.* [44] in the pre-vaccine era and Pilishvili *et al.* [35] in the vaccine era found an

association between IPD and parental smoking. A meta-analysis of 30 case-control studies found no association between high exposure to smoking and IPD in children [45].

Serotype 1 was associated with the 24-59 months age group, pneumonia, and pneumonia with empyema. All cases due to serotype 1 caused pneumonia and were penicillin-susceptible. As noted, the increase in IPD due to serotype 1 was mainly caused by the ST306 clone [14]. Other studies [26] have found associations between serotypes 1, 3 and 7F and pneumonia in children aged <5 years or between serotype 1 and pneumonia with empyema in persons aged <18 years [46]. Serotype 1, unlike other serotypes, has been associated with daycare or school attendance. The presence of clone ST306 strains of serotype 1 in our community is a possible explanation [14]. This clone is penicillin-susceptible and can produce outbreaks in high-density populations such as daycare or schools [47]. Daycare or school attendance helps maintain the circulation of pneumococci and increases carrier status [48, 49], which may favor the development of micro-epidemics [47].

Serotype 19A was associated with age <2 years and with pneumonia with empyema. The association between serotype 1 and the 24-59 months age group and between serotype 19A and children aged <2 years has been found by other authors [26, 46]. Serotype 19A has been described in a large percentage of carriers, with different clonal expressions [14, 50, 51] and has an elevated capacity to produce IPD. In addition, it was associated with respiratory infection in the month prior to IPD. This may explain, at least in part, why an imbalance in the ability of host defense against respiratory infection [50,52] and tissue damage caused by the virus, favors the invasion of cells and produces IPD more easily than other serotypes. We found an association between serotype 19A and non-susceptibility to penicillin. The association of non-penicillin susceptible strains with serotype 19A may be explained by the introduction of the multidrug-resistant clonal complex ST320 expressed by serotype 19A [14]. The fact that these strains are associated with taking antibiotics in the month prior to IPD reinforces the need to recommend correct antibiotic administration to patients to prevent the emergence of resistant strains. This is especially relevant in our setting, where there is a high consumption of antibiotics [13].

Serotype 3 was the third most frequent serotype in children aged <5 years and was associated with pneumonia with empyema and receiving ≥1 dose of PCV7. Most cases due to serotype 3 were diagnosed by PCR. When PCR is not used, few cases of serotype 3 are observed as it is more difficult

to grow in cultures [19]. A large percentage of carriers present serotype 3, which has low invasive potential [53] but produces complications such as parapneumonic empyema [28], necrotizing pneumonia [54] and hemolytic uremic syndrome, [55] with a high case fatality rate [56]. We found an association between serotype 3 and PCV7 vaccination. Bender *et al.* [54] found that a large percentage of children with necrotizing pneumonia due to serotype 3 had received ≥1 dose of PCV7 compared with children with pneumonia caused by other serotypes, although the differences were not significant. PCV7 exercises natural selection in the ecological niche of the nasopharynx and decreases the percentage of carriers of vaccine serotypes, which has been described as one of the main factors responsible for the replacement of PCV7 serotypes by non-PCV7 serotypes [57]. Other authors have described an association between PCV7 administration and serotype 19A [26, 58]. We found an intermediate PCV7 coverage and, therefore, the results may differ in regions with high coverages.

Serotype 7F/A was the fourth most frequently observed in children aged <5 years but no association with the factors studied was found, possibly due to the number of cases. Although the presentation included all clinical forms, the most frequent was pneumonia. In this serotype 1/20 cases (5%) resulted in death. Rückinger *et al.* [56] found the highest case fatality rate for serotype 7F (14.8%), serotype 3 (8.3%), and serotype 23F (8.3%). Serotype 7F has also emerged after the introduction of PCV7 in children. Some studies suggest it is a serotype with high invasive potential that acts as a primary pathogen, like serotype 1 [53]. In our study, serotype 7F/A strains studied were penicillin-susceptible, as observed by Aguiar *et al.* [46].

In Catalonia, the strategy of vaccinating with PCV7 vaccination of children aged <5 years with risk diseases and private vaccination by parents according to the AEP recommendations explains why 47.1% of controls were correctly vaccinated according to age and 58.7% had received at ≥1 vaccine dose. The vaccination coverages found in our study were similar to those of other Spanish studies [27, 57] but lower than those found in other countries that include PCV7 in the routine immunization schedule [59-61].

In our study, children attending daycare or school had received PCV7 more frequently than those who did not, possibly due to pediatricians' recommendations [11, 62]. Children aged 24-59 months and children living with > 4 cohabitants were less frequently vaccinated than those aged <2 years and those cohabiting with ≤ 4 people. In addition, children of low social class were less frequently vaccinated than those of high social class, possibly due to lesser parental purchasing power.

The case fatality rate in children aged <5 years was 1%, slightly lower than that observed by Ingels *et al.* [63]. However, IPD preferably presents in people with risk medical conditions [15]. Pilishvili *et al.* [35], in the US, found that 11.3% of children aged <5 years with IPD had risk medical conditions. In our study, only four cases (1.4%) had diseases predisposing to IPD.

The study of VE is of great interest in public health. We found that complete PCV7 vaccination had a VE of 93.7% (95% CI 51.8% -99.2%) in avoiding cases of IPD caused by vaccine serotypes. There were differences in VE between age groups. In the 7-23 months age group, PCV7 was effective (VE 92.5%; 95% CI 39.3% -99.1%), while in children aged 24-59 months there was a nonsignificant trend to protection (VE 79.4%; 95% CI -84.0% to 97.7%). However, the statistical power of the study in this age group was only 40.5%, due to the low number of subjects included (10 cases of IPD caused by vaccine serotypes) and the high proportion of serotype 1 cases. Studies in different regions and countries have shown the effectiveness of PCV7. The case-control study by Whitney *et al.* [4] in the US, with 782 cases and 2,512 controls, found a VE for ≥1 doses of PCV7 of 96% (95% CI 93%-98%) in healthy children and 81% (95% CI 57-92%) in children with chronic disease. In Quebec province (Canada), Deceuninck *et al.* [5] conducted a study with 180 cases and 897 controls, and obtained an VE for IPD caused by vaccine serotypes of 92% (95% CI 83%-96%) and for IPD caused by any serotype of 60% (95% CI 38%-75%). Barricarte *et al.* [64], in Navarre, with 85 cases and 425 controls, found a VE for ≥1 doses of 88% (95% CI: 9%-98%) for vaccine serotypes. Mahon *et al.* [65] in the US, found a VE of 90.5% (95% CI 17.7%-98.9%) for complete vaccination (three doses plus booster). In children aged <7 months old, the VE was 76.6% (95% CI 50.4%-88.9%) when three doses were administered and 70.5% (95% CI 28.8%-87.9%) in children aged <5 months with two doses. No effectiveness was shown for one vaccine dose in children aged <3 months (VE 38.8%; 95% CI: -79.7% to 79.1%). The results of the schedule started in the second year of life were inconclusive, possibly due to the small sample number.

Rückinger *et al.* [66] in a German indirect cohort study found a VE for complete vaccination of 94.6% (95% CI 69.7%-99.5%). VE for ≥1 doses of PCV7 was 88.3% (95% CI 64.0%-96.6%) and VE for one, two and three doses in the first 7 months were 78.1% (95% CI 3.4%-96.1%), 89.8% (95% CI 20.6%-100%) and 94.6% (95% CI 69.7%-99.5%), respectively.

Our study, like all observational studies, has strengths and limitations. One strength is the study design and methodology, which minimized potential selection and information bias. To minimize selection bias, cases and controls were matched using variables such as age, sex, underlying disease, the hospital and the date of hospitalization. Social class was not used for matching, but no significant differences between cases and controls were found.

Possible information bias was minimized by collecting information on the vaccination status from individual health records (vaccination card, medical record or health center records) with information recorded prior to the study. Sociodemographic and epidemiological variables were collected using a single questionnaire for cases and controls administered to parents. Moreover, the use of PCR and culture as diagnostic techniques increased the ability to detect IPD, as PCR provided the most cases. The low proportion of cases caused by vaccine serotypes (8.9%) made it difficult to obtain significant results in the 24-59 months age group, but this could not have been predicted beforehand.

Finally, we made a conditional logistic regression analysis including variables whose clinical relevance could have had a confounding effect, such vaccination, daycare or school attendance, and antibiotic treatment, and the variables in the bivariate analysis associated with the dependent variable. Therefore, although some residual confusion cannot be ruled out, the results can be considered as reasonably valid.

4. Conclusions

In children aged <5 years, PCV7, PCV10 and PCV13 vaccine serotypes represented 8.9%, 39.2% and 70.0% of cases, respectively. The most common serotypes were: 1, 19A, 3 and 7F/A. Molecular PCR allowed identification of IPD cases not identified by conventional culture. This was particularly relevant in IPD caused by serotypes 1 and 3. Serotype 1 was associated with the 2-4 years age group, pneumonia with empyema and daycare or school attendance. Serotype 19A was associated with age < 2 years, pneumonia with empyema, previous respiratory infection, and non-penicillin susceptible strains.

Children attending daycare had the highest PCV7 vaccination coverage (76.4%) and those with a lower social class the lowest coverage (32.0%). PCV7 vaccine effectiveness was very high in children aged 7-59 months (93.7%; 95% CI 51.8%-99.2%). In children aged 24-59 months VE was

lower (79.4%; 95% CI -84.0% to 97.7%) than in children aged 7-23 months (92.5%; 95% CI 39.3%-99.1%).

Accurate monitoring that allows the identification of emerging strains and patterns of antibiotic sensitivity patterns is necessary to prevent IPD.

Acknowledgements

The authors thank the other members of the Working Group for the Study of PI 06/1507: Laura Selva, Cristina Esteva, Mariona F. De Sevilla, Juan José García-García (University Hospital Sant Joan de Deu), Fernando Moraga, Ana Maria Planes, Gemma Codina, Francis Coll (University Hospital Valle Hebron), Joan Batalla, Neus Cardeñosa [Department of Health, Generalitat of Catalonia and CIBER Epidemiología y Salud Pública (CIBERESP), Spain].

This study was supported by grants from Fondo de Investigaciones Sanitarias del Instituto de Salud Carlos III (PI 06/1507), Agencia de Gestión de ayudas a la investigación de la Universidad (AGAUR 2009/SGR 0042 and 2009/SGR 00136) and Fundación Caja Navarra.

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Research Signpost
37/661 (2), Fort P.O.
Trivandrum-695 023
Kerala, India

Recent Advances in Pharmaceutical Sciences VI, 2016: 111-129 ISBN: 978-81-308-0566-5
Editors: Diego Muñoz-Torrero, Àngela Domínguez and Àngels Manresa

7. Neuropsychopharmacologic and neurotoxicologic effects of the combination of ethanol with mephedrone in adolescent mice

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Abstract. In the last decade, a new family of synthetic psychostimulant drugs, under the name of cathinones, broke into the market. These drugs are mainly consumed by adolescents and young adults with recreational purposes, in most cases combined with alcoholic drinks. Although a number of works about new cathinones have been recently published, none explored the consequences of such combination. Because adolescence is a crucial period in brain development, we sought to study the effects of the combination of mephedrone plus ethanol in adolescent mice, focusing on psychostimulant and conditioning effects, as well as on neurotoxicity markers. Ethanol increased both locomotor activity and conditioned place preference (CPP) induced by mephedrone. RNA microarray assays after CPP test yielded significant alterations

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in neuronal plasticity-related genes and a key role of BDNF and dopamine D3 receptors in CPP acquisition was found. Ethanol potentiated the oxidative stress as well as the decreases in dopaminergic and serotonergic markers in frontal cortex and hippocampus respectively, after a binge treatment with mephedrone. Moreover, the drug combination impaired spatial learning and memory, as well as neurogenesis to a higher extent than mephedrone alone.

Introduction

Drug abuse is a matter of concern at all life stages but its occurrence at earlier ages is especially worrisome, as it can determine the social outcome of an individual [1]. While adolescence is a crucial stage in brain maturation, experimentation with alcohol and other drugs is common; teenagers are not aware of the risks they are taking, as the regions of the brain that control impulses are still immature. Substance use during adolescence has been associated with alterations in brain structure, function, and neurocognition (reviewed by [2]). Moreover, it has been reported in studies with humans that drug consumption during adolescence increases the likelihood of drug abuse in adulthood [3]. Specifically, alterations in the prefrontal regions and limbic systems are thought to contribute to increased risk-taking and novelty/sensation seeking behaviors [4-6].

Currently, most drug use during adolescence occurs in leisure environments, such as dance clubs and parties [7]. Alcohol is omnipresent due to its legal drug status [8] while other drugs such as cannabis, cocaine, and amphetamine derivatives are often associated with it [9].

Moreover, adolescents are less sensitive than adults to the depressant effects of alcohol, as well as to the subsequent hangover (reviewed by [10]), which facilitates the intake of higher amounts. Numerous studies report neurotoxic effects of alcohol itself in consumption models using adolescent rodents (reviewed by [11]), mainly leading to impairment in memory and visual and verbal tasks [12]. Excitotoxicity and neuroinflammation seem to be involved in such deleterious effects [13].

During the last decade, a miriad of new designer drugs broke into the market. These substances structurally differred from existing banned drugs and took profit of this legal loophole to be sold through licit media such as the Internet, smart shops, gas stations, etc., always with the disclaimer “not for human use” and packaged as “bath salts”, “plant food” or “research chemical”. For this reason, these substances where generically called “legal highs”. Some of them are currently banned in many countries, but

the pace at which new substances appear exceeds that of the legal machinery to illegalize them [14].

Among these new substances, mephedrone (4-methylmethcathinone, Meph) is an increasingly consumed synthetic psychostimulant compound, which first appeared for sale on the Internet around 2007. It belongs to the β-ketoamphetamines group (Fig. 1), also known as cathinones, and is commonly taken orally or insufflated [15]. Preclinical studies have shown that mephedrone stimulates the release of dopamine (DA), serotonin (5-HT) and norepinephrine and inhibits their re-uptake in the CNS [16-19]. These actions explain the psychostimulation and the effects on perceptions reported by human consumers [20]. Experiments in rodents have demonstrated the psychostimulant (measured as hyperlocomotion) and rewarding (measured as conditioned place preference, CPP) effects of mephedrone, which are indicative of its abuse liability [18,21].

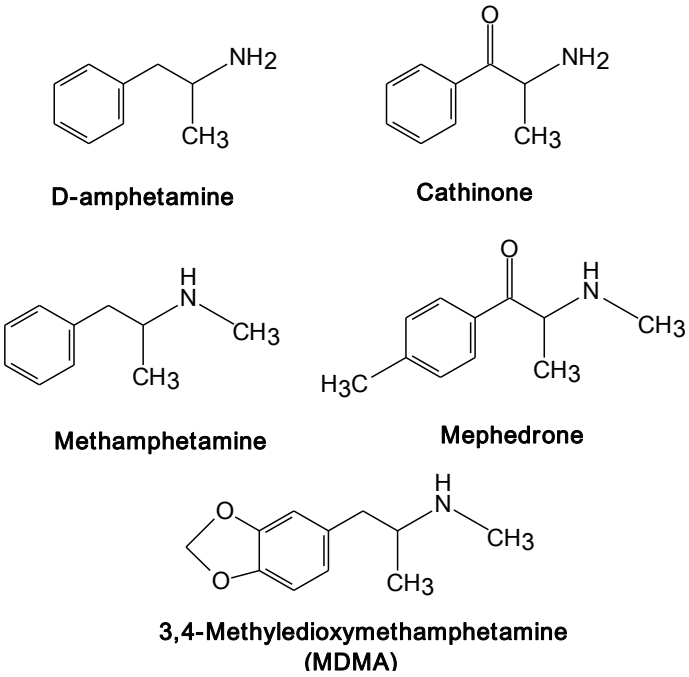

Figure 1. Chemical structures of classic amphetamine derivatives, (D-amphetamine, methamphetamine and MDMA) cathinone and mephedrone. Please notice the β-keto group in both cathinones.

Mephedrone is also commonly combined with many other drugs, but mainly alcohol [9,22] which, in turn, is the most consumed drug. The study of the interactions between drugs of abuse is of interest because potentiation of the effects could result in increased abuse liability.

In fact, ethanol can effectively potentiate the psychostimulant and rewarding effects of another widely abused amphetamine derivative, 3,4-methylenedioxy-methamphetamine (MDMA) in rodents [23] by a combination of both pharmacokinetic and pharmacodynamic interactions. Given the resemblance between mephedrone's mechanism of action and that of MDMA [9,18,24], a similar profile should be expected when combined with alcohol.

Moreover, interaction between drugs could also result in increased deleterious effects. Hernández-Rabaza *et al.* [25] described that combination of ethanol with MDMA produces cognitive impairment in adolescent rats at doses that do not when administered alone. This impairment is accompanied by a decrease in survival of neuronal precursor cells as well as a decrease in the presence of mature cells in the dentate gyrus (DG) of the hippocampus. Furthermore, Izco *et al.* [26] found that ethanol potentiates MDMA neurotoxicity through the production of hydroxyl radicals.

These antecedents justify the need for studies about the pharmacological and neurotoxicological effects of the combination of mephedrone plus ethanol. In this chapter we summarize the works we performed on this subject using adolescent mice, owing to the prevalent consumption of these drugs in this population group and the reasons explained above. Results show increased psychostimulant and rewarding effects of the combination, as well as potentiation of the neurotoxicity markers and the impaired learning and memory. Such preclinical evidences deserve to be investigated in humans in order to, if also occurred in this species, transmit a pertinent warning to the population.

1. Psychostimulant effects: Locomotor activity

In rodents, psychostimulants produce increased locomotor activity; therefore measurement of locomotion is a widely employed technique to study the acute behavioural effects of new drugs such as mephedrone. The technique consists of placing the animals in a cage equipped with infrared photocells so that ambulation produces occlusions of the photo beams, which are recorded and sent to a computerized system. The more

interruption counts (measured over blocks of 10 min during 120 min or longer), the higher locomotor activity. Previous works had demonstrated that mephedrone induces robust hyperlocomotion [16,18,21] after a single injection of 5-25 mg/kg. To assess the effect of the combination with ethanol, mice were administered with mephedrone (10 or 25 mg/kg, s.c.) alone or combined with ethanol and immediately placed in the activity box. Since ethanol, at certain doses, can modify locomotion, it was administered at doses reported to not affect basal activity (0.5 or 1 g/kg, s.c.; [27,28]).

As can be seen in Fig. 2A, the hyperlocomotion induced by mephedrone was significantly increased when administered concominantly with ethanol [29]. In order to investigate the neurotransmitter responsible for such potentiation, the experiments were repeated administering previously ketanserin (a serotonin 5-HT_2 receptor antagonist) or haloperidol (a dopaminergic antagonist with predominant D_2 activity). The result showed that, although expectedly both antagonists reduced locomotor activity, only haloperidol prevented the locomotor enhancement of the drug combination, indicating that this occurs through a dopaminergic mechanism.

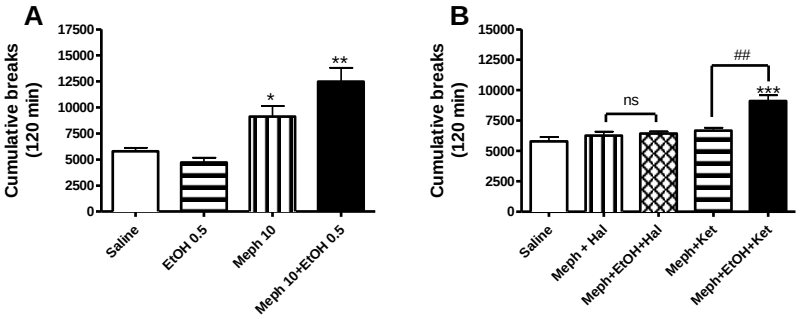

Figure 2. A. Locomotor activity of adolescent CD-1 mice treated with ethanol (EtOH, 0.5 g/kg, s.c.), mephedrone (Meph, 10 mg/kg, s.c.) or their combination, expressed as the cumulative interruptions of the infrared beams (breaks) in the activity cage during 120 min. B. Effects of the 5-HT_2 antagonist ketanserin (Ket, 1 mg/kg, i.p.) and D_2 antagonist haloperidol (Hal, 0.25 mg/kg), on the hyperlocomotion induced by mephedrone (10 mg/kg) and its combination with ethanol. Although both antagonists reduced hyperlocomotion, only haloperidol abolished the potentiation by ethanol. *P<0.05, **P<0.01, ***P<0.001 *vs.* saline; ##P<0.01 between the indicated groups; ns, non significant differences; one-way ANOVA. Graphs modified from [29].

The effects of ethanol in the brain are numerous as it easily crosses biological membranes and interacts on several molecular targets (i.e. ligand-gated ion channels). One of the main mechanisms by which it is capable of increasing hyperlocomotion is the inhibition of GABAergic interneurons in the substantia nigra reticulata, which leads to disinhibition and increased burst firing of dopamine neurons in the nucleus accumbens, but it also directly increases DA release in other areas of mesocortical pathways (see [30] for a review). These mechanisms are different from those of mephedrone but, in turn, would converge in increased DA release and/or desinhibition in certain brain areas, which could explain the observed increased effect.

2. Rewarding effects: conditioned place preference (CPP) and associated transcriptional changes

A rewarding stimulus is defined as a stimulus that is considered likeable and thus is worthy of being desired and pursued [31]. Rewards (both natural and exogenous) trigger two important biological processes:

- Assignment of a hedonic value. This is defined as how much the reward is "pleasurable" or "liked".
- Assignment of an incentive salience, which is defined as a motivational value or "wanting" or a given rewarding stimulus [32].

This distinction is important, since rewarding stimuli modulate behavior through an increase in dopamine in the nucleus accumbens (NAc), whereas dopamine is not a mediator of the hedonic state elicited by a rewarding stimulus [33]. Therefore, the rewarding effects can be indicative of its abuse liability.

The CPP test is performed in an apparatus composed of three distinct areas (two well distinguished compartments communicated by a central corridor) separated by manually operated doors [34]. The procedure is performed in three phases: pre-conditioning, conditioning and post-conditioning test. During the pre-conditioning phase (day 1), the mice are placed in the middle of the corridor and have free access and roam among the compartments of the apparatus. The time spent in each compartment is recorded through a zenithal camera and computerized tracking software. During the conditioning phase, mice are randomly assigned to receive the drug in one of the compartments and the vehicle in the opposite so that they receive in alternate days the drug or the vehicle (4 days each

treatment), and are confined to the assigned compartment for 30 min. In the post-conditioning day, the mice are left to freely wander through both compartments, and the time spent in each one is also registered. If the drug induced reward, the mouse will prefer to stay in the drug-matched compartment, attempting to repeat the experience produced by the drug, so an increased time (preference score) with respect to that measured in day 1 will be recorded.

Results showed that the combination of ethanol, at a dose non conditioning on its own (0.75 g/kg), increased the preference score induced by mephedrone (Fig. 3), which could, in turn, indicate increased abuse liability.

The mesolimbic pathway is involved in the acquisition of CPP, so addictive drugs are expected to evoke synaptic plasticity in the areas that it comprises including the NAc, the ventral tegmental area, the hippocampus and the medial prefrontal cortex [35]. For this reason, we aimed to characterize these changes by determining major transcriptional modifications in the ventral striatum (comprising the NAc) after completing the whole conditioning process, by means of RNA microarray assays.

A number of studies using the microarray approach with psychostimulants (mainly cocaine, methamphetamine and amphetamine) in rodents have been published (reviewed by [36]). More recently, similar studies have been carried out with alcohol [37] or heroin and methamphetamine [38]. From these studies it is concluded that differential gene expression for a given drug depends on many factors such as dose, schedule, mode of administration (non-contingent or self-administration), studied tissue, animal strain and time of withdrawal or at which timepoint the expression is measured. In this study, we focused on the remaining expression changes in the ventral striatum 48 h after the end of a conditioning treatment, an approach that had not been yet taken for any drug of abuse.

Results showed significant changes in mRNAs involved in neuronal plasticity [29], which is in line with CPP acquisition. These included Syt10 and Muted, which were only significantly increased in the groups receiving mephedrone; and Arpc5, whose expression was increased in all drug-treated groups and potentiated in the Meph+EtOH group. Its product, ARPC5, plays an important role in maintaining the ARP2/3 activity (see below). Syt10 encodes synaptotagmin 10, a calcium sensor involved in the regulation of neuron size and arborization [39]. Furthermore, the Muted gene codifies for a subunit of the BLOC-1 complex, which is involved in

the activation of ARP2/3 [40], whose complex nucleating capability is essential for actin remodeling and synaptic plasticity at a pre- and post-synaptic level [41,42]. The ARP2/3 complex is associated with F-actin in the spinoskeleton core and acts to nucleate new actin filament branches from existing actin filaments. It is therefore essential in the activity-dependent enlargement of dendritic spines. BLOC-1 also plays a key role in endosomal trafficking and as such has been found to regulate cell-surface abundance of the D_2 dopamine receptor, the biogenesis and fusion of synaptic vesicles, and neurite outgrowth. Similarly, Camkk1, whose codified protein plays an important role in actin dynamics, was significantly up-regulated.

Moreover, the D_3 dopamine receptor gene (Drd3) was one of the most marked and similarly increased in all drug-treated animals. D_3 dopamine receptors (D_3Rs, see [43] for a review) are a subtype of D_2-like receptors with both pre- and postsynaptic locations, negatively coupled to adenilyl-cyclase and acting as autoreceptors modulating dopamine release and/or synthesis. D_3 receptors are known to be involved in reinforcement and reward induced by many drugs, including ethanol [44], cocaine [45, 46],

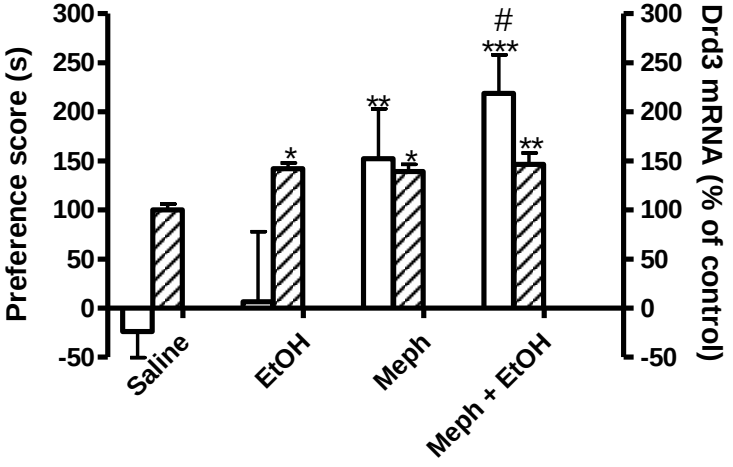

Figure 3. Place preference score (open bars, left axis) and Drd3 mRNA expression (dashed bars, right axis) in adolescent mice subjected to CPP being treated according to the schedule stated in the text with saline, ethanol (EtOH, 0.75 mg/kg) and mephedrone (Meph, 25 mg/kg) plus/minus ethanol. *P<0.05, **P<0.01, ***P<0.001 *vs*. saline; #P<0.05 *vs*. Meph.; one-way ANOVA. Data taken from [29].

morphine [47] and methamphetamine [48]. They are mainly localized in limbic brain regions, especially the nucleus accumbens [49]. Ethanol, morphine or cocaine, are also capable of upregulating Drd3 mRNA in rodents [50-52] and in human addicts [53].

Based on these antecedents, we tested whether blocking D_3 receptors with the D_3 antagonist SB-277011A affected CPP and Drd3 up-regulation induced by Meph and its combination with ethanol. The antagonist was given before the drugs each conditioning session, and was capable of completely blocking mephedrone-induced CPP and Drd3 mRNA up-regulation (Ref BJP). The fact that Drd3 was also increased in the EtOH group, which did not show CPP at the dose used, suggests that it is not the sole player in establishing conditioning but that other partners, probably among the other modified genes reported above, are needed.

However, due to the robust blockade obtained with the D_3 antagonist, we further explored the mechanisms involved in mephedrone-induced CPP and Drd3 up-regulation. BDNF controls dopamine D_3 receptor expression [54] and its levels are increased by psychostimulants [55]. In fact, BDNF and D_3 receptors share common pathways in their respective signalling cascades (reviewed by [56]). Furthermore, Le Foll *et al.* [51] described that Drd3 mRNA and D_3 receptor binding are significantly increased after a single dose of cocaine and preceded by a transient increase in BDNF mRNA. Thus increased BDNF expression has been suggested to alter the response to drug-associated cues by affecting the D_3 receptors in the nucleus accumbens.

In order to assess a possible role of BDNF in the effects of mephedrone, we measured its mRNA in medial prefrontal cortex after an acute dose of this cathinone (25 mg/kg), finding a progressive increase along 4 h after the injection [29] up to two-fold. Further, the role of BDNF was pharmacologically confirmed by using ANA-12, a selective trkB (BDNF receptor) antagonist. When administered before the drugs each conditioning session, ANA-12 blocked both CPP and Drd3 up-regulation induced by mephedone. This confirms that D_3 receptor differential expression can be mediated by BDNF, and points to the fact that blocking their signalling can reduce the rewarding properties of mephedrone.

3. Dopaminergic and serotonergic toxicity

Neurotoxicity of amphetamine derivatives (i.e. methamphetamine, MDMA) is a matter of concern and has been subject of a great amount of

research. This led to undertake studies exploring a possible neurotoxic effect of new drugs such as mephedrone in rodents [57,58]. Reported research evidences the need to perform neurotoxicity assays under different administration schedules and controlled room temperature. Our group described mephedrone neurotoxicity using dosing schedules which agreed with mephedrone pharmacokinetics and exploring cerebral areas others than the striatum [59,60]. From these antecedents and in order to investigate the effects when combined with ethanol, we chose administering four doses of 25 mg/kg (s.c.) in one day, every two hours, given alone or combined with ethanol at changing doses calculated to cause blood ethanol concentration leveling around 1.5 g/L during the whole duration of the treatment [61]. Also, during the treatment, room temperature was set at 26 ± 2 ºC, at which mephedrone has been reported to induce signs of neurotoxicity [60], in order to reproduce the common hot conditions found in crowded dance clubs.

Decreases in the density of monoamine transporters and synthetic enzymes are characteristic markers of amphetamines' neurotoxicity. Thus, we measured the density of transporters by means of radioligand binding experiments and the enzymes by Western blotting in brain areas of mice 7 days after receiving the above treatment. The results showed that, as expected, mephedrone reduced the levels of dopamine transporters (DAT, binding of [^{3}H]WIN 35428) in the frontal cortex and of serotonin transporters (SERT, binding of [^{3}H]paroxetine) in the hippocampus (Fig. 4A, B), while DAT in the striatum and SERT in the frontal cortex were unaffected [61]. Accordingly, the levels of tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH) were reduced in frontal cortex and hippocampus, respectively. Moreover, the decreases were higher when mephedrone was combined with ethanol, which indicates that the combination potentiates the dopaminergic and serotonergic toxicities of these drugs.

4. Oxidative stress

Oxidative stress has been classically associated with amphetamines' neurotoxicity and previous evidence indicates that this also occurs with mephedrone. Therefore lipid peroxidation and oxidative stress-related enzymes were assessed in frontal cortex and hippocampus from mice sacrificed 24 h after receiving the above treatment.

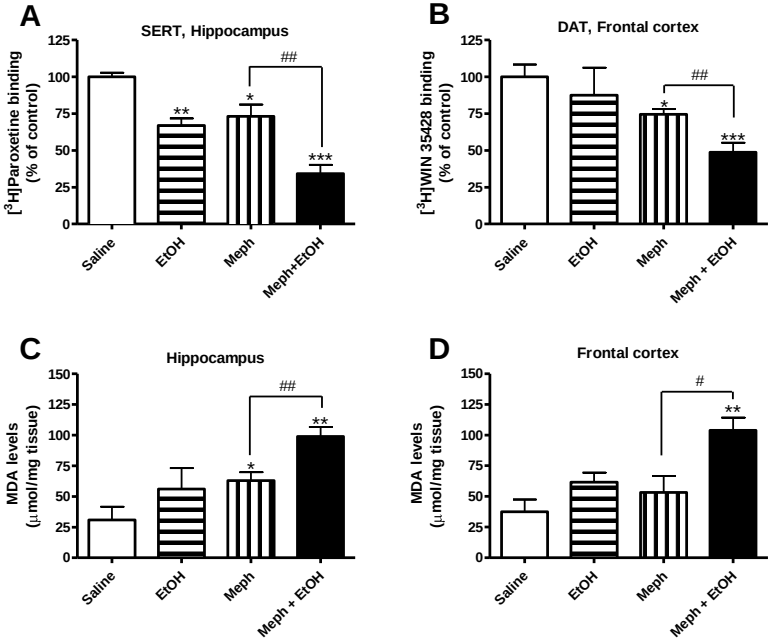

Figure 4. Assessment of the levels of serotonin (SERT) and dopamine (DAT) transporters (panels A and B) and lipid peroxidation measured as levels of malondialdehyde (MDA, panels C and D) in hippocampus and frontal cortex, respectively. Adolescent CD-1 mice were treated with either saline, ethanol, mephedrone, or their combination, following the schedule described above. Monoamine transporters were measured 7 days after the treatment, while MDA was determined in samples from mice killed 24 h after the last dose. Values represent means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 vs. saline; #P < 0.05, ##P < 0.01 between the indicated groups. Data obtained from [61].

Lipid peroxidation was measured as a raise in the MDA levels, a general indicator of the decomposition of polyunsaturated fatty acids. Mephedrone alone only significantly increased MDA levels in the hippocampus. By contrast, the combination of mephedrone with ethanol caused higher and significant increases in the levels of MDA (Fig. 4B, C).

As far as antioxidant activity-related enzymes is concerned, glutathion peroxydase and catalase were significantly and similarly overexpressed (around two-fold increase) in both mephedrone-treated groups [61]. Regarding superoxide dismutase expression, there was also an increase

(around 85%), although statistically non-significant in both mephedrone-treated groups. This indicates that an antioxidant response is triggered after the treatment with mephedrone plus/minus ethanol, which is not further increased in the mephedrone+ethanol group. When comparing these with the lipid peroxidation data, it can be suggested that there is a potentiation in oxidative stress-related damage in the mephedrone+ethanol group, where the effects of the drug combination exceed the antioxidant response leading to increased effect of generated ROS.

5. Effects on learning and spatial memory

Neuronal oxidative stress and serotonergic impairment can have consequences on learning and memory, so we investigated the performance of mice in the Morris water maze seven days after receiving the treatments stated above [61]. The maze consists of a circular pool filled with water (22 ± 1º C) and rendered opaque by the addition of a non-toxic latex solution. The pool must be in an isolated room and black curtains are closed around it to suppress room cues [62]. Four positions around the edge of the tank are designated as north (N), south (S), east (E), and west (W) and also define the division of the tank into four quadrants: NE, SE, SW, and NW, providing alternative start positions. Four extra-maze distal cues are located equidistantly around the pool, labeling the N, S, E and W locations. A Plexiglas escape platform is submerged in the water so that it is not visible at the surface level.

The test measures the ability of each mouse to learn the position of the hidden platform (always the same) in relation to the distal cues, after several training sessions being placed in the water from an alternate semi-random set of start locations. The path taken by each mouse and the escape latency (the time needed by each mouse to find the platform, in s) is recorded by a zenithal video camera connected to a computer running a tracking software. Mice were trained throughout six days, receiving five trials per day [61]. A trial was started by placing the mouse in the desired start position of the pool, facing the tank wall. The mice were allowed to swim to the hidden platform, and the escape latency was determined. If an animal did not escape within 60 s, it was gently placed on the platform or guided to it. The mice were allowed to rest for 30 s (inter-trial interval) on the platform (even those that failed to locate it). Then, learning curves of the four treatment groups were plotted as the time-course evolution of the latency to reach the hidden platform. To assess reference memory at the

end of learning, a probe trial (free swimming without platform for 60 s), was given 24 h after the last training session. Different parameters of each mouse's performance are analyzed: the total time and distance spent swimming in each quadrant, entries in each quadrant and time elapsed (latency) until the mouse first reached the target zone (area where the platform was formerly located).

The learning curves of the four treatment groups (saline, mephedrone, ethanol and mephedrone+ethanol) showed that the mice pre-treated with saline or ethanol reduced the latency along the training days, which indicates learning, while those pre-treated with mephedrone and, moreover, those that received mephedrone+ethanol did not show such a progression, suggesting learning impairment [61].

As far as memory is concerned, in the probe trial, the mice that had received mephedrone and those pre-treated with the drug combination spent significantly less time than the saline and ethanol groups in the quadrant where the platform was located (Fig. 5), pointing to impaired memory. In this case, no significant differences were found between mephedrone and mephedrone+ethanol groups. As spatial memory is mainly attributable to hippocampal activity, the serotonergic impairment previously detected in the hippocampus could account for this memory affection.

6. Effects on hippocampal neurogenesis

The subgranular zone of the dentate gyrus (DG) of the hippocampus is one of the two regions in the adult brain containing neural stem cells that underlie adult neurogenesis [63,64]. It is currently accepted that hippocampal structure and function relies upon hippocampal stem cells and constitutive neurogenesis [65,66]. The thousands of new cells added daily to the DG suggest its role in hippocampal structure and/or function [67]. Neurogenesis consists of four main components: neural stem cells proliferation followed by newborn cell migration, differentiation, and survival.

It has been widely suggested that the generation of new neurons is implicated in correct learning and memory processes, including MWM performance in rodents [68]. Furthermore, neurotoxic processes are closely related to a decrease in cell proliferation and an increase in cell death. Serotonin input to the hippocampus positively regulates adult neurogenesis [69]. In this sense, serotonin reuptake inhibitors increase

hippocampal neurogenesis [70] while repeated exposure to high doses of MDMA, which produces serotonergic neurotoxicity, causes the opposite effect [71].

Neurogenesis from the granular layer of the DG is impaired following treatment with ethanol [72-74] and adolescents are more sensitive than adults to such effects [75]. Therefore, combination of alcohol with serotonergic amphetamines could account for an increased deleterious effect on neurogenesis. In fact, Hernández-Rabaza el al. [25] described that the cognitive impairment produced by the combination of MDMA with ethanol in adolescent rats was accompanied by a decrease in survival of neuronal precursor cells as well as a decrease in the presence of mature cells in the DG of the hippocampus.

With these antecedents, the effects of the combination of ethanol with mephedrone on neurogenesis deserved to be studied. For this reason, the mice that were tested in the MWM had received two injections of bromo-deoxyuridine (BrdU) 2 and 12 h after the last dose of treatment [61]. BrdU is a thymidine analog that is incorporated into cells in place of a thymine base pair as the cell undergoes DNA replication during the S phase of the mitotic cell cycle and is transmitted to the newly generated cells. BrdU can be labeled with specific antibodies so that it can be used as a measure of cell proliferation.

Twenty eight days after receiving the binge drug treatment (14 days post-MWM test) the animals were sacrificed and their sectioned brains were stained for BrdU and NeuN (a marker of neurons) and visualized under a confocal microscope. The cells colocalizing the two labels (newborn neurons) were counted and the results from the different treatment groups compared. A significant decrease in newly formed cells in the DG of mice administered with mephedrone and mephedrone+ethanol was found, with respect to saline. Furthermore, the group treated with the drug combination showed significantly less new neurons than that treated with mephedrone alone, indicating an increased deleterious effect of the combination. BrdU count in animals treated with ethanol alone was unaffected with respect to saline (Fig. 5B). Moreover, there was a good correlation between the total amount of new cells and overall MWM performance (Fig. 5).

5-HT input to the hippocampus positively regulates adult neurogenesis [69]. In this sense, 5-HT reuptake inhibitors increase hippocampal neurogenesis [70]. Furthermore, repeated exposure to high doses of MDMA causes the opposite effect [71]. Similarly to what occurs with

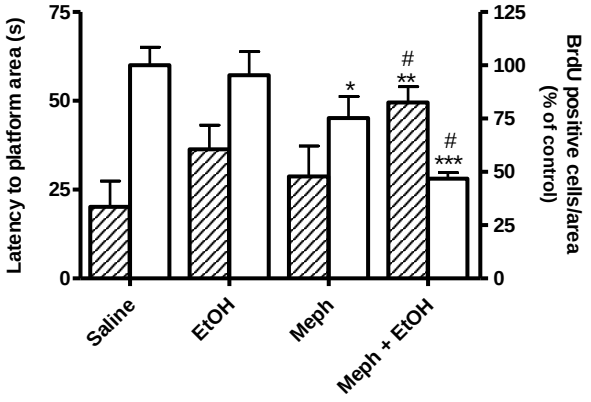

Figure 5. Effects of treatment with saline, ethanol, mephedrone or their combination on spatial memory and hippocampal neurogenesis. Adolescent mice were treated as described above and received two injections of BrdU. Seven days after, they were submitted to the Morris water maze paradigm, consisting of 6 days of training and 1 day of trial. Dashed bars represent, on the probe test day (day 7), the latency to first reach the area where the platform had been located during the training period. The animals were killed 28 days after the treatment and their brains were fixed, sliced and immunostained for BrdU (proliferating cells) and NeuN (neuronal marker). Open bars show overall quantification and means of BrdU-positive neurons per area. Data are means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 vs. saline; #P < 0.05 *vs.* mephedrone group. Data obtained from [61]

mephedrone in the present study, MDMA is known to produce a depletion of serotonergic markers in the hippocampus 7 days after repeated treatment [76]; this 5-HT depletion can, in turn, cause decreased cell survival in the dentate gyrus [77].

7. Conclusions

To sum up, the co-administration of ethanol to adolescent mice potentiates the psychostimulant and conditioning effects of mephedrone, but also its neurotoxic properties.

All this suggests an increased risk if translated to humans. On the one hand, enhancement of psychostimulant and rewarding effects could promote increased abuse liability and addiction-related disorders whereas, on the other hand, binge abuse of the drug combination could carry more severe neural damage involving dopaminergic and serotonergic impairment, decreased neurogenesis and cognitive deficits.

The preclinical studies reviewed here are the first performed on polyabuse with cathinones, which are becoming increasingly popular among adolescents. Their importance lies in that cathinones are mostly used in combination with alcoholic drinks, and are generally regarded as "safe" drugs. Thus, an experimental-based warning concerning the risks regarding the combined consumption of these drugs should be conveyed to the population at large. Nonetheless, although adolescent brains are exceptionally vulnerable, studies in adult mice would be necessary to determine whether adults could be also susceptible to these effects.

Acknowledgements

This work was supported by grants from the Spanish drug initiative "Plan Nacional sobre Drogas" (2012I102), "Generalitat de Catalunya" (2009SGR977) and from "Plan Nacional de Investigación Científica" (SAF2013-46135-P). Our group holds the Quality Mention from the "Generalitat de Catalunya" (2014SGR1081). AC was recipient of a fellowship from the "Plan Nacional sobre Drogas".

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Research Signpost
37/661 (2), Fort P.O.
Trivandrum-695 023
Kerala, India

Recent Advances in Pharmaceutical Sciences VI, 2016: 131-145 ISBN: 978-81-308-0566-5
Editors: Diego Muñoz-Torrero, Àngela Domínguez and Àngels Manresa

8. Bioavailability and metabolism of maslinic acid, a natural pentacyclic triterpene

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Abstract. Maslinic acid is a pentacyclic triterpene found in plants used in traditional medicine with antidiabetic, antitumor, antioxidant and parasitostatic activities. A diet rich in foods of plant origin could provide a regular supply of this bioactive compound since it has been detected in olives, spinaches, eggplants, chickpeas and pomegranates. The multiple biological effects elicited by maslinic acid suggest its putative use as nutraceutical. This review summarizes our study on the safety, pharmacokinetics, and metabolism after the oral administration of maslinic acid, conducted with the aim of increasing the knowledge about the bioavailability of this bioactive food component.

Introduction

The association between nutrition and human health is not a novel concept, since Ayurveda and Chinese medicine had already endorsed food as medicine, either as prevention for the development of certain ailments or as a treatment. This association was promoted by Hippocrates (460–377 BC) in

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his principle "Let food be thy medicine, and medicine be thy food". The relationship between nutrition and health has encouraged research on bioactive food components that could improve the physical and mental well-being and reduce the risk of disease. Among the different food from the diet, those from vegetable origin have been strongly associated with a reduced risk of developing chronic diseases, due to the presence of phytochemicals that are non-nutritive secondary metabolites. These bioactive compounds may have a lower potency if they are compared to drugs. However, phytochemicals could impact on health given that they are ingested regularly as part of the diet [1]. Different dietary molecules have been associated with physiological effects, such as glucosinolates, carotenoids, polyphenols and triterpenoids. Among the latter, stands out maslinic acid, which is currently in the early stages of the pre-clinical phase of research, as evidenced by the fact that the vast majority of published references mainly consisted of *in vitro* studies, with only a few studies conducted *in vivo*. Maslinic acid has been described to exert several biological activities, such as antidiabetic, antitumor, antioxidant and parasitostatic, among others [2]. Therefore, the present chapter focuses on the recent research in our group that has been aimed at assessing the safety of maslinic acid and at evaluating its bioavailability after oral administration.

1. Chemistry of maslinic acid

Maslinic acid or (2α,3β)-2,3-dihydroxyolean-12-en-28-oic acid (Fig. 1) was first isolated in 1927 from the leaves of *Crataegus oxyacantha* L. and was named "crategolic acid" [3].

The identification of this compound in *Olea europaea* L. came in 1961 when Caglioti *et al.* [4] found in olive husk a triterpenic compound with the same molecular formula and chemical structure as crategolic acid and termed it maslinic acid. The same year, Vioque and Morris [5] detected this pentacyclic triterpene in olive pomace. However, it was not until 1994 that Bianchi and coworkers [6] established that maslinic acid was the main pentacyclic triterpene in olives.

The biosynthetic pathway has been recently postulated in *Olea europaea* L. [7], where it has been suggested that sterols (primary metabolites) and non-steroidal triterpenoids (secondary metabolites) share 2,3-oxidosqualene as a common precursor. The cyclization of 2,3-oxidosqualene leads to the oleanyl cation as a precursor of erythrodiol, which is the first derivative of ß-amyrin. Further oxidation steps yield to the formation of

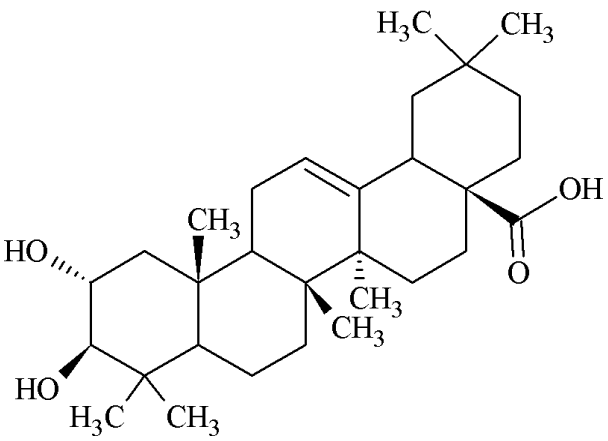

Figure 1. Chemical structure of maslinic acid.

oleanolic acid and its isomer maslinic acid. In the plant, maslinic acid acts as a phytoalexin that is a secondary metabolite involved in the protection against pathogens [8].

2. Sources of maslinic acid

Maslinic acid is broadly distributed in the plant kingdom, being detected in more than 30 species worldwide. This pentacyclic triterpene has been found in plants used in traditional medicine for the treatment of diverse affections. The leaves of banaba or *Lagerstroemia speciose* L. are especially rich in maslinic acid (4.96 ± 0.13 mg/g) [9]. The extracts of the banaba have been used for many years in folk medicine for the treatment of diabetes. *Crataegus monogyna* L., commonly known as hawthorn, contain 0.93 ± 0.07 mg/g of maslinic acid [9] and has been used as a remedy for the cardiovascular system given its hypotensive, antioxidant, anti-inflammatory, and vasodilating effects, among others. *Ortosiphon stamineus* L. possesses several pharmacological activities such as diuretic, hepatoprotective, antidiabetic, antihyperlipidemic, and has been described to have 0.84 ± 0.06 mg/g of maslinic acid [9]. Moreover, this pentacyclic triterpene has been described in *Eriobotrya japonica*, employed as antitussive and anti-inflammatory for chronic bronquitis as well as diuretic [10], *Geum japonicum* employed as diuretic [11], and *Agastache rugose* used in the treatment of intestinal disorders [12].

Maslinic acid has also been described in several edible plants that could provide a constant supply of this compound, especially in the countries bordering the Mediterranean Sea where the diet is rich in fruits and vegetables, olive oil is used for cooking as well as salad dressing and table olives are consumed as appetizer. The latter are a food with a particularly high content in maslinic acid although the concentration depend on the fruit variety and the method of elaboration. In fruits processed with natural fermentation, the concentrations of maslinic acid ranged from 1.32 ± 0.4 g/g fresh olive pulp in Kalamata to 0.28 ± 0.07 g/g fresh olive pulp in Manzanilla variety [13]. Virgin olive oil is obtained in a process involving pressing, which may disrupt the surface waxes on the fruit. Therefore, part of maslinic acid contained in the olive may be transferred to the oil. However, the amount of this pentacyclic triterpene in the oil is much lower than in the fruit, and depends on the oil quality. Extra virgin olive oil, with an acidity under 1% contains 64.2 ± 8.1 mg/kg of maslinic acid, an amount that increases to 193.9 ± 14.0 mg/kg in virgin olive oil, since the hydrolytic process that takes place during the extraction enables the release of this compound from the cuticular layer [14]. The presence of this compound has been reported in spinach (1260 mg/kg dry weight), eggplants (840 mg/kg dry weight), chickpeas (62 mg/kg dry weight), large lentils (40 mg/kg dry weight), and aromatic herbs such as basil (350 mg/kg dry weight) [15,16]. Maslinic acid has also been found in fruits, like kiwi (17.3 mg/kg), pomegranate (10.8 mg/kg), or mandarin (10.8 mg/kg) [17].

3. Toxicology

The beneficial effects on health described for maslinic acid granted the use of this compound as a possible nutraceutical. However, the safety of this bioactive compound needed to be assessed. Accordingly, the effect of high doses of maslinic acid on Swiss CD-1 mice were evaluated following the indications given by the Organization for Economic Cooperation and Development (OECD) guidelines [18] for the evaluation of preclinical acute and subacute toxicities. In first place, the effect of the single oral administration of maslinic acid was assessed by administering 1000 mg/kg to two adult Swiss CD-1 mice. The acute toxicity study was conducted in order to evaluate if the administration of a single high dose of this pentacyclic triterpene exerted any adverse effect on the animals over the immediately consecutive days. This evaluation of the acute toxicity showed that this pentacyclic triterpene did not have any negative effect on the

animals after the 15-day observation period in which body weight, food consumption as well as the general state of the animals was controlled, indicating that maslinic acid is practically nontoxic under these conditions [19].

In second place, the subacute toxicological evaluation was performed and consisted of the repeated oral administration for 28 days of maslinic acid at 50 mg/kg [19]. The dose selected corresponded to approximately 125 times the amount that may be consumed by a person eating 40 g or 10-medium sized olives and 33 g of olive oil a day. The results showed that no death or hazardous signs of toxicity were recorded during the experimental period. A decrease of a 13% was observed in the food intake of the treated mice compared to the controls, without affecting the body growth, which was equal in both groups [19]. A similar effect was indicated for the gilthead sea bream (*Sparus aurata*) that were fed with 100 mg/kg of maslinic acid for 210, reporting a decrease in the relative intake of diet 18% lower in the fish fed with the triterpene with respect to the control group and an increase of body weight in the treated group of only a 5% [20]. No effect on body weight was observed after the administration of 20, 40, and 80 mg/kg to juvenile dentex (*Dentex dentex*) during 49 days [21]. By contrast, maslinic acid has been described to stimulate body growth in healthy fish thus being proposed as a feed additive in aquiculture [22]. Rainbow trout (*Oncorhynchus mykiss*) fed with 25 and 250 mg/kg of maslinic acid in the diet during 225 days increased body weight, compared to the control group, in 19.3% and 29.2%, respectively. This effect was attributed to a higher growth rate of white muscle, which was correlated with hyperplasia and hypertrophy in the tissue [22].

The safety of maslinic acid was further evaluated by the assessment of hematology and clinical chemistry, which showed no differences between the control and the treated groups [19]. The absence of negative results obtained after the clinical chemistry evaluation corroborated that the treatment of mice with 50 mg/kg of maslinic acid did not compromise the overall health status of the animals since no alterations in metabolic processes in target organs were observed [19]. Worth mentioning is the point that maslinic acid did not have any significant effect on blood glucose in physiological conditions, despite being considered an anti-diabetic agent in different models of hyperglycemia [23,24]. It could be considered a positive outcome, since it would imply that maslinic acid could avoid the side effects caused by hypoglycemic treatments. No effects were observed on the lipid profile [19].

The lack of harmful effects exerted by the oral administration of 50 mg/kg of maslinic acid for 28 days was verified with a gross necropsy with the measurement of the organ weights and the histopathologic evaluation of the tissues [19]. The relative weight of liver, kidney, heart, lungs, and spleen were not different from controls. Remarkable the fact that no liver hyperplasia was observed in our study, although it was reported that rainbow trout that ingested maslinic acid at the dose of 25 and 250 mg/kg for 225 days displayed an increase of the liver size of 52% and 40% with respect to the controls [22]. The treatment of mice with 50 mg/kg of maslinic acid, induced an increase on the relative weight of the brain of 11% ($P < 0.05$) higher than the control group, but changes in relative brain weight are rarely associated with neurotoxicity [25].

Overall, the results obtained in the toxicological study that showed an absence of harmful effects found in the hematology, clinical biochemistry, and histopathology evaluation suggest a large safety margin for this pentacyclic triterpene after oral administration.

4. Oral bioavailability of maslinic acid

The absence of adverse effects demonstrated for maslinic acid constituted a promising starting point for its future use as a nutraceutical due to the biological activities described for this pentacyclic triterpene. However, this information needed to be completed with a detailed study about its absorption, distribution, and metabolism either in humans or in animals after the consumption of this molecule. Bioactive compounds from food must be bioavailable in order to perform their beneficial effects on health. Therefore, bioavailability and metabolism of maslinic acid had to be explored *in vivo* in order to elucidate its mode of action and clarify the extent of its absorption and metabolism.

4.1. Plasmatic concentrations of maslinic acid

The bioavailability of maslinic acid was assessed after the p.o. administration of 50 mg/kg and the i.v. administration of 1 mg/kg to overnight fasted male Sprague-Dawley rats. Blood was taken from the lateral saphenous vein at different time points from 1 min to 24 h in order to cover the whole plasma concentration *versus* time curve [26]. Sampling was performed following a sparse design with three to six replicates for each time point. Blood was centrifuged and plasma was separated from

cells, and frozen at -20 ºC until analysis. The method to determine maslinic acid from plasma consisted of two extractions with ethyl acetate, prior to evaporation to dryness and reconstitution with methanol 80%. This pentacyclic triterpene was detected by liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS) [26].

The method to determine maslinic acid from plasma was validated according to the EMA Guidelines on Bioanalytical Method Validation [27]. The validation was performed with blank plasma samples spiked with maslinic acid at eight different concentrations (0.05-10 µM) and the parameters evaluated were recovery, matrix effect, linearity, sensitivity, precision, accuracy, selectivity, and carry-over [26].

The validated analytical method yielded excellent separation and detection of maslinic acid and the I.S. (betulinic acid) in plasma samples, as can be seen in Fig. 2.

The recovery of maslinic acid was calculated as the ratio between the mean peak area of extracted calibration standard samples and the mean peak area of the blank plasma samples spiked with this pentacyclic triterpene after extraction. Eight different calibration standards in the range 0.05 ± 10 µM were evaluated and the mean recovery was 99.0 ± 0.9% (n = 31). The sensitivity was evaluated on the basis of a signal to noise ratio of 3 for the LOD and 10 for the LOQ. The analytical method had an adequate sensitivity for the measurement of maslinic acid in plasma samples,

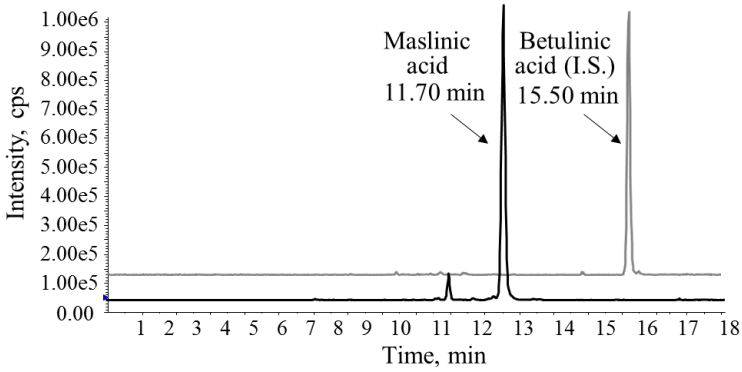

Figure 2. Representative HPLC-APCI-MS chromatogram obtained in negative mode with SIM acquisition at m/z 471.3 for maslinic acid and m/z at 455.5 for betulinic acid (I.S.). Rat plasma was obtained 45 min after the oral administration of 50 mg/kg of maslinic acid.

since the LOD was 2 nM and the LOQ was 5 nM. The LOD, verified by analyzing six blank plasma samples to which the theoretical concentration had been added, yield a concentration of 5.03 ± 0.13 nM with a precision of 6.22%. The linearity was assayed within the concentration range of 0.010-30 µM, which covered the expected concentration range for both the i.v. and p.o. administration routes. Linearity was observed in the calibration curves over the range 0.010-20 µM with a correlation coefficient up to 0.99, losing the linear tendency at 30 µM. The intra-day and inter-day precisions of the method were below 5.46% and 8.38%, respectively, in all the concentrations evaluated (0.05-20 µM). Finally, accuracy was established by comparing the nominal concentrations and the calculated concentrations. The deviation was lower than 4.82%.

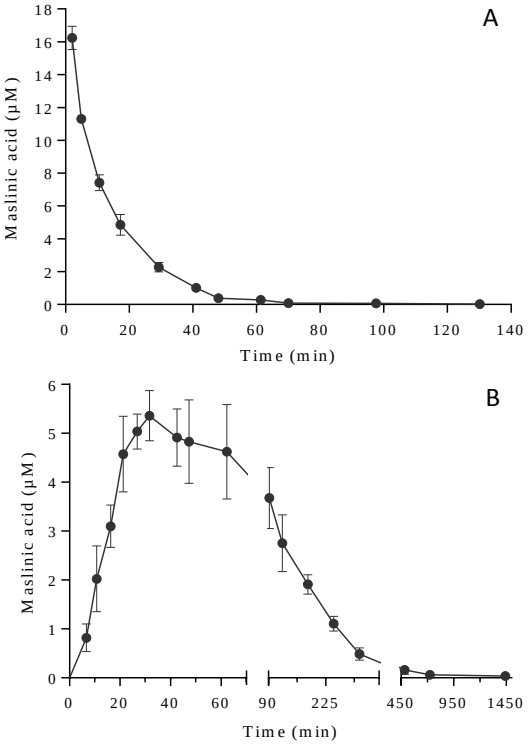

Figure 3. Plasmatic concentrations *versus* time profile of maslinic acid after single i.v. (1 mg/kg) (A) and p.o (50 mg/kg) (B) administration to overnight fasted rats.

The analytical method allowed the detection of maslinic acid after p.o. and i.v. administration, and the sampling times were adequate since in both cases the elimination phase was properly characterized. Fig. 3 shows the plasmatic concentrations *versus* time of maslinic acid after i.v. and p.o. administrations. After p.o. dosing maslinic acid was adequately quantified up to 24 h. Nevertheless, in the i.v. administration the blood collected at 8, 10, and 12 h yielded concentrations of maslinic acid below the LLOQ and were below the LOD at 24 h. Therefore, these concentrations were not included in the subsequent pharmacokinetic analysis [26].

4.2. Pharmacokinetic analysis of maslinic acid

The plasmatic concentrations *versus* time data were analyzed with the WinNonlin software to calculate the pharmacokinetic parameters. Non-compartimental and compartimental analyses were performed. Plasmatic data was best adjusted to a two-open compartments with first order absorption and linear elimination process (Fig. 4) [26].

Following p.o. administration, the peak plasma concentration after oral dosing (Cmax) was 4.82 µM and the time to peak plasma concentration (Tmax) was 30 min with an oral bioavailability of 6.25% [26]. The low oral bioavailability observed was in accordance with the results obtained for similar pentacyclic triterpenes [28,29]. A value of 2.3% was reported for 23-hydroxybetulinic acid after the p.o. administration of 200 mg/kg to mice [29]

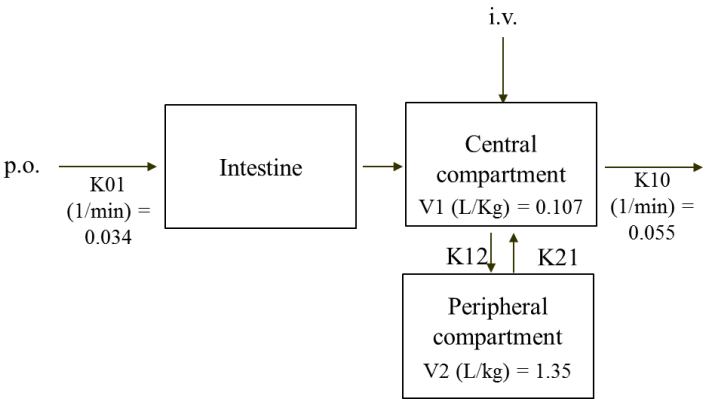

Figure 4. Schematic representation of the pharmacokinetic model that best describes the data of plasmatic concentrations of maslinic acid *versus* time after p.o. and i.v. administration.

and a 0.7% was indicated for oleanolic acid after the oral dosing of 25 and 50 mg/kg to mice [28]. The low oral bioavailability found for maslinic acid could be attributed to either a poor gastrointestinal absorption or a first-pass effect of the compound at the intestine or the liver. Maslinic acid have been described to exert a chemopreventive activity on colon cancer either *in vitro* in HT-29 colon cancer cells [30] or *in vivo* in Apc(Min/+) mice [31]. Then, if maslinic acid is poorly absorbed in the gut wall, then, the large intestine would face important quantities of this pentacyclic triterpene, thus becoming a target organ where maslinic acid could elicit the described protective activity against colon cancer.

The absorbed maslinic acid that reached the blood was distributed to the central (0.107 L/kg) and peripheral (1.35 L/kg) compartments. These results suggest an extensive distribution into tissues, since the total distribution volume (1.46 L/kg) is higher than the total body water in the rat (0.17 L/kg, for a mean weight of 0.28 kg). Similar distribution values were indicated for oleanolic acid (0.451 L/kg) after the i.v. injection of 1 mg/kg [28]. The estimated total plasma clearance (CL, 0.348 L/h/kg) was slightly lower than that of oleanolic acid (1.98 L/h/kg) [28]. The clearance value could be attributed to small hepatic and renal metabolism with unaltered renal excretion of this triterpene. Noteworthy, oleanolic, ursolic, and maslinic acids have been determined in several organs after a 4-week consumption of a diet with 0.5% of these triterpenes, and the liver had the largest concentrations followed by the kidney [32]. This results suggest that, liver could be an organ for maslinic acid storage, where this pentacyclic triterpene could exhibit a protective activity against hepatic diseases, as it has been demonstrated in different *in vitro* experiments [16,33].

5. Metabolism

The results obtained in the pharmacokinetic study showed a low oral bioavailability that could be attributed, in part, to metabolism. Therefore, the identification of the biotransformation of maslinic acid constitutes an important step in the investigation of this bioactive compound. In addition to the role that metabolism may have in affecting the bioavailability by influencing the distribution and the rate or the route of excretion, metabolites could either contribute to the biological activities or have toxicological effects independent of the parent compound [34]. Moreover, the identification of metabolites is important since they could act as

biomarkers of intake in dietary intervention studies [35]. With the aim of gaining insight into the bioavailability of maslinic acid, the metabolism was studied in rat plasma obtained after the oral administration of a dose of 50 mg/kg. Plasma samples were extracted with ethyl acetate prior to liquid-chromatography-atmospheric pressure ionization-linear trap quadrupole-ORBITRAP-mass spectrometry (LC-LTQ-ORBITRAP-MS) analysis [36]. This high-resolution mass spectrometer provides robust, accurate mass data that enables the unambiguous identification of metabolites. Plasma was screened for metabolites with the assumption that this bioactive compound from the diet could be considered as a xenobiotic by the organism and consequently, the detoxification processes that increased the hydrophilicity of maslinic acid could be activated [37]. Hence, metabolites arising from phase I reactions, which are based on oxidation, reduction, and hydrolysis processes, and phase II biotransformations, such as glucoronidation, sulfation, methylation, acetylation, and glutathione conjugation were searched in the samples. The results obtained showed that only compounds arising from phase I reactions were found. The molecule of maslinic acid was modified in order to yield four monohydroxylated derivatives (M1a-M1d), one monohydroxylated and dehydrogenated metabolite (M2) as well as two dihydroxylated and dehydrogenated compounds (M3a-M3b) [37].

Metabolism of maslinic acid has only been investigated *in vitro* with the use of fungi such as *Cunninghamella* [38] and *Rhizomucor* [39,40]. These microorganisms are commonly used in the evaluation of the metabolism of drugs and xenobiotics since they express cytochrome P450 (CYP) enzymes that allow equivalent phase I reactions to mammalian metabolism. In these studies, the chemical structure of the derivatives was elucidated based on the use of nuclear magnetic resonance and high-resolution mass spectra analyses. Therefore, the hydroxyl of the mono-hydroxytaled metabolite (M1-a, M1-b, M1-c, M1-d) could be compatible with the metabolites previously described [38,39,40]. The transformations found after incubations with *Cunninghamella blakesleana* were three different monohydroxylated derivatives (7β-hydroxy, 15α-hydroxy, 13β-hydroxy with double bound migration) [38]. When *Rhizomucor miehei* was used, monohydroxylation was also observed, but in this case the addition of the hydroxyl group took place at the angular methyl group on position 30 [39].

Moreover, the monohydroxylated and dehydrogenated metabolite (M2) could be formed by a hydroxylation at C-11, followed by a

dehydrogenation to ketone or by the formation of an epoxy group between C-11 and C-12, as previously suggested [39,40]. The dihydroxylated and dehydrogenated metabolite (M3-a, M3-b) could be compatible with the hydroxylation at C-30 and the epoxy between C-11 and C-12 [39]. However, the two observed metabolites could also be due to other structures not detected in the incubations of maslinic acid with fungi [38-40] such as the combinations of these functionalizations or the presence of a hydroxyl group in one position and a ketone in another carbon.

The only *in vivo* studies were performed with pentacyclic triterpenes similar to maslinic acid and confirmed that the biotransformation of these compounds occurs mainly by phase I reactions [41,42]. The oral administration of 12.5 mg/kg of boswellic acids to female albino Wistar rats gave in plasma two monohydroxylated derivatives [56]. Incubation of oleanolic acid [28] and boswellic acids [41,42] with rat and human liver microsomes also indicated that hydroxylation is the main metabolic route for pentacyclic triterpenes.

The quantification by LC-APCI-MS showed that maslinic acid was the most abundant compound in plasma (81.8%), which indicates that metabolism is low. It was followed by metabolite M2 with a 13.2% and M1-a with a 2.5%. The other metabolites (M1-b, M1-c, M1-d, M3-a, M3-b) were found in minor amounts since all of them accounted for only a 2.5% [37]. Overall, the study gives a comprehensive insight into the metabolite profile of maslinic acid, thus increasing our knowledge about the bioavailability of this bioactive food component.

6. Conclusion

Maslinic acid is a bioactive compound that targets a wide variety of molecules or metabolic pathways suggesting its putative use as nutraceutical. However, research on maslinic acid is currently in the early stages of the pre-clinical phase. Consequently, the present chapter summarizes the current knowledge about the safety and the bioavailability of maslinic acid after oral administration. A liquid-liquid extraction method followed by LC-APCI-MS was validated to determine of maslinic acid in blood. Analysis of plasmatic concentrations indicated that this bioactive compound was absorbed with a peak plasmatic concentration at 30 min and oral bioavailability of 6.25%. Metabolism was subsequently screened and revealed that maslinic acid is the main compound in plasma although it undergoes mainly phase I metabolism through hydroxylation

and oxidation reactions, leading to seven metabolites. In summary, the present results show that maslinic acid can be safely administered by the oral route and provides a comprehensive knowledge on the bioavailability and metabolism as a first step on the use of this bioactive compound as a nutraceutical.

Acknowledgements

This work was supported by grants AGL2009-12866 and AGL2013-41188 from the Ministerio de Economía y Competitividad and 2009SGR471 and 2014SGR1221 from the Generalitat de Catalunya, Spain.

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Research Signpost
37/661 (2), Fort P.O.
Trivandrum-695 023
Kerala, India

Recent Advances in Pharmaceutical Sciences VI, 2016: 147-163 ISBN: 978-81-308-0566-5
Editors: Diego Muñoz-Torrero, Àngela Domínguez and Àngels Manresa

9. Lichenysin production and application in the pharmaceutical field

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Abstract. Lipopeptides such as lichenysin are biosurfactants of great interest, due to the demand for natural surface-active agents with low toxicity. *Bacillus licheniformis* AL 1.1 produces a lipopeptide characterized as lichenysin ($Lch_{AL1.1}$), which acts as a powerful surfactant, able to reduce surface tension to 28.5 mN m^{-1} and with a critical micelle concentration of 15 mg L^{-1}. $Lch_{AL1.1}$ is particularly effective in preventing biofilm formation by pathogenic strains, has an emulsifying capacity and permeabilizes membranes by a colloid-osmotic process. The production of lipopeptides from agro-industrial residues, particularly molasses, is a sustainable process of great potential for the development of economic bioprocesses.

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Introduction

Surfactants are amphiphilic compounds with a hydrophobic and hydrophilic domain. They have the ability to concentrate at interfaces and increase the aqueous solubility of non-aqueous liquids. Bio-surfactants (BS) are produced by microorganisms on surfaces to enhance access to nutrients or facilitate growth in the environment, BS have many advantages compared with their chemically synthesized counterparts, including biodegradability, low toxicity, availability from renewable resources, resistance to environmental factors, and high surface and interfacial activity. In short, they are molecules with a promising future [1].

BS can be classified based on molecular weight. Those of a low molecular weight, such as glycolipids and lipopetides, effectively reduce surface and interfacial tension. High molecular weight BS, or bioemulsifiers, are more effective at stabilizing oil in water emulsions and include polymeric surfactants such as polysaccharides, proteins, lipopolysaccharides and complex mixtures [2]. According to their polar group, BS are anionic or neutral [1].

BS are also grouped according to the chemical structure of the hydrophilic moiety. (i) Glycolipids are carbohydrates combined with long-chain aliphatic or hydroxyaliphatic acids. This group includes the most studied BS, the rhamnolipids, trehaloselipids, sophorolipids, cellobioselipids and mannosylerytroil lipids. (ii) Lipopeptides consist of cyclic peptides or unattached lipidic chains and are characterized by remarkable surfactant and antimicrobial properties. Examples are gramicidin, surfactin, polymixin, subtilisin, iturin, mycosubtilin, fengycin, and viscosin. (iii) Phospholipids and fatty acids with surfactant activity, such as phosphatidylethanolamine, are overproduced by several bacteria during growth on n-alkanes [3]. (iv) Polymeric surfactants consist of heteropolysaccharides combined with proteins. Commercially important compounds included in this group are emulsan, liposan, biodispersan, alasan, and manoprotein. (v) Particulate BS formed by extracellular membrane vesicles are able to form stable emulsions, important for microbial alkane uptake [1,4].

Microbial BS are secreted or attached to cellular walls. They are usually produced in the presence of water-insoluble substrates, but not always, which is an impediment in explaining the bacterial benefits associated with their production. Numerous bacteria and yeasts of diverse

genera produce BS of varying chemical nature. Cyclic lipopetides, produced as secondary metabolites by different species of *Bacillus*, are remarkable surfactants with high surface activity and antimicrobial properties. Considering the high demand for new products with health applications, the lipopeptide lichenysin, with its surface activity, emulsifying capacity, and anti-adherent and antiproliferative properties, is of particular interest.

1. *Bacillus licheniformis*

Bacillus licheniformis is an endospore-forming bacterium widespread in soils and other environments, including food and clinical and veterinary samples. It grows in a wide range of temperatures, from 15ºC to a maximum of 68ºC (strain AL1.1 was isolated from a geothermal zone in the Antarctic). Its rapid growth, low nutritional requirements, resistance and capacity to produce enzymes (proteases and amylases), polysaccharides and biosurfactants, make this bacterial species interesting for the fermentation industry as a productive microorganism or probiotic [5,6,7].

B. licheniformis has occasionally been reported as an opportunistic pathogen in man and animals and a cause of food poisoning, with large amounts being associated with intoxication in a few cases. *B. licheniformis* has been described as a contaminant of dairy products, and toxin-producing isolates have been found in raw milk and baby food [8]. Lichenysin has been proposed as a virulence factor, although the mechanism of action is unknown [7].

In contrast, *Bacillus* species have been used as probiotics, or live microbes, which when administered confer a health benefit to the host. Spore probiotics are being used in humans (dietary supplements), animals (competitive exclusion agents) and in aquaculture (to increase disease resistance). *B. licheniformis* is used in combination with *B. subtilis* in two commercial products, the animal feed BioPlus®2B in the Ukraine and the medicine Biosporin in Russia. Its probiotic effect is associated with Amicoumacin production, with activity against *Helicobacter pylori*. The easy production and stability of spores and their immune stimulation, antimicrobial and competitive exclusion properties suggest potential application as probiotic dietary supplements, although more clinical studies are required to confirm the absence of adverse effects [9, 10].

Exopolysaccarides with antioxidant and anti-aging activity produced by *B. licheniformis* KS-17 and KS-20 may be used as functional ingredients in novel probiotics [11]. However, results suggest the toxigenic potential of *Bacillus* species used in nutrition needs to be revised [7].

2. Lichenysin characterization and production

Lipopeptides are a class of microbial surfactants with a growing attraction for the therapeutic, cosmetic and food industries. They occur across the whole spectra of microorganisms, but above all in *Bacillus* sp. The basic structure of lipopeptides consists of a specific fatty acid combined with an amino acid moiety. They are usually found in mixtures of closely related compounds with slight variations in their lipid part and amino acid composition. Lipopeptide activities include antibiotic, antiviral, antitumor, inmunomodulator and inhibition of specific toxins and enzymes. These properties make them potential agents for therapeutic applications [12, 13].

The first lipopeptide to be isolated was surfactin [14]. Produced by *Bacillus subtilis*, it is among the most powerful surfactants, along with iturin, fengycin and lichenysin, whose exceptional surface activity endows them with powerful biological effects [15]. The mechanisms of action of lipopetides have not been clarified in detail, but their different activities are clearly due to their surface and membrane properties. Surfactin, as the first lipopetide described, is the most studied. Produced as a mixture of isoforms, it has a molecular weight of 1007-1035 Da and is formed by one heptapeptide with the amino acid sequence Glu-Leu-Leu-Val-Asp-Leu-Leu [16]. Lichenysin is the most potent anionic cyclic lipoheptapeptide BS reported to date [17]. It is produced by most, if not all, *B. licheniformis* strains on media containing glucose as the carbon source [16, 18]. Lichenysin production has recently been described in *B. lichenyformis* AL 1.1, isolated from an extreme Antarctica environment [19]. Lichenysin consists of a peptide moiety with seven amino acids and a β-hydroxy fatty acid of 12-17 carbon atoms, with normal iso and anteiso branching. Several lichenysin isoforms and homologues are found in nature, due to modifications in the length and branching of the fatty acid chain and amino acid substitutions. Six variations are accepted, named lichenysin A, B, C, D, G and surfactant BL86, lichenysin A being the most abundant isoform. Lichenysins are anionic surfactants due to the presence of Asp and/or Glu residues. Lichenysin$_{AL1.1}$ (Lch$_{AL1.1}$) is a mixture of lipopeptide homologues,

with a molecular weight between 1006 and 1034. The peptide moiety consists of glutamine as the N-terminal amino acid, two leucines, valine, aspartic acid and iso-leucine as the C-terminal amino acid. The lipid moiety is formed by β-hydroxy fatty acids ranging in size from C_{14} to C_{16}, with high similarity to lichenysin groups A, D, and G [19]. In conclusion, lichenysin A is very similar to surfactin, differing only by 1 Da in molecular mass, a consequence of the substitution of glutamic acid for glutamine in the first amino acid position. This small difference significantly modifies the physicochemical properties of lichenysin, notably the surface tension [7].

Unlike surfactin, lichenysin is synthesized during growth under an aerobic or anaerobic atmosphere. It is synthesized by lichenysin synthetase, a multiple enzyme complex, encoded by lichenysin operon lchA (26.6 Kb). The structure of lichenysin and its operon indicate a nonribosomal biosynthesis with the same multifunctional modular arrangement as observed in surfactin synthetase SrfA [16]. The nature of the peptide and fatty acids dictate the activity of BS, which can be tailor-made to have the desired attributes using engineered synthetases. The industrial production of environmentally friendly BS remains a pending subject, due to factors such as low-yield, high cost of raw materials, and inefficient purification processes [17, 21]. A reduction in production cost could be achieved by two approaches: i) the development of hyper-producer microbial strains and ii) the design of the production medium and optimization of the culture conditions with a highly efficient recovery process, or combining different strategies. Generally, *Bacillus* species co-produce various families of lipopeptides with different homologues and isoforms [21, 22]. When lichenysin production was qualitatively examined in 53 different *B. licheniformis* strains, all of them produced the same isoforms but in varying ratios. Rønning et al. [7] reported that lichenysin production is more dependent on growth conditions (physical or chemical) than genotypes. It was demonstrated by Coronel et al. [19] that environmental factors such as temperature, pH and aeration are very important for product yield. *B. licheniformis* strain AL1.1, a fast-growing thermophilic isolate with an optimal growth temperature of 65ºC, shows visible colonies in TSA after 3-4 h incubation; nevertheless, at this temperature BS production is inhibited, being optimum at 30-37ºC, when growth is much lower.

The nature of the carbon and nitrogen sources and other micronutrients can influence the amount of BS produced, as well as the cost of the

process. Pure carbon sources such as glucose, sucrose, and glycerol, and above all hydrophobic compounds such as n-alkanes, vegetal oils are used for BS production. When *B. lichenyformis* AL 1.1 was studied, neither growth nor BS production were obtained when oils were used as the carbon source, but carbohydrates or glycerol gave a remarkable BS production. In contrast, microorganisms such as *Rhodococcus erythropolis* [23] or *Sphingobacterium detergens* need n-alkanes, alone or in combination with carbohydrates, as a carbon source for BS production [24]. To improve BS yields and reduce the initial costs of raw materials, the use of local and cheap agro-industrial wastes is proposed. The use of residual substrates can have a double benefit, providing a solution to an environmental problem while allowing the development of a new product with added value. Various substrates, including frying oil, peanut oil cake, molasses, whey, sugarcane bagasse, potato peel and rice straw, have been tested for BS production [25, 26, 27, 28, 29, 30, 31]. *B. licheniformis* AL1.1 growth and lichenysin production are supported by cassava water, cassava starch and whey. Regarding the nitrogen source, pure compounds such as nitrate, ammonium salts and urea can be used since they are inexpensive [19, 32].

Downstream processing is an important step in biomolecule production processes, accounting for 50-80% of the total production cost. Its study and optimization is an important stage in the overall optimization process and constitutes an obstacle to a reasonable economical production [33]. When lichenysin is produced in a bulk medium, the first crucial step to obtain a highly pure compound is often acid precipitation, followed by solvent extraction. Alternative systems involve foam fractionation and membrane filtration, the choice depending on cost and effectiveness [34].

Response surface methodology (RSM), which includes factorial experimental design and regression analysis, is suitable for multifactor experiments, such as kinetics studies of microbial production, since it avoids having to consider one variable at a time. RSM with a central composite rotatable design (CCRD) constitutes a simple and economical method for designing experiments and evaluating the effect of factors and desirable responses. The production of $Lch_{AL1.1}$ using sugar-cane molasses was optimized using RSM. Molasses is a cheap substrate with a high content in sugars; its complex composition includes a variety of micronutrients, allowing the development of a medium that only requires

the addition of a low amount of nitrogen and phosphorous sources. To optimize the medium composition for $Lch_{AL1.1}$ production and bacterial growth, different concentrations of molasses, nitrate and phosphates were tested. The two variables examined were biomass formation (Equation 1) and $Lch_{AL1.1}$ production (Equation 2). The design matrix of the variables allowed the construction of an empirical second-order polynomial model for biomass and $Lch_{AL1.1}$ production [32]. The functional form of the models for the two response variables is:

Equation 1

$\mathbf{Y_1}$**=** $8.32-2.15\ x_1^2 + 2.03\ x_2 + 2.64\ x_1\ x_2 + 1.39\ x_1 - 0.59\ x_3^2 - 0.46\ x_2^2 - 0.48 x_2 x_3 - 0.13 x_3$

Equation 2

$\mathbf{Y_2}$**=** $3.14 - 0.78 x_1^2 - 0.50\ x_3^2 - 0.45 x_2^2 - + 0.33 x_3 + 0.13 x_1 - 0.17\ x_1 x_3 - 0.16 x_x x_3$

An F-test (ANOVA) was used to check the statistical significance of the second-order model equations. Table 1 shows the results of the ANOVA for both models. As can be seen, there is no significant lack of fit of the regression models. The results of Fisher's F test for the regression models were highly significant ($p<0.05$). Besides, the R^2 of the biomass polynomial model and $Lch_{AL1.1}$ polynomial model was calculated to be 0.998 and 0.97 [32], respectively, indicating that 99.8 and 97%, respectively, of the variability in the responses could be explained by the second-order polynomial prediction equations given above (Equations 1 and 2).

In Figure 1, contour plots show the effect of the concentrations of molasses (x_1), nitrate (x_2) and phosphates (x_3) on biomass production (Y_1) and $Lch_{AL1.1}$ accumulation (Y_2). The horizontal and vertical axes correspond to two significant factors for response variables x_1, x_2, and x_3, and the other axes are equal to response variables Y_1 and Y_2, respectively.

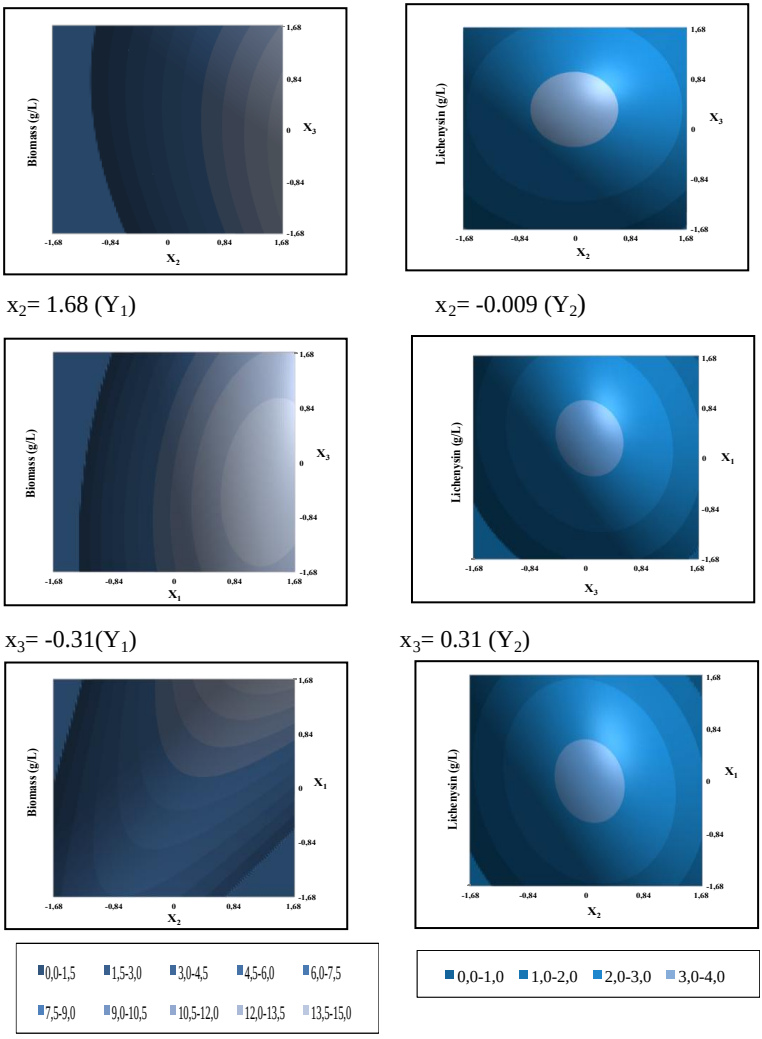
The optimum components for biomass production (g l^{-1}) were molasses 180.2, nitrate 12 and phosphate 7.5. The predicted maximum production value for biomass corresponding to these values was 14.5 g L^{-1} and the obtained production was 13.7 g L^{-1}, after 72 h of incubation, whereas in the initial non-optimized conditions, biomass production was found to be 3.5 g L^{-1}.

Table 1. Analysis of variance (ANOVA) for the nine-term equation for biomass (Y_1) production and for the seven-term equation 2 for $Lch_{AL1.1}$ production (Y_2).

Analysis of Variance for Biomass Production (Y_1)					
Source	Sum squares	Degrees freedom	Men square	F_0	Probability P(>F)
Regression	224.57	9	24.95	1025.8	$4.81e^{-17}$
Residual	0.316	13	0.243		
LOF error	0.147	5	0.029	1.39	0.323
Pure error	0.169	8	0.021		
Total	224.89	22			
Analysis of variance for $lichenysin_{AL1.1}$ production (Y_2)					
Regression	19.17	7	2.739	79.3	$1.04\ e^{-10}$
Residual	0.518	15	0.034		
LOF error	0.189	6	0.031	0.864	0.555
Pure error	0.329	9	0.036		
Total	19.69	22			

Thus, optimizing the medium composition using RSM increased the biomass yield 4-fold. Unlike the study of BS production, the concentrations of molasses and nitrate were the most important factors for bacterial growth, with high levels favoring biomass production. In contrast, the phosphate concentration had little influence. When *B. licheniformis* AL1.1 was grown under optimal production conditions, it was possible to enhance biomass from 3.5 g L^{-1} to 13.7 gL^{-1} . Previously published data on biomass production show that increasing the concentration of molasses from 10 to 100 g l^{-1} (1% to 10% (w/v)) leads to a gradual increase in biomass production for strains of *B. licheniformis* TR7 and *B. subtilis* SA9 [27]. This is consistent with the results obtained in our study, in which biomass production was favored by an increase in the molasses concentration up to the optimal value of 180 g L^{-1}, above which microbial growth declined. It is also important to note that at molasses concentrations greater than 107.8 g L^{-1}, $Lch_{AL1.1}$ production by *B. licheniformis* AL1.1 was inhibited.

a) x_1= 1.33 (Y_1) x_1= 0.0515 (Y_2)



b) x_2= 1.68 (Y_1) x_2= -0.009 (Y_2)

c) x_3= -0.31(Y_1) x_3= 0.31 (Y_2)

Figure 1. Contour plot graphs showing the effect of the molasses concentration (x_1), nitrate concentration (x_2) and phosphate concentration (x_3) at the optimum conditions for biomass (Y_1, left column) and lichenysin$_{AL1.1}$ production (Y_2, right column).

On the other hand, optimum components (g L^{-1}) for $Lch_{AL1.1}$ production (Y_2) were molasses 107.8, nitrate 6.5 and phosphate 9.7. The predicted and obtained maximum production of $Lch_{AL1.1}$ was 3.2 g L^{-1}, after 72 h of incubation, 4.5-fold higher than the initial production, 0.7 g L^{-1}. It is noteworthy that the production of $Lch_{AL1.1}$ (Y_2) was not affected by the concentration of sodium nitrate (x_2) added to the medium. This suggests that the nitrogen content of the raw material was enough to support bacterial growth (Y_1) and production. In contrast, phosphate (x_3) addition was crucial, being essential for growth and production, and its buffering effect was necessary for the BS yield [32].

The optimal concentration of molasses for lichenysin production varies with the microorganisms studied, being 4% for *B. licheniformis* TR7 and *B. subtilis* SA9 [29], 7% for *B. subtilis* and *Bacillus* HS3 20B [28], and 10% for *B. licheniformis* AL 1.1 [32]. This variation might be due to the molasses composition, which can depend on the cultivation conditions and treatment of the sugarcane. When glucose was used as a carbon source, the maximum $Lch_{AL1.1}$ production, which was linked with bacterial growth, was 0.86 g L^{-1} after 24 h incubation [19]. In contrast, when molasses were used as the carbon source, production peaked after 72h and was only partially associated with bacterial growth (Figure 2). Accumulation began during the exponential phase and continued after growth ceased. Under these conditions, the 3.2g L^{-1} obtained represented a remarkable increase over the initial production [32]. Similar results (3.3 g L^{-1} BS) have been reported for *B. licheniformis* TR7 when using a molasses medium [27].

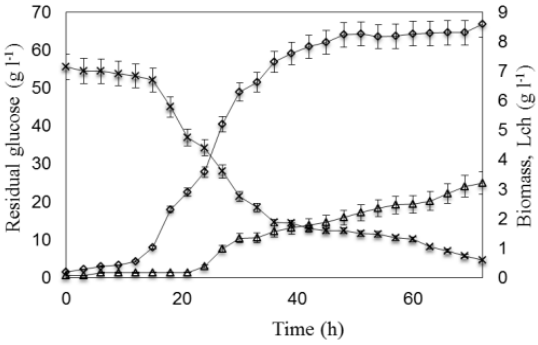


Figure 2. Time course of growth and $lichenysin_{AL1.1}$ production by *B. licheniformis.* (Δ) lichenysin; (◊) biomass; (x) residual glucose.

This work thus demonstrates the feasibility of using molasses as a component of a minimal mineral medium to produce BS (Table 2). This substrate has also been successfully used for the production of surfactin in *B. subtilis* B20 [35] and, diluted in tap water, in different strains of *Bacillus* [28]. Molasses, a by-product of the sugar industry with little commercial value, therefore has potential as a cost-saving tool, not only for the production of BS, but also for the development of other biotechnological processes, allowing the pursuit of sustainable development.

Table 2. Production data of the media used in the optimization experiments for $Lch_{AL1.1}$. Cellular yield of product formation ($Y_{P/X}$); volumetric productivity of product formation (P).

Parameter	Initial medium	Developed medium	
		Theoretical	Experimental
Lichenysin (g L^{-1})	0.73	3.20	3.20
Biomass (g L^{-1})	3.52	8.50	8.40
$Y_{p/x}$ (g g^{-1})	0.20	0.37	0.38
P (mgL^{-1} h^{-1})	10.13	44.3	44.4

Having confirmed the excellent qualities of molasses as a culture medium, and acquired knowledge about the effect of two other medium components, will be explored different strategies with the goal of fully exploiting the potential of this raw material, and if possible increasing $Lch_{AL1.1}$ production.

3. Physiological role of lichenysin

Many physiological roles are attributed to BS, which are produced by microorganisms living in a wide range of environments. Their most important function seems to be a capacity to produce emulsions to enhance the accessibility of non-water-soluble substrates. Yet the production of high surface-active compounds, like lichenysin by *B. licheniformis* AL 1.1, has been achieved with soluble nutrients, but not hydrophobic carbon sources such as n-alkanes and olive oil [19]. Further research is required to explain these results. Microbial surfactants also play an important role in the regulation of attachment-detachment of microorganisms from surfaces in natural environments. Adhesion is a physiological mechanism for growth and survival on abiotic surfaces or water-insoluble hydrocarbons

affecting bacterial transport. Other advantageous properties associated with BS are their antimicrobial activities associated with defense mechanisms and virulence factors. The advantages of BS over synthetic surfactants lie in their activity, specificity, versatility and biodegradability [13].

4. Physiochemical properties of lichenysin

Lipopeptides are characterized by high surface activity, an ability to effectively reduce surface tension, and a very low cmc. Surfactin produced by *B. subtilis* reduces surface tension of water to 27.9 - 29.5 mN m^{-1} with a cmc of 17 mg L^{-1} [36, 37], or 30 mg L^{-1} when using a molasses medium [27]. Lichenysin is more active, and has the capacity to lower the surface tension of water from 72 to 27- 28.5 mN m^{-1} [16, 19, 27, 38]. Acid precipitation of lichenysin B produces the lowest known interfacial tension against decane (0.006 mN m^{-1}) [16]. The cmc of $Lch_{AL1.1}$ is 12- 15 mg L^{-1}, while BL86 and lichenysin B have the lowest known cmc (10 mg L^{-1}) of any known surfactants under optimal conditions [16].

5. Biomedical and environmental applications of lichenysin

At the beginning of the XXI century, the world production of surfactants was 17 million metric tonnes, with an expected growth of 3-4% per year. Their most important application is in the cleaning industry (54%), followed by the textile, leather and paper industries. Only 10% of their usage is in pharmaceuticals and cosmetics, but recent studies have revealed interesting properties with potential new applications [1]. Research to find new products in order to develop new treatments has become a priority for the pharmaceutical industry. BS are considered relevant molecules with application in the treatment of many diseases. $Lch_{AL1.1}$ is stable under a wide pH range (6-11), high temperatures (up to 100ºC) and different salt concentrations (up to 20%), which are beneficial properties for exploitation in industrial and environmental processes [19], with potential applications in healthcare, cosmetics or food products with high added value.

Environment remediation. Oil remains a predominant source of energy and its transport causes accidents in marine environments. BS can be applied in environment bioremediation for oil dispersion and degradation after an accident at sea or for heavy metal mobilization after soil contamination. Saimmai et al. [27] reported that *B. lichenyformis* TR7 and

B. subtilis SA9 can enhance the solubility of polyaromatic hydrocarbons and therefore have the potential to remove oils from the environment. Surfactant production cost indicates that *in situ* production by microorganisms is a more economical strategy than the use of purified BS.

Antimicrobial activity. The use of BS as antimicrobial agents has been documented [39, 40, 41]. According to their structure, BS exert their toxicity on cell membrane permeability with a similar effect to that of detergents. The antimicrobial properties of Lichenysin A produced by *B. licheniformis* BAS50 and surfactin have been studied and compared. Surfactin is clearly more active against both Gram positive and Gram negative bacteria than lichenysin. A native form of lichenysin A presented antimicrobial activity against *Acinetobacter calcoaceticus*, *Alcaligenes eutrophus*, *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas fluorescens* cells [42]. BS produced by *B. subtilis* SPB1 showed high antimicrobial activity against Gram negative cocci such as *Enterococcus faecalis* and *S. aureus*. These results are of interest, since these microorganisms are naturally resistant to many commonly used antibiotics. BS activity against Gram negative bacteria is lower. An important antifungal activity against *Penicillium notatum*, *Penicillium italicum*, *Aspergillus niger* and *Candida albicans* has also been observed [43]. Additionally, lichenysin has anti-inflammatory, antitumor and inmunosupressive properties, but its use is hampered by its hemolytic activity [44].

Emulsion capacity. Many promising new drug candidates, active components or food additives tend to have low water solubility, and consequently fail to enter industrial development processes. The incorporation of lipophilic compounds in O/W emulsions is an attractive solution to solubility problems. The capacity of $Lch_{AL1.1}$ to emulsify oils used in cosmetic preparations (isopropyl palmitate and myristate, octyldodecanol, cetearyl ethylhexaonate and caprylic triglycerides) has been demonstrated. The thermic resistance and stability of BS favors their application in industrial fabrication processes.

Biofilms. A biofilm is an organized ecosystem formed when microorganism growth is strongly adhered to a surface. The advantages of this ecosystem for the bacteria include more stability, synergism, and increased resistance to antibiotics and disinfectants. Biofilms may cause biodeterioration of materials and can act as a reservoir of contaminants with potential health problems. Among new approaches to the control of biofilm formation, BS application may be considered as a green strategy

because of their natural origin, simple production and biodegradability. The effect of surface pre- and post-treatment by $Lch_{AL1.1}$ on microbial adhesion has been studied by Coronel et al. [32]. When a polystyrene surface was covered with $Lch_{AL1.1}$, a decrease in microbial adhesion was observed in *Candida albicans* and *Staphylococcus aureus* (>60%). With *Escherichia coli*, *Yersinia enterocolytica*, *Listeria monocytogenes* and *Campylobacter jejuni*, an adhesion decrease of 40% was measured. The anionic nature of lichenysin may be responsible for the adhesion reduction in negatively charged surface microorganisms, due to forces of electrostatic repulsion. When the detergent effect of $Lch_{AL1.1}$ was studied, an adhesion decrease between 50-30% was observed. This result could be a consequence of BS penetration and absorption at the interface between the solid surface and the attached biofilm-forming bacteria, which reduced the interfacial tension and favored the bacterial detachment. According to these results, lichenysin could be an interesting alternative for controlling microbial biofilm growth on critical surfaces, including the protection of medical materials during use. Pathogen implantation in industrial and medical equipment or products is generally controlled by cleaning and disinfection procedures, but microorganisms possess a certain degree of resistance to the chemical-based products used [45].

Biomembranes. The molecular relationship established between $Lch_{AL1.1}$ and biomembranes has been explored in a recent interesting study. Hemolysis can be due to membrane permeabilization caused by pore formation or by disruption/solubilization of the membrane. In presence of human erythrocytes and $Lch_{AL1.1}$ at concentrations below its cmc, a slow process of hemolysis was developed. The release of K^+ before the hemoglobin leakage and hemolysis inhibition by PEG 3350 suggests that $Lch_{AL1.1}$ induced hemolysis by a colloid-osmotic mechanism, producing pores close to 34Å. These pores seem to be formed by clusters of lichenysin surrounded by phospholipids. Additionally, it was observed that the lipid membrane composition plays a role in the target membrane selectivity, since a high cholesterol ratio decreased the extent of leakage. The absence of cholesterol in bacterial membranes compared to eukaryotic membranes may be related with BS activity. The authors conclude that the presence of $Lch_{AL1.1}$ in the membrane increased the permeability to hydrophilic molecules, facilitating its flux across the lipid palisade [46]. Considering the interesting potential applications of BS in medicine as drug vehicles, as well as in the cosmetics and food industries, this study of BS hemolytic activity and behavior at membranes is of great importance.

6. Conclusion

$Lch_{AL1.1}$, the anionic BS produced by *B. licheniformis* AL1.1, has notable anti-adhesion activity, being able to prevent and eliminate biofilm formation by pathogenic strains. $Lch_{AL1.1}$ also produces colloid-osmotic hemolysis by pore induction and permeabilizes1-palmitoyl-2-oleoyl-*sn*-glycerol-3-hosphocholine (POPC) membranes to small-sized solutes, by the formation of lichenysin clusters surrounded by phospholipids. Notably, $Lch_{AL1.1}$ action is related with the presence of cholesterol, an important component of eukaryotic but not bacterial membranes. Optimizing the production of $Lch_{AL1.1}$ has confirmed that molasses can be regarded as a useful resource for biotechnological applications. The use of agro-industrial substrates has an important role in the sustainable and competitive development of several industrial sectors, as well as in industrial residues management. This new growth medium resulted in a 4-fold increase in production compared with the non-optimized medium. Nevertheless, despite their attractive properties for application in different fields, the commercial production of microbial surfactants such as lichenysin is still not a reality and more studies are necessary to explore their properties and disadvantages in more depth.

Acknowledgements

Financial support was received from the Spanish Ministerio de Economia y Competitividad, project CTQ2014-59632-R and the Generalitat de Catalunya Project 2014-SGR-534 and the Universitat de Barcelona. We thank the Government of Ecuador for a SENESCYT-ESPOL grant.

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Research Signpost
37/661 (2), Fort P.O.
Trivandrum-695 023
Kerala, India

Recent Advances in Pharmaceutical Sciences VI, 2016: 165-188 ISBN: 978-81-308-0566-5
Editors: Diego Muñoz-Torrero, Àngela Domínguez and Àngels Manresa

10. Advances in the research of new genetic markers for the detection of *Helicobacter pylori* infection

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Abstract. *Helicobacter pylori* is one of the human pathogens with highest prevalence around the world. Colonizing the human stomach, *H. pylori* could lead to peptic ulceration, gastric adenocarcinoma and gastric lymphoma. *H. pylori* is a genetically diverse bacterial species, and variability in virulence factors has a role in bacterial pathogenesis and progression to gastric cancer, although bacterium and host factors of progression are not completely understood. In a recent study, we have demonstrated that six housekeeping genes related to *H. pylori* pathogenesis were specifically amplified for *H. pylori* in a total of 52 *H. pylori* clones isolated from 11 patients. Although most clones isolated from the same patient showed identical gene sequences, events of multiple infection and microevolution were detected. We consider that housekeeping genes could be useful for *H. pylori* detection and to elucidate the mode of transmission and the relevance of the

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multiple infection. Further genetic studies are required to provide powerful tools to face all current unmet challenges of *H. pylori* infection, such as the elucidation of mode of transmission, the development of new sensitive and specific PCR methods for *H. pylori* detection, and implication of *H. pylori* in other diseases.

Introduction

H. pylori is one of the human pathogens with highest prevalence around the world (50% of the world´s population, with proportions as high as 80% in developing countries) [1,2], which was first isolated in culture media by Warren and Marshall in 1982. This Gram negative, spiral-shaped, microaerophilic bacterium, with positive findings for urease, oxidase and catalase, colonizes the human gastric epithelium [2,3], where it can be found in two forms, the bacillary and coccoid forms [4]. *H. pylori* is difficult to culture *in vitro*, particularly in liquid media, being its growth demonstrated in several nutrient-rich media at 37ºC [5].

H. pylori is a genetically diverse bacterial species, and variability in virulence factors has a role in bacterial pathogenesis [6,7]. Accumulating evidences support that *H. pylori* infection cause a list of diseases, ranging from gastric to extra-gastric diseases, from chronic gastritis to gastric carcinoma, and thus this bacterium is recognized as Class I carcinogenic pathogen in humans [8,9]. Gastric adenocarcinoma is the second highest cause of cancer deaths worldwide. The nearly one million deaths per year are due to a combination of high incidence, aggressive disease course, and lack of effective treatment options. *H. pylori* causes distal but not proximal gastric adenocarcinoma, distal being the most common form. *H. pylori* also causes B cell mucosa-associated lymphoid tissue (MALT) lymphoma of the stomach [3,10].

Progression from initial gastritis toward more severe disease such as gastric cancer occurs only in a small proportion of the infected individuals and is likely to depend on host factors, exposure to lifestyle factors, and bacterial factors [1,7,10]. Furthermore, epidemiological and eradication studies have demonstrated a casual relationship between *H. pylori* infections and endothelial dysfunction, leading to vascular diseases [3,11]. It has also been suggested the possible association of *H. pylori* infection with several extragastric effects, including hepatobiliary and pancreatic diseases, although further research is needed in this issue [12].

H. pylori colonization usually occurs in childhood, particularly in developing areas, and usually in the same family for a cohort effect [3], but infection persists lifelong in the absence of treatment. *H. pylori* persistence is central to pathogenesis; ulcers occur mainly in mid- or late adulthood after

many years of infection and inflammation, and gastric adenocarcinoma occurs in late adulthood after an even longer period of chronic inflammation and epithelial damage [10].

H. pylori strains appear to be spread by person-to-person contact and DNA fingerprinting has provided evidence of transmission between family members [13]. To date, however, the exact mode of transmission is still uncertain. While this organism is isolated from the human stomach, it has not been consistently isolated from other niches, and thus the mechanism by which it colonizes the human stomach remains largely unknown [2].

Different invasive and non-invasive diagnostic tests are available for the diagnosis of *H. pylori* in the individual patient. The non-invasive tests obviate the need for endoscopy and can be surely more accepted by the subjects [3]. Therefore, further research is needed to improve non-invasive methods, mainly based on PCR methods.

The goal of *H. pylori* treatment is the complete elimination of the organism. Once this has been achieved, reinfection rates are low; thus, the benefit of treatment is durable [14]. Curing the infection interferes with the precancerous cascade if accomplished early in the process, and can prevent cancer development [15]. The so-called triple therapies, combinations of one antisecretory agent with two antimicrobial agents for 7 to 14 days, have been extensively evaluated and approved [14]. Failure of antibiotic treatment, often caused by antibiotic resistant *H. pylori* strains, however, is frequent [14] and appears to be increasing, so susceptibility testing for antibiotics plays an important role in treatment [16].

The recent findings on the bacterial virulence factors, effects of *H. pylori* on epithelial cells, genetic polymorphism of both the bacterium and its host, and the environmental factors can help to understand the role of this bacterium in gastric carcinogenesis [9]. However, more research is needed to globally face this prevalent infection, such as: to screen and treat only strains that are known to cause disease, the elucidation of mode of transmission, implication of *H. pylori* in other diseases, the role of mixed infections, the development of new sensitive and specific PCR methods for *H. pylori* detection in gastric samples and other specimens, and the development of a safe and efficient preventive vaccine that could address the wide diversity of strains.

1. Epidemiology and population genetics of *H. pylori*

H. pylori is one of the most common bacterial infectious agents; it inhabits the stomachs of more than half of the world's population. The prevalence of *H. pylori* infection is decreasing in developed countries as

showed by the lower prevalence in the younger generations. In north European and North American populations, about one-third of adults are still infected, whereas in south and east Europe, South America, Africa, and Asia, the prevalence of *H. pylori* is often higher than 50%. These prevalences may differ between different ethnic, social or age groups within the same country [2,10,17]. The prevalence of infection seems to mostly depend on the rate of acquisition, but also on the rate of loss of infection and the length of the persistence period between acquisition and loss [2]. Several factors have been associated with *H. pylori* infection as a low socioeconomic status, living in a rural area, in crowded homes, and having contaminated sources of drinking water [17].

Despite the high prevalence of *H. pylori* infection in Africa and South Asia, the incidence of gastric cancer in these areas is much lower than in other countries. Furthermore, the incidence of gastric cancer tends to decrease from north to south in East Asia. Such geographic differences in the pathology can be explained, at least in part, by the presence of different types of *H. pylori* virulence factors determinants, especially *cagA*, *vacA* genes, and the right end of the *cag* pathogenicity island. The genotype of the virulence genes is useful not only for the study of gastroduodenal diseases related to *H. pylori* but also as a tool to track human migration although with less accuracy than the multilocus sequence typing (MLST) [18].

MLST analysis using seven housekeeping genes (*atpA*, *efp*, *mutY*, *ppa*, *trpC*, *ureI*, and *yphC*) is useful to predict the history of human migrations and may be at least as informative as most human genetic markers [18,19]. *H. pylori* is ubiquitous and possesses strong phylogeographic structure, suggesting that bacterial polymorphisms reflect human phylogeography and historical migrations. Population structure analysis based on MLST has revealed seven modern population types of *H. pylori* (Fig. 1): hpEurope, hpAfrica1 (with two subpopulations, hspSAfrica, and hspWAfrica), hpAfrica2, hpAsia2, hpEastAsia (with three subpopulations, hspAmerind hspEAsia, and hspMaori), hpNEAfrica, and hpSahul [18,20,21,22,23].

The geographic sources of the different strains isolated reflect major events in human settlement history (Fig. 1), such as the colonization of Polynesia and the Americas and the African Bantu migrations. Like humans, simulations indicate that *H. pylori* seems to have spread from east Africa around 58,000 years ago. Therefore, these data indicate that anatomically modern humans were already infected by *H. pylori* before their migrations from Africa and demonstrate that *H. pylori* has remained intimately associated with their human host populations ever since [20,21,22,23].

The existing European population of *H. pylori* is known to be a hybrid between Asian and African bacteria, although there are hypotheses about

when and where the hybridization took place. In a recent study, a 5300-year-old *H. pylori* genome from a European Copper age glacier mummy was determined and reconstructed. Results obtained showed that the "Iceman" *H. pylori* was a nearly representative of the bacterial population of Asian origin that existed in Europe before hybridization, suggesting that the African population arrived in Europe within the past few thousand years [24].

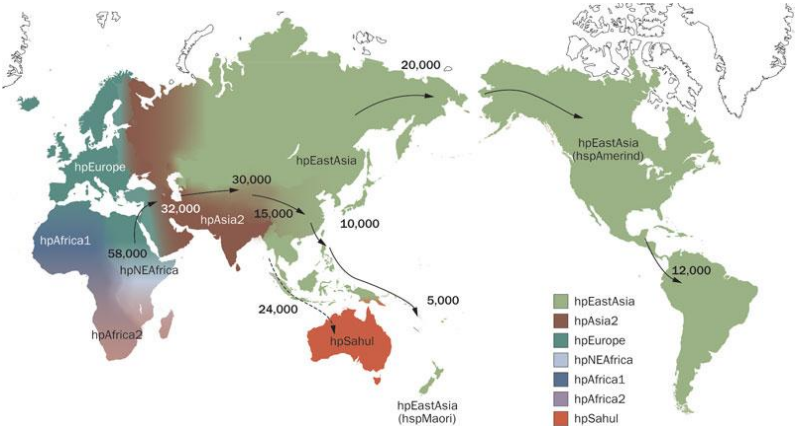

Figure 1. Distribution of *Helicobacter pylori* genotypes before Columbus found the New World and human migration to America and Oceania began. There are seven modern *H. pylori* population types—hpEurope, hpEastAsia, hpAfrica1, hpAfrica2, hpAsia2, hpNEAfrica and hpSahul. hpEurope includes almost all *H. pylori* strains isolated from ethnic Europeans, including people from countries colonized by Europeans. Most *H. pylori* isolates from East Asia belong to hpEastAsia, which includes hspMaori (Polynesians, Melanesians, and native Taiwanese), hspAmerind (American Indians) and hspEAsia (East Asia) subpopulations. hpAsia2 strains are isolated in South, Southeast and Central Asia; hpAfrica1 in west Africa, South Africa and African Americans. hpNEAfrica is predominantly made up of isolates from Northeast Africa. hpAfrica2 is very distinct from any other type and has currently only been isolated in South Africa. hpSahul is a novel group specific to *H. pylori* strains isolated from Australian Aborigines and Highlanders of New Guinea. *H. pylori* is predicted to have spread from East Africa over the same time period as anatomically modern humans (~58,000 years ago), and has remained intimately associated with their human hosts ever since. Estimated global patterns of *H. pylori* migration are indicated by arrows and the numbers show the estimated time since they migrated (years ago). The broken arrow indicates an unconfirmed migration pattern. (reprinted by permission from Macmillan Publishers Ltd: ref. [23], copyright 2010).

Elucidation of the pattern of population subdivision is also of medical interest. Geographically variable results regarding the association of putative virulence factors with disease might well reflect differences in the local prevalence of the individual *H. pylori* populations. Similarly, the development of diagnostic tests, antibiotics, and vaccines needs to account for global diversity and will be aided by the availability of representative isolates [20].

2. Transmission of *H. pylori* infection

Currently, the majority of available evidence points to the transmission of *H. pylori* from human-to-human. The exact route of transmission from person-to-person is still unknown. Ingestion of the bacteria, which is the most likely portal of entry, may occur by one or a combination of three means: oral-oral, gastro-oral, or fecal-oral. However, determination of the dominant route has not been possible to date [2].

H. pylori has been detected in dental plaque and saliva, suggesting that the oral cavity may be an extra-gastric reservoir and play an important role in both transmission and recurrence [25]. Since the human stomach is the primary niche of *H. pylori*, it is reasonable to suggest a direct gastro-oral route of transmission mediated by refluxed gastric juice. This hypothesis is supported by studies showing the presence of *H. pylori* in the gastric juice, as vomitus [2,26,27].

Although *H. pylori* is sensitive to the bile's bactericidal effect, different studies have detected viable *H. pylori* in stools [2,28,29], thus supporting the fecal-oral route of transmission.

H. pylori has also been detected in environmental or animal reservoirs, although it has not been demonstrated yet if they are natural or primary vehicles of transmission [2].

Presence of *H. pylori* has been detected in un-washed vegetables, as leek, traditional salad, basil or lettuce [30]. More research is needed to establish different foods at high risk of *H. pylori* presence and transmission.

Moreover, the demonstration of transmission of viable cells is even more complicated taking into account the difficulties of the bacterium to grow *in vitro* [5].

3. Role of *Helicobacter pylori* infection in pathogenesis of gastric carcinoma

The gastric mucosa is well protected against bacterial infections because of its acidic milieu that acts as a first line of defense against food-borne

microbes. In addition, the reflux of bile acids in the stomach, the thickness of the mucus layer, and the effectiveness of gastric peristalsis might have impeded bacterial colonization of the stomach. Nevertheless, *H. pylori* is highly adapted to this ecological niche, with a unique array of features that permit entry into the mucus, swimming and spatial orientation in the mucus, attachment to epithelial cells, evasion of the immune response, and, as a result, persistent colonization and transmission [14].

For colonization and survival in the human stomach all *Helicobacter* isolates require urease and flagella. Urease metabolizes urea to carbon dioxide and ammonia to buffer the gastric acid. Flagella allow the bacterium to swim across the viscous gastric mucus and reach the more neutral pH below the mucus. *H. pylori* may use at least five different adhesins to attach to gastric epithelial cells. Adhesion by adhesins to epithelial gastric cells is important for the beginning of infection and for the enhanced inflammatory response. Following colonization, *H. pylori* must acquire nutrients from the gastric mucosa, in which the acquisition of iron from the host is particularly important [3].

In most infected people, the bacterium acts as a commensal organism inducing chronic asymptomatic gastritis that can last for life. In other cases, however, it is responsible for a high morbidity and mortality as a consequence of peptic ulcers and gastric cancer. Chronic gastritis may progress to intestinal metaplasia, dysplasia and eventually gastric cancer (Fig. 2), a multi-step process known as the Correa pathway [15,31]. *H. pylori* infection is the strongest known risk factor for gastric cancer, as supported by epidemiological, preclinical and clinical studies [9,14]. In fact, gastric cancer is the fourth cause of death because of a cancer in Europe (GLOBOCAN 2012; http://globocan.iarc.fr/).

However, the mechanism of *H. pylori* developing gastric carcinoma has not been well defined. Among infected individuals, approximately 10% develop severe gastric lesions such as peptic ulcer disease and 1–3% progresses to gastric cancer. The outcomes of *H. pylori* infection are determined by bacterial virulence, genetic polymorphism of both hosts and bacteria, as well as environmental factors [9,31]. Environmental factors, such as cigarette smoking, are major risk factors for duodenal ulceration among *H. pylori*-infected people [3]. Other important factors include stress, childhood living conditions, diet, alcohol and use of non-steroidal anti-inflammatory drugs (NSAIDs) [3,31,32].

H. pylori infection commonly lasts for decades, provoking a series of histological changes including destruction of intercellular junctions, apoptosis and proliferation of epithelial cells and malignant transformation (Fig. 3) [9,33,34]. The genotypes of *H. pylori* strains, host genetic

polymorphisms, environmental factors like high salt diet, smoking habit and certain gastric commensal organisms have been determined to be associated with occurrence of gastric cancer. *H. pylori* genetic polymorphisms, effects of specific *H. pylori* products on gastric epithelium and cellular signaling process have been intensively investigated in recent decades [9,35].

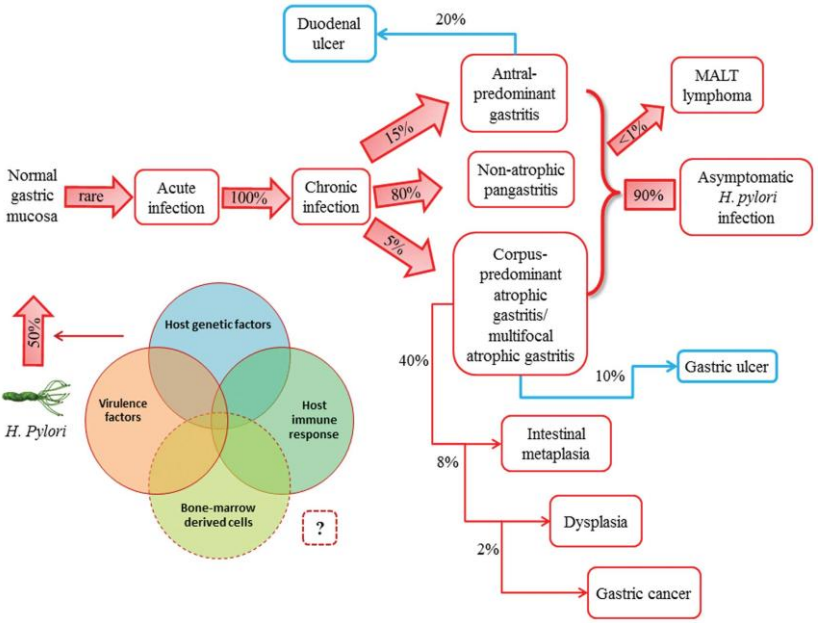

Figure 2. Natural history of *H. pylori* infection. *H. pylori* is usually acquired in childhood, whereas acute infection with the bacterium is rarely diagnosed. Instead, chronic gastritis develops in almost all persistently colonized individuals, 90% of whom will remain asymptomatic. The clinical course of *H. pylori* infection is highly variable depending on bacterial and host (genetic and immune) factors. Recent studies have supported the possible role of bone marrow-derived cells (i.e., gastric stem cells) in tumor progression. Patients with increased acid secretion are more likely to have antral-predominant gastritis, which predisposes to duodenal ulcers. Patients with low acid secretion will more likely develop gastritis in the body of the stomach and are thus more likely to develop gastric ulcer, leading to gastric atrophy, intestinal metaplasia, dysplasia and, finally, in rare cases, gastric carcinoma. This sequence of events is more frequent in people of advanced age. *H. pylori* infection induces the formation of mucosa-associated lymphoid tissue (MALT) in the gastric mucosa and MALT lymphoma is another rare complication of *H. pylori* infection. (reprinted by permission from Spandidos Publications: ref. [31], copyright 2013).

Besides its arsenal of virulence factors, persistence of *H. pylori* is strongly influenced by the ability of the bacterium to evade, overthrow and manipulate the host's immune system. This bacterium can evade detection by several innate immune receptors through target modification and it can subvert other innate recognition pathways through the suppression of downstream signal transduction, whereas evasion of adaptive immunity is achieved by the modulation of effector T cell functions [36].

Despite the host's vigorous immune response, *H. pylori* is capable of persisting for decades in its human host. Interestingly, coccoid forms appear to be the persisting form, allowing *H. pylori* to spread between human hosts. It has been shown that the morphological transition from bacillary to coccoid forms is accompanied by modifications of the bacterial cell wall peptidoglycan, in which AmiA protein is essential. Cell wall modifications and morphological transition allow the coccoid forms to escape detection by the immune system and therefore could participate in the persistence of *H. pylori* infection during the lifetime of its human host [4].

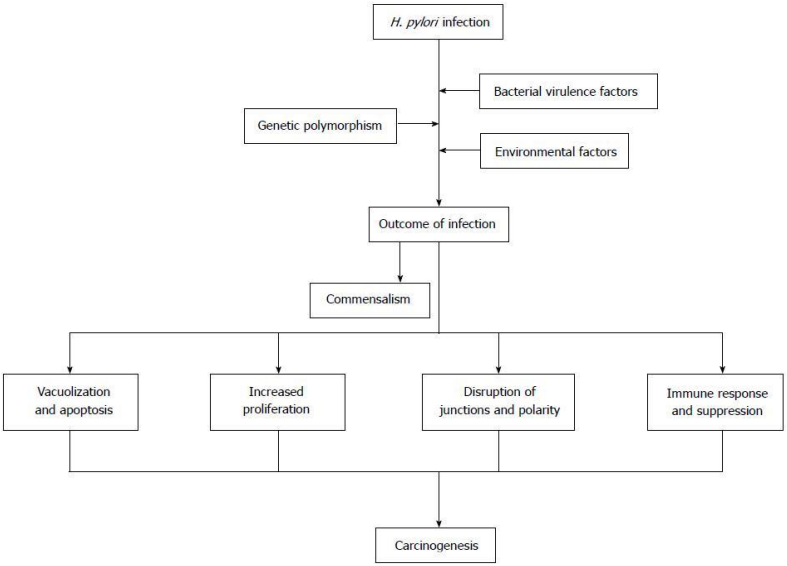

Figure 3. The pathogenesis of *Helicobacter pylori*-associated gastric cancer is a multi-factorial process, its development depends on a combination of host, bacterial and environmental factors, and the pathological changes might progress in steps. (reprinted by permission from Baishideng Publishing Group Inc.: ref. [9], copyright 2016).

4. *H. pylori* virulence factors

Crucial bacterial factors associated with pathogenicity comprise flagella, urease, peptidoglycan, lipopolysaccharide (LPS), Hsp60 (= Cpn60) chaperone, a type IV secretion system encoded by the *cag* pathogenicity island containing the effector protein CagA, the vacuolating cytotoxin (VacA), and others [37].

The high number of these factors and allelic variation of the involved genes generates a highly complex scenario and reveals the difficulties in testing the contribution of each individual factor. Much effort has been put into identifying the molecular mechanisms associated with *H. pylori*-associated pathogenesis using human primary tissues, Mongolian gerbils, transgenic, knockout and other mice, as well as *in vitro* cell model systems [37].

Studies on *H. pylori* heterogeneity have proved that the strongest virulence factors were amongst the genes within the *cag* pathogenicity island (PAI). The mechanisms underlying *H. pylori*-related gastric carcinogenesis, however, remain unclear [9], with the virulence factors CagA and VacA having a prominent role.

Development of gastric and hematological carcinoma has been observed in the mice that were genetically modified to express CagA [9,38]. Studies revealed that *cagA+*/*vacAs1+*/*vacAm1+ H. pylori* strains promoted pathogenesis of intestinal metaplasia and gastric carcinoma [9,39].

4.1. Flagella

H. pylori possess two to six sheathed unipolar flagella, which confer motility and constitute a virulence factor shown to be absolutely essential for colonization [40]. Non-motile mutants lacking flagella are unable to establish persistent infection in animal models [40,41,42], although it has also been shown that the presence of flagella alone is not sufficient for colonization [40,43]

4.2. Urease

Urease can play a broad role in the pathogenesis associated with *H. pylori* infection. Although urease is known for its enzymatic activity (converts urea into ammonia and bicarbonate to counteract the low acidity of the stomach), it is also correlated with the dual function of adhesivity and immunogenicity [3,44].

4.3. Peptidoglycan

The mechanism by which *H. pylori* generates helical shape is unknown. A novel study identified four genes being involved: peptidoglycan endopeptidases (*csd1-3*) and *ccmA*. The findings suggest that the coordinated action of multiple proteins relaxes peptidoglycan crosslinking, enabling helical cell curvature and twist, which is required for robust bacterial colonization in the stomach [35,45].

4.4. Lipopolysaccharide

O polysaccharides of the gastric pathogen *H. pylori* contain Lewis antigens, mimicking glycan structures produced by human cells. The interaction of Lewis antigens with human dendritic cells induces a modulation of the immune response, contributing to the *H. pylori* virulence. The amount and position of Lewis antigens in the LPS varies among *H. pylori* isolates, indicating an adaptation to the host [46].

Two new enzymes typically involved in LPS biosynthesis were discovered in *H. pylori*, glycosyltransferase WecA and O-antigen ligase WaaL, but a flippase enzyme, normally involved in O-antigen synthesis, could not be detected. Instead, *H. pylori* uses a translocase named Wzk in a novel LPS biosynthetic pathway, evolutionarily connected to protein N-glycosylation [35,46]. In addition, 3-deoxy-d-manno-octulosonic acid (Kdo) hydrolase genes (HP0579 and HP0580) were shown to affect the expression of O-antigen Lewis epitopes [47]. Finally, an ADP-l-glycero-d-manno-heptose-6-epimerase ortholog (HP0859) was characterized. DHP0859 mutants exhibited severe truncation of LPS, decreased growth rate, higher susceptibility to novobiocin, and failed to induce the AGS elongation phenotype in AGS cells. Genetic complementation restored these phenotypes, revealing HP0859 essential for LPS biosynthesis and virulence [35,48].

4.5. Hsp60 superficial protein

H. pylori produces two heat shock proteins (Hsps): a groEs-like HspsA (size 13 kD) and groEL-like HspsB (size 54–60 kD, Hsps60). Hsps60 is in particular shared by *H. pylori* and eukaryotic cells [3,49]. The homologies between bacteria and human Hsp60 antigens leads to an antibody response directed to both the bacteria and to human tissues that express Hsps, including vascular endothelial cells (autoimmune response) [3].

4.6. CagA

CagA, a highly immunogenic protein, is encoded at one end of the *cag* PAI, which encode the components to form the type IV secretion system (T4SS) [7,9,50]. As a component of T4SS, CagL protein binds to and activates the integrin α5β1 receptor on gastric epithelial cells and triggers CagA delivery into the target cells [9,51].

H. pylori strains harboring the cag PAI or producing CagA are related to enhanced inflammation and risk of ulcers and carcinoma [9,52,53]. Moreover, the C-terminal EPIYA-containing region of CagA is polymorphic and isolates from gastric cancer patients frequently have a high number of EPIYA motifs [54]. CagA contributes to myriad signaling alterations, which profoundly affects physiology of host epithelial cells [9].

Once inside host cells, CagA is tyrosine phosphorylated, triggering the cellular signaling pathways leading to expression of proinflammatory cytokines and chemokines, and deregulates the signaling pathways that control host cell shape, adhesion and transformation [9,35,55].

Unphosphorylated CagA interacts with certain intracellular proteins, up-regulate production of proinflammatory cytokines, provoke mitogenic responses and disrupt intercellular junctions and epithelial cell polarity [9,55,56]. Additionally, CagA intoxicates dendritic cells leading to impaired activation, decreased inflammatory cytokine production and Th1 immune response [9,57]. Recently, it was confirmed that *H. pylori* infection resulted in rapid association of the virulence factor CagA with the c-Met receptor, activation of signaling and epithelial proliferation [9,58].

4.7. Vacuolating cytotoxin A

Vacuolating cytotoxin A (VacA) is a bacterial toxin with multiple activities, contributing to multiple structural and functional alterations of epithelial cells [7,9]. After secretion by the bacterium through the type V secretion system, VacA binds to host cells interfering with endosomal maturation and leading to vacuolation, enhances leakage of nutrients by destruction of barrier function at tight junctions of epithelial cells, provokes mitochondrial damage and cell apoptosis, which improves *H. pylori* growth [9,59,60,61]. Recent studies proved that VacA could disrupt phagocytosis, interfere with antigen presentation, restrain T cell activation in vitro and inhibit T cell proliferation independing of NFAT

(nuclear factor of activated T cells) activation or IL-2 expression [9,62,63,64]. These effects of VacA on the immune system may explain how *H. pylori* evades adaptive immune responses to establish persistent infection [9].

The secreted mature form of VacA is a 88 kDa monomer comprising two domains designated p33 (residues 1–311) and p55 (residues 312–821) [65]. VacA harbours different allelic variants that modulate its activity. The amino-terminal end of VacA (residues -33–1), designated as the signal peptide or "s-region", carrying the s1 or s2 alleles; the intermediate region or "i-region" that encodes part of the p33 (residues 190–223) with the allelic variants i1 or i2; and the mid region or "m-region", existing as an m1 or m2 allele [7,65,66]. Mosaic combinations of the s-, i- and m-regions exist and relate to the in vitro cytotoxin activity. Strains with alleles s1/i1/m1 have been associated with peptic ulcer disease and gastric cancer [67]. González *et al*. (2011) [7] showed that patients infected with high virulence strains (*cagA*+, *vacA* s1i1m1) have a higher risk of progression of the preneoplastic lesions to gastric cancer in relation to patients infected with low virulence strains.

4.8. Other virulence factors

Adherence to epithelial cells is essential for *H. pylori* colonization and delivery of virulence factors to host cells. Some *H. pylori* adhesins have been described, including the sialic acid-binding adhesins BabA and SabA [9,68,69], and OipA, an inflammation-related outer membrane protein [9,70]. Other outer membrane proteins (OMPs) as HomA and HomB have been associated with peptic ulcer disease (PUD) [3,71,72]. The development of duodenal ulcer has also been correlated to the presence of the duodenal ulcer promoting gene A (*dupA*), but its role in gastric cancer is controversial [70].

Other *H. pylori* virulence factors described are HrgA, GGT, HP-NAP, HopQ, etc. Restriction endonuclease-replacing gene A (*hrgA*) is considered as an important virulence determinant in *H. pylori*-associated gastric diseases such as carcinoma [3,73,74]. GGT is a gamma-glutamyl transpeptidase related to increased levels of hydrogen peroxide and IL-8 in epithelial cells and *H. pylori*-associated diseases [9,75,76,77]. The *H. pylori* neutrophil-activating protein (HP-NAP) is another virulence determinant that stimulates neutrophil high production of oxygen radicals and adhesion to endothelial cells [3,78]. It has also been shown that the outer membrane

protein HopQ is involved in leukocyte migration inhibition, together with CagA, suggesting an unprecedented role of the HopQ-CagA axis in immune modulation [79].

5. Currently used diagnosis methods

In patients with *H. pylori*-related diseases, a reliable diagnosis of infection with this bacterium is crucial, but no single test can be considered the gold standard [80,81]. Testing for *H. pylori* infection is indicated in patients with active peptic ulcer disease, a past history of documented peptic ulcer, or gastric MALT lymphoma [82].

Different invasive and non-invasive diagnostic tests are available for the diagnosis of *H. pylori* in the individual patient. The non-invasive tests obviate the need for endoscopy and can be surely more accepted by the subjects [3].

Histology is usually considered to be the gold standard in the direct detection of *H. pylori* infection and is also the first method used for the detection of *H. pylori*. Several factors, however, influence the diagnostic accuracy of histology, such as site, size and number of biopsies, staining methods, proton pump inhibitor, antibiotics and experience of the examining pathologist [81]. The rapid urease test (RUT) is the most commonly used biopsy-based method to diagnose *H. pylori* infection because it is simple, rapid and accurate. However, it requires a high density of bacteria, and anything that reduces the bacterial load may produce false-negative tests [80,83]. Culturing of *H. pylori* from gastric biopsy specimen is a highly specific but less sensitive method. In general, culturing has almost 100% specificity, but the sensitivity of culture shows significant variation, between 85-95% [81].

The non-invasive tests as diagnostic tool in *H. pylori* infections of patients with various gastrointestinal disorders are strongly important because they make the endoscopy unnecessary in different situations [3]. Urea breath test (UBT) has been used for almost 30 years and is still the most popular and accurate noninvasive test for diagnosis of *H. pylori* infection. By the urease activity of *H. pylori*, the ^{13}C- or ^{14}C-labeled urea ingested by the patient is hydrolyzed to labeled CO_2 in stomach, then labeled CO_2 is absorbed in the blood and exhaled by breathing in which labeled CO_2 is measured [81]. Stools antigen test (SAT) is the other noninvasive method with good sensitivity and specificity (94% and 97%, respectively) in the diagnosis of *H. pylori* infection [81].

The pre-endoscopy screening may be performed principally through serological markers (detection of different kinds of immunoglobulins). For CagA detection, serology has proved to be useful, being CagA protein a factor with good antigenic properties, easy and reliable to perform and prone to reveal the presence of cag pathogenicity island [3,84]. Hsp60 is also a good antigen so that its detection can be performed through the appearance of specific antibodies against it [3,85].

The rapid and easy detection of virulent strains using novel PCR methods has been suggested as a mean to avoid the consequences of a long-lasting untreated infection [3,80]. Real-time PCR is a sensitive, accurate method of diagnosing *H. pylori* infection. Compared to other available methods for diagnosing *H. pylori* for clinical and research purposes, PCR yields high sensitivity and specificity for *H. pylori* in frozen samples from patients with nonbleeding and bleeding peptic ulcer [86].

A useful approach is to use the new and advanced multiplex PCR methods in gastric tissue samples, which could contribute to gain insights into the genotypic variability exhibited by this pathogen [3,80,87]. Novel ultra-sensitive methods as digital PCR could also contribute to detect cases of "occult infection", which were traditionally considered as false-positive results in the ^{13}C-urea breath test [88].

6. Bacterial genetic diversity and role of mixed infections

The *H. pylori* genome (1.65 Mbp) codes for about 1,500 proteins [14]. *H. pylori* has been reported to be genetically extremely variable and this heterogeneity is proposed to be involved in the ability of *H. pylori* to cause different diseases [3,89,90], detrimental and non-detrimental chronic infections [3,7,91].

The remarkable adaptive capacity of *H. pylori* can be partly attributed to the enormous plasticity of its genome, caused by high mutation and recombination rates, and by the ability for aberrant genomic rearrangements and incorporation of non-homologous DNA. This results in an exceptionally high sequence diversity among strains that were isolated from unrelated human hosts and in an *H. pylori* core genome that consists of only approximately 1,100 genes, while the other approximately 400–500 genes per strain are strain-specific and variably present [92].

Strains of *H. pylori* exhibit considerable genetic diversity following a panmictic (non-clonal) population structure due to horizontal gene transfer and frequent recombination [21,22,93]. The estimated mutation rate reveals a mutation burst during the acute infection phase that is over 10 times faster

than the mutation rate during chronic infection, and orders of magnitude faster than mutation rates in any other bacteria. The elevated frequency of mutations in outer membrane protein genes suggests that the mutation burst facilitates rapid host adaptation of the bacteria [92].

In a recent study, it has been suggested that prolonged *in vivo* exposure of *H. pylori* to the gastric environmental conditions associated with a high-salt diet can lead to the emergence of strains adapted to these conditions. It has been shown that the production of proteins involved in iron acquisition and oxidative-stress resistance in *H. pylori* strains cultured from animals on high-salt diet differs from that of the input strain and strains isolated from animals on a regular diet [94].

It seems that the number of *H. pylori* clones isolated from a single host varies depending on the geographic region and other factors as age, socioeconomic and hygienic status [16]. It has also been demonstrated the importance of mixed infections for the genetic diversification of *H. pylori* through recombination [95]. *H. pylori* mixed infections with facilitate interstrain gene transfer and the maintenance of genetic diversity for adaptation to the gastric environment, but whether mixed infections with histological significance and tissue tropism occur in the human stomach is still unclear [96].

Recent data suggest that around 23% of *H. pylori*-infected patients have mixed infections, with different dominant strains isolated from the antrum and the corpus specimens, and being mixed infection significantly related to the appearance of intestinal metaplasia in the antrum [96]. The role of mixed infections in the development of gastric cancer, however, deserves further investigation.

Mixed infection has also been described in the pediatric population [52]. It has been shown that the panmictic population structure of *H. pylori* results from very frequent recombination during mixed colonization by unrelated strains [97].

Results of genetic studies comparing sequences of housekeeping genes have shown natural mixed infection in family members. Identical alleles found in some strains isolated from the children and parents, but not from unrelated patients, demonstrated that strains had circulated within the family. Several mechanisms, such as point mutations, intragenic recombination, and introduction of foreign alleles, were shown to enhance strain diversity within the family [13].

Using enzyme-linked immunosorbent assay and line probe assay (LiPA) techniques, the prevalence of mixed *H. pylori* colonization was analyzed in 127 subjects from Venezuela, a country of high *H. pylori* prevalence, from three regions representing different population groups: the Andes (Merida),

where Caucasian mestizos predominate, a major city near the coast (Caracas), where Amerindian-Caucasian-African mestizos predominate, and an Amazonian community (Puerto Ayacucho), where Amerindians predominate and mestizos reflect Amerindian and Caucasian ancestry. Among 121 *H. pylori*-positive persons, the prevalence of *cagA*-positive strains varied from 50% (Merida) to 86% (Puerto Ayacucho) by LiPA. Rates of mixed colonization also varied, as assessed by LiPA of the *vacA* s (mean, 49%) and m (mean, 26%) regions. In total, 55% of the individuals had genotypic evidence of mixed colonization. *vacA s1c*, a marker of Amerindian (East Asian) origin, was present in all three populations, especially from Puerto Ayacucho (86%). These results demonstrate the high prevalence of mixed colonization and indicate that the *H. pylori* East Asian *vacA* genotype has survived in all three populations tested [1].

7. Research on new markers of *H. pylori* infection and progression

The low incidence of severe disease associated with infection has suggested that there may be "beneficial" *H. pylori* organisms in addition to those that cause disease. In fact, since humans have co-evolved with *H. pylori*, they might derive some benefits from them [3,98]. Certainly, man adapts physiologically to infection during his lifetime and treatment can lead to problems such as reflux oesophagitis. In view of these real problems, it would seem preferable to screen for and treat only strains that are known to cause disease. In *H. pylori* a lot of virulence determinants have been detected affecting the infection course [3]. To date, despite intensive research, not enough knowledge exit on clear risk or disease-specific markers [99].

Recent studies have analysed miRNA expression in human *H. pylori*-infected patients with or without duodenal ulcer disease. Results obtained have shown that a set of miRNA are deregulated during chronic gastric inflammation and could be useful as a surrogate marker for determining the presence of *H. pylori* [99].

In our research group, we have amplified and sequenced six housekeeping genes (*amiA*, *cgt*, *cpn60*, *cpn70*, *dnaJ*, and *luxS*) related to *H. pylori* pathogenesis to evaluate their usefulness for the specific detection of *H. pylori*, the genetic discrimination at strain level and the detection of multiple infection. A total of 52 *H. pylori* clones, isolated from 14 gastric biopsies from 11 patients, were analyzed for this purpose [93].

All genes were specifically amplified for *H. pylori* and all clones isolated from different patients were discriminated, with gene distances ranged from 0.9 to 7.8%. Although most clones isolated from the same

patient showed identical gene sequences, an event of multiple infection was detected in all the genes and microevolution events were showed for *amiA* and *cpn60* genes [93].

These results suggest that housekeeping genes could be useful for *H. pylori* detection and to elucidate the mode of transmission and the relevance of the multiple infection [93]. These genes are potential candidates to detect *H. pylori* infection in gastric biopsies and other specimens (as gastric juice, stool, saliva, dental plaque, water, and food samples), together with others, mainly used for gastric biopsies, as *ureA*, *vacA*, 16S rRNA, and 23S rRNA genes, previously described [81,86,93].

In our study, two different strains isolated from the antral biopsy B657A were clearly detected by all genes with high distance values (1.6–7.3%). One strain was represented by the identical clones B657A-1, -2 and -3, and the other strain by the clone B657A-4, which was identical to clones isolated from B657C (corpus biopsy from the same patient), except in the case of *amiA*. This high divergence observed between both strains indicates an event of multiple infection in the antrum of the stomach [93]. Since the role of multiple infections on disease outcome and progression is still unclear [1,13,93,96], we consider that the genes sequenced in our study could be used in further research in this issue.

Of note, concatenation of *amiA*, *cgt*, *cpn60*, *cpn70*, *dnaJ*, and *luxS* partial sequences allowed us to conduct a multilocus sequence analyses (MLSA) approach, which is useful to elucidate intra- and interspecies phylogenetic relationships [93,100,101] and, in the case of *H. pylori*, the phylogeographic differentiation of bacterial populations associated to the migration of human populations [18,21,93]. Most of these genes had never been used for these purposes before, except *dnaJ* and, especially, *cpn60*, a gene useful for microbial phylogeny, detection and identification, ecology, and evolution through the analysis of the 555 bp region known as universal target (UT) analyzed in our study [93,102,103].

In conclusion, our results provide new tools for the detection of *H. pylori*, with potential applications for *H. pylori* detection in different specimens and to assess the mode of transmission, the role associated to virulence or the phylogeographic differentiation of *H. pylori* populations [93].

8. Conclusions

Stomach cancer is one of the leading causes of cancer death worldwide, despite its incidence and mortality falling in many places. The discovery in 1984 that a bacterial infection with *H. pylori* could cause stomach and

duodenal ulcers prompted work in its role in causing gastritis, and led to the first prospective study in 1991 by Forman et al., showing that infection with *H. pylori* increased the risk of stomach cancer in those infected by almost three-fold [104].

Efforts to reduce the incidence of gastric cancer by means of eradicating *H. pylori* have been variable (because of re-infections) and there is still the debate around population screening and opportunistic "seek and treat" of *H. pylori* in the absence of symptoms [104]. Instead, it would seem preferable to screen for and treat only strains that are known to cause disease, as a promise targeted intervention [3,104].

Research on the bacterial virulence factors, effects of *H. pylori* on epithelial cells, genetic polymorphism of both the bacterium and its host, and the environmental factors can help to understand the role of this bacterium in gastric carcinogenesis [9].

Other challenging issues, needing further research, are the elucidation of transmission, implication of *H. pylori* in other diseases, the role of mixed infections, the development of new sensitive and specific PCR methods for *H. pylori* detection in gastric samples and other specimens, and the development of a safe and efficient preventive vaccine that could address the wide diversity of strains.

In this context, results of our genetic study from *H. pylori* clones isolated from gastric biopsies can contribute to the advances in all these fields. Our results suggest that housekeeping genes could be useful for *H. pylori* detection and to elucidate the mode of transmission and the relevance of multiple infection [93]. We consider that further genetic studies, including sequencing of complete genomes of virulent strains and metagenomics, will provide powerful tools to face all current unmet challenges of *H. pylori* infection.

Acknowledgements

This work has been supported by a project from the Fundació La Marató de TV3 (1007/C/2013), Catalonia.

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Research Signpost
37/661 (2), Fort P.O.
Trivandrum-695 023
Kerala, India

Recent Advances in Pharmaceutical Sciences VI, 2016: 189-211 ISBN: 978-81-308-0566-5
Editors: Diego Muñoz-Torrero, Àngela Domínguez and Àngels Manresa

11. Current status of Leishmaniosis in the Balearic Islands

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Abstract. Data on leishmaniosis and its vectors (sand flies) in the Balearic Islands are scarce and restricted mainly to Majorca. According to the official data, the overall rate of human leishmaniosis (HL) is 0.7-3.5 cases per year/100,000 inhabitants (for the period 2001-2015), and the reported prevalence of canine leishmaniosis (CanL) varies between 0 and 45%, depending on the island and the dog population tested. In the present study, we investigated the sand fly fauna and current status of CanL in the Balearic Islands. Four sand fly species were captured: *Phlebotomus perniciosus*, a known vector in the Mediterranean area, *P. sergenti*, *P. papatasi* and *Sergentomyia minuta*. *P. perniciosus* was found throughout the island of Majorca, from sea level to the mountains, being detected in 70% of the capture sites and with a density of 6.7 specimens/m^2. The global density of *P. perniciosus* in Minorca was of 3.4 specimens/m^2, which constitutes a significant decrease

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compared to the results of a previous study performed 20 years ago. The influence of environmental factors on the presence or density of *P. perniciosus* differed according to the physiography of the area studied. A standard questionnaire sent to the local veterinarians in the Balearic Islands revealed that 73.8% of veterinarians had confirmed CanL cases in the previous 12 months and thought the disease was increasing in Minorca. The global seroprevalence of CanL in Minorca was 24%, being 31% among animals who had never left the island, which shows the existence of an autochthonous focus of CanL unrelated with an increasing vector density.

Introduction

Leishmaniosis is one of the world's most neglected diseases [1]. A total of 98 countries and 3 territories on 5 continents have reported endemic leishmaniosis transmission [2]. Caused by infection with a protozoan parasite of the genus *Leishmania*, this parasitic disease affects man and other mammals, and maintains its cycle by transmission through the bite of sand flies [1]. The presence of sand flies is critical for the development of the disease and one of the factors influencing its heterogeneous distribution [3].

In the Mediterranean region, leishmaniosis is caused by *L. infantum* and is considered a zoonotic disease with a secondary epidemiological cycle [4]; dogs act as the main host and parasite reservoir, and man as a secondary host [5 – 7]. The prevalence of leishmaniosis has recently increased in established endemic regions [8 – 10] and cases have emerged in areas previously considered non-endemic [11 – 14]. This increase may be due to various factors, including environmental changes and global warming [15 – 17], as indicated in predictive risk map models of leishmaniosis vectors and reservoir hosts [17 – 18].

In the Balearic Islands, previous studies have shown a heterogeneous distribution of both human and canine leishmaniosis [19 – 28]. Human leishmaniosis (HL) was designated as a notifiable disease ("Enfermedad de Declaración Obligatoria") in Spain from 1982 to 1996 [29]. Thereafter it was considered a regionally endemic disease, and each autonomous community with devolved powers over health could decide whether or not to keep it in its notifiable disease list. The Ministry of Health of the Govern de les Illes Balears decreed HL to be a "Malaltia de Declaració Obligatoria" (MDO) requiring individual numerical declaration [30]. In 2015, the disease was once again given mandatory notification status throughout the Spanish territory [31].

Leishmaniosis is included in the list of communicable diseases of importance for public health and the international trade of animals and animal products in the World Organisation for Animal Health (OIE). In Spain, canine leishmaniosis (CanL) was declared a notifiable disease by the Real Decreto 526/2014 [32], which regulates its notification.

Two main clinical forms of HL are found in Spain, as in other Mediterranean regions: cutaneous and visceral. Cases of mucosal involvement (mucocutaneous leishmaniosis) are rare [33]. Dogs usually show clinical signs of skin and visceral involvement concomitantly [5 – 6]. Entomological studies have demonstrated the presence of *Phlebotomus perniciosus*, a proven vector of leishmaniosis in Spain, in all the Balearic Islands [26 – 28, 34 – 36], but without considering the factors that could influence its distribution in the area.

The aim of the current work was to gain new epidemiological data on leishmaniosis in the Balearic Islands by studying the distribution of human and canine leishmaniosis and their vectors in the islands of Minorca and Majorca, where previous data indicate different degrees of endemicity [19 – 28].

1. Area of study

The study was carried out in the Balearic Islands (Spain), an archipelago in the western Mediterranean comprising four main islands (Majorca, Minorca, Ibiza and Formentera). Geologically, the Balearic Islands are a continuation of the Betica Mountains, with the exception of Minorca, which is a continuation of the Pyrenees [37]. The Balearic Islands have a Mediterranean climate, with a mean annual temperature of about 16-17 ºC, except in the high mountains of Majorca, the Serra de Tramuntana, where it drops to 13 ºC. Annual rainfall oscillates from a maximum in autumn (66.9mm) to a minimum in summer (8.6 mm), with considerable differences between the mountainous north and the arid south in Majorca, where altitudes range from sea level to 1,445 m.a.s.l. [38].

In 2011, Majorca had a population of 862,425 inhabitants, Ibiza 129,562, Minorca 92,434 and Formentera 9,147 [39]. The canine census in 2012, according to the data provided by the *Col.legi de Veterinaris de les Illes Balears*, was of 250,596 dogs, with 166,858 on the island of Majorca, 13,956 on Minorca, and the rest on Ibiza and Formentera.

2. Entomological survey in the Balearic Islands

Entomological studies of sand flies on the Balearic Islands are scarce. The first was conducted by Pittaluga and de Buen (1918) [40], who detected *Phlebotomus perniciosus*, *P. papatasi* and *Sergentomyia minuta* in Majorca. Later, Gil Collado (1977) [41] reported the occurrence of these three species in the Balearic Islands, but without distinguishing between the islands. In an update of sand fly species in Spain, *P. ariasi* was added to those present in Majorca [34]. More recent maps elaborated by different authors also show this distribution [35, 42 – 47] (Fig .1).

Our entomological study was focused on the islands of Majorca and Minorca. Prior entomological data were available for both islands [26, 47], but they were incomplete, especially in the case of Majorca, and failed to take into account the possible influence of environmental and climatic variables on the presence and distribution of leishmaniosis vectors.

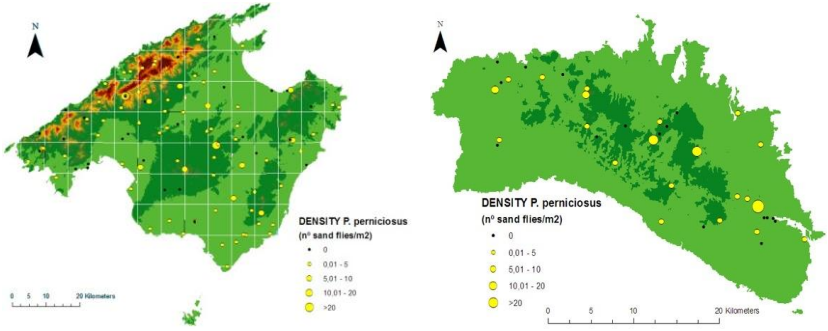
In July 2008, sand fly captures were carried out in Majorca with sticky castor oil traps (20x20 cm) according to a standardized methodology (Fig. 2) [15, 48, 49]. The sampling sites consisted of holes used to drain embankments or containment walls, and were distributed among 77 grids (5x5 km square) covering the entire island (Fig. 3a). Sand fly captures were carried out in Minorca between 2 and 4 July 2009, placing sticky traps in the

Figure 1. Distribution of sand flies in Spain (modified from Gállego et al., 1992) [35]. (⊙: Dubious appointment of *P. ariasi*, ⬬: *P. perniciosus*, ✱: *P. sergenti*, △: *P. papatasi*, ☒: *S. minuta*).

same 39 stations as in a previous study in 1988 [26], but now using the standardized methodology (Fig. 3b). The characteristics of the stations and sampling methodology (site location, type of trap, number of traps placed and collected, and meteorological data for the days of capture) and ecological and environmental factors were recorded in a PDA, using the Pendragon Forms v.5.0 software, associated with a GPS to record the coordinates of the sampling site. Sand flies were removed from the sticky traps with a brush and fixed in 96% ethanol to remove the castor oil, and then placed in 70% ethanol until identification. Males of all the species and *Sergentomyia* females were observed and identified under a stereomicroscope. Females of the genus *Phlebotomus* were mounted on glass slides in Hoyer medium and identified in an optical microscope using the keys of Gállego et al. [35].

(a) **(b)** **(c)**

Figure 2. Sticky traps used in the capture of sand flies (a); example of a sampling site (b), adhesive paper with captured sand flies (c).



(a) **(b)**

Figure 3. Sampling sites showing the density of *P. perniciosus*, in Majorca (2008) (a) and in Minorca (2009) (b).

2.1. Qualitative and quantitative analysis of the sand flies

On the island of Majorca, 88.2% of the traps were recovered, representing a surface of 135.68 m^2. A total of 14,412 specimens were captured, with 4 species identified (Table 1): *P. perniciosus*, *P. sergenti*, *P. papatasi* and *S. minuta*. *P. perniciosus* was captured throughout the island in 77 of the 111 stations prospected (69.36%), at 6 to 772 m.a.s.l. *P. sergenti* and *P. papatasi* were captured in only 14 and 1 of the stations, respectively, and always in a low number. The entomological surveys carried out in Majorca did not confirm the presence of *P. ariasi*, a proven vector of *L. infantum*, as this species was not found, despite sampling the whole island from 0 to 772 m.a.s.l and using a large number of traps.

On the island of Minorca, 92.38% of the traps were recovered, representing a surface of 54.32 m^2. The qualitative composition of sand flies in Minorca in 2009 showed no change had occurred over the 20 years since the previous study of 1988) [28], the same 3 species being identified on both occasions (Table 1): *P. perniciosus*, *P. sergenti* and *S. minuta*. On the contrary, changes in the density of the species were observed, with a significant decrease regarding *P. perniciosus*.

No environmental changes (newly urbanized areas, road construction, changes in land use) between 1988 and 2009 were observed. Meteorological factors were also analysed (mean daily temperature, average rainfall, wind speed, and humidity, all in different periods, and winter temperature) (Table 2), since climate change is another risk factor for the emergence or re-emergence of leishmaniosis foci [17, 52]. The meteorological data provided by the Spanish Meteorological Agency (AEMet) for the years of the two studies were compared using a linear regression model. This analysis revealed a decrease in average temperature of 0.9 °C in the month before the capture period in 2009 compared to 1988, thus showing a negative

Table 1. The sand fly fauna of Majorca (2008) and Minorca (1988, 2009)

		Sex ratio (M:F)	Number	Density (n/m^2)
Majorca (2008) [50] 135.56 m^2	*P. perniciosus*	4:1	921	6.72
	P. sergenti	24:1	25	0.18
	P. papatasi	3:0	3	0.02
	S. minuta	1.4:1	14,412	99.3
Minorca (1988) [28] 65.52 m^2	*P. perniciosus*	16.5:1	421	6.4
	P. sergenti	7.8:1	167	2.5
	S. minuta	1:1.4	937	14.3
Minorca (2009) [51] 54.32 m^2	*P. perniciosus*	8.5:1	179	3.4
	P. sergenti	9.5:1	21	0.4
	S. minuta	1.1:1	1,090	20.5

correlation with *P. perniciosus* density. These results disagree with Kuhn's model [53], which predicts that an increase in temperature would reduce the density of this species. Another difference observed between the two years was the average wind speed during capture, which was significantly lower (by 2.4 km/h) in 2009 than in 1988. Several authors have mentioned the negative influence of this meteorological variable on both the activity [54] and density [55] of sand flies. Thus, while less wind would not have led to an increase in the density of *P. perniciosus* in Minorca, it could have favoured sand flies leaving their resting places and biting animals.

Table 2. Results of the analysis of the meteorological factors associated with the decrease of *P. perniciosus* density in Minorca (Modified from Alcover et al., 2013) [51].

	Bivariate analysis		Linear model analysis	
Variable 2009	**IRR (CI)**	**p-Value**	**IRR (CI)**	**P-Value**
Mean daily temperature (ºC) (period2)	0.95	0.016	-0.91	0.002
Wind speed (Km/h) (period1)	0.91	0.058	-2.4	3.434×10^{-5}

Period 1: sampling Day 1 (traps set) to Day 4 (traps recovered)

Period 2: the month before sampling Day 1

Abbreviations: IRR, incidence risk ratio; p-Value, Pr(>z); CI, confidence interval; ºC, degree Celsius; Km/h, kilometres per hour

2.2. Factors influencing the presence or density of *Phlebotomus perniciosus*

To determine the predictors of the presence of *P. perniciosus* on the island of Majorca, a logistic regression analysis was performed using the presence/absence of the species as dependent variables. For independent variables, a panel of 57 ecological, epidemiological, environmental and meteorological characteristics was used. The independent variables of a preliminary bivariate analysis with a p-value ≤ 0.2 were used for a multivariate analysis. In the final multivariate model, only the independent variables with $p \leq 0.05$ were considered. SPSS v.20 was used (Table 2). The effect of meteorological and capture station variables on *P. perniciosus* density in Minorca was evaluated by generalized linear models based on negative binomial distribution [56] using the glm.nb function of the MASS statistical package, available on free software R [57]. After an initial bivariate analysis, the resulting independent variables with a value of

$p \leq 0.2$ were used in a multivariate analysis. In the multivariate model, independent variables with $p \leq 0.05$ were considered (Table 3).

Among climatic variables, temperature is known to affect sand fly activity. In the Balearic Islands, the summer temperatures trigger the end of sand fly diapause, after which they leave their resting sites at dusk and night-time hours, when the females bite vertebrate hosts [44, 58]. According to the results [26, 49], the capture of *P. perniciosus* on the islands of Majorca and Minorca was not influenced by meteorological variables, in contrast with other studies [15, 48, 55, 59]. In Majorca, *P. perniciosus* was captured at average temperatures ranging between 19.6 ºC and 27.4 ºC, regardless of the altitude of the trap location, while in Minorca the temperature ranged from 25.04 ºC to 25.08 ºC. This species was observed in a wide range of temperatures at which sand flies are reported to be active (above 15.6 ºC) [58]. The short period of capture, only during the month of July in both Minorca (just four days) and Majorca (full month), together with the geographical homogeneity of the capture sites, prevented the finding of significant differences in density (Minorca) or presence/absence (Majorca) of vectors between different capture points. In Majorca, it was difficult to find places suitable for traps in mountainous areas above 700 m.a.s.l.

Locations away from inhabited areas were positively correlated with the presence or density of *P. perniciosus* in both islands, as in other studies [15, 48, 59]. Rural and remote locations away from urban centres provide more suitable conditions for the vector to develop its terrestrial cycle [11, 60 – 61]. In Majorca, the species increased in areas with adjacent garrigue shrub vegetation, and in Minorca vector density was higher in natural environments far from human populations than in cultivated areas, where the use of pesticides could decrease sand fly density. Altitude is a variable that is positively or negatively related to the presence of sand flies, depending on the species considered [59]. In our case, we analysed its influence only on the populations of *P. perniciosus* in the island of Majorca, where there is more altitudinal variation. A greater abundance of *P. perniciosus* was detected in remote capture sites over 50 m.a.s.l. Stations at 0-50 m.a.s.l. were located in humid and breezy coastal areas and sand flies are very sensitive to windy conditions [11, 58, 60].

The presence of a specific type of farm or animal species can also favour the capture of *P. perniciosus.* Thus, a nearby sheep farm favoured the presence of this species in Majorca, as did cats in Minorca. Sheep farms provide sand flies with feeding opportunities, since they contain a large number of animals that spend the night outside, the time when sand flies are active.

Table 3. Bivariate and multivariate analysis of the environmental factors associated with *P. perniciosus* presence/absence in Majorca (2008) and its density in Minorca (2009). (Modified from Alcover et al., 2013 and 2014) [50 – 51].

MAJORCA	Bivariate analysis			Multivariate analysis		
	IRR	**p-Value**	**C.I. (95%)**	**IRR**	**p-Value**	**C.I. (95%)**
Altitude (m.a.s.l.)						
0-50	ref.			ref.		
51-150	3.133	0.020	1.195-8.214	8.653	0.015	1.514-49.441
>150	1.625	0.402	0.522-5.055	0.805	0.816	0.131-4.964
Settlement						
Within settlement	ref.			ref.		
Edge of / between settlement	5.339	0.001	1.950-14.617	8.080	0.008	1.737-37.596
Site category						
Embankment drainage holes	ref.					
Wall drainage holes (not embankment)	2.111	0.031	0.204-0.843			
Other holes in walls (not embankment)	0.308	0.148	0.062-1.522			
Natural rock crevices	0.235	0.313	0.014-3.917			
Farm building (holes)	0.264	0.166	0.040-1.735			
Sewer/drainage openings	-	0.999	-			
Wall construction						
Stone without mortar	ref.					
Stone / mortar	0.338	0.079	0.101-1.133			
Brick/mortar	0.263	0.009	0.097-0.714			
Other	1.974	0.414	0.386-10.089			
General environment						
Rural village	ref.					
Rural agriculture and forestry	2.977	0.032	1.095-8.091			
Coastal village	0.548	0.435	0.122-2.475			
Other settlement (non rural or non coastal village)	0.366	0.158	0.090-1.478			
Adjacent flora						
Aleppo pine and evergreen oaks	ref.			ref.		
Garrigue shrubs	14.529	0.001	2.949-71.587	38.051	0.001	4.900-295.469
None	0.935	0.904	0.313-2.795	1.308	0.707	0.323-5.307
Land cover (Corine)						
Urban area	ref.					
Agricultural area	5.525	<0.001	2.113-14.448			
Forest area	1.594	0.461	0.462-5.497			
Humid area	-	1.000	-			
Arable						
Cereals	ref.					
Root crop	0.167	0.231	0.009-3.118			
Other (not cereal or root crop)	0.333	0.268	0.048-2.328			
None	0.269	0.016	0.093-0.781			
Sheep farm animals near						
No	ref.			ref.		
Yes	2.720	0.019	1.177-6.289	19.989	0.001	3.557-112.322
Pigeon farm near						
No	ref.					
Yes	0.155	0.031	0.028-0.842			
Orientation						
Other (all orientations except south-east- and west-facing)	ref.			ref.		
South-east-facing	2.990	0.171	0.623-14.350	34.975	0.018	1.817-673.425
West-facing	0.716	0.500	0.271-1.892	0.457	0.263	0.116-1.798
Drain hole construction						
Plastic pipe	ref.			ref.		
Other (unlined, cement pipe)	2.250	0.061	0.964-5.249	3.451	0.05	1.002-11-880

Table 3. Continued

MINORCA	Bivariate analysis			Multivariate analysis		
	IRR	**p-Value**	**C.I. (95%)**	**IRR**	**p-Value**	**C.I. (95%)**
Site category						
Urban	ref.					
Rural (includes edge of and between locations)	8.7	0.015	1.371-52.959			
Orientation						
North-facing	ref.			ref.		
West-facing	0.45	0.405	0.064 – 3.168	0.08	0.017	0.009-0.771
South-facing	0.43	0.244	0.100- 1.869	1.02	0.955	0.431-2.427
East-facing	2.11×10^{-9}	0.996	0-Inf	0	1	0-Inf
Other (not applicable)						
Water course nearby						
No	ref.					
Yes	0.07	0.0252	0.004-0.751			
Wall construction						
Stone without mortar/Dry stone wall	ref.					
Stone/mortar	5.61	0.006	1.793-22.746	4.13	0.002	1.549-11.024
Stone/mortar (plaster/white)	0.18	0.207	0.007-2.773	0	1	0-Inf
Adjacent flora (within 100m)						
Natural vegetation	ref.			ref.		
Arable	0.24	0.0319	0.061-0.927	0.29	0.035	0.081-1.024
Cats						
Not seen	ref.			ref.		
Seen	9.59	0.011	2.242-91.704	16.39	0.001	2.616-102.669
Capture *S. minuta*						
No	ref.					
Yes	1.02	0.0001	1.006-1.039			

In Minorca, this variable was not taken into account, since livestock on the island is mainly cattle. However, an increase in the density of *P. perniciosus* was related with the presence of cats, which has epidemiological importance, since cats can act as reservoirs of *L. infantum* [62 – 63].

The type of wall was only an influencing factor in Minorca, where a positive correlation with sand fly density was observed when sticky traps were placed on stone mortar walls. Dry stone walls, very common in the Balearic Islands, have numerous interconnected holes, which facilitate air currents and consequently disturb sheltering sand flies and hamper sampling [11, 58, 60].

3. Canine leishmaniosis (CanL) in the Balearic Islands

In 2015 leishmaniosis was included among the diseases of zoonotic origin in the list of diseases of official notification by the Government of the Balearic Islands [64].

The first study on CanL in the Balearic Islands dates from 1989 [19], when a prevalence of 14% was reported in Majorca. Subsequently, several studies have been published on the different islands [19 – 21, 25 – 28, 65 – 66], from which it was inferred that CanL was endemic in Majorca, Ibiza and Formentera, with a seroprevalence ranging between 6 and 45%

[19, 21, 25 – 28, 43]. The prevalence rose to 67% when molecular diagnostic techniques were used [66]. With respect to Minorca, Seguí [26 – 28] could not demonstrate the presence of an autochthonous focus of CanL in the island. The few positive cases detected (8/813, 0.98%) were regarded as imported, since they involved dogs introduced to the island from well-established foci. The characterization of the strains isolated from dogs in Majorca by Multilocus Isoenzyme Analysis (MLEE), led to the identification exclusively of zymodeme MON-1 of *L. infantum* [67].

In order to know the trends of CanL in the area, a standard questionnaire [12, 18] was sent to local veterinarians in 2009. Of the 111 questionnaires sent out to veterinary clinics, 42 were returned completed (a reply rate of 38%). Over 80% of surveyed veterinary clinics attended more than 20 dogs a week, and of these, 73.8% had confirmed CanL in more than 10 dogs in the previous 12 months. While 50% of the veterinarians thought that the incidence of CanL had not changed over time (Fig. 4), 26.2% perceived an increasing trend, mainly those from Minorca (3/6). Most veterinarians considered the new diagnosed cases as autochthonous (88.1%), including all those from Minorca (6/6).

In order to investigate the possibility of an autochthonous focus of CanL in Minorca, a cross-sectional study on CanL was performed in the island in April-June 2010 with the help of the practitioners of three veterinary clinics located in different areas of the island (Mercadal, Ciutadella and Sant Lluís) (Fig. 5). Veterinarians were asked to randomly select animals born on the island and without any history of travelling abroad, regardless of the presence of clinical signs.

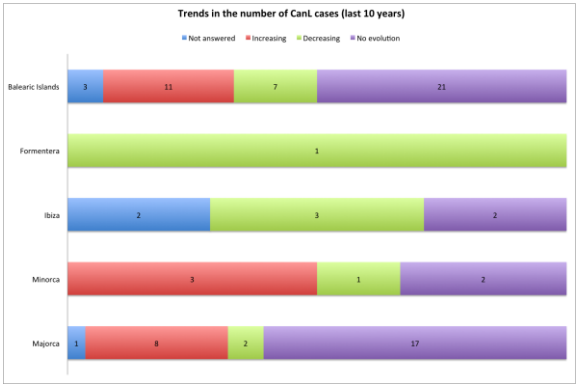


Figure 4. Veterinary questionnaire on CanL trends in the Balearic Islands in the last 10 years (number of replies).

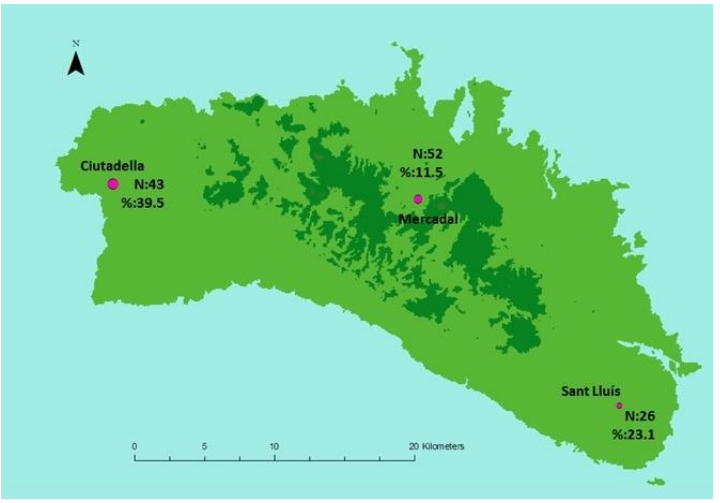


Figure 5. Prospective study of CanL in Minorca. N: number of dogs, %: seroprevalence.

In total, 121 dogs were selected with permission from the owners. The prospective study of CanL was carried out by serological analysis. Blood samples were obtained by cephalic vein puncture and sera were frozen and conserved at -40ºC until use. Serology was performed, with minor modifications, according to the protocols of "in-house" ELISA and Western Blot techniques [68], IFI [69] and a commercial ICF technique (Speed®Leish K, BVTGroup, Virbac) (Fig. 6). Dogs that tested clearly positive with at least two immunological methods were considered seropositive and probably infected [12]. Dogs that tested positive with at least two techniques but were borderline were considered doubtful.

The global seroprevalence was of 23.96%, and 31% among animals of known local origin and with no history of movements to endemic areas. The high seroprevalence in different localities in Minorca (Fig. 5) was similar to that found in known endemic areas in Spain [8, 70 – 72], indicating that dogs with suspected *Leishmania* infection had been included in the sample. Our results point to the emergence of an autochthonous focus of CanL in Minorca, which could be related with the continuous introduction of infected animals from endemic areas to the island while the vector is present, as reported by Seguí [27 – 28], rather than an increase in *P. perniciosus* density.

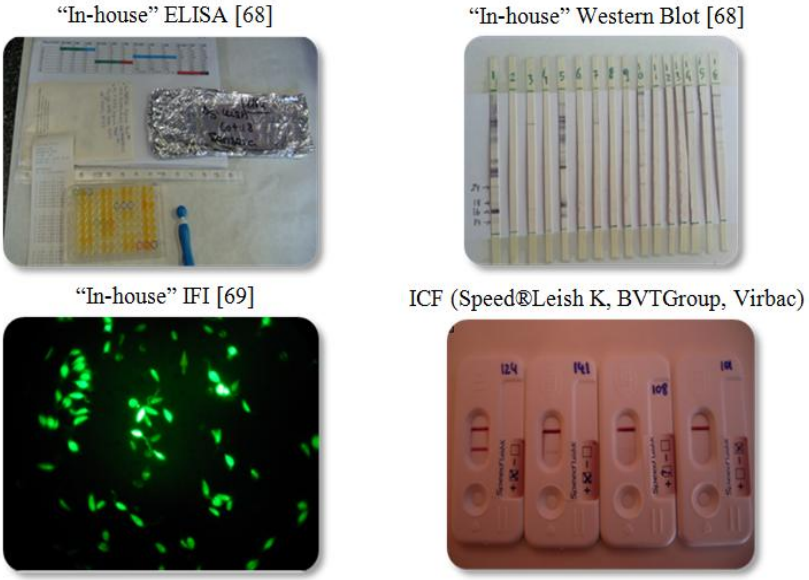


Figure 6. Leishmaniosis diagnostic techniques used to study CanL seroprevalence

4. Human leishmaniosis (HL)

The source of HL data in the Balearic Islands is a literature review of published cases, together with officially reported cases. The first description of a HL case in the area was in 1925, namely visceral leishmaniosis on the island of Majorca [73]. In 1926, a child was diagnosed with visceral leishmaniosis (VL) by Drs. Cervera and Darder, also in Majorca [26], and in 1935, a report on VL and cutaneous leishmaniosis (CL) in Spain by Dr. Najera Angulo [74] includes three cases in Majorca between 1925 and 1934. Finally, Gil Collado [41] reviewed the data from dermatological services in the islands from 1961 to 1973 and found only one case of cutaneous leishmaniosis during this period.

The first officially reported case of HL we could find dates from 5-11 September, 1982 [75], the year that HL became a notifiable disease in Spain. From 1982 to 1996, the Health Department of the Spanish Government collected data on HL in the Balearic Islands but without differentiating between islands (Table 4). In 1981 began a decentralization

Table 4. Distribution of human leishmaniosis cases reported in the Balearic Islands. CL: Cutaneous Leishmaniosis. VL: Visceral Leishmaniosis.

	Year	CL	VL	Majorca	Minorca	Ibiza-Formentera	Cases not assigned to Islands	Total	Report (year)
Notifiable disease, nationwide Boletines Epidemiológicos Semanales del Ministerio de Sanidad y Consumo.	**1982**	--	--	--	--	--	1	1	BES 1741 (1986)
	1983	--	--	--	--	--	1	1	BES 1741 (1986)
	1984	--	--	--	--	--	2	2	BES 1741 (1986)
	1985	--	--	--	--	--	3	3	BES 1741 (1986)
	1986	--	--	--	--	--	4	4	BES 1880 (1991)
	1987	--	--	--	--	--	0	0	BES 1880 (1991)
	1988	--	--	--	--	--	9	9	BES 1880 (1991)
	1989	--	--	--	--	--	8	8	BES 1880 (1991)
	1990	--	--	--	--	--	11	11	BES 1880 (1991)
	1991	--	--	--	--	--	8	8	SNE
	1992	--	--	--	--	--	22	22	SNE
	1993	--	--	--	--	--	5	5	SNE
	1994	--	--	--	--	--	0	0	SNE
	1995	--	--	--	--	--	0	0	SNE
	1996	--	--	--	--	--	0	0	SNE
Notifiable disease, regional Xarxa de Vigilancia Epidemiológica de les Illes Balears	**1997**	--	--	--	--	--	12	12	Full Set. 39/2005*
	1998	--	--	--	--	--	14	14	Full Set. 39/2005* +
	1999	4	8	--	--	--	12	12	Full Set. 39/2005*
	2000	0	2	--	--	--	2	2	Full Set. 39/2005*
	2001	0	6	5	0	1	0	6	Full Set. 8/2002*
	2002	2	2	3	0	1	0	4	Full Set. 20/2003*
	2003	2	8	10	0	0	0	10	Full Set. 37/2004*
	2004	14	7	17	0	3	1	21	Full Set. 22/2005*
	2005	25	11	36	0	0	0	36	Full Set. 49/2006*
	2006	25	10	33	0	1	1	35	Informe 2006*
	2007	9	13	19	0	2	0	22	Informe 2007*
	2008	6	5	11	0	0	0	11	Informe 2008*
	2009	6	8	11	0	3	0	14	Informe 2009*
	2010	7	1	7	0	1	0	8	Informe 2010*
	2011	17	3	17	1	2	0	20	Informe 2011*
	2012	14	6	20	0	2	0	20	Informe 2012*
	2013	12	4	14	0	2	0	16	Informe 2013*
	2014	17	7	20	0	4	0	24	Informe 2014*
	2015	--	--	26	0	2	0	28	Full Set. 14/2016*
	TOTAL National (1982 – 1996)						74	74	
	TOTAL Regional (1997 – 2015)	160	101	247	1	24	43	315	

* Accessible at http://www.caib.es/sacmicrofront/noticias.do?idsite=337&tipo=12245&mcont=84507

SNE: Data provided by the SNE (Sistema Nacional de Epidemiología)

BES: Boletin Epidemiológico Semanal (weekly report from Spanish Government)

Full Set.: Full Setmanal (Weekly report from Balearic Government)

\+ Data modified from the Full Set. and provided by the SNE Informe (Report from Balearic Government)

in Social Security healthcare, and consequently, in 1996 the responsibility for the declaration of HL cases was transferred to the Autonomous Communities [76]. Each region was made responsible for its own list of notifiable diseases, and the *Conselleria de Salut del Govern de les Illes Balears* included leishmaniosis in the list [30].

Since 1982 it have been notified 377 cases of HL in the Balearic Islands being the majority reported in Majorca (78.2% of these cases from 1997 to 2015) (obtained from data in Table 4).

From 1997 to 2015, 315 cases were notified at a regional level (Table 4). Nevertheless, the disease is underreported in the Balearic Islands. As an example, from 1999 to 2014, 261 cases were notified 85 of which were detected through active searches, which represents 32.6% of underreporting (Fig. 7). In the years 2003, 2007 and 2013, the percentage of underreporting reached levels of 70, 54.5 and 62.5%, respectively.

An active search for HL cases in Minorca by Seguí [26 – 28] revealed two cases in the Hospital Virgen de Monte Toro (Mahon), but both were considered imported. One was a case of VL diagnosed in 1983 in a 23-year-old woman of Minorcan origin, but living in Almeria. The second case was of CL, diagnosed in a man who had spent some years living in Valencia. In 1994, Portús et al. [36] published a case of VL involving a

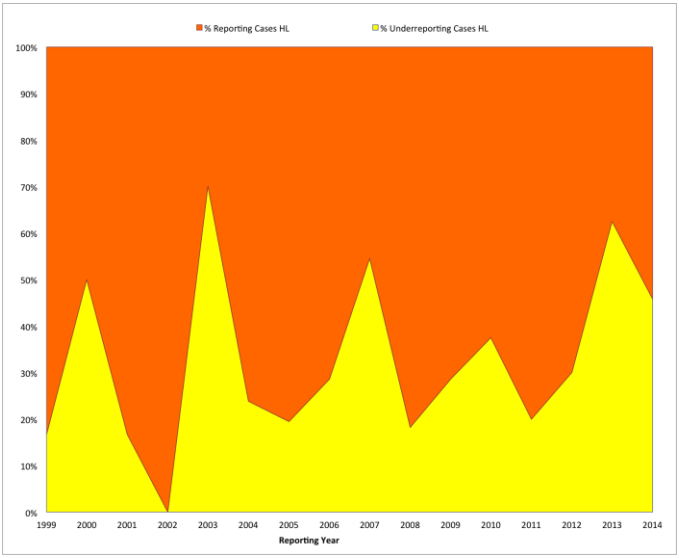


Figure 7. Percentage of reported and underreported HL cases in "*Xarxa de Vigilància Epidemiològica*" [24].

44-year-old woman who had not left the island for 13 years, her last trip outside being in 1978 to the Costa Brava. The possibility of a cryptic form of leishmaniosis acquired 13 years earlier was suggested. And finally, one case of CL was notified in 2011 [24] in an 11-month-old baby who had previously travelled to Ibiza, suggesting that it was an introduced case (Table 4).

The characterization of the strains isolated from humans in Majorca showed the presence of four zymodemes of *L. infantum* in the island (MON-1, MON-24, MON-34 and MON-108) [67].

5. General considerations

Entomological studies are often used as indicators of leishmaniosis vectors and therefore of the disease risk. Such studies are being standardized to determine which factors influence the incidence of the disease [14, 15, 48, 50, 51, 60, 61,77]. Most of these studies are performed using sticky traps, which allow sand flies to be caught in a relatively large area over a short period of time, but other traps, such as light traps (CDC traps), are often used in Europe. Sticky traps do not use any attraction device [78 – 79] and are placed mainly in crevices of sheltered walls used by sand flies as resting sites [80].

Two basic types of methodology have been used for the placement of these traps: transects, which aim to capture sand flies in the maximum number of places in the chosen routes [48, 59, 61, 79], or in at least one spot within each grid over a given area [18].

Herein, we report entomological studies carried out on the islands of Majorca and Minorca. The use of different methodologies, type of traps, and criteria for choosing capture sites makes it difficult to compare results from different studies.

Since the study objectives in the two islands were not identical, the methodology used also differed. In Minorca, the aim was to observe any changes in the density of *P. perniciosus* that might explain the emergence of CanL in the island, and therefore the traps were set along the same transects as in a previous study in 1988 [28]. However, while the traps in 1988 were left for a variable number of consecutive nights (from 2 to 8), in 2009 traps were placed on four nights, as stipulated in the EDEN (Emerging Diseases in a changing European Environment) methodology, and as performed in recent studies in Spain and other countries [15, 48, 60 – 61, 77], as well as in our study in Majorca.

With respect to the island of Majorca, and in contrast with Minorca, data are available on an autochthonous focus of HL and CanL, but information on the sand fly fauna, in particular *P. perniciosus*, is very fragmented and incomplete. Thus, the aim of the current work was to study the distribution of the leishmaniosis vector in the island, and the factors influencing its occurrence.

The variables analyzed also differed between islands, with fewer analyzed in Minorca (28) than Majorca (57). In Majorca, the study was more extensive, covering the entire island, and was focused on the distribution of *P. perniciosus* and the factors that influence it. Additionally, since Majorca is larger and more geographically diverse than Minorca, it was possible to study the effect of a higher number of variables on the occurrence of *P. perniciosus*.

Another difference between the two studies was the statistical approach. Thus, in Minorca we used the density of *P. perniciosus* as the dependent variable, since we wanted data that was comparable with those of a previous study [28], while for Majorca, the variable was the absence/presence of the vector, which has also been used in other studies carried out in Spain [15, 48].

Among the variables finally included in the bivariate analysis of data collected on both islands (14), three were found to have a statistically significant positive and/or negative influence on *P. perniciosus*: the location of the sampling site (defined in relation to the nearest settlement: inside, at the edge or between settlements); the type of wall construction (stone without mortar, stone/mortar, brick/mortar, other); and the adjacent flora (nearest vegetation <100 m to the sampling site).

A location away from settlements was positively correlated with *P. perniciosus* presence and/or density, as in other studies [18, 48, 60]. Rural locations away from urban areas would provide the right conditions for the vector to develop its terrestrial cycle [11, 60 – 61]. Moreover, the use of insecticides in urbanized or cultivated areas would reduce the population of sand flies [1, 63].

We also detected a positive correlation in sampling sites where sticky traps were placed on walls constructed of bricks or stone with mortar. Dry stone walls are also very widespread in the two islands, but their numerous holes impede a comprehensive sampling and therefore the provision of representative data. Furthermore, the holes in dry stone walls are often interconnected, facilitating air currents that would be unfavorable for sand flies [11, 60 – 61].

The "adjacent flora" variable had a positive influence on the vector presence in both islands, but an accurate comparison of results was hindered by different categorization of the data. Thus, in Majorca the presence of the vector was linked to scrub vegetation, whereas in Minorca it can only be concluded that a natural, uncultivated habitat had a positive influence. Natural environments are located away from human populations, while in cultivated areas pesticide use may decrease the presence and/or density of *P. perniciosus*.

In our studies, the possible presence of animals, including dogs, was not generally related with *P. perniciosus* detection, coinciding with other studies [15, 48, 59], which may be explained by the opportunistic feeding behaviour of this species [81 – 82]. Also, the type of trap influenced what sampling sites could be used and therefore could have affected the results, as sticky traps are usually placed far from kennels or livestock.

As mentioned above, vector studies are essential to understand the epidemiology of leishmaniosis outbreaks in different areas and apply control measures. The complexity of the vector-parasite-host-environment-climate relationships in the biological ecosystem requires the development of prevention programs that integrate the surveillance and control of the parasite, vector and reservoir of leishmaniosis [1, 11, 65].

Wide-ranging strategies for controlling leishmaniosis have been proposed, and traditional approaches are discussed by various authors [83 – 84]. Currently, these strategies focus primarily on the early diagnosis of the disease [85], treatment of infected humans and animals [1, 83 – 84], finding suitable molecules for the treatment and control of the disease [86], vaccine development [7, 85] and the application of different methods of vector control [87].

Regarding HL, the number of cases being reported in the Balearic Islands is likely to increase [45], given the current level of underreporting. This would provide more reliable data on its incidence and distribution, of particular importance in the Balearic Islands. Despite the diagnosis of some cases of HL in Minorca, the displacements of such patients to endemic areas makes it difficult to establish the geographical origin of the infection. The islands of Ibiza, Formentera and Majorca, which are very close and well connected to Minorca, are highly endemic areas of both CanL [19 – 21] and HL [22 – 24].

The aforementioned emergence of CanL on the island of Minorca is probably due to a lack of measures to prevent infected dogs entering the island from endemic areas. The recent inclusion of leishmaniosis among

diseases to be notified by veterinarians in the Balearic Islands will provide more accurate data on current distribution and prevalence of the disease.

6. Conclusion

P. perniciosus is the only vector of *L. infantum* species found in the islands of Majorca and Minorca. This sand fly species was captured throughout both islands, which points to a risk of leishmaniosis transmission. In Majorca, *P. perniciosus* is most likely to be found at altitudes of 51-150 m.s.a.l., in garrigue vegetation, between and at the edge of settlements, and in the proximity of sheep farms. The global density of *P. perniciosus* in Minorca has decreased significantly since the study performed 20 years ago, specifically in rural areas. The emergence and establishment of an autochthonous focus of CanL in Minorca is apparently related with the continuous introduction of *Leishmania*-infected dogs rather than an increase in *P. perniciosus* density. Although HL is a notifiable disease, it is clearly underreported in the Balearic Islands.

Acknowledgements

This work was supported by grants of the Ministerio de Educación y Ciencia of Spain (CGL2007-66943-C02-01/BOS), and Departament d'Universitats, Recerca i Societat de la Informació de la Generalitat de Catalunya (Spain) (2009SGR385). The Spanish Meteorological Agency (AEMet) provided the meteorological data for the study. The *Sistema Nacional de Epidemiología* confirmed the HL cases. Grateful acknowledgement is given to M.G. Seguí for helping in the location of the capture stations and A. Lanau for their active participation in the sand fly captures. We thank the veterinarians R. Aranda, M. Huguet and E. Sintes, for their active participation in collecting blood samples of dogs, and the farm owners that allowed us to collect blood samples from their dogs and to capture the sand flies. We are also grateful for the help of the Col.legi de Veterinaris of Balearic Islands, particularly R. García and A. Figueroa, as well as the veterinarians who completed the questionnaires. Thanks are due to J. Abellán, from the Centre Insular Sanitat Menorca, who provided data on the human case of leishmaniosis declared in Minorca. Thanks are due to J. Martín-Sánchez, X. Castells and A. Picado for helping in the statistical study.

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