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# ANTISECRETORY FACTOR AND ITS BIOLOGICAL ACTIVITIES

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## SUMMARY

The Antisecretory Factor, AF is a phylogenetically ancient, multifunctional protein that mediates clinical protection against various forms of diarrhoeal diseases and intestinal inflammation. The cDNA of AF has been cloned and sequenced, and AF antisecretory activity can be mapped to a peptide located between position 35 and 50 of the full length AF protein sequence. AF is present in all mammalian tissues investigated so far, and is secreted into blood, bile and breast milk. Many of the effects of AF observed clinically as well as experimentally seem to be nerve-mediated.

Dietary intake of Specially Processed Cereals, (SPC-flakes®), increases endogenous AF synthesis in man. Thus, positive effects of treatment with SPC-flakes® have been shown in patients suffering from inflammatory bowel disease and Ménière's disease. Intake of SPC-flakes® also prevented mastitis in lactating women. In a controlled study treatment with AF-rich egg yolk, B221® (Salovum®) was shown to reduce diarrhoeal disease in infants and children in a developing country. Recently, we have demonstrated that AF-16, i.e. a peptide representing the antisecretory/anti-inflammatory AF domain is capable of preventing neurological malfunctions by reducing an experimentally induced raise of the intracranial pressure. Together, these results advocate that the AF concept can be used clinically both by stimulation of the endogenous synthesis via intake of SPC-flakes®, and also by administration of the AF-16 peptide.

## INTRODUCTION

Fluid, ions and nutrients in the gut are transported across or between the epithelial linings as the results of a most intricate action of the enteric nervous system, which include co-operation of chemoreceptors, mechanoreceptors, and thermoreceptors. Intestinal motility is also regulated by neuronal activity (*Booth*, 1992; *Cook,* 1994), which, in turn, is significantly influenced by a multitude of gastrointestinal peptides, capable of inhibiting as well as stimulating the transports of ions, nutrients and water (*Hansen and Skadhauge*, 1995; *Vagne-Descroix* et al., 1991).

By using Cholera Toxin, CT, as the intestinal secretagogue in a rat diarrhoea model we discovered an endogenous factor, a protein, of central im-

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portance for the intestinal resistance to various forms of experimental diarrhoeal diseases, but also inflammatory diseases. This protein was named Antisecretory Factor, AF. The endogenous synthesis of AF increases in several tissues after intestinal challenge with enterotoxins such as cholera toxin, *Escherichia coli* LT and *Clostridium difficile* toxin A. These findings initiated a thorough characterization of AF and evaluation of its biological actions in experimental and clinical studies (reviewed in *Lange and Lönnroth*, 2001). The present publication points out various aspects in human medicine where functional capacities of AF play an important role.

## RESULTS

### Chemical characterization of the AF protein and AF peptides

Extracts from rodent and pig intestinal mucosa and pituitary gland were originally used for the purification and characterization of AF. The procedures used included isoelectric focusing, gel filtration, and affinity chromatography in agarose gels. The antisecretory potency of the purified products was determined in rat jejunal ligated loops subjected to CT challenge and suitable products were used for production of rabbit polyclonal antibodies. After screening a cDNA library from human pituitary glands, cloning of AF was performed (*Johansson* et al., 1995). Sequencing of the full-length protein revealed that human AF cDNA consisted of 1309 base pairs, of which 1131 compromised the coding sequence. The human AF protein demonstrated a molecular weight of 41 kDa with an isoelectric point of 4.9. Treatment of the full-length AF with trypsin resulted in smaller peptide fragments with preserved antisecretory and anti-inflammatory effects. The shortest peptide fragment with a preserved effect was an eight amino acid long sequence, $^{35}$IVCHSKTR$^{42}$, located in the N-terminal part of the protein (*Johansson* et al., 1997 a and b).

AF has later been shown to be a component of the proteosome and it is therefore also named S5a (*Ferell* et al., 1996). Expression of AF has been demonstrated in mammals as well as in non-mammals, and it can definitely be concluded that AF is a unique protein mediating a multitude of biological functions.

We produced polyclonal rabbit AF antibodies, and determined their attachments to different portions of the AF-molecule (Figure 1). Western blot and 2D gels showed that all of these antisera detected a single protein with a similar molecular mass and pI, while immunohistochemistry performed on various tissues resulted in an epitope-specific sub-cellular staining pattern (Jennische et al., 2006). Antisera with affinity to epitopes in the N-terminal part of the AF protein, which represent the antisecretory activity, demonstrated a more restricted localisation than antisera with an affinity to the C-terminal part that include the ubiquitin binding sites. We suggest that AF can exist in several conformational variants, probably related to functional importance which might be related to differences in redox state and/or pH in the various cellular compartments. Furthermore, chromosome 1, 19 and 23 in the human genome contain genes with a potential AF code. In mouse only one AF-gene, called rpn10, has been sequenced entirely (*Kawahara* et al., 2000).

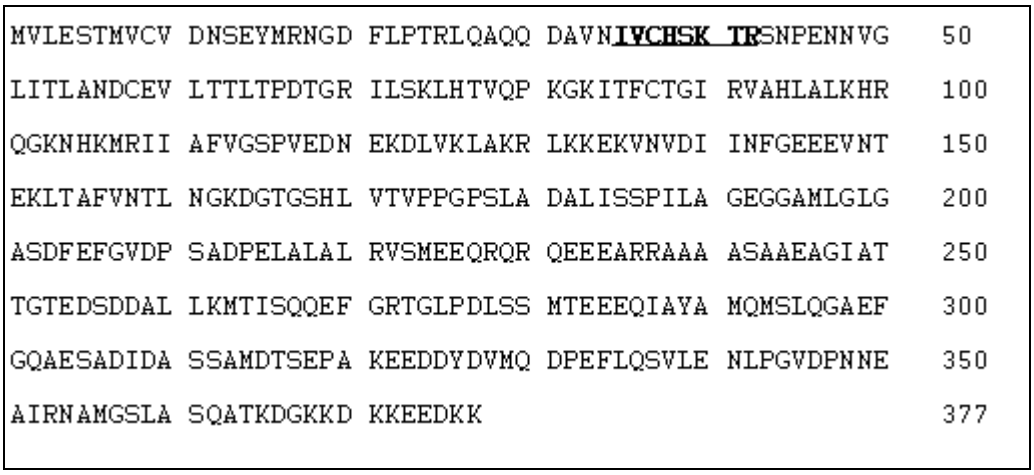


**Figure 1:** The amino acid sequence of AF. The antisecretory and anti-inflammatory sequence is marked with bold letters.

## AF cellular distribution and expression

Immunohistochemistry and mRNA *in situ* hybridization were used to enable differentiation between AF synthesis (*in situ* hybridization) and AF uptake and storage (immunohistochemistry). AF was demonstrated in the epithelial lining of the nasal mucosa, the trachea, the bronchial tree, in the alveolar type II cells, but not in the type I or squamous alveolar cells. This suggests a role for AF in the regulation of a pulmonary surfactant. AF was distinctly present in all of the surface epithelium of the gastrointestinal canal, but also in mononuclear cells located in the lamina propria. AF probably participates in urine production since AF expression was evident in the thick ascending limb of Henle. The adenohypophysis displayed AF-positive cells, while no stained cells were seen in the pars intermedia or in the neurohypophysis (*Lange* et al., 1999). AF expression in all tissues so far examined has been restricted to a most specific and defined cell population, in most cases compatible with a postulated role of AF as a regulator of water and ion transport processes (Figure 2).

## AF effects determined by *in vitro* methods

A two-chamber system separated by isolated membranes of Deiter's cells was used for studies of AF influence on the passage of Gamma-Amino-Butyric Acid ($^{3}$H-GABA) or $^{36}$Cl- between the two compartments. AF was demonstrated to inhibit $^{3}$H-GABA diffusion across the membrane in a dose dependent fashion (*Lange* et al., 1985), but we also demonstrated that $10^{-13}$ M of AF suppressed permeation of $^{36}Cl^{-}$ (*Lange* et al., 1987). All results consistently demonstrated an interaction between AF/$^{3}$H-GABA/$^{36}$Cl- suggesting a multiphase AF action on the membrane most probably mediated by a direct effect of AF on the $Cl^{-}$ channels not under GABA regulation. We have extended these studies by investigating whether AF can modulate neuronal synaptic transmission in a model system using brain slices from adult rats. Extracellular recordings were performed in the CA1 region of the hippocampus, and

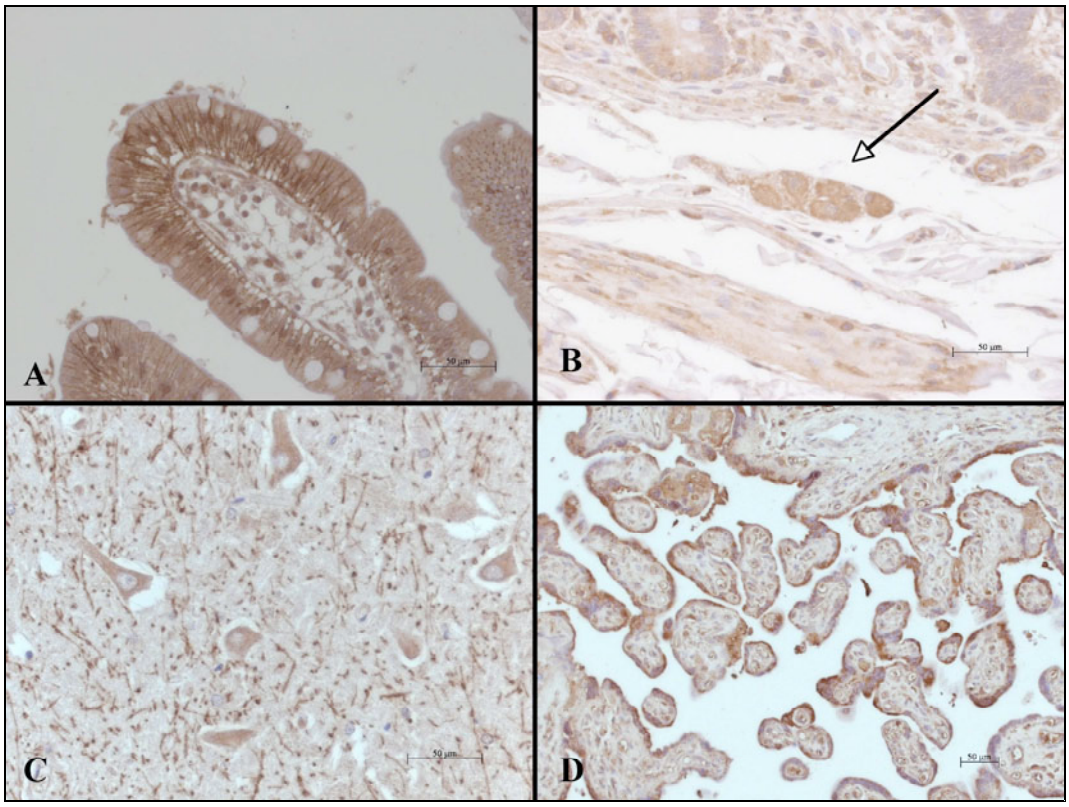


**Figure 2:** Paraffin sections from some human tissues processed to demonstrate AF-immunoreactivity.
Positive signal brown, nuclei are counterstained blue with hematoxylin. Bars = 50 µm
A. Small intestine. The epithelium and immune cells in lamina propria are stained. B. Nerve cells in Meissner's plexus (arrow) are stained. C. CNS. Positive staining is seen in neuronal bodies and processes. D. Placenta. The syncytiotrophoblast layer is stained.

addition of AF was followed by a 40% suppression of the $GABA_A$-mediated synaptic transmission. No influence on glutamatergic transmission could, however, be registered. A suppression of hippocampal $GABA_A$-mediated transmission (*Kim* et al., 2005) was also seen after treatment with cholera toxin per os or by feeding the animals an AF-inducing feed (SPC-flakes®). These results strongly suggest that AF acts as a neuromodulator, but also indicate that AF regulates intestinal secretion and inflammation via a neuronal gut-brain loop system.

## AF effects determined by *in vivo* experimental methods

A. *Intestinal inflammation*

*Clostridium difficile* is the most frequently identified pathogen in patients with antibiotic-associated diarrhoea and colitis. Its pathogenicity is mediated via the two enterotoxins toxin A and toxin B, respectively (*Bartlett*, 1994). Toxin A induces increased AF synthesis (*Torres* et al., 1991), but we have also shown that AF production is stimulated in patients suffering from acute colitis of unknown aetiology (*Torres* et al., 1993). Toxin A seemed relevant to use for studies of the anti-inflammatory

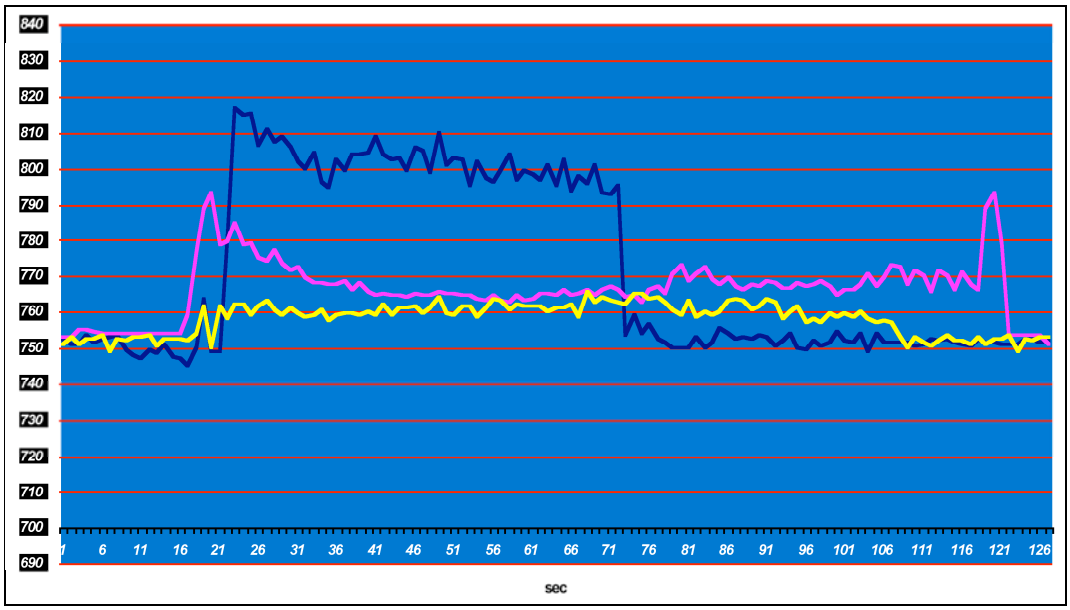


**Figure 3**. Intracranial pressure (ICP) in anaesthetized rats. The blue line visualizes the strongly raised ICP, approaching 65 mm Hg, in a rat with Herpes simplex encephalitis 6 days after intranasal instillation of Herpes simplex virus type 1. Such a high ICP is likely to prevent blood circulation and to cause herniation, i.e. persistent severe brain damage. The red line reveals that the ICP is decreasing to tolerable levels, 10-15 mm Hg, after intranasal instillation of the peptide AF-16, thereby preventing the otherwise unavoidable brain injury. For comparison, the ICP in a normal, uninfected rat is illustrated by the yellow line, fluctuating between 4 to 8 mm Hg.

effects of AF, and the results revealed a strong anti-inflammatory effect along with a diminished bleeding in the small intestinal submucosa as demonstrated by histological examination (*Johansson* et al., 1997a).

B. *AF and the intestinal capillary permeability*

CT was used for challenge in rat intestinal ligated loops, followed by an intravenous injection of the azo dye Evans blue. The results demonstrated CT-induced extravasation of the dye in the upper parts of the villi, but not in the crypts. This extravasation of the transcapillary albumin flux (due to the binding between Evans blue and albumin) was reduced back to normal after intravenous AF administration (*Lange* et al., 1998).

C. *AF and intracranial pressure*

Elevated intracranial pressure (ICP) is an aggravating factor in infectious encephalitis, and high ICP levels may be fatal or cause persistent neurological and psychiatric malfunctions. The means of pharmacological treatments are limited. We used the peptide AF-16, comprising 16 of the amino acids located to the amino terminal part of the endogenous AF protein, for regulating the raised ICP in rats with experimental herpes simplex encephalitis (Figure 3). Intranasal instillation of the AF-16 peptide counteracted the increment of ICP, while it at the same time abrogated the neurological morbidity and the mortality in a dose-dependent manner. AF-16 achieved this effect most probably by restoring the hampered outflow of cerebrospinal fluid along the olfactory nerve bundles through the cribriform

plate to the nasal lymphatic vessels. No effects on viral replication or antigen distribution within the CNS tissue were found. Thus, AF-16 abolished the prevalence of symptoms, ICP elevation, neurological malfunctions and deaths (*Jennische* et al., 2008). Further studies of AF-16 in situations with brain inflammation and elevated ICP seem warranted.

## CLINICAL STUDIES

### AF activity in patients suffering from diarrhoea, studies in Mexico and Pakistan

AF was found to rapidly increase after onset of diarrhoea in Mexican patients (*Torres* et al., 1993), the highest increment registered in patients suffering from infection with *Gardia lamblia*. However, in patients not suffering from diarrhoea, circulating AF was low irrespective of colonization or not with enteropathogens. This finding suggests that intestinal hypersecretion rather than the mere presence of intestinal pathogens is activating the endogenous AF production.

High levels of AF were determined in the breast milk of Pakistani women, probably reflecting a high prevalence of enteric pathogens in the environment. Swedish women demonstrated significantly lower AF milk levels (*Hanson* et al., 2000; *Hanson* et al., 2008; *Svensson* et al., 2004). The high AF level in milk probably provides protection against diarrhoeal diseases in the suckling child. Demographic studies of the AF levels in milk in developing countries are presently performed in our laboratory.

### Use of the AF concept in human diseases

In clinical studies we have used two forms of AF-therapy:

1. Intake of SPC-flakes® which stimulates to increased endogenous synthesis of AF.
2. Salovum® (B221®) which consists of egg yolk with a high content of AF (*Lange* et al., 1994).

### AF-inducing food

Intake of SPC-flakes®, in healthy volunteers convincingly demonstrated an increase in endogenous plasma AF activity. The most significant AF increase was attained after 3-4 daily meals with a total administration of 1 gram of SPC-flakes® per kg body weight (*Lange* et al., 2003). Furthermore, a 12-16 days long period of intake of SPC-flakes® is necessary for obtaining a significant AF plasma concentration, commonly followed by a positive clinical outcome. However, an increased AF response is achieved after only 2-3 days after a second period of intake of SPC-flakes®. Thus, there exists some sort of a "biological memory" for the human capability of AF synthesis, and the first period of intake of SPC-flakes®, seems to be responsible for "priming" of the secondary, enhanced AF response. A sensitive ELISA has been developed which permits studies of dose response relationships and follow up of compliance to AF-inducing diet (*Johansson* et al. manuscript in preparation).

### AF-inducing food and Inflammatory Bowel Diseases (IBD)

We performed a double blind placebo controlled clinical trial using SPC-flakes® in a group of patients suffering from *ulcerative colitis* and *Crohn´s disease*. All medication was kept unchanged during the 4 week long diet period. The clinical outcome of the disease was significantly improved by the diet of SPC-flakes®, and a positive correlation to plasma AF activity

($p<0.001$) was also demonstrated (*Björck* et al., 2000).

**AF and chronic diarrhoea due to intestinal resections**

The clinical effects of SPC-flakes® during a fourteen day long period were tested in patients suffering from chronic diarrhoea due to extensive intestinal resections. Subjects with signs of active intestinal inflammation were excluded from the study. Only two out of six patients responded with increased plasma AF activity at the end of the test period, and both of these patients had a remaining small intestinal length of at least 300 cm, i.e. longer than the other four patients. Thus, an increase in plasma AF activity by dietary means requires a remaining small intestine of a certain length. This finding indicates that the small intestine is the main source of active AF (*Lange* et al., 2003).

**AF administrated to patients suffering from severe *colitis ulcerosa***

In a double blind placebo controlled study Salovum® was given as a supplement to patients with acute onset of severe colitis ulcerosa. The histological and clinical laboratory outcome was studied. In mid rectum biopsies from patients treated with AF there was a less severe inflammatory reaction than in biopsies from patients treated with placebo. There was also a lowering in the inflammatory blood parameters erythrocyte sedimentation rate and C-reactive protein. The results demonstrate that Salovum® mediates an anti-inflammatory effect in cases of acute onset of *colitis ulcerosa* (*Eriksson* et al., 2003a).

**AF and patients with Crohn's disease**

A 38-year-old patient with a 20 year history of severe and progressive Crohn's colitis, combined with a continuously declining response to conventional pharmacological medication was selected for complementary treatment according to the AF concept. Thus, initially he was supplemented with Salovum®, followed by intake SPC-flakes® in order to increase endogenous AF synthesis. The clinical, endoscopic, biochemical and histological outcome was rapidly improved (*Eriksson* et al. 2003b). We have also demonstrated such a persistent positive effect of AF treatment in other patients with Crohn's disease refractory to conventional medical treatment (to be published). The pathophysiological reasons behind these exceptionally positive effects of AF treatment in patients suffering from Crohn's disease remains to be elucidated.

**AF and severe Ménière's disease**

The pathogenesis behind Ménière's disease is unknown, but impaired production and/or transport of endolymph seems to be of central importance. The clinical symptoms of this disease include attacks of fluctuating sensorineural hearing loss, rotatory vertigo, tinnitus and feeling of fullness in the affected ear. The influence of increased AF activity in patients with incapacitating Ménierè's disease was studied for a 14-30 day long period in an open pilot study. Intake of SPC-flakes® was followed by increased AF levels in 80 per cent of the cases. The attacks of rotatory vertigo were reduced in 54 per cent of the patients**,** and in 12.5 per cent the hearing was normalized and vertigo completely cured. Patients who experienced a reduction of the vertigo attacks had significantly higher final AF-levels ($p<0.01$) than those with no effects of the treatment (*Hanner* et al. 2003, 2004). Consequently, we suggest that AF might play an important part in the regulation of endolymph turnover,

thereby improving the clinical outcome of Ménière's disease. This hypothesis is further supported by immunohistochemical demonstration of AF in the epithelial lining of the endolymphatic space in the inner ear (*Hanner* et al 2004).

A double blind, placebo-controlled study was thereafter performed in 51 patients with long-standing and well-documented Ménière's disease. The patients were randomized to intake of SPC-flakes® or control cereals for 3 months, and they were examined by otoneurological methods before and after the treatment. The results show that vertigo decreased significantly in the group treated with SPC-flakes®, while no influence on the hearing capacity was registered (*Hanner* et al. 2008, in preparation). We conclude that in patients suffering from Ménière's disease intake of SPC-flakes® may improve their clinical performance especially concerning the vertigo component, and therefore could be initially recommended for treatment of this disease.

**AF and diarrhoea induced by carcinoid tumours**

In Sweden 50-60 new cases of carcinoid tumours are diagnosed each year (*Wilander* et al., 1989). These tumours are clinically associated with severe diarrhoea, and often there is a persistent hypersecretory state in the small intestine despite adequate surgical and medical therapy. A diet of SPC-flakes® combined with Salovum®, was therefore offered to carcinoid patients with residual intestinal hypersecretion. In an initial, open part of the study all of the patients received a four week long period with Salovum®, followed by a double blind cross-over period with SPC-flakes® or control cereals for 6 weeks each. A significant decrease of bowel movements in response to Salovum®, was registered, combined with a further reduction of bowel movements during the SPC-flakes® period. It seems that patients with carcinoid tumours may improve clinically following use of the AF concept, since significant reduction of bowel movements was registered in response to both forms of AF-therapy (*Laurenius et al*. 2003).

**AF and mastitis.**

The influence on mastitis of AF activity in milk was studied in lactating women during and after a 5 week long period of SPC-flakes® /placebo intake in a double blind study. The frequency of acute mastitis in the group receiving SPC-flakes® was significantly reduced when compared with the control group. The clinical outcome was also reflected by the high AF levels in milk in the SPC-flakes® group. We can conclude that intake of SPC-flakes® in lactating women increase AF in milk, and this increase is followed by significant protection against clinically manifest, acute mastitis (*Svensson et al.* 2004). The diminished inflammation in the lactating mammary gland might also protect against transfer of HIV-1 from the mother to her breast fed offspring, but also against diarrhoeal disease via the passively transferred AF. We are presently testing these hypotheses.

Subclinical mastitis is a condition which has been linked to an increased risk of HIV-1 transfer from the mother to her breastfed infant. In a Pakistani study of milk samples from 107 mothers with or without subclinical mastitis, measured as the Na/K ratio in the milk we did not see any difference between mothers receiving AF-stimulating SPC-flakes® or placebo cereals (*Jalil et al*., to be published).

**AF and acute and prolonged paediatric diarrhoea.**

Salovum®, was used for treatment of acute (<7 days) or prolonged (>7 days) diarrhoea in children 6-24 months of age in a double blind randomized study in Pakistan. The children (N=240) were randomly given 2 g Salovum®, or placebo every 5 h for 3 days, in addition to an oral rehydration salt solution. Patients receiving Salovum®, and suffering from acute diarrhoea improved with a successful clinical outcome in 83% of the cases, while in the placebo group 54% of the patients improved similarly within 3 days. The children suffering from prolonged diarrhoea treated with Salovum®, improved clinically in 91% of the cases within 3 days, compared to the 63% improvement registered in the placebo-treated group. Salovum®, seems to significantly improve the clinical condition of children suffering from diarrhoea, caused by a broad range of undefined pathogens (*Zaman et al.,* 2007).

## CONCLUDING REMARKS

The AF protein regulates transports of water and ions across various forms of biological membranes, and is capable of mediating and modulating a multitude of biological reactions.

Our studies show that a peptide situated between position 35 and 50 in the AF protein sequence has potent anti-inflammatory and anti-secretory actions, and consequently stands out as an interesting candidate for future drug development.

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