Fibrosis is defined as the abnormal accumulation of extracellular matrix. It is a multifactorial process, in which several cell types are involved, by being "activated" in order to respond to injurious stimuli. It is a common situation, affecting several organs and it constitutes the underlying cause of substantial increase in morbidity and mortality in all modern societies.
Renal fibrosis can be observed in all anatomical compartment of the kidney and can be caused by a variety of conditions and diseases, both renal (glomerulonephritis, interstitial nephritis, cystic diseases, etc) and extra-renal (diabetes, systemic lupus erythematosus, etc). Despite differences in etiology, the development of renal fibrosis leads to chronic kidney disease (CKD), which represents a major health problem worldwide. Several studies indicate that between and 10% and 13% of the adult population suffers from CKD. In view of the high incidence and the severity of the problem, it is imperative to better understand the fibrotic process at the molecular level and to uncover markers for early stages of renal fibrosis; such markers will help to perform extensive screening of the general population and especially of the population considered at high risk (individuals with diabetes, hypertension or family history of renal disease) leading to improvement of the health of the population and substantially reducing the costs of health care.

Since renal fibrosis is a complicated, multifactorial process involving several cell types and renal compartments, the group of the Principal Investigator has applied proteomic approaches, in order to gain a more holistic understanding of the macromolecules involved. This approach, applied in a widely used animal model of renal fibrosis, the unilateral ureteric obstruction model in rats and mice, has generated a list of macromolecules differentially expressed during the development of renal fibrosis. Two of them, calreticulin and transgelin, not suspected to play a role in fibrosis up to recently, are the focus of the proposed research.

Calreticulin is a 46 kDa protein, found in all eukaryotic organisms, being a product of a single gene whith no alternative splicing. Originally, it was described as an exclusive endoplasmic reticulum resident protein, able to bind Ca++ and involved in Ca++ homeostasis. Eventually, it was found that calreticulin may be involved in other non-ER cellular functions like intracellular signal transduction, cell adhesion, cell migration, phagocytosis. Following the findings from the proteomic analysis, the group of Dr. Charonis has confirmed: first, biochemically by Western blotting, that calreticulin is elevated in the rat renal fibrosis model at early time intervals and is further elevated at later time intervals and second, by Real Time PCR, that the increase is due to increase in calreticulin gene transcription. Furthermore, in the renal fibrosis model studied, morphological studies by immunocytochemistry and immunofluorescence identified that calreticulin up-regulation is almost exclusively observed in tubular epithelial cells, but not in other cell types of the renal parenchyma. However, several aspects of the role and the significance of calreticulin in the fibrotic process remain unanswered, mainly regarding the mechanism of up-regulation, the effect of the up-regulation of the cellular phenotype and the possible usefulness of detection of calreticulin as an early fibrotic marker.

Transgelin (also known as SM22) is a 22 kDa protein present in smooth muscle cells (it is actually one of the earlier markers of smooth muscle cell differentiation). The family of transgelins consists of 3 highly homologous genes (A, B and C). Proteomic studies indicated that transgelin A is upregulated. Again, until recently, the main functions assigned to transgelin A were the actin-binding ability and the ability to function as a tumor suppressor. Following the findings from the proteomic analysis, the group of Dr. Charonis has confirmed: first, biochemically by Western blotting, that transgelin is elevated in the rat renal fibrosis model at

early time intervals and is further elevated at later time intervals; second, by Real Time PCR, that the increase is due to increase in transgelin A gene transcription; and third, by using immunocytochemistry, that transgelin up-regulation is mainly observed in spindle-shaped cells localized preferentially in interstitial areas surrounding glomeruli and not in other cell types of the renal parenchyma. In addition, preliminary results with biopsy material from a group of patients suffering from glomerulonephritis of variable etiology has indicated the possible usefulness of detection of transgelin as a fibrotic marker.

Based on the above findings, these two proteins need to be further studied, in order to establish their role in the early stages of renal fibrosis and their possible utility as early markers for the development of renal fibrosis.

Consequently, the current proposal has three major aims:
First, to understand at the molecular level the mechanism(s) underlying up-regulation of calreticulin and transgelin during the development of renal fibrosis, especially at the early stages of the process. Bioinformatic and biochemical approaches will be used in order to accomplish this aim. Findings from this approach will allow us to design in the future more specific therapeutic interventions.
Second, to examine the effect of the up-regulation of calreticulin and transgelin in the specific renal cell types that it is observed to happen. More specifically to uncover and confirm the phenotypic changes resulting from the transcriptional alterations, including cell adhesion, cell motility, cellular secretory profile, cell viability, cell stress etc and to correlate these changes with the renal pathology observed. Culture of specific cell types, proteomic analysis of their alterations, cellular assays for specific properties and morphological studies on sections from animal models will be used in order to accomplish this aim. Findings from this approach will allow a better explanation of patho-physiological observations and will contribute to a better understanding of renal pathology.
Third, to explore the possibility that calreticulin and transgelin could be detected in patients suffering from renal diseases of variable etiology. Material to be used will be sections from renal biopsies and urine samples. Findings from this approach might contribute to the design of novel, more accurate staging and prediction of the evolution of renal diseases.

In order to achieve the above mentioned aims, the following Work Packages (WP) are proposed:
WP1: Collection of experimental material
WP2: Mechanisms of calreticulin and transgelin up-regulation
WP3: Phenotypic alterations following calreticulin and transgelin up-regulation
WP4: Detection of calreticulin and transgelin in patients suffering from renal diseases
WP5: Administration of the project

In WP1, appropriate renal cell lines will be purchased, cultured and transfected in order to establish the stable overexpression of calreticulin and transgelin in them. Renal parenchyma from rats and/or mice subjected to unilateral ureteric ligation at different time intervals (2 and 8 days) will be collected for morphological and biochemical studies. Material from specific cell types expressing either calreticulin or transgelin will be collected by the technique of Laser Capture Microdissection and further processed for proteomic analysis. Biopsy material and urine samples from patients suffering from renal diseases having a strong fibrotic component and material from appropriate controls will be collected, appropriately processed and stored.

In WP2, combining bioinformatic and experimental approaches, regulatory elements at the transcription start site of the calreticulin and the transgelin gene will be examined, transcriptional regulators will be mapped and the significance of their contribution will be further tested using appropriate vectors, several constructs and relevant human renal cell lines. Furthermore, transcription factors giving promising results will be tested for in vivo occupancy of the corresponding genomic sites by chromatin immunoprecipitation methods. The results will then be analyzed using Ingenuity software, in order to identify putative signaling pathways involved and the involvement of these pathways will be tested pharmacologically.

In WP3, specific renal cell types over-expressing either calreticulin or transgelin will be studied for generation of their overall proteomic profile. Following that, specific aspects of the cell phenotype will be further evaluated by appropriate well established assays. These include cellular differentiation markers for the epithelial and/or the mesenchymal phenotype, cell adhesion and motility, the cellular secretory profile (with emphasis on basement membrane or matrix proteins), cell viability, proliferation and apoptosis, signaling pathways (with emphasis on wnt pathway and beta catenin translocation) and cellular stress. Based on the results from these assays, selected markers will then be examined in sections from the animal model and from human biopsies.

In WP4, biopsy material from patients suffering from renal diseases and appropriate control material will be examined for expression of calreticulin and transgelin using appropriate imaging software. Furthermore, urine samples will be studied, using either the soluble peptide fraction or after isolation of exosomes and the results will be compared to standardized samples from healthy individuals.

In WP5, the scientific and the financial administration of the project will be carried on, in collaboration with the grants management office of BRFAA and the funding agency. In addition, the dissemination of the results will be realized, including participation in meetings, publications in high visibility journals, generation and constant update of a related website and organization of a special one-day workshop to update nephrologists and basic scientists with related interests.

The description provided above does not deal with the techniques to be used, since these techniques are being extensively used by the group of the Principal Investigator and by the groups of the Collaborating Investigators (all experts in their fields), as can be confirmed by their publication record.

In summary, this is a proposal focusing on understanding the development of renal fibrosis and developing molecular markers in order to be able to perform early diagnosis. It focuses on two such proteins observed to have a possible role in the molecular mechanism(s) and to have the potential to become in the future novel markers of renal fibrosis, calreticulin and transgelin. Key characteristics of the proposal are its multidisciplinary nature (combination of discovery-driven and hypothesis-driven research), its strong translational aspect (from bench to bedside) and the fact that already existing preliminary data strongly support the need to perform the proposed studies.