The maintenance of gastrointestinal tissue integrity is physiologically essential in the presence of the persistent harassment of microbial flora and injurious agents. Even though the repair of the gastric epithelium may be modulated by several factors, the epithelial continuity also depends on a family of small peptides called trefoil factors (TFFs). The trefoil factors family comprises the gastric peptides pS2/TFF1, the spasmolytic peptide (SP)/TFF2 and the intestinal trefoil factor (ITF)/TFF3; they are characterized by a three looped domain, the "trefoil domain", stabilized by three disulphide bridges. TFF1 and TFF3 also have a seventh cysteine that allows the formation of omo- and/or hetero-dimers. On the other hand TFF2 presents only a monomeric form, containing two trefoil domains in the same polypeptide chain. TFFs are small protease-resistant proteins that are abundantly produced by mucus-secreting cells of the gastrointestinal tract onto the mucosal surface. TFFs are essential in the protection of the mucosal epithelia against a wide range of biological threats, thus contributing to the mucosal repair. The signaling events that mediate the cellular responses elicited by TFFs are only partially understood. Moreover there are convincing evidence that TFFs do play an important role in tumorigenesis, even though their specific roles in cancer are still unclear.

TFF1 expression is strongly induced after mucosal injury and it has been proposed that TFF1 functions as a gastric tumor suppressor gene. Several studies confirm that TFF1 expression is frequently lost in gastric cancer because of deletions, mutations or methylation of the TFF1 gene. Infection by *Helicobacter pylori*, a class 1 carcinogen according to WHO classification, is thought to promote stomach carcinogenesis through induction of aberrant DNA methylation. Samples from infected patients show lower expression of TFF1. Recent studies have also shown that there is a direct relationship between *Helicobacter pylori* and the dimeric form of the protein. In fact, it was demonstrated that the core oligosaccharide portion of *H. pylori* lipopolysaccharide (RF-LPS) is able to bind to TFF1.

It also seems that the loss of TFF1 is an important event in shaping the NF-kB-mediated inflammatory response during the progression to gastric tumorigenesis, being TFF1 a negative regulator of NF-kB signalling. It is thus emerging a clear correlation between loss of TFF1, the development of inflammatory disease and the neoplastic process.

Recent analyses made by our research group allowed us to point out the up-regulation of TFF1 gene expression in rats fed on copper deficient diets, and allowed us to find out the unexpected ability of TFF1 to bind copper ions. The presence of a cysteine surrounded by several negatively charged residues in the carboxy-terminus of the protein suggested the presence of a copper-binding site. Afterwards, it was shown that Cys58 and at least three Glu surrounding residues are essential to efficiently bind copper. Moreover, the incubation of the native peptide with copper salts increases the fraction of peptide omodimers produced by inter-molecular oxidation of Cys58 and disulphide bond formation.

The Ph.D. research project was aimed at characterising the structure-function relationship of the TFF1-Cu complex. Briefly, we studied the influence of copper on known TFF1 biological activities and on its gene regulation, then we investigated its involvement in the TFF1 mediated mechanisms of *Helicobacter pylori* virulence and infection.

A preliminary Real Time PCR quantitative analysis showed that copper deficiency positively modulates *tff1* expression in an adenocarcinoma cell line (AGS), thus confirming our previous data obtained *in vivo* in copper deficient rat intestine.

In order to map possible copper responsive elements in the proximal promoter sequence, we analysed the expression of a reporter gene (Luciferase) driven by deletion constructs of the *tff1* gene promoter. AGS cells transfected with the deletion constructs allowed us to identify the upstream 5' gene sequence -583/-435 as a promoter region sensitive to the changes of copper concentration. In fact, copper chelation treatments with bathocuproine disulfonate (BCS) were able to stimulate an increase of the promoter activity of the corresponding deletion construct. Following the sequence analysis (*Transfac* software) we focused our attention on a putative SP1 binding site identified in this region, whose binding ability was then confirmed by electrophoretic mobility shift assay (-561/-552).

In agreemente with our *in vitro* results, it was also observed that copper favours the native TFF1 dimer formation in the culture medium of MCF-7 and HT29-MTX cells (a mucus-secreting clone obtained from the HT29 colon cancer cell line), thus confirming a possible role of the metal in the balance between the monomeric and the dimeric forms

To evaluate the effect of copper-TFF1 interaction on the well known motogenic activity of the protein, we performed wound healing assays on an inducible clone of gastric cancer cells (AGS) able to overexpress the peptide (AGS-AC1). As expected, the overexpression of TFF1 stimulates an appreciable increase of cell migration, and copper chelation (BCS) undo the benefits of the increased peptide level.

Our previous results showed that copper treatments decreased the amount of secreted protein in culture medium. Further experiments demonstrated that induced AGS-AC1 cells are able to store intracellularly higher amount of copper if compared to uninduced AGS-AC1 cells. This evidence suggests that TFF1 levels may also play a role also in the uptake/traffic of copper in this *in vitro* model.

Finally, we studied the combined influence of TFF1 and copper in *Helicobacter pylori* infections. Our results demonstrate that Cu-TFF1 complex promotes *H. pylori* colonization of AGS cells. In fact, AGS-AC1

cells overexpressing TFF1 are more efficiently colonised by *H. pylori* wild-type (str. P12) if compared to uninduced cells. The presence of copper in a duplicate experiment further increases the colonization, as well as copper chelation by bathocuproine disulfonate (BCS) reduces the observed effect. The same result was obtained with *H. pylori* str. P12Δ479*,* an isogenic mutant expressing a truncated LPS core still able to bind to TFF1. On the other hand, *H. pylori* P12Δ1191, unable to bind TFF1, is not affected by copper levels in the culture medium. Parallel experiments were carried out on mucus secreting HT29-E12 goblet cells, to compare and/or confirm the results obtained in AGS-AC1. The results show that also in HT29-E12 cells the *H. pylori* colonization follows a similar trend, increasing when incubated in the presence of Cu and decreasing after BCS treatment.

The present work contributed interesting results in the field of the biochemistry of the epithelia, in the wake of the research in progress in our laboratory aimed at studying the biological activities of the newly identified metalloprotein Cu-TFF1, whose properties are still poorly characterized. On the basis of the previous structural pieces of evidence we observed that the protein level and the balance of its oligomeric forms can be affected and regulated by copper ions. In turn, this delicate equilibrium is able to affect the integrity and the rheological properties of the epithelial barrier, thus representing a fine tuner, or an Achille's heel, through which pathogenic microrganisms and deregulated proliferation of neoplastic cells may take advantage for their invasiveness. The role of copper in the TFFs biochemistry represents a new finding in the puzzling and versatile functions of this interesting peptide family, whose thorough comprehension still reserves many questions and surprises.