Global skin action of a laminaria extract

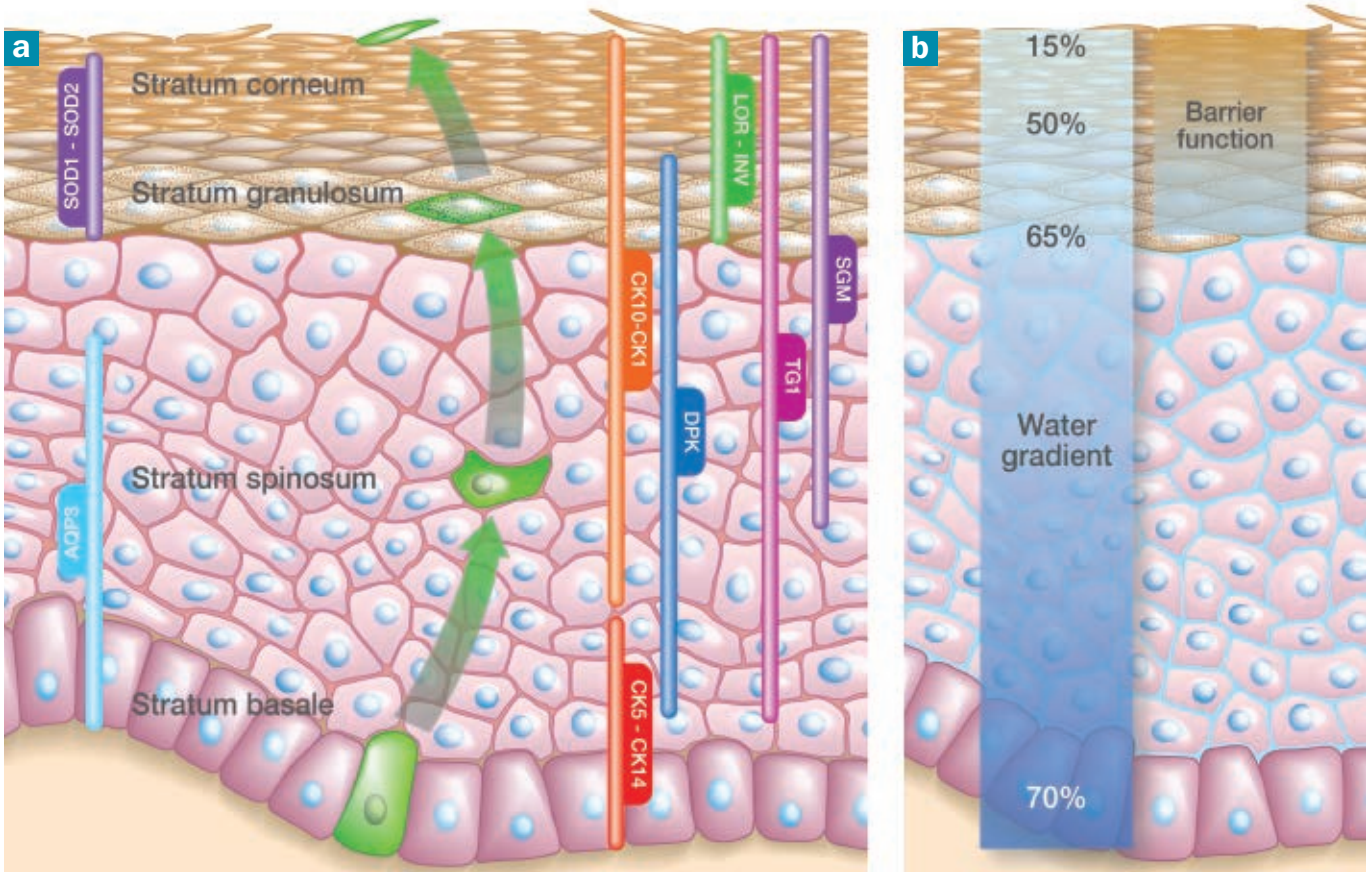

Figure 1: *Epidermal site of expression/function of various gene/proteins.* **a)** *Localisation of the site of gene expression and protein function of different constituents of the epidermis. SOD1 (superoxide dismutase-1), SOD2 (superoxide dismutase-2), AQP3 (aquaporine-3), CK14 (cytokeratin-14), CK5 (cytokeratin-5), CK1 (cytokeratin-1), CK10 (cytokeratin-10), DPK (desmoplakin), LOR, (loricrin), INV (involucrin), TG1 (transglutaminase-1) and SGM (sphingomyelinase).Green cells and arrows represent epidermal cell renewal.* **b)** *Formation of the epidermal water gradient and localisation of the skin barrier function.*

Skin is a large and complex tissue where the orchestrated actions of resident cells are necessary to support its structural and metabolic integrity. Cells of the epidermis (mainly keratinocytes) play a role in protecting from environmental stress such as UV exposure, mechanical damage and pro-oxidative attacks. Perhaps more importantly, top layers of the epidermis along with a lipid-rich intercellular matrix form the skin barrier. It is no secret that the skin barrier is fundamental in preventing excessive water evaporation thereby supporting normal skin hydration levels. A functional barrier also prevents penetration of allergens that sometimes can exacerbate sensitive skin conditions. With age, the rate of epidermal cell renewal and the ability of the skin barrier to repair from a physical insult decrease.

Ageing not only weakens epidermal structures and functions but also deregulates the metabolic activity of fibroblasts. Indeed, some metabolic pathways are increased and others are decreased in aged skin fibroblasts. The best example is the general reduction in collagen synthesis and the augmentation in the production of matrix metalloproteases (MMPs) causing a gradual dismantling of the extracellular matrix (ECM) organisation.

We tested a laminaria extract (commercial name: Regenesea™-LS, INCI Name: Glycerin (and) Water (and) Laminaria saccharina extract) for its effects on epidermal molecular pathways using cDNA experiments on human skin equivalents. We also verified the ability of the laminaria extract in rejuvenating aged skin fibroblasts at the gene expression level. The actions of the extract were confirmed clinically by measuring various parameters including skin barrier function, hydration, skin smoothness, wrinkle density and volume as well as visco-elastic properties.

Results and discussion

SkinEthic reconstructed epidermis underlaid with a high density human dermal fibroblast culture were incubated with the laminaria extract. Results obtained with this reconstructed skin assay are shown in Table 1. The epidermal site of

Table 1: Effect of the laminaria extract on the expression of epidermal constituents.

Gene	Expression level	Cellular/skin functions
SOD 1 (Cu/Zn)	▲46%	Cytosolic, scavenges O_2^-, prevents accelerated ageing
SOD 2 (Mn)	▲21%	Mitochondrial antioxidant defense, prevents DNA damage
Aquaporine-3	▲17%	Water/glycerol transporter, epidermal hydration, skin elasticity
Cytokeratin-14	▲46%	Epidermal basal layer, mechanical integrity
Cytokeratin-5	▲10%	Basal layer – stem cells, physical stability
Cytokeratin-1	▲30%	Major cytokeratin of epidermis, Important for skin mechanical integrity
Cytokeratin-10	▲37%	Major cytokeratin of epidermis, Complete differentiation marker
Desmoplakin	▲22%	Most abundant component of desmosomes, resistance to shear forces
Loricrin	▲22%	Predominant proteins (80%) of the cornified envelop (*stratum corneum*)
Involucrin	▲14%	Important component of the cornified envelop (*stratum corneum*)
Transglutaminase-1	▲24%	Cross-links loricrin and involucrin in the cornified envelop
Sphingomyelinase	▲25%	Ceramide synthesis, most important lipid of *stratum corneum* barrier

cDNA array with skin reconstructs. Coloured squares are to indicate sites of expression/function of proteins.
Please refer to Figure 1 for histological localisation as per the corresponding coloured squares.

expression/function of each gene product studied here is depicted in Figure 1 with corresponding coloured bars. Based on the genes targeted, the action of the laminaria extract can be grouped in four categories such as: protection, hydration, differentiation and barrier function. Laminaria has a positive action of the expression of the antioxidant enzymes superoxide dismutase (SOD)-1 and SOD-2. SOD enzymes are present in almost all cellular systems as a protection against the superoxide radical O_2^-. SOD-1 is located in the cytoplasmic compartment while SOD-2 is localised in the mitochondria – the cell powerhouse – where the production of chemical energy (ATP) also generates deleterious oxygenated metabolites. Therefore, the upregulation of SOD-1 and SOD-2 will help epidermal cells to scavenge O_2^- reducing external (pollution, chemical stress) and internal (mitochondrial age-related oxidative stress) oxidative damage.

The increased expression of the water and glycerol transporter aquaporine-3 in the presence of laminaria has also been observed. This action will most likely support the distribution of the water gradient in the epidermal layers. The water holding capacity is a multifactorial phenomenon but the skin barrier function is certainly a key element. The formation of the barrier function would not be possible without keratinocyte differentiation from epidermal basal (*stratum basale*) to the upmost external layer, *stratum corneum*. Along the differentiation and migration path, keratinocyte metabolic activity will progressively become specialised in the production and secretion of a lipid matrix filling out spaces between keratinocytes forming the well characterised brick-and-mortar model of the skin barrier. The laminaria extract supports the integral differentiation of epidermal keratinocytes by an increased expression of markers of early differentiation (cytokeratins 5 and 14) and late or terminal differentiation (cytokeratins 1 and 10). The expression of other key components of the epidermal cell cohesion and the cornified envelope such as desmoplakin, loricrin, involucrin and transglutaminase-1 (TG-1) is also upregulated by laminaria. Loricrin, involucrin and TG-1 are also considered as markers of terminal keratinocyte differentiation. A very interesting action of laminaria is demonstrated with the augmentation of sphingomyelinase expression. This enzyme is involved in the synthesis of ceramide, the most important lipid constituent of the skin barrier function.

The global epidermal action of the laminaria extract points toward an increased skin barrier function and skin water holding capacity (Fig. 1b). This was verified clinically with twenty subjects in a single-blind, split-face, randomised protocol with an active formulation containing 3% of the laminaria extract applied twice daily for up to 60 days. In this trial, trans-epidermal water loss (TEWL) (Tewameter 300, Courage+Khazaka GmbH), skin hydration (Corneometer, Courage+Khazaka GmbH) and skin surface smoothness (Visioscan VC98, Courage+Khazaka GmbH) were

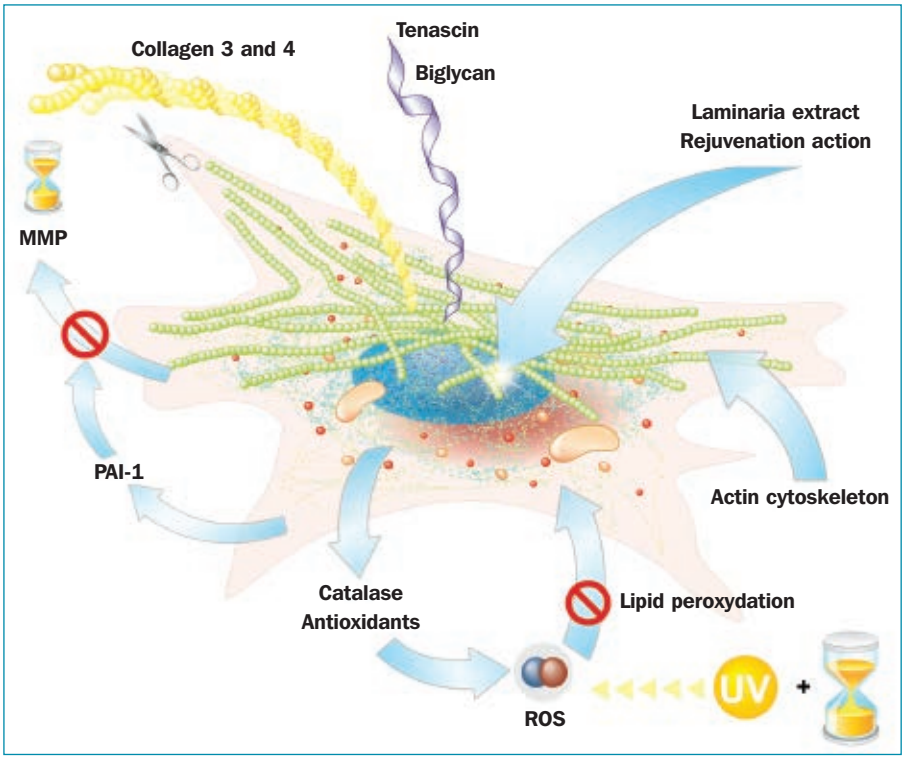

Figure 2: *Metabolic pathways regulated upon ageing and rejuvenated in the presence of the laminaria extract.*

measured. The side of the face where the active formulation containing laminaria was applied revealed a significant reduction in TEWL (–12.1% and –13.4% after 30 and 60 days respectively; $p<0.0001$ compared to baseline) and a significant increase in skin hydration (+16.8% and +24.0% after 30 and 60 days respectively; $p<0.0001$ compared to baseline) (Table 2).

As skin ages, the cellular metabolic activity generally decreases causing a progressive dismantlement of skin structures. The extracellular matrix and the dermo-epidermal junction (DEJ) are such structures weakened by a reduction in the synthesis and excessive degradation of macromolecules like collagen and glycosaminoglycans. We examined the efficacy of the laminaria extract in 'rejuvenating' aged skin fibroblasts by measuring the expression of specific genes.

Normal human dermal fibroblasts were obtained after the 8th 'young cells' or the 14th 'aged cells' passage *in vitro* culture conditions. Fibroblasts were incubated in the presence, or the absence, of 0.75% of the laminaria extract for a period of 24 hours. Analysis of gene expression was performed using standard minichips with dedicated cDNA (BIOalternatives, Gençay, France).

Aged cells display the typical 'age-related expression profile' that is overall catabolic (Table 3). A reduction in the expression of actin, collagens, glycosaminoglycans, antioxidant-associated enzymes is observed. On the other hand, there is an obvious upregulation of MMP-1 and MMP-3 that will cause the breakdown of structural components of the ECM. Ultimately, this age-related gene expression profile will cause the appearance of cutaneous signs of ageing. Very interestingly, the addition of the laminaria extract 'reverses' the age-related gene expression profile. Indeed, laminaria reactivates, or rejuvenates, the expression of collagens type 3 and 4, of glycosaminoglycans tenascin and biglycan while down-regulating that of MMP-1 and MMP-3. A very unique action of laminaria is the upregulation of the plasminogen activator inhibitor type 1 (PAI-1) that is an endogenous inhibitor of MMP. Altogether, this concerted action is likely to support the build-up of the collagen network and prevent excessive degradation of it. Along with the increased expression of tenascin and biglycan, collagen fibres (especially type 4) would improve the integrity of the DEJ reinforcing the anchoring of the epidermis onto the dermis.

The antioxidant capacity weakens with age, compromising the ability of aged cells to protect themselves against reactive oxygen species. This is demonstrated in our study by a reduced expression in catalase, glutathione reductase and thioredoxin reductase in aged cells. The addition of the laminaria extract to aged cells clearly reactivates the expression of those antioxidant network components (Table 3). Catalase is a direct scavenger of hydrogen peroxide dissociating the deleterious pro-oxidant in water and oxygen. Glutathione reductase and thioredoxin reductase are not antioxidants *per se* but they recycle the pool of antioxidants present in the skin. Glutathione reductase can recycle (from the oxidised form back to the active reduced form) the antioxidants glutathione

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Table 2: Clinical benefits of the laminaria extract based on epidermal actions.

	TEWL	Hydration	Smoothness
Day 30	–12.1%	+16.8%	+13.6%
Day 60	–13.4%	+24.0%	+17.8%

All values are statistically significant at $p<0.0001$ compared to baseline.
The placebo did not provide statistically significant effect.

Table 3: Gene expression profile during ageing and effects of laminaria.

	Expression level		
Gene	Aged cells *vs.* young cells	Aged cells + Laminaria	Functions
αSM-actin	▼68%	▲214% (1.0)*	Fibroblast contraction, wound healing
Collagen 3	▼42%	▲107% (1.2)	Collagen fibres dermis; DEJ
Collagen 4	▼10%	▲102% (1.8	Basement membrane; DEJ
MMP-1	▲205%	▼23% (2.3)	Degrades collagen III, I
MMP-3	▲2000% (20x)	▼64% (7.3)	Degrades ECM, activates pro-MMP
PAI-1	▼12%	▲167% (2.3)	MMP inhibitor (Plasminogen Activator Inhibitor-1)
Tenascin	▼39%	▲172% (1.7)	ECM; dermo-epidermal junction
Biglycan	▼37%	▲46% (0.9)	Collagen fibre organisation
Catalase	▼17%	▲163% (2.2)	Eliminates H_2O_2
Glutathione reductase	▼61%	▲739% (3.2)	Recycles glutathione and vitamin E
Thioredoxin reductase	▼ 54%	▲150% (1.2)	Recycles thioredoxin and vitamin C

cDNA array with aged skin cells (fibroblasts). Values next to coloured arrows indicate level of gene expression.
*Values in parentheses represent expression level ratios compared to that of young cells.

and vitamin E while thioredoxin reductase does so for thioredoxin and vitamin C. In this way the laminaria extract can potentiate the antioxidant network of aged cells. The rejuvenating action of the laminaria extract at the cellular level can be summarised as shown in Figure 2.

The laminaria extract provides a rejuvenating action to aged cells by regulating various metabolic pathways. The synthesis of collagen (types 3 and 4) and glycosaminoglycans (tenascin and biglycan) is increased. The upregulation of MMP is a hallmark of the ageing process (illustrated by the hour glass in Figure 2) and causes excessive degradation of collagen fibres. Laminaria directly inhibits MMP expression and upregulates the expression of the endogenous MMP inhibitor PAI-1. Reactive oxygen species (ROS) generated during ageing and upon UV exposure can be inactivated by the increase in catalase, glutathione reductase and thioredoxin reductase triggered by laminaria. The laminaria extract also acts positively on the cellular integrity and contraction by increasing the expression of α-smooth muscle actin (actin cytoskeleton).

The rejuvenating of aged skin cells in the presence of the laminaria extract translates in clinical benefits. Indeed, the application of the active formulation containing laminaria significantly reduced the wrinkle density (–7.5% and –11.5% after 30 and 60 days respectively; $p<0.0001$ compared to baseline) and wrinkle volume (–11.2% and –15.7% after 30 and 60 days respectively; $p<0.0001$ compared to baseline) (Table 4) when assessed using the Visioscan VC98 (Courage+Khazaka GmbH). There is a parallel increase in skin visco-elastic properties (+12.7% and +13.0% after 30 and 60 days respectively; $p<0.0001$ compared to baseline) that relies on an adequate ECM integrity and normal levels of hydration. At the skin level, the clinical benefits of laminaria are compatible with an improved integrity of the extracellular matrix especially at the DEJ (Fig. 3). The activation of aged fibroblasts by laminaria would bring new collagen and glycosaminoglycan materials at the dermo-epidermal junction reinforcing the anchoring of the epidermis to the dermis. The chelation of reactive oxygen species (ROS) would prevent oxidative damage to cellular constituents and components of the extracellular matrix.

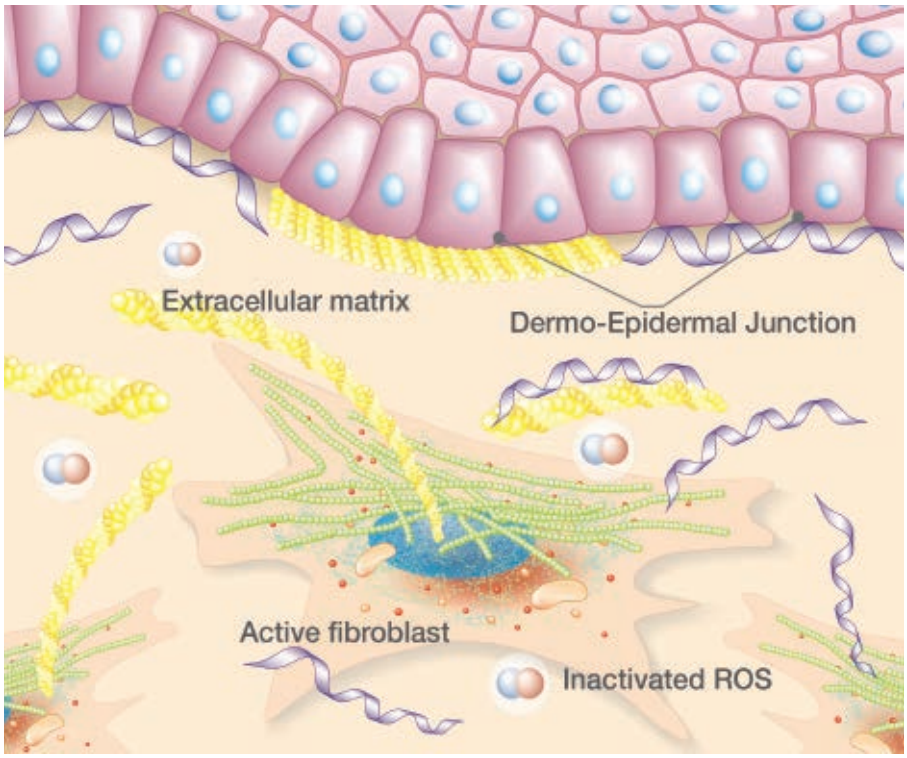

Figure 3: *Proposed anti-wrinkle mechanisms of action of the laminaria extract.*

Conclusion

Skin ageing is dictated by external insults (actinic ageing) such as UV radiation and by internal damage (intrinsic ageing) mostly caused by accumulation of noxious metabolites such as reactive oxygen species and a deregulation of skin cell metabolism. Skin ageing is thus a process involving various pathways present in both, the epidermis and the underlying dermis. The end result is a deterioration of skin ultrastructure which leads to the appearance of cutaneous alterations like dry skin, fine lines and wrinkles. At the histological level, a diminished density in collagen and glycosaminoglycans, thinning of the epidermis, decline in subcutaneous fat volume, reduced water holding capacity and flattening of the DEJ proceed as skin ages.

Regenesea-LS provides a global skin action targeting various pathways associated with epidermal keratinocyte differentiation (which slows down with age), water transport, skin barrier function, antioxidant protection and extracellular matrix synthesis. Altogether, those actions would contribute to the clinical benefits obtained with the active formulation containing Regenesea-LS such as reduction of TEWL, increase in hydration and a diminution of wrinkle appearance. Improvement in skin surface smoothness and visco-elastic property was also demonstrated in this study.

Both skin layers, epidermis and dermis, are closely juxtaposed and this lead to a close cooperation or cross-talking between corresponding cells keratinocytes and fibroblasts. Laminaria was tested in skin equivalents made of both cell types trying to mimic the *in vivo* skin environment. Results have shown an activation of the gene expression of markers of differentiation and more functional proteins such as aquaporine-3, SOD and the ceramide-making sphingomyelinase. When added to cultures of aged fibroblasts, Laminaria reset metabolic activity to a profile close to what observed in young cells. Laminaria succeeded to switch cell metabolism from a catalytic mode to a more anabolic one improving synthesis of collagen 3 and 4 (DEJ), glycosaminoglycans (collagen organization and DEJ), α-smooth muscle actin (cell motility and contraction) and antioxidants while reducing that of degradation MMP enzymes. Cellular metabolic pathways 'rejuvenated' by Regenesea-LS are likely to help skin fighting against external and internal insults and support its use as an active ingredient aimed at reducing the appearance of signs of ageing. PC

Table 4: Clinical benefits of the laminaria extract based on aged fibroblast metabolism rejuvenations.

	Wrinkle density	Wrinkle volume	Visco-elasticity
Day 30	–7.5%	–11.2%	+12.7%
Day 60	–11.5%	–15.7%	+13.0%

All values are statistically significant at $p<0.0001$ compared to baseline.
The placebo did not provide statistically significant effect.