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Box 1 beginning ---The algae compounds' family -----

Seaweeds belong to a rather ill-defined group of plants called algae. The term “seaweed” itself has no taxonomic value but is rather a popular term used to describe common large attached (benthic) marine algae that live on, in or near the seabed. They belong to the groups *Chlorophyceae* (4500 species), *Rhodophyceae* (6500 species) and *Phaeophyceae* (1800 species), commonly known as green, red and brown algae, respectively (Figure 1 Box 1). Rhodophyceae and Phaeophyceae are exclusively found in oceans and seas, whereas chlorophyceae are common in freshwater rivers and lakes [1].

Fucan is the collective name for a family of sulphated polysaccharides rich in L-fucose and found in brown seaweed (Phaeophyceae) cell walls. These polysaccharides were first isolated by Kylin et al. [2] at the beginning of the 20th century, and were originally named fucoidins. They are found in the intercellular mucilage of marine algae, where they play a key role in wall architecture, by cross-linking cellulose and alginates [3]. Fucans are arbitrarily divided into three main families: homofucans (or fucoidans), xylogucoglucuronans (or ascophyllans) and glucuronofucoglycans (or sargassans) (Table Box 1). Fucoidans are based on L-fucose, with a repeating structure of alternating $\alpha(1\rightarrow3)$ and $\alpha(1\rightarrow4)$ glycoside bonds and sulphate groups at position 2 or 4, and additional monosaccharides (D-xylose, D-galactose and D-mannose) [4,5]. Ascophyllans consist of a polyuronide backbone (mainly poly β -(1,4)-D-mannuronic acid) branched with short chains of neutral or sulphated residues of galactose, xylose and fucose. Sargassans consist largely of linear chains of (1,4)-linked D-galactose or glucose branched at C-5 with L-fucosyl-3-sulphate or, occasionally, a uronic acid (usually D-glucuronic acid).

Table box 1 : Analytical composition (%) of the different fucans [67].

	L-fucose %	Uronic acid %	Other sugars %	Sulfate groups %	Proteins %
Fucoidans	50-90	<8	5-47	35-45	<4
Ascophyllans	≈ 25	≈ 25	≈ 25	≈ 13	≈ 12
Sargassans	25-45	≈ 12	≈ 36	15-21	4

For a given species of algae, the relative proportions of fucoidan, ascophyllan and sargassan depend on the tissue and many other factors such as age, habitat and season. The exact composition varies with the species. These variations affect the properties and quality of extracts. The biological properties and quality of fucoidan derived from rockweeds (*Fucus*, *Ascophyllum*) depend on three factors: 1) the L-fucose composition, 2) the amount of ascophyllan or alginate, and 3) the amount of phenolic compounds [6].

A high-quality fucoidan fraction has been commercially available in France for almost 20 years, from the company Algues et Mer (<http://www.algueset-mer.com/>), being obtained by oxidative-reductive

depolymerisation with H₂O₂ of a crude high-molecular-weight fucoidan. Algues et Mer is the world's largest producer of pure LMWF from *Ascophyllum nodosum*. It sells the product mainly for cosmetic and research purposes.

Box 2. Beginning --Oligosaccharides in use as drugs or in development -----

Carbohydrates represent the most abundant group of naturally occurring biological molecules, being produced in billions of tons every year by plants and photosynthetic bacteria. They are mainly produced as polysaccharides or oligosaccharides -- macromolecules composed of a large or moderate number of monosaccharide residues, respectively. The use of carbohydrate-based drugs is in its infancy, although there are several well-known examples: one is the "monosaccharide-inspired" drug oseltamivir ((Tamiflu[®], Roche), an anti-influenza treatment extracted from Chinese star anise. There are also two "blockbuster" drugs: an acarbose of microbial origin sold by Bayer AG (Precose[®] in Europe and China; Glucobay[®] in the USA) and used in the management of type 2 diabetes mellitus; and heparin, a drug derived from porcine intestinal or bovine pulmonary mucosa, and used to treat and prevent deep vein thrombosis and pulmonary embolism, along with its low-molecular-weight derivatives such as enoxaparin (Lovenox), dalteparin (Fragmin, Pfizer Inc.), and tinzaparin (Innohep, Leo Pharmaceutical Company). The drugs listed in **Table Box 2** have overcome some of the perceived limitations of sugar-based molecules in terms of their synthesis, delivery and immunogenicity. Although many of these drugs still require intravenous or subcutaneous delivery, several are available for oral administration. Ongoing research is seeking to improve oral bioavailability by reducing the compounds to their smallest active components, or by combining them with other molecules such as sulodexide. Small active components can be selected or modified to improve efficacy. No major immunogenicity problems have been reported (except for thrombocytopenia induced by heparin), either in animal trials or in routine clinical use of synthetic or animal-derived materials, including those obtained from marine invertebrates.

Table Box 2:

Examples of carbohydrate-based drugs in use or in development AML Acute Myeloid Leukemia, CJD Creutzfeldt Jakob disease, COPD chronic obstructive pulmonary, DVT Deep Venous Thrombosis. (GlycoMar www.glycomar.com).

Company	Compound	Activity / disorder	Development
Numerous	Heparin and derivatives: in particular low molecular weight forms	Anticoagulants	Since 1940's
Astellas Pharma France	Auranofin (Ridaura) organogold monosaccharide	Rheumatoid and juvenile arthritis	1983
Aspri Pharmaceuticals Canada	Arixtra (fondraparinux) synthetic heparin Pentasaccharide	DVT & pulmonary embolism	1982
GlaxoSmithKline UK	Zanamivir (Relenza) modified polysaccharide	Anti-influenza (neuraminidase inhibitor)	1992
Johnson & Johnson USA	Topiramate (Topamax) : modified polysaccharide	Anti-epileptic	1987
Bayer USA	Acarbose (Glucobay) (Pseudo-oligosaccharide)	Type II diabetes Alpha-glucosidase, alpha-amylase inhibitor.	1990
Ortho-McNeil (USA) Janssen (Belgium) Pharmaceuticals (Johnson & Johnson)	Elmiron (Pentosan polysulphate)	Cystitis (interest in development for CJD)	1996
Alfa Wassermann Italy	Sulodexide (Vessel™)	Arterial antithrombotic	Marketed since 1980's
Merrion Pharmaceuticals Ireland	MER-102: oral uLMW heparin	DVT	Phase IIa
Sanofi Adventis France	Idrabiotaparinux, bioninylated synthetic heparin pentasaccharide	DVT Pulmonary embolism	Phase III
Cantex Pharmaceuticals USA	PGX100: O-desulphated heparin	Cardiac ischemia-reperfusion injury, COPD, use in AML	Phase I / Phase II
Progen Australia	PG545 fully synthetic heparan sulphate mimetic	Anti-angiogenic Anti-metastatic	Phase I
	PI-88 phosphomannopentaose sulphate)	Anti-angiogenic Anti-metastatic	Phase III
Glycomimetics Inc	GMI1070	Vaso-occlusive crisis of sickle cell disease	Phase I
	GM1010/1011/1043	Anti-inflammatroy / pulmonary inflammation	Preclinical
GlycoMar / Verona Pharma UK	GLY145 - glycosaminoglycan	Anti-inflammatory	Preclinical

Box 3: beginning ----Fucoidan a promising product? -----

D. Letourneur's team showed that fucoidan could serve as a platform for the development of thrombus-targeting contrast agents. A LMW fucoidan fraction prevents P-selectin binding to Sialyl Lewis X with an IC(50) of 20 nM, as compared to 400 nM for heparin and >25 000 nM for dextran sulphate. It exhibits the highest affinity for immobilized P-selectin comparing to these polysaccharides. As a consequence, fucoidan binds to platelets with an intensity that depends on the level of platelet activation [7]. The interaction is highly specific, as shown by competition assays between fucoidan and an anti-P-selectin antibody. D. Letourneur et al. radiolabeled fucoidan with ^{99m}Tc for scintigraphy, and with ultrasmall superparamagnetic iron oxide particles (USPIO agents) for magnetic resonance imaging. This allowed the detection of areas of platelet activation and subsequent thrombus formation *in vivo* [8]. USPIO-LMWF conjugates were shown to target P-selectin. Flow cytometry showed interaction with activated platelets in human whole blood *in vivo*. [9]. USPIO-Fuco is now being developed as an MRI contrast agent for thrombus imaging (<http://www.google.com/patents/WO2010116209A1?cl=en>); <http://www.google.ch/patents/US20140134102>).

Table 1 (§2 history of natural marine products):

Examples of marine-derived carbohydrate-based therapeutic molecules (GlycoMar www.glycomar.com). HIV / AIDS Human immunodeficiency virus infection and acquired immune deficiency syndrome, Herpes Simplex Virus type 1.

Compound	Source	Activity	Status
Chondroitin sulphates	Mussels Nutraceutical	Joint repair / maintenance	Clinical
Galactofucan sulphated (GFS)	Seaweed Marinova Australia	Herpes virus infections Mobilization of stem cells	Preclinical
Fuoidan		Immunity, Osteoarthritis, HSV-1, antioxidant,	Phase I & II
Oligo G	Alginate AlgiPharma Norway	Cystic fibrosis	Phase I & II
Heparinoid 916	Chitosan Ocean University of China	Atherosclerosis	Phase III
Propylene glycol alginate sodium sulphated PSS)	Alginate Ocean University of China	Antiangiocardopathy	Available in China
Propylene glycol mannate sulphated (PGMS)		Posterior capsular opacification	Available in China
Heparinoid 971		Alzheimer's disease	Phase IIb
Polymannuronic acid propyl sulphate (PMS)		Cerebral ischemia	Phase IIa
Heparinoid 911		HIV / AIDS , HBV	Phase IIa

Figure 1 box 1: Colorful seaweed in rockpool on the coast of Brittany in France.

From right bottom to the upper left: brown algae *Laminaria digitata*, green algae *Ulva lactuca*, red algae *Palmaria palmate*, green algae *Enteromorpha intestinalis*, brown algae *Laminaria digitata* and *Fucus vesiculosus*. Courtesy of S. La Barre.

Figure 2 (§3 Peripheral arterial disease and cardiovascular risks) :

Peripheral Arterial Disease in the lower limb is characterized by chronic obstruction of the arteries supplying the leg, gradually leading to CLI. High blood pressure, smoking and high cholesterol can damage the endothelium and lead to the development of a fatty streak under the endothelium. An unstable plaque develops with a fatty core that can sometimes rupture into the bloodstream. Clotting of the blood begins at the site of the plaque rupture. The artery becomes partially blocked and blood flow is diminished. Accordingly, there is insufficient blood flow to redirect towards the collaterals. Furthermore, the acute arterial occlusion leads to distal tissue hypoxia and causes the activation of an inflammatory response against ischemic tissue injury owing to endothelium dysfunction and several factors. The insufficient collaterals and angiogenic signaling limit tissue regeneration and can lead to necrosis and loss of tissue function if revascularization is not re-established.

Figure 3 (§3.4.2: how do new blood vessels form?)

The angiogenesis and vasculogenesis processes. Recruitment of the endothelial cells from preexisting vessels plays a critical role in the regulation of angiogenesis. **1)** Mobilized bone marrow endothelial progenitor cells (EPCs) with high proliferative capacity migrate and **2)** home to the site of angiogenesis where **3)** they deliver signals that activate pericytes, which then in turn play an active role by secreting mediators that stimulate migration and replication of local endothelial cells [10,11]. A small amount differentiates and incorporates in the new vessel wall. EPC endothelial progenitor cells, VEGF vascular endothelial growth factor, EPO erythropoietin, MMPs matrix metalloproteinases, NO nitric oxide.

Figure 4 (§3.4.2 How do new blood vessels form ?)

Activation of local angiogenesis is a promising approach to patients with critical limb ischemia with a high risk of amputation. Stem/progenitors cells isolated from bone marrow or peripheral blood can be pre-activated with SDF-1, VEGF, fucoidan, hypoxia... or modified to improve survival, homing, functional engraftment and functional activity (e.g. differentiation) before being infused or injected in patients. Infusion of these in vitro-expanded bone marrow cells enhances neovascularization in animal models of hindlimb ischemia.

Figure 5 (§5.1.3 Fucoidan exhibit venous and arterial antithrombotic...)

Fucoidan can prevent thrombosis, catalysing thrombin inhibition and promoting fibrinolysis. It reduces circulating level of thrombin by catalyzing its inhibition: Tissue factor (TF) bound to the endothelium initiates the coagulation cascade. It binds to activate circulating factor VII (FVIIa) and catalyzes the activation of factor X. Then factor Xa (FXa) binds to factor V (FV) present on the phospholipid surface of platelets or endothelial cells to catalyze the conversion of prothrombin to activate thrombin (IIa). Thrombin cleaves fibrinogen to form fibrin that leads to the formation of fibrin-clot then a thrombus. The propagation of the cross-linked fibrin clot is limited by plasmin which cleaves fibrin. Endothelial cells secrete tissue plasminogen activator (tPA) to activate plasmin and control the expansion of the clot on the injured endothelial cell surface. Fucoidan (Fuc) is a direct inhibitor of thrombin and catalyze the inhibition of serine proteases Xa and thrombin by antithrombin (AT) and the inhibition of thrombin by heparin cofactor II (HCII). Fucoidan promotes fibrinolysis by potentiating plasminogen activators such as tissue-plasminogen activator (tPA) and reducing plasminogen activator inhibitor-1 (PAI-1) release by endothelial cells.

Figure 6 (§5.2.1) : Fucoidan induces a proangiogenic phenotype in human EPC.

1 - Fucoidan enhances EPC proliferation in a concentration dependent manner starting at 1 µg/ml. **2** – Fucoidan-pretreatment promotes EPC motility (no chemoattractant) and enhances EPC chemotaxis towards VEGF. **3** – Fucoidan pre-treatment enhances basic fibroblast growth factor (FGF2)-induced vascular tube formation by EPC: **3A** EPC do not form tubular structures in control medium, **3B** 18h after seeding, FGF2-pre-treated cells are elongated and interconnected, **3C** The tubular network is significantly more extensive in the presence of FGF-2 and fucoidan. **4** – Fucoidan-pretreatment enhances EPC adhesion on activated endothelium under static and dynamic conditions, and their extravasation towards VEGFs (40 ng/ml). **5** – Fucoidan induces mobilization of immature CD34⁺CD31⁺CD45⁻ murine progenitors. Wild mice were intraperitoneal injected with PBS (negative control, CTRL), 5 mg/kg of low molecular weight fucoidan (LMWF) or 0.5 µg/kg VEGF (positive control, VEGF) and bled 30 min after the injection. Blood was assayed for CD34⁺CD31⁺CD45⁻ cells and plasma samples were analyzed for SDF-1 concentration by enzyme-linked immunosorbent assay test. **5A** – Progenitors mobilization after intraperitoneal injection of PBS (negative CTRL), LMW fucoidan or VEGF. **5B** – SDF-1 level in mice plasma after intraperitoneal injection of fucoidan compared to controls. Results are expressed as the means +/- SEM * p<0.05, ** p<0.01, *** p<0.001.

Figure 7 (§5.2.1) : Fucoidan protects ischemic tissue against necrosis.

Fucoidan-pretreatment of cultured EPC (Endothelial colony-forming cells, ECFC) increases neovascularization in hindlimb ischemia of mice with PAD 14 days after artery ligation and intravenous injection of pretreated ECFC. Macroscopic aspects of ECFC-injected mice (**A**) and fucoidan-ECFC-injected mice (**B**). Cumulative incidence of clinical necrosis (**C**), foot perfusion (**D**) and quantitative analysis of angiographic score (**E**) of normal-saline (CTRL, ■), untreated ECFC (ECFC, □)

and fucoidan-stimulated ECFC (ECFC, green □) transplanted mice. Hematoxylin and eosin staining of the same distal gastrocnemius muscle sections on day 14 and quantification of histologically preserved area, ischemic infiltrated area and necrotic area type surface **(F)**. The surface of each area type is reported as a percentage of the entire histological section surface.

(G) To evaluate the effect of fucoidan on critical ischemia, 2 groups of animals underwent the surgical procedure and received two intramuscular injections 1 and 2 days after surgery of normal saline and fucoidan solution (15 mg/kg Fuc). Quantification of histologically preserved area, ischemic infiltrated area and necrotic area types surface 14 days after surgery and intramuscular bolus administration of fucoidan

Values are expressed as means +/- SEM (n=15) * p<0.05, ** p<0.01 and *** p<0.001 vs normal saline-injected mice (CTRL). NI, non ischemic hindlimb, isch ischemic hindlimb.

Figure 8: Proposed mechanisms involved in beneficial effects of fucoidan in peripheral ischemia.

A Fucoidan can bind to, potentiate and inactivate cationic proteins such as adherent proteins expressed in extracellular matrix (ECM) and bone marrow, enzymes, growth factors, cytokines.... **B**) fucoidan can prevent thrombosis, catalysing thrombin inhibition, promoting fibrinolysis and stimulating the release of tissue factor pathway inhibitor (TFPI). **C**) Fucoidan can promote EPC recruitment by displacing sequestered SDF-1 from HSPG. It can also compete with EPC for binding heparan sulphate proteoglycans (HSPG) or ECM proteins. **D**) Fucoidan can bind to EPC and induce a proangiogenic phenotype to EPC, by enhancing their mobility. **E**) Fucoidan enhances EPC chemotaxis toward VEGF, EPC attachment to laminin and growth factor-induced vascular tube formation, improving revascularization of ischemic tissue.

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About the authors

Catherine Boisson-Vidal obtained her PhD degrees from the Paris Nord University (France), where she studied the isolation, structural elucidation and structure-activity relationships of biologically active marine fucoïdanes. She undertook postdoctoral research in biomaterials at McMaster University (Hamilton, Canada) and took a lectureship at Paris Nord (1986-2003) and Paris Descartes University, where she is currently a CNRS research director and deputy director of Inserm research unit 1140.

Over the period 1989-2000, her main research focus was the development methods to prepare reproducible antithrombotic fractions of low-molecular-weight fucoïdan and their structure-function relationship. She is currently developing new therapeutic approaches based on stem cell therapy, notably by attempting to enhance the proangiogenic potential of adult stem cells through their incorporation into sites of neovascularization, in both physiological and pathophysiological conditions. Her main focus is on the proangiogenic activity of fucoïdan and its uses in vascular biotherapy and tissue regeneration.