AGEING: A MYSTERY UNRAVELED.

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She's 23, her make-up is done by one of the best visagists, and she claims in the advertisement for an anti-ageing cream: "I say no to wrinkles." Ageing is always correlated with wrinkles, and these have to be avoided at any time. However, good cosmetic chemists will resist that there is a direct relation between age and wrinkles, but it is nonetheless a significant sales and marketing tool. Hopefully your esteemed marketers will one day see the light.

The population of the civilised countries is growing quickly and thus a market is in development for anti-ageing products, dedicated to those who have money in their pocket. Everybody wants to get old, but nobody wants to look old. In virtually all western countries the percentage people older than 65 years of age is quickly growing. Since 1950 the average age of the people in the industrial countries has drastically increased, Japan being the exponent of that statement: from 5% in 1950 to an expected 26% in the year 2020.

	1950	2000	2020
Japan	5,1	18,2	25,8
Italy	8,6	17,4	23,2
France	11,5	16,0	20,9
Germany	9,5	17,2	20,2
United Kingdom	10,4	15,4	18,5

Percentage of people older than 65 years of age.

In 20th century the above mentioned countries the average age has increased with ~25 years. Not at all surprising that also the number of centenarians is swiftly increasing. Medical healthcare has given a tremendous contribution to the increasing age (although one could wonder whether also the quality of life has grown with a similar speed). On December 30th,1999 Sarah Knauss died at an age of 119 in New Jersey; Elizabeth Israel was reported to be born in the year 1875 in the Dominican Republic, and died in 2000. It is said that the number of centenarians in the Caucasus is significantly higher compared to the rest of the world because of high lithium contents in the drinking water; no scientific proof can be generated from the birth registers.

The famous Paracelsus, Theophrastus Bombastus von Hohenheim, was searching in the 16th century for the eternal life, and he was certainly not the first person who did so. The Sumerian epic Gilgamesh, written down on clay tablets, already describes the search for eternal life, unfortunately and predictably without success. To answer the question "how can we influence ageing ?" first the answer has to be given to the question "what is ageing ?". The spin-off is than also "how can we contribute with cosmetic chemistry when it comes to ageing ?" We're not discussing increased life expectation (we leave that to molecular biology and medical science) but we're talking about quality of life and to delay the signs of ageing from a cosmetic point of view.

Ageing goes hand-in-hand with a number of syndromes: reduced hearing and sight, reduced taste (especially salt & bitter), osteoarthritis and osteoporosis, reduced sensitivity for

growth factors & growth hormones, reduced body weight & muscle volume and a reduced REM sleep (Rolling Eye Movement). It appears that people are subjected to a gradual decay of body functions, something which is far less pronounced in the animal and the plant kingdom. The symptoms of ageing are usually also correlated with an increased mortality chance. Greying hair and baldness are certainly not life threatening, but a decreasing insulin production and cholesterol deposition in the arteries are without doubt reducing life expectation and quality of life. Because of greatly improved medical science and understanding of our dietary needs it is in many cases possible to suppress (not to eliminate !) many of the signs of ageing. These will anyway appear, but a number of years later. In the 60's of the 20th century somebody of 55 was considered old. Today an individual in her/his 60's is frequently still playing tennis, goes to the gym or plays golf. It is nonetheless unavoidable that sooner or later the signs of ageing will show up.
Although it is not very likely that the 21st century will show another growth of average age of 25 years, gerontologists do not exclude such a phenomenon. Imagine yourself playing polo on your 84th birthday !!

At one time gerontologists were looking for so-called "biomarkers", products that are indicative for the biological age. Examples are the relative of cross-linking of collagen, insulin resistance, urea secretion or lung capacity. Some people considered that the number of heartbeats in a lifetime was constant for mammals and birds. This model worked reasonably well, but not for humans and parrots. According to this model parrots are getting much to old; the story goes that a parrot was the last living creature that spoke with Napoleon Bonaparte. The contents of the discussion is, however, not reported. So, none of these biomarkers were satisfying, and they in fact raised more questions than generated answers.
The awareness was growing that the skin is also subject to ageing, like all of our organs. It's not so long ago that the skin was considered to be a plastic back filled with bones, muscles, organs, etc. The skin shall be approached in a similar fashion as our liver, kidneys or spleen. The whole organism, inclusive of the skin, is subject to ageing, until the final journey comes.

Mortality in Nature is a necessity; dying is pre-programmed. Salmons live in the ocean for 2-3 years before they return to their spawning grounds to take care about their offspring. The journey is dangerous and many salmons do not reach their destination. Those salmons that succeed deposit their eggs and immediately afterwards they start producing so much corticosteroids in their adrenal glands that ageing start almost spontaneously, with death as the final result. Programmed dying is normal for animals and plants that reproduce themselves only once in their life.

Humans have, compared to other mammals and birds, a long life expectation. Until recently gerontologists were convinced that 120 was the maximum achievable age. Part of a potential explanation is the relatively large amount of enzymes with anti-oxidant qualities such as superoxide dismutases, glutathione and catalases. Anti-oxidant qualities can be translated as the ability to repair damaged genetic material (DNA) and to deactivate pathogenic free radicals. Glutathione is a rather simple enzyme composed of three amino acids: {glutamic acid ←→ cysteine ←→ glycine}. Reaction of glutathione with a free radical results in abstraction of a hydrogen atom from the thiol group of the amino acid cysteine, followed by dimerisation of two glutathione radicals to form glutathione dimer; the free radical scavenging activity of glutathione is largely steered by ascorbic acid. This does not mean that glutathione used is lost for the future: another enzyme, the selenium-containing enzyme glutathione reductase regenerates glutathione and the cycle can start all over again.

Being able to repairing damaged DNA is probably one of the achievements for humans. On a daily basis some 70.000 genetic mistakes are made during duplication of cells

and thus the genetic material. If these mistakes would not be corrected our chances for survival would be minimal. On a cellular level "repair enzymes" are active that contain a blueprint of the genetic code. The enzyme folds itself around the DNA and controls whether all nucleic acids are in the correct position. If mistakes are detected these will be repaired, and only 5-10 insignificant mistakes will remain. If functional mistakes are still present the cell is condemned to apoptosis, self-destruction. The first repair enzymes were discovered in 90's, and since than several hundreds have been found (Science, 291,1284-1289, (2001)). Probably folic acid (vitamin B7), pyridoxine (vitamin B6) and cyanocobalamine (vitamin B12) are some of the most important co-factors for these repair enzymes.

There are already commercial repair enzymes available containing such as T4 Endonuclease V, encapsulated in liposomes. Reactive oxygen species (ROS), produced during regular cellular activities or by ionising radiation (Röntgen, γ-radiation), or by oxidation and/or alkylation reactions, can modify the DNA bases by disrupting the phosphodiester backbone, and it does not require discussion that repair of damaged DNA is essential to prevent mutations and cell death; non-repaired damaged/modified DNA has also been implicated in ageing and development of cancer. A number of proteins involved in base excision repair have been identified in Escherischia coli, showing a very high degree of structural similarity with human proteins. Using recombinant techniques there is a sincere possibility to producing practical amounts of the proteins, endonucleases, for topical application as repair enzymes.
It shall be kept in mind that each individual endonuclease is only involved in one enzymatic reaction. Thus, to contribute to the delay of the signs of ageing a cocktail of endonucleases is required. Such an endonuclease cocktail would be complementary to what the organism is doing already by itself, having said that the efficacy of endonucleases that humans produce themselves is decreasing with increasing age. Isn't that an interesting (partial) definition of ageing ???

Talking about repair of DNA: one of major causes of DNA damage is irradiation of the skin by UV light: skin damage, sunburn, skin alterations and ultimately skin cancer. UV-light induced DNA damage can be inhibited using repair enzymes, as shown by Wolf et.al. (J.Invest.Dermatology, **114**, 149 (2000)). In a double blind study human volunteers were exposed to UV radiation, whether or not treated with a liposome preparation containing T4 endonuclease V. On the untreated irradiated areas in all cases carcinoma developed. On the other hand, T4 Endonuclease V offered full protection for genetic damage. Quite spectacular indeed ! The research of Wolf and co-workers is the start of a new era for sun care products, and our present UV filters will be history in the foreseeable future. Recombinant T4 endonuclease V is already commercially available, as well as some other endonucleases. For the short future their price is an absolute barrier for cosmetic application, but that will be just a matter of time. The research after endonucleases is very important as it also will enable to properly treat patients suffering from xeroderma pigmentosum (XP). This disorder is due to ineffective excision or post-replication repair of DNA following exposure to UV light. Patients with xeroderma pigmentosum exhibit extreme sun-sensitivity by age 1 and generally develop skin cancer by the age of 8. Also DNA damage induced by other forms of radiation exposition might be curable by means of endonucleases, such as gamma radiation and Röntgen radiation. Many endonucleases use the Mg^{2+}-ion as their cofactor.

Apoptosis, self-destruction, is the fate of cells from which the genetic material cannot be repaired. But sometimes cells that should be destroyed do survive, and a particular protein is responsible for that: survivin. Badly affected cells that should be eliminate easily survive due to the presence of this protein. Survivin is coded by a gene that is used only active during foetal development; it is a large protein with a molecular weight of 16,5 kDa. The 3-D structure of survivin has been fully elucidated by means of crystallography (M.A.Verdecia, H.Huang,

E.Dutil, D.A.Kaiser, T.Hunter,J.P Noel, Nat.Struct.Biol., **602**,7,(2000). At the end of the foetal development period the gene is switched off and no more survivin is produced. Survivin is only found in the placenta and the thymus, but is also abundantly present in cancer cells (lung, intestine, pancreas, prostate, breast) and other quickly reproducing cells. Survivin even resists chemotherapeutics such as doxorubicine. Why some cells suddenly start to develop survivin is still a mystery but also a crucial question in the treatment of cancer.

REPRODUCTION.

For short living organisms it does not make sense to invest in enzymes with anti-oxidant qualities or DNA repair enzymes. Their short life expectation gives only a limited statistical chance for DNA damage. An interesting question is immediately popping up: "Could this be the definition of a mutation ?".
If ageing and mortality are pre-programmed and coded in the genetic material, than it is inevitable that the reproduction system plays a dominant role. Only by reproduction the genetic information can be secured for the future. In the reproduction process a large number of hormones play an important role, and the processes are largely steered by the hypothalamus.

Human females are noticeable faced with ageing at an age of 25-30. The production of FSH (Follicle Stimulating Hormone), LH (Leutinising Hormone), estradiol and progesterone starts to decay until the production of these hormones comes to a full stop at an age of 45-60 years of age. Discontinuation of hormone production leads to massive changes in the body, and all kinds of processes will start to be manifest: cardiovascular diseases, diabetes mellitus type II and osteoporosis. Delay of the undesirable side effects of the menopause can be suppressed by HRT (Hormone Replacement Therapy), making use of a cocktail of steroid hormones (estrogens).
A few years ago medical doctors described HRT without restrictions. Today one considers that the menopausal period is a verdict by Nature, and not without a reason: intervening in the hormonal system carries sincere risks. Thus, the HRT "solution" is not a real solution, and suddenly survivin may show up again: an increased risk for breast, uterine, bladder and colon cancer.

Many naturally occurring substances exhibit estrogenic properties, and they are therefore identified as phyto-estrogens. They are much safer (but also weaker !) compared to the steroid estrogen hormones, but lack the undesired side effects. Especially a number of flavonoids are significant phyto-estrogens, such as genistein, daidzein, formonometin and biochanin-A; significant sources for these phyto-estrogenic flavonoids are soy and red clover. Their estrogenicity compares to 17-β-estradiol in a range of 0,005-2%, and that is considered highly significant. Also a number of phytosterols exhibit estrogenic activity (e.g. β-sitosterol, brassicasterol) and not to forget about the pentacyclic triterpenes (oleanolic acid, asiatic acid, lupeol). Virtually all menopausal females suffer from a quickly degrading quality of skin and may take benefit from the use of phyto-estrogens.

Without doubt one of the breakthroughs in gerontology is the discovery of the telomeres and telomerases. The human cell kernel has 23 pairs of chromosomes, and at the end of each chromosome a strand of DNA is present composed from six nucleic acids: TTAGGG. "T" stands for thymine, "A" stands for adenine and "G" stands for guanine. With every cell division some of the telomeres are lost, until a telomere level is reached whereby the cell cannot make the required proteins anymore and cell division becomes impossible: the Hayflick limit, which is defined as the maximum number of possible cell divisions. Loss of

telomeres is not applicable to stem cells such as egg cells, sperm cells and nerve cells. For all other cells is valid:

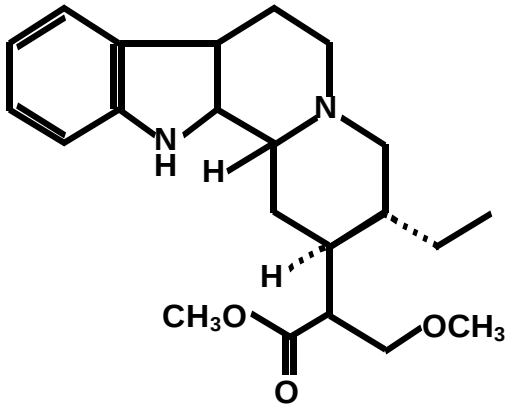
AGEING STARTS AT THE VERY MOMENT OF CONCEPTION

On the day of birth already 50% of our telomeres are lost, but fortunately the decay curve is exponential. If you're free coming Saturday afternoon, it will be raining anyway, you certainly will be able to calculate the magic achievable age of 120. Diseases go on the account of the maximum achievable age. One of our friends, an internationally accredited gerontologist, once raised the idea to giving points to a particular disease: flue costs you six weeks, a simple cold costs you a week, etc. Sounds frightening !!

Stem cells, such as sperm cells and cancer cells, are able to reconstitute the telomeres, in fact making them immortal. To do so they make use of telomerases, enzymes that act upon the telomeres. On a laboratory scale it has been shown that using telomerases the Hayflick factor can be dramatically increased from 20-25 to 40-45. A fruit fly (Drosophila), normally living for a one day, suddenly survived for 3-5 days, while mice who usually won't get older than 1½year became 3-5 years old ! This looks like what has been written in Gilgamesh or what Paracelsus was looking for. It's certainly the achievement of biochemistry, and the future of these developments cannot be forecasted. From a cosmetic point of view one could wonder how our cosmetic science will be able to contribute and how to take advantage on a consumer level. One conclusion must be drawn: we will not be able to stop ageing.

Commercial application of telomere reconstitution and the application of endonucleases is still far away, but with the knowledge generated by biochemistry we are already able to delay the signs of ageing, while maintaining the quality of life. The key factor is indeed the human enzyme system. The human genome is decoding a tremendous number of enzymes from which only a small percentage has been isolated and identified. Based on genomic information it is predictable that particular enzymes will be present, but in many cases we haven't the foggiest what there function is. Literally thousands of enzymes are active in our body, and a significant number of those are active on the skin and in the subcutaneous tissue. What we can do at present is to try to manipulate the activity of some of those enzymes that are responsible for the signs of ageing. Enzymes are best described as proteins, frequently composed from a large number of amino acids, and they require a cofactor to become active. These cofactors may be metal ions, such the Mg^{2+}-ion in the case of the endonucleases, or may be vitamins, flavonoids, alkaloids steroids & pentacyclic triterpenoids, or other naturally occurring substances.

A good example is Cat's Claw (Uncaria Tomentosa), a Peruvian vine that we discussed with you before in quite some details. Cat's Claw is a very rich source for alkaloids, more than 55 have been identified. They are roughly categorised in three groups. There are present tetracyclic oxindole alkaloids (TOA's), pentacyclic oxindole alkaloids (POA's) and also indole alkaloids. From the group of indole alkaloids hirsutine is the most important representative, which is only reported to be present in Cat's Claw. These alkaloids are all physiologically active products. TOA's mainly affect the nervous system, both peripheral and central, while the POA's affect the immunologic system cells, respecting the cellular immunity. A major interest has been raised for POA's because of their anti-proliferative properties to HL60 and U937 leukaemic cell lines, not inhibiting the growth of healthy blood cells, and do not show cytotoxic effects. Uncarine F and isopteropodine show the greatest activity; profound testing is ongoing for non-invasive treatment of leukaemia.



HIRSUTINE

In recent studies it has been shown that particular TOA's are potent survivin suppressors enabling the affected cells for apoptosis. Quite spectacular indeed has been the observation that Cat's Claw extracts also had DNA repair capacity. It is not known whether this relates to improved endonuclease activity (Y.Sheng, et.al., Induction of apoptosis and inhibition of proliferation in human cells treated with extracts of Uncaria tomentosa. Anticancer Res., 1998, 3363-8; Y.Sheng, et.al., DNA repair enhancement of aqueous extracts of Uncaria tomentosa. Phytomedicine, 8(4), 267-274, (2001)). Since 1995 approximately 700 scientific papers have been published on Cat's Claw.

Another beautiful example is Great Burnet (Sanguisorba Officinalis), used since time memorial for the treatment of menopausal problems that relate to a changing hormone balance. Aqeous extracts of the great burnet contain pentacyclic triterpenoids identified as tomentosolic acid and ziyu-glucoside. These are known to inhibit a number of enzymes that relate to the signs of ageing, such as tyrosinases, hyaluronidases and elastases. The extract inhibits UV-induced photodamage. Tsukahara et.al. (Biol.Pharm.Bull., **24**(9), 998,(2001)) reported that Sanguisorba Officinalis greatly reduced UV-B wrinkle formation, and this was shown to be related to the presence of a peptide identified as N-phenetyl-leucyl-tryptophane, an agent that was shown to specifically inhibit fibroplast derived elastase.

In actual fact, in many cases particular enzymes become over-active, such as collagenases, tyrosinases, elastases, phosphodiesterases, c-AMP isomerases and hyaluronidases. In the case of hyaluronidases the degradation of hyaluronic acid by hyaluronidases (seven are known) is to quick compared to the production of hyaluronic acid. Than an option is to slow down the hyaluronidases by means of partial inhibition. Flavonoids are quite good in that (kaempferol, luteolin, avicularin, apigenin, baicalin, gossypetin, taxifolin, etc.).

On the other hand, it would have also been an option to improve the activity of hyaluronic acid synthases, the enzymes that are responsible for the production of hyaluronic acid. Ginsenoides, steroids coming from Panax Ginseng, are able to promote hyaluronic acid synthases, but an equally nice example is the use of majoranin (5,6,4'-trihydroxy-7,8,3'-trimethoxyflavone, also named thymonin), a flavonoid occurring in sweet majoram (Origanum Majorana). Both ginsenoides and majoranin promote the production of hyaluronic acid. There is a second advantage of improved hyaluronic acid production, as the degradation products of hyaluronic acid are used elsewhere in the body, for example to produce lubricants for the joints. In the same time sweet majoram inhibits also c-AMP phosphodiesterase, one of those enzymes that "make age visible".

If your product design would make use of enzyme inhibition to delay the signs of ageing, this is not a license to use reported botanical properties as consumer product claims without further substantiation. The majority of suppliers of botanical extracts do not properly specify the active matter in particular extracts, and that makes these extracts rather untouchable. We have always advocated that the percentage active matter shall be part of the specifications rather than taking for granted what is claimed by the supplier. Our opinion on that

was recently shown to be justified as the 100% Aloe Barbadensis powder that was offered to us contained only 12-15% Aloe Barbadensis, replenished with maltodextrin. Of course we would have had to pay for 100% material !