UNIVERSITÀ DEGLI STUDI DI CATANIA

DIPARTIMENTO DI SCIENZE BIOMEDICHE E BIOTECNOLOGICHE

DOTTORATO DI RICERCA IN BASIC AND APPLIED BIOMEDICAL SCIENCES

XXXII CICLO

TESI DI DOTTORATO

Role of Neutrophil Gelatinase-Associated Lipocalin (NGAL) as a Therapeutic Target in Urologic Cancer

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Anno accademico 2018/2019

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ABSTRACT

Neutrophil gelatinase-associated lipocalin (NGAL) positively modulates the activity of matrix metalloproteinase-9 (MMP-9) through a protein-protein interaction, resulting in the formation of the NGAL/MMP-9 complex. This complex is stabilized by a disulfide bond between the Cysteine 107 (Cys107) of NGAL and one of the available cysteines of MMP-9. By binding NGAL, MMP-9 is also protected from degradation.

NGAL and MMP-9 play a key role in the degradation of the extracellular matrix (ECM), promoting metastasis formation.

The role of NGAL, MMP-9 and of the NGAL/MMP-9 complex has been investigated in several cancers of the urinary tract, including bladder cancer, kidney cancer and prostate cancer.

We have previously demonstrated that the TP53 mutation and NF-kB over-expression lead to NGAL over-expression in advanced prostate cancer cells. In particular, we found that NGAL over-expression in the prostate cancer cell lines that express low NGAL basal levels (22Rv-1 and LNCaP) is associated to enhanced tumor cell growth in soft agar assay. Conversely, NGAL silencing induced in the PC3 and DU145 cells, that over-express NGAL, results in a lower growth rate. These results prompted us to investigate the interaction

mechanisms between NGAL and MMP-9 putatively responsible for the enhancement of MMP-9 proteolytic activity and, in turn, for the aggressiveness of cancer cells. In particular, the functional effect of disulfide bond depletion in the stability of the NGAL/MMP-9 complex was evaluated through the modification of Cys107 obtained by the substitution of the cysteine residue with a glycine (NGALC107G).

We demonstrated that the clones co-transfected with plasmids coding for MMP-9 and NGAL show higher MMP-9 protein levels than those transfected with the plasmid for MMP-9 alone. In addition, there are no differences in cells co-transfected with NGALWT vs. NGALC107G plasmid. Moreover, the invasion assay has shown that cells overexpressing MMP-9 have a higher invasive capacity as compared to controls (cells transfected with empty plasmid) and that cells transfected with NGALWT are more aggressive than cells transfected with NGALC107G and controls.

Taken together, the results show that NGAL over-expression enhances cells invasion independently and through the modulation of MMP-9 activity. Furthermore, the induced NGALC107G mutation and the disulfide bond depletion did not appear necessary for the activation of MMP-9, thus suggesting that this activation may be mediated by other interaction mechanisms between NGAL and MMP-9 not yet identified.

Considering that the inhibition of NGAL, MMP-9 or the complex NGAL/MMP-9 may result in decreased cancer cell growth and invasiveness, the possible use of tailored-targeted therapeutic strategies in metastatic patients seems to be promising.

Further studies are warranted in order to better characterize and target the interaction mechanisms between NGAL and MMP-9 and to test NGAL-MMP9-targeted cancer therapy, both in preclinical models and in clinical trials.

INTRODUCTION

Neutrophil gelatinase-associated lipocalin (NGAL), as well as matrix metalloproteinase-9 (MMP)-9 and the NGAL/MMP-9 complex, have been investigated in several cancers of the urinary tract, such as bladder cancer, kidney cancer and prostate cancer.

We have previously shown that the microRNAs (miRNAs) hsa-miR-145-5p and hsa-miR-214-3p are downregulated in bladder cancer and may modulate both the epithelial-mesenchymal transition (EMT) and the NGAL/MMP-9 pathways [1].

Moreover, it has been demonstrated that the NGAL/MMP-9 pathway is associated with an aggressive phenotype of bladder cancer [2] and that urinary NGAL concentrations significantly correlate with bladder cancer stages and grade [3]. In addition, it has been proposed that serum NGAL could be a useful non-invasive biomarker for bladder cancer [4].

Yu et al. have demonstrated that NGAL enhances renal cell carcinoma (RCC) growth and it is involved in the mechanisms of resistance to sunitinib treatment in RCC [5]. Furthermore, the combined analysis of the urinary level of NGAL and KIM-1 could predict the histologic subtype of the radiographic-detected masses among cases with kidney cancer [6].

On the basis of these data, in this study we focused on the role of NGAL in prostate cancer and on the identification of the interaction mechanisms between NGAL and MMP-9 that lead to the enhancement of MMP-9 proteolytic activity, thus enhancing cancer cells aggressiveness.

Prostate cancer

Epidemiology and etiology

After lung cancer, prostate cancer is the second most frequent cancer among males worldwide [7]. In Italy, prostate cancer is currently the most common cancer in men and represents over 20% of all cancers diagnosed from the age of 50 years [8].

In recent decades, particularly since the 2000s, the incidence of prostate cancer has shown a steady upward trend, probably due to the diffusion of the prostate specific antigen (PSA) test as a tool for screening. The incidence shows a North-South gradient: compared to 144.4 cases per 100,000/year among residents of Northern Italy, the regions of the Center do record a –3% (140.0/100,000) and those of the South even a –25% (109.00/100,000). These differences, besides the use of PSA as a screening test [8], are probably due to the different incidence of susceptibility factors and above all, to the different lifestyle, in particular the diet and the lower income of protective factors, such as antioxidants.

Prostate cancer, despite being in the first place for incidence, in Italy occupies the third place in the mortality rate, with almost all cases involving males over 70 years. However, prostate cancer has been a cause of death in constant moderate decrease (–1.9% per year) for more than the last twenty years [8].

Despite the differences in the incidence in Northern Italy, compared to the Center and the South, there are no substantial differences in mortality for this neoplasm between the various areas of the country, with 30-35 deaths per 100,000 inhabitants/year, slightly higher in the South [8].

The survival of patients with prostate cancer, not considering mortality from other causes, is currently at 91.4% at 5 years following diagnosis, and it is in constant and sensitive growth [8]. The main factor related to this temporal trend is given by the diagnostic anticipation and the progressive diffusion of screening [8].

The etiology of prostate cancer is multifactorial and is the result of a complex interaction between genetic factors (responsible for the familiarity and the different incidence in human races) and environmental factors (diet, carcinogens present in the environment).

The risk factors for prostate cancer include: age (the incidence of cancer increases with age)[9]; race (the black race is most at risk for the highest circulating levels of androgens, androgen dihydrotestosterone and 5-alpha reductase)[9–11]; hormonal factors (high circulating levels of testosterone and

IGF-1 predispose to tumor onset)[12]; family history of prostate cancer (about 25% of patients)/genetic factors (9% of inherited forms; 43% in patients aged <55 years)[10,11]; lifestyle, including diet (excessive caloric and fat intake)[13,14].

As far as familiarity is concerned, it is estimated that the risk is at least doubled if a first-degree family member suffers from this neoplasm [11]. If two or more first-degree relatives are affected, the risk increases by 5-11 times [11]. Actually, only a small subgroup of patients with prostate cancer (less than 15%) has a disease on a hereditary basis (among the inheritance criteria: presence of three or more affected family members, or at least two family members who developed the disease before the age of 55). Hereditary familial carcinoma is usually diagnosed earlier than sporadic carcinoma [11].

Genetic factors together with hormonal factors and those related to lifestyle probably explain the wide variability in the expression of neoplasia in the various geographical areas of the globe. The incidence of prostate cancer is in fact highest in African-Americans residing in the San Francisco bay, in the USA, and minimum among the resident population of South-East Asia [10]. On the other hand, the prevalence of tumors detected at autopsy is about 100 times higher than the clinical incidence of the disease and it is almost identical in all geographical areas, reflecting the fact that environmental factors, in particular those related to diet and lifestyle, are probably more relevant than

the genetic factors. This would also seem to be demonstrated by studies on migrants. For example, it is interesting to note that among the inhabitants of Japan who emigrated to Hawaii, the risk of illness increases, and even more so in the case of the Japanese who emigrate to California, reaching incidence rates, especially from the second generation, almost comparable to those of the resident population [10]. Factors such as food and alcohol consumption, sexual behavior, chronic inflammation and occupational exposure have all been correlated with the etiopathogenesis of the disease and with the neoplastic progression [14]. In particular, a key etiopathogenetic role is currently attributed to the reduced exposure to possible protective factors, such as Vitamins C and D, trace elements, and antioxidants [14,15]. However, the currently available evidence does not allow us to recommend specific changes in lifestyle, and in particular nutrition, in order to reduce the risk of developing this disease, even if a reduced consumption of animal fats and an increase in the consumption of fruit, cereals and vegetables, and an increase in physical activity may in any case be advisable.

Chemoprevention, early diagnosis and screening

Prostate cancer can be considered an ideal candidate for dietary and pharmacological chemoprevention, due to specific features such as the high incidence of the disease, long latency, endocrine dependence and the presence of potential pre-neoplastic lesions carcinoma precursors (prostatic

intraepithelial neoplasia [PIN], especially in the high-grade form). The real usefulness of corrective measures of diet and lifestyles is still a subject of controversy, since most of the information available on the subject derives from case-control studies [16–19].

In 2003, the first large-scale chemoprevention study was conducted on a population of 18,882 men, asymptomatic, with digital rectal examination (DRE) negative and PSA < 3 ng/ml, which were randomized to receive finasteride (5mg/day) or placebo for 7 years (PCPT trial) [20]. This study showed a reduction in the number of incident cases of prostate cancer in the group treated with finasteride (18.4% vs 24.4%). However, in the treated group, in addition to higher toxicity, a significant increase in the number of tumors with a high Gleason score (> 7) was observed.

More recently, in another clinical trial a reduction in the hazard ratio for incidence of prostate cancer of 21.1% was observed in the group of patients treated with finasteride compared to the placebo group (HR 0.79; 95% CI 0.74-0.84 p <0.001). However, due to the nature and source of the collected data, it was not possible to obtain information regarding the Gleason of the diagnosed tumors. Furthermore, no mortality data were obtained from this study [21].

A subsequent study (called REDUCE) evaluated the use of another 5 alpha reductase inhibitor, dutasteride, in men at risk of developing prostate cancer. This study enrolled about 6,300 men, ranging in age from 50 to 75 years, with

negative prostate biopsy performed in the 6 months prior to randomization and PSA between 2.5 and 10 ng/ml (if < 60 years), or between 3 and 10 ng/ml (if ≥ 60 years). The subjects were randomized to receive dutasteride (0.5 mg/day) or placebo and subsequently evaluated by repeating prostate biopsies at 2 and 4 years from the start of treatment (a biopsy was also performed every time the presence of a prostate tumor is suspected) [22]. In dutasteride-treated patients there was a 22.8% reduction in the risk of developing prostate cancer, without a significant increase in the percentage of tumors with high Gleason score in the treatment group and with an acceptable toxicity profile. It should be emphasized that, even in this case, no differences in mortality risk have emerged, up to now [22].

However, since primary prevention cannot be used to reduce the incidence of the disease, there is no doubt that secondary prevention remains, theoretically, the most appropriate instrument to influence the natural history of the disease and to reduce its lethality. The screening test that appeared to be potentially more suitable for the purpose, for overall considerations of cost, convenience and diagnostic accuracy, is the periodic dosage of the PSA. In order to propose a screening procedure, both individually and in the population, it is necessary that its efficacy (reduction of mortality) and cost/benefit ratio are confirmed by randomized prospective studies. Similar studies have been conducted in Europe (ERSPC) and in the USA (PLCO) and in 2009 these studies produced

the first data on the impact of mortality screening [23,24]. The European Randomized Study of Screening for Prostate Cancer (ERSPC) was started in the early 1990s in seven European countries and enrolled a total of 182,000 subjects, aged between 50 and 74, who were randomized to be subjected to a periodic dosage of PSA (on average every 4 years, but with differences between the protocols applied in the different participating countries as regards the periodicity of the execution of the test, the cut-offs used and the possible simultaneous execution of the DRE and/or transrectal ultrasound) or to be part of the control group. According to Schröder et al., despite a reduction in cancer-specific mortality of nearly 20%, PSA screening was associated with a high risk of overdiagnosis, and therefore of over treatment of nearly 50%. These conclusions are still valid, although subsequent analysis of the study showed an increase in the "yield" of the screening procedure over time [25].

In the last decade, much emphasis has been placed on the reduction of cases with metastatic disease at the onset correlated with the use of PSA for screening purposes in asymptomatic men. In fact, at 12 years of follow-up, the ERSPC study showed a reduction of 3 cases with metastatic disease per 1000 men screened compared to controls [26]. However, this reduction seems mainly due to the initial recognition of cases with high PSA still asymptomatic, but with subclinical disease probably already widespread, since in the

subsequent monitoring the frequency of metastatic progression is not different in men subjected to screening (135 cases) and in controls (130 cases) [26].

At the present state of knowledge, the guidelines recommend against the adoption of population screening policies [27–29].

As regards, instead, the "spontaneous" use of the PSA dosage as a screening test in asymptomatic men [27] it has been suggested that: individuals above the age of 75 and/or with a life expectancy of less than 10 years should not be screened with PSA as any benefits are marginal compared to the risks; the PSA dosage can be offered to men with a life expectancy higher than 10 years if they wish, as long as they are informed of the (prevalent) risks and (limited) benefits linked to the administration of the test and the actions resulting from the result of the same; the dosage of PSA as a screening test in asymptomatic men should not be recommended under 50 years for men without risk factors; in men between the ages of 40 and 50, with risk factors such as family or ethnicity, the opportunity for PSA monitoring should be discussed on a case-by-case basis, explaining to the person concerned the potential risks (over-diagnosis, over-treatment) and the possible benefits; in the absence of symptoms and in any case of suspected diagnosis, the PSA dosage should not be included in routine blood tests.

Finally, it should be remembered that: with the commonly used PSA threshold values, false negative results can be found in 20-25% of clinically significant initial tumors, and that the most undifferentiated tumors may present with low

PSA values. Therefore, the risk of a possible "false reassurance" given by a negative test result must be taken into consideration.

Faced with the request of the individual, the doctor must therefore always be cautious and ensure that the interested party is provided with the most adequate information, not only on the risks and benefits but also on the diagnostic limits of the test.

Anatomo-pathology and classification

Adenocarcinoma of the prostate usually originates in the peripheral portion of the gland (70%), and is therefore often appreciable also for rectal exploration. Less commonly it starts from the anteromedial portion of the organ, the so-called transition zone (20%), distant from the rectal wall, which represents the typical site of benign prostatic hypertrophy.

The central area of the prostate is rarely the site of origin of the tumor (5%), but more often it is invaded by large tumors originating in the neighboring portions of the organ. The prostate cancer is mostly multifocal [30].

The local lymph nodes are in the small pelvis, and include the pelvic lymph nodes distal from the bifurcation of the common iliac vessels. The following groups are considered: pelvic; hypogastric; shutters; iliacs (internal, external); sacral (lateral, presacral, of the promontory [of Gerota]).

The involvement of one or both sides does not affect the classification N [30,31]. The distant lymph nodes are located beyond the small pelvis. Metastases in the extraregional lymph nodes are classified as M1a [30,31]. The distant lymph nodes are: aortic (lumbar para-aortic); common iliac; deep inguinal; superficial inguinal (femoral); supraclavicular; cervical; scalene; retroperitoneal.

Clinical TNM classification of prostate cancer

The extension and the location of the tumor are categorized according to the TNM classification preceded by the letter c (clinical) (UICC 2009) (Table 1) [31].

Primitive tumor (T)

TX The primitive tumor cannot be defined (categorized)

T0 No evidence of primitive tumor

T1 Clinically not appreciable tumor, not palpable nor visible with images

- **T1a** Tumor discovered casually in 5% or less of the tissue removed following TURP/adenomectomy
- **T1b** Tumor discovered casually in more than 5% of the tissue removed following TURP/adenomectomy
- **T1c** Tumor diagnosed by needle biopsy (for example, due to high PSA)

T2 Tumor limited to prostate*

- **T2a** Tumor affecting half or less of a lobe
- **T2b** Tumor that affects more than half of a lobe but not both lobes
- **T2c** Tumor affecting both lobes

T3 Tumor that extends outside the prostate **

- **T3a** Extraprostatic, unilateral or bilateral extension, including invasion of the bladder neck.
- **T3b** Tumor invading the seminal vesicle(s)

T4 The tumor is fixed or invades adjacent structures besides the seminal vesicles: bladder neck, external sphincter, rectum, elevator muscles and/or pelvic wall.

* Note: A tumor discovered in one or both lobes by needle biopsy, but not palpable or visible through imaging, is classified as T1c; ** Note: The invasion of the prostatic apex or prostatic capsule (without exceeding it) is not classified as T3 but as T2.

Metastasis to regional lymph nodes (N)

NX Regional lymph nodes have not been clinically evaluated

N0 No clinically evident metastases in regional lymph nodes

N1 Metastasis in regional lymph node(s)

Distant metastases (M)

M0 Not distant metastases

M1 Distance metastasis

- **M1a** Metastasis in extra-regional lymph node (s)
- **M1b** Bone metastases
- **M1c** Metastasis in other sites with or without bone metastases

Stage I	T1a	N0	M0	G1
Stage II	T1a	N0	M0	G2, G3-4
	T1b	N0	M0	Every G
	T1c	N0	M0	Every G
	T2	N0	M0	Every G
Stage III	T3	N0	M0	Every G
Stage IV	T4	N0	M0	Every G
	Every T	N1	M0	Every G
	Every T	Every N	M1	Every G

Table 1. Staging of prostate cancer.

Histological classification of prostate cancer

The reference classification for evaluation of the histological subtypes of prostate cancer is the one indicated by the World Health Organization (WHO) in 2016 [32].

Glandular neoplasms

• Acinar adenocarcinoma

-Atrophic

-Pseudoiperplastic

-Microcistic

- Foam cells

- Colloid

- Ring-shaped cells

- Pleomorphic giant cells

-Sarcomatoid

• High grade prostatic intraepithelial neoplasia

• Intraductal carcinoma

• Ductal carcinoma

Squamous neoplasms

• Adenosquamous carcinoma

• Squamous carcinoma

Neuroendocrine tumors

• Adenocarcinoma with neuroendocrine differentiation

• Well differentiated neuroendocrine tumor

• Small-cell neuroendocrine carcinoma

• Large-cell neuroendocrine carcinoma

Transitional cell carcinoma*

Prostate stroma tumors and mesenchymal tumors*

* TNM staging should not be applied to this histotype

In the new classification a new entity has been introduced consisting of the intraductal carcinoma defined as: "intra-acinar/intra-tubular neoplastic epithelial proliferation" which has the same aspects as high-grade prostatic intraepithelial neoplasia (HG-PIN) but shows a cytological atypia/major architectural, typically associated with high-grade and high-level adenocarcinoma. It represents the final event of the evolution of prostate cancer with an intraductal growth of aggressive carcinoma and the cancerization of ducts and acini by a high-grade adenocarcinoma.

Differential diagnosis with HG-PIN is important and for this purpose the use of immunohistochemical investigations to evaluate the expression of PTEN and ERG may be helpful; in fact intraductal carcinoma shows the absence of PTEN and ERG expression while the lack of PTEN is rare in HG-PIN and the expression of ERG is not common [33].

It is important to remember that intraductal carcinoma should not be assigned a Gleason grade [34].

Up to 2004, the most commonly used immunophenotypic markers have been PSA, prostate-specific acid phosphatase (PAP), high molecular weight cytokeratin (34betaE12), p63 and racemase (AMACR). In the 2016 WHO classification, new markers such as prostein (PS501S) and NKX3.1 [35–38] have been considered. This last antigen is particularly useful in the differential diagnosis of urothelial carcinoma and metastases in cases of PSA and PAP negativity, to confirm the prostate origin. PSA, PAP, prostein and NKX3.1 are highly sensitive (over 94%) in the diagnosis of metastasis of carcinoma of prostate origin. It should be remembered that the positivity for PSA and PAP decreases after antiandrogen therapy, so in these cases it is advisable to use prostein or NKX3.1 which instead maintain the positive expression.

The Gleason score is considered an international standard for prostate cancer gradation: it should be emphasized that, almost 40 years after the introduction

of this classification system, it remains one of the most important independent prognostic factors [39,40].

The Gleason grading system takes into consideration the degree of cytoarchitectural differentiation of the glands and the ratios of the neoplasm with the stroma, i.e. the type of infiltration. The Gleason classification identifies five architectural glandular aspects to which a score of increasing malignancy is attributed. The score is assigned to the two structural aspects most represented in the neoplasm. Initially, the Gleason score envisaged the sum of the primary grade, i.e. the most present, and the secondary grade (second pattern represented > 5% but in lesser quantity than the primary pattern). According to the ISUP 2005 classification, when examining a prostatic biopsy, the primary pattern will always be the most represented, while the secondary Gleason will be the worst among the other patterns. When there is no secondary grade, the primary grade must be doubled to obtain the Gleason score. The primary and secondary grade can be reported before the Gleason score which appears to be the sum, e.g.: Gleason score 3 + 4 = 7, or be reported in brackets after the Gleason score, e.g.: Gleason score 7 (3 + 4).

The Gleason grades attributed to the neoplastic tissue according to the ISUP 2005 classification are the following:

• Grade 1: circumscribed nodule of dense but distinct, uniform, oval, medium-sized berries (larger glands than pattern 3).

• Grade 2: as for model 1, a relatively circumscribed nodule, but minimal infiltrations may be present at the margins. The glands are arranged less tightly and uniformly than the pattern 1.

• Grade 3: discrete gland units; in general, the glands are smaller than those seen in models 1 and 2. Infiltrates are present among non-neoplastic acini. Considerable variability in shape and size, sometimes with cribriform aspects.

• Grade 4: confluent, undefinable micro-acinary glands with poorly formed glandular lumen; cribriform glands, also with irregular edges; sometimes hypernephromatoid aspects.

• Grade 5: relative absence of glandular differentiation; composite solid aggregates or single cells; comedocarcinoma with central necrosis surrounded by papillary, cribriform or solid masses.

Five different grade groups, defined according to Gleason, have recently been identified [35].

The groups are as follows:

• grade group 1: Gleason score lower/equal to 6

• grade group 2: Gleason score 3 + 4 = 7

• grade group 3: Gleason score 4 + 3 = 7

• grade group 4: Gleason score 4 + 4 = 8, 3 + 5 = 8; 5 + 3 = 8

• Grade group 5: Gleason score 9-10

The prognostic impact of these five groups was validated in a multi-institutional study comprising 20,000 radical prostatectomies, 16,000 prostate biopsies and 5,000 prostatic needle biopsies in patients who underwent radiotherapy treatment [34]. Genomic correlations were also observed and molecular biology data were obtained to support this differentiation into prognostic groups [41].

As can be seen from the classification, the Gleason score less than or equal to 6 indicates the grade group 1, with excellent prognosis [34,42]; for this reason, also considering those that are other factors, such as the PSA value, the neoplasms belonging to this group can be candidates for active surveillance.

PIN is a precancerous lesion consisting of a cellular proliferation within the ducts and acini of the prostate. Initially, three PIN grades were considered, but currently two PIN grades are considered (a low grade PIN, corresponding to the grade 1 PIN, and a high grade PIN, that includes grades 2 and 3) [43–45].

The diagnosis of low grade PIN should not be reported in the histopathological report as it has a poor reproducibility among the various observers [46] and in any case does not seem to have a correlation with the risk of cancer [47].

The diagnosis of high grade PIN, on the contrary, must always be reported in the histopathological report of needle biopsies since, although it does not have

a prognostic significance, its presence is strongly predictive of the subsequent identification of carcinoma in 27-31% of patients [47–50]. Whether or not the extension of high-grade PIN in biopsies is a predictor of subsequent prostate cancer is still controversial [50,51]. The morphological aspect of the high grade PIN (micropapillary, cribriform) is not correlated with the subsequent development of neoplasia [51]. In patients with high-grade PIN on needle biopsy a close clinical follow-up is indicated with intervals of three to six months for two years and an annual check in the following years.

Diagnosis

The diagnosis of prostate cancer is based essentially on the following investigations: DRE; dosage of PSA; image techniques; prostate needle biopsy.

DRE

The DRE must be the first diagnostic approach to the patient that presents symptoms referable to a possible prostatic pathology. Given that prostate cancer occurs in more than 70% of cases at the peripheral portion of the gland, the neoplastic nodule can often be detected already with the simple palpation. In particular, this is easier when the lesion has a volume of 0.2 mL or greater. About 18% of prostatic neoplasms are detected by the DRE alone, regardless of PSA values [52]. A suspected palpatory to DRE, associated with a PSA > 2

ng/ml, presents a positive predictive value between 5 and 30% [53]. A 'dubious' DRE is also associated with an increased risk of disease with a high Gleason score and therefore leads to the consideration of prostatic biopsy [53,54].

However, the DRE, although indispensable in the evaluation of the patient, cannot be used as the only diagnostic method, since it presents low levels of sensitivity (positive predictive value), and specificity. In many patients, it has been shown that the DRE fails the diagnosis of cancer in about half of the cases, as there is no demonstrated correspondence between a palpatory alteration and the presence of tumor in this site [55,56].

Dosage of PSA and other markers

PSA is a glycoprotein produced mainly by prostate gland tissue [57]. PSA is secreted in the seminal fluid and, under physiological conditions, only minimal amounts of antigen reach the bloodstream. The subversion of the normal prostatic histoarchitecture, as in the case of benign pathology (prostatic hypertrophy, prostatitis) or malignant prostate, determines an increase in blood levels of PSA, which must therefore be considered a marker of prostatic pathology. PSA is present in the circulation both in free form and conjugated to enzyme inhibitors, such as antichimotripsin and α-2-macroglobulin. Free PSA is made up of a mixture of different molecules, which include BPSA, iPSA and proPSA. BPSA and iPSA would be predominantly expressed by benign prostate tissue, while proPSA is most frequently associated with

prostate cancer [58]. Three truncated forms of proPSA have been identified and studied, [−2] proPSA, [−4] proPSA and [−5, −7] proPSA [58]. Among these, [−2] proPSA represents the form more stable and has been extensively studied both as an individual test and combined in algorithms with total PSA and free PSA [59]. The result of [−2] proPSA can be expressed both as a percentage with respect to the free PSA, and through an index called Prostate Health Index (phi), calculated using an algorithm that also includes total PSA and free PSA [phi = ([-2] proPSA / free PSA) x (square root of the total PSA)] [60].

PSA can be elevated in circulation not only in the presence of malignant prostate disease, but also in physiological conditions (e.g. recent ejaculation, intense physical activity), in the case of benign pathology (e.g. prostatic hypertrophy, prostatitis, prostatic infarction, urinary retention), as well as after performing some diagnostic maneuvers, such as cystoscopy or prostatic biopsy (in the latter case, increments up to 50 times are described, with a return to pre-biopsy values even in 30-60 days). The effect of DRE seems limited and mostly restricted to cases with PSA > 10 ng/mL; however, when it is intended to evaluate the changes in PSA induced by a given treatment, it is recommended to take the PSA sample before DRE, or at least 24 hours after the maneuver.

Conversely, PSA levels may decrease in circulation following the use of 5-α-reductase inhibitors (finasteride, dutasteride). In the case of finasteride an

average decrease is reported around 50% after about 6 months of treatment, so that the rule of multiplying the PSA value by 2 to find out what would be the level of the biomarker in the absence of treatment was proposed. This approach (the multiplication rule for 2) is strongly discouraging, as extensive individual variations in the effect of finasteride on PSA have been described [61–64]. Even in the case of dutasteride significant reductions in circulating PSA are described (around 40-60% of the baseline value after at least 6 months of treatment). Preliminary data show that dutasteride would have a greater effect than finasteride in reducing PSA [65]. Other studies show that the administration of dutasteride does not reduce the diagnostic value of an increase in PSA, even in patients monitored after a first negative biopsy [66].

PSA is generally evaluated with reference to a positive/negative threshold value (cut-off) calculated on the basis of the distribution of the marker in normal subjects. The traditionally used threshold value of 4 ng/mL must be considered conventional and is characterized by a low predictive value, both positive and negative. In fact, there is a large overlap between subjects with neoplasia confined to the organ and subjects with prostatic hypertrophy, which often present values between 4 and 10 ng/mL. By contrast, approximately 20% of patients with cancer confined to the organ have PSA values below 3 ng/mL [67,68].

Most of the guidelines [27] agree on the following points: the PSA cannot be considered the only criterion for deciding whether or not to do the biopsy; the choice whether to perform the biopsy must be based on the clinical suspicion obtained from DRE and/or PSA integrated with additional clinical information and with the assessment of any risk factors; a single high PSA value should not lead to clinical decisions immediately, but should be confirmed by a new detection after a few weeks.

Among the approaches used to improve the diagnostic accuracy of PSA are PSA velocity, PSA density (PSAD) and the free/total PSA ratio, conventionally called "PSA derivatives".

PSA velocity expresses the rate of PSA increase in time [69] and would have the ability to predict the onset of cancer with significant diagnostic advance compared to exceeding the threshold value [70] and would also be a potential indicator of aggressiveness of the neoplasm [71]. However, other lines of evidence confirm neither the diagnostic value nor the prognostic significance of PSA velocity [72,73]. In any case, PSA velocity should not be considered in cases with PSA values < 2.0 or > 10.0 ng/mL.

PSAD expresses the relationship between circulating PSA and gland size measured on ultrasound scans and is based on the observation that the amount of PSA produced and released in circulation per gram of glandular tissue is much higher in cancer than in prostatic hypertrophy. Numerous pieces of

evidence show that PSAD has better diagnostic accuracy than total PSA and has similar performance to the free/total PSA ratio in cases with total PSA between 4 and 10 ng/mL. It should be emphasized that in patients with low total PSA (e.g. between 2 and 4 ng/mL), in which the determination of free PSA can be less accurate, PSAD has a better diagnostic accuracy than the free/total PSA ratio [62,74,75].

The position of the guidelines on the use of the free PSA/total [27] ratio can be summarized in the following key points: the free/total PSA ratio can be taken into consideration in cases where the probability of neoplasia is better defined before the initial biopsy, but the evidence is still insufficient to formulate specific clinical practice recommendations; the determination of free PSA should however be limited to cases with total PSA between 2 and 10 ng/mL; the free/total PSA ratio has value only in the diagnostic phase and is not applied in staging, in follow-up after primary therapy and in monitoring therapy for advanced disease.

Numerous markers have been identified with the aim of improving the diagnostic accuracy of PSA and derivatives; some of them belong to the kallikrein family ([−2] proPSA, 4Kscore), others are represented by molecular alterations (Prostate Cancer Antigen 3, TMPRSS: ERG) [76,77].

In the last two decades, several PSA isoforms have been studied, some of which would show a high specificity for prostate cancer. Among them the most

widely evaluated is [−2] proPSA [78]. The result of [−2] proPSA is commonly expressed through an index called Prostate Health Index (phi), calculated using an algorithm that includes total PSA and free PSA [60]. Overall, the results of the various studies on [−2] proPSA, suggest that [−2] proPSA, and in particular phi, have a better diagnostic performance in cases with PSA between 2 and 10 ng/mL compared to derivatives of PSA and the free/total PSA ratio [60,79,80]. Furthermore, some studies have shown an association between [−2] proPSA and the aggressiveness of the disease [80].

In the case of free PSA and [−2] proPSA, the ratio of the different PSA isoforms in the blood was more informative than the concentration of the single isoform.

4Kscore is a further approach to combine the information provided by molecules connected with the regulation of circulating PSA isoforms. The 4Kscore combines, in a dedicated algorithm, the values of a kallikrein battery (Total PSA, Free PSA, iPSA and human kallikrein 2, a glycoprotein with strong homology to PSA) with clinical data (age, rectal exploration, biopsy results previous one). Several clinical studies have shown that 4Kscore would have excellent ability to predict the presence of a high-grade neoplasm [81–83] and the risk of metastatic progression [84].

PCA3 (also known as DD3) is a gene located on chromosome 9q21–22 [85]. The non-coding PCA3 mRNA is overexpressed in 95% of prostatic tumors,

while a low level of expression has been described in normal prostate tissue and benign prostate hypertrophy. PCA3 is measurable in urine with a standardized commercial method. The result is expressed as PCA3 score, which links PCA3 with PSA mRNA in urine samples [86]. PCA3 was approved by the FDA in 2012 as a support to decide whether to repeat a biopsy in men with a previous negative biopsy. According to the most recent studies, PCA3 has a sensitivity and specificity higher than PSA and the free/total PSA ratio. However, the threshold value that should be used it is still not clear. With a cut-off of 35, a risk of missing the diagnosis of 26% of aggressive neoplasms is described, while false positive results seem to remain a consistent problem even when using cut-offs of 100 [25,87,88].

Gene rearrangement phenomena have been described in numerous neoplasms, including prostate cancer. The most commonly identified alterations in the genome of prostate cancer are fusions of androgen-regulated promoters with the ERG (ETS-related gene) or other members of the ETS (E26 transformation-specific) transcription factor family [89]. Among these, the most frequently identified alteration in prostate cancer is TMPRSS2: ERG, a fusion between ERG and the TMPRSS2 gene, which codes for a transmembrane protease (serine 2) [90]. The TMPRSS2: ERG rearrangement can be measured in urine after prostate massage and is expressed in relation to the PSA mRNA in the sample, used as a normalization criterion. Some studies

have evaluated the diagnostic accuracy of TMPRSS2: ERG in comparison or in association with the PCA3 score, showing that the inclusion of molecular markers improves the predictive ability for biopsy positivity and for the aggressiveness of the neoplasm compared to the "risk calculator ”developed by the ERSPC [90,91].

The position of the guidelines towards these new markers is still cautious [27,28,91,92]. The guidelines acknowledge that today there are several additional tests (phi, 4Kscore, PCA3, TMPRSS2: ERG) in addition to PSA that have shown sensitivity and specificity superior to PSA and are potentially useful to avoid unnecessary biopsies and generically indicate that these tests could be taken into consideration in cases where it is better to define the probability of neoplasia before the initial biopsy or after a first negative biopsy. Moreover, according to the guidelines the evidence is still insufficient to make specific clinical practice recommendations.

Treatment of prostate cancer

Generalities

The treatment of prostate cancer pursues different goals, depending on the anatomical extension and aggressiveness of the disease, but also on patient's life expectancy and on the presence of comorbidities that may represent a risk of death greater than that represented by the same prostatic neoplasm. We must

not, indeed, neglect that about 40% of patients diagnosed with prostate cancer is destined to die "with" and not "for" their cancer and that this percentage also includes patients with locally advanced or metastatic disease [93–95].

For this reason, in patients with short life expectancy (generally less than 10 years), due to advanced age or the presence of comorbidity with higher lethality than prostate cancer, a policy of watchful waiting can be indicated. Similarly, patients suffering from a very low-low grade disease, even in the presence of a good life expectancy, can be directed towards an active surveillance policy (active surveillance) [28,96–100].

The concept of watchful waiting differs from that of active surveillance. The watchful waiting policy is, in fact, a policy of surveillance (in the absence, however, of systematic checks) of those patients in whom it is considered reasonable to think that the immediate treatment of the tumor is not able to have an impact on their life expectancy and for whom therefore, any therapies can be delayed until the appearance of symptoms, with almost exclusively palliative purposes. Active surveillance, on the other hand, is a strategy of possible deferred treatment, which is offered to patients with very low-low-risk disease at the diagnosis to whom, instead of immediate treatment, the option of close monitoring is offered, through the periodic repetition of prostatic biopsies, the clinical examinations, PSA monitoring and in some

specific cases, of multiparametric magnetic resonance imaging, in order to promptly detect the eventual progression of the disease.

Patients at intermediate-high or very high risk should undergo to treatments with radical aims, as it is necessary to do everything possible to pursue an adequate control of the disease both locally and at a systemic level, with the use of multimodal treatments, because, if properly treated, they can however benefit from a longer survival.

In patients with metastatic disease, palliation remains the most concretely pursuable objective, especially if symptomatic. For these patients, there are currently various hormonal therapy and chemotherapy options which, together with the latest forms of radiometabolic therapy (alpha emitters) and bone-targeted therapies, can significantly impact both their quality and their life expectancy. Moreover, also in this case, there are cases (biochemical progression after failure of the first and/or second line hormone therapy) in which the absence of symptoms can authorize a conduct of attendance, delaying the possible treatment to the appearance of symptoms, even if some more recent studies suggest that these patients may benefit from immediate treatment [28,101].

The following table (Table 2) shows the risk classes currently used to select the various treatment options:

Risk Classes	Characteristics
Very low	T1c; Gleason score ≤6 / Grade Group 1 sec. ISUP/WHO 2016; PSA <10 ng/ml; less than 3 positive bioptic frustules with ≤50% neoplasia in each frustule; PSA density <0.15 ng/ml/g
Low	T1-T2a; Gleason score ≤6 / Grade Group 1 sec. ISUP/WHO 2016; PSA <10 ng/ml
Intermediate	FAVORABLE: T2b-T2c or Gleason score 3 + 4 = 7 / Grade Group 2 sec. ISUP/WHO 2016 or PSA 10-20 ng/ml. The percentage of positive biopsy frustules is <50% UNFAVORABLE: T2b-T2c or Gleason score 3 + 4 = 7 / Grade Group 2 sec. ISUP/WHO 2016; or Gleason score 4 + 3 = 7/Grade Group 3 sec. ISUP/WHO 2016; or PSA between 10 and 20 ng/ml
High	T3a or Gleason score 8/Grade Group 4 sec. ISUP/WHO 2016; or Gleason score 4 + 5 = 9 / Grade Group 5 sec. ISUP/WHO 2016 or PSA > 20 ng/ml
Very High	T3b-T4; or primary Gleason pattern 5; > 4 biopsies with Gleason score 8-10/Grade Group 4 or 5 sec. ISUP/WHO 2016

Table 2. Risk classes in prostate cancer.

Local treatments with radical aims

Surgery

Radical prostatectomy (PR) refers to the surgical removal of the prostate and seminal vesicles, including surrounding tissue, sufficient to achieve negative surgical margins. The continuity of the urinary excretory pathway is restored with the packaging of a vesicourethral anastomosis. To date, the staging lymphadenectomy, if provided, accompanies the PR intervention.

The stated objective of surgery in organ-confined disease is the eradication of the pathology itself and, when possible, the preservation of continence and erectile function, elements which, however, remain subordinate to the oncological purpose [102,103].

These three objectives, associated with a reduction to the minimum of any postoperative complications and the presence of negative margins, best define the ideal objectives of surgical therapy [104].

PR can be performed with the classic open technique or through minimally invasive techniques: laparoscopic PR and robot-assisted laparoscopic PR. These two techniques have represented a remarkable change in the field of radical prostate surgery in the last two decades. The clinical studies concerning these methods are now extremely numerous and include metanalysis,

randomized studies, studies based on data provided by the tumor registers, prospective monocentric series, multi-institutional series [105–112].

Radiotherapy

Radiation therapy is a therapeutic option for the treatment of localized prostate cancer with the aim of radicality. The most frequently used technique is the external beam technique. However, some patients may benefit from brachytherapy. Unfortunately, the lack of randomized studies with long-term data does not allow to identify patients who should be sent to radiotherapy rather than to other therapeutic alternatives, in particular to RP. The PROTECT study [11,12], showed that in patients at very low, low and intermediate risk, PR, external beam RT and brachytherapy produce 10-year results comparable in terms of overall survival, but not in terms of biochemical relapse and distant relapse of the disease. In some centers, radiation treatment is reserved for older patients, those with co-morbidities that should not undergo to a major surgical procedure, or those that, regardless of age, want to avoid the side effects most frequently caused by surgery (urinary incontinence, erectile dysfunction). In general, brachytherapy is proposed as a therapeutic alternative to radical surgical treatment of low-risk prostate cancer (T1-T2a, Gleason $\leq$ 6 / Group Gleason grade 1, PSA $\leq$ 10 Prognostic). The clinical results related to the biochemical relapse period, in retrospective mono-institutional studies, are equal to 70-82% at 5 years. These values are similar to those of surgery and

external beam radiotherapy [113,114]. There are no randomized trials demonstrating a difference in local control between brachytherapy, surgery and external radiotherapy and unfortunately the ACOSOG-Z007 [115] study, started in October 2001, in which patients were randomized to brachytherapy or surgery, was closed in 2009 for lack of recruitment.

Multimodal treatments

Radiotherapy and hormone therapy

Three types of studies have taken into consideration the potential superiority of a combined treatment with radiotherapy and hormone therapy in patients with locally advanced disease (cT3-cT4/cN1), or at least at high or very high risk. These include: studies in which hormonal therapy was used concurrently and subsequently to radiotherapy; studies in which hormonal therapy was started for neo-adjuvant purpose before radiotherapy and continued until the end of radiotherapy itself (in both cases the therapy associated with RT and hormone therapy was compared with exclusive RT); and studies in which the combination of radiotherapy and hormone therapy was compared with hormone therapy alone.

The results of these studies [116–123] show a substantial advantage of the multimodal approach compared to radiotherapy alone or hormone therapy

alone, thus highlighting the importance of associating adequate local systemic control with the disease and vice versa.

Radical surgery and hormone therapy

The efficacy of hormonal treatment in association with surgery was evaluated both in terms of neoadjuvant and adjuvant treatment. At the current state of knowledge, there is no indication for the use of endocrine therapy with neoadjuvant intent, as none of the studies performed to answer this clinical question has shown a statistically significant impact on survival by this therapeutic strategy [124–127].

As for the use of hormone therapy in the adjuvant phase, after surgery, there is the recommendation to use the androgen-deprivative therapy for adjuvant purposes, in patients at risk for the presence at histological examination of metastases in the regional lymph nodes [123,128,129].

Adjuvant radiotherapy after radical surgery

Regardless of the starting stage, a non-negligible percentage of patients subjected to PR, at the definitive histological examination, shows unfavorable prognostic characteristics and in any case potential indicators of ineffective radical surgery. Among them: a) capsular penetration, provided it is complete and extensive; b) infiltration of seminal vesicles; c) the positive surgical margins. In these patients, when the persistence of disease at the biochemical

level and the presence of clinically evident pelvic and/or distant metastases can be ruled out in a reasonable way, it is still questioned which is the role of a radiation therapy of the prostatic loggia, extended or not to the pelvis, and in particular what benefits can be obtained from the adjuvant use of radiation therapy. Pending the results of the phase III Adjuvant Versus Early Salvage (RAVES) study, which intends to demonstrate that early rescue RT is not inferior to the adjuvant RT for pT3 patients with or without positive surgical margins [130], it can therefore be concluded that the irradiation of the prostatic loggia for adjuvant purposes may be considered in patients with pathological stage pT3pN0M0 and/or with positive margins, although not all studies agree on the possible benefit in terms of OS.

Clinically detectable recurrent disease

To date, besides some limited cases that have been sent for surgical or radiotherapeutic procedures in the presence of limited clinical recurrence of disease, there is no certain evidence to suggest, as a clinical standard, the opportunity for local treatment (radiant or surgical). Patients suffering from relapse at the loggia and/or lymph node level of the disease, even if limited to the lymph nodes of the small pelvis, should therefore be initiated, at least in the first instance, at hormonal therapy [131,132]. Local treatment of these patients with definitive surgery or radiotherapy should be performed only in selected cases, better in the context of controlled clinical trials.

Metastatic disease

In accordance with the most recent version of the TNM classification, within the M1 category we must also consider patients with juxta-regional lymph node involvement (common iliac, para-aortic, inguinal), whose clinical course, unlike what was considered in the past, it is to be considered comparable to that of patients with disseminated disease. In principle, M1a patients should be treated like other metastatic patients or endocrine therapy. However, even in the absence of prospective studies, retrospective analyzes indicate that, in selected cases, which have responded to first-line endocrine therapy, external RT can be used, possibly with "boost" on the sites of illness, in analogy to what happens for cN1 extra-prostate tumors [133]. With regard to patients with metastatic disease the choice of treatments, their sequence, the possible association of bone-targeting therapies, the possible palliative radiotherapy of bone metastases and, more general, supportive therapies, must take into account the age of the patient, his co-morbidities, the presence or absence of symptoms, the overall clinical picture, as well as the expectations of the patient and family members.

Chemotherapy

Prostate cancer has been considered a not chemo-sensitive tumor for a long time. However, in the early 2000s, some controlled studies demonstrated the efficacy of Docetaxel in patients suffering from castration-resistant disease.

Recently the efficacy of Docetaxel has also been tested in "hormone-naive" disease in combination with androgen-suppressive therapy.

The recent results of the CHAARTED [134] and STAMPEDE [135] studies provide solid support for the possibility of improving the survival of metastatic hormone-naive patients at diagnosis by adding docetaxel to androgen-suppressive therapy within 12 weeks.

However, at the moment, it remains to be clarified which factors can identify patients destined to benefit most from this therapeutic strategy compared to androgen-suppressive therapy alone, also considering the greater incidence of side effects related to the use of chemotherapy compared to the androgen deprivation therapy alone.

Treatment of bone metastases

Bone tissue is the predominant, and sometimes exclusive, site of secondary localizations due to prostatic neoplasia [136]. The specific treatment of bone metastases is aimed at controlling pain and preventing or delaying skeletal complications over time. Current possibilities include the use of selective treatments (external radiotherapy, metabolic radiotherapy) and that of "osteoprotective" drugs (dysphosphonates, RANKL inhibitors).

Lipocalin family

Lipocalins belong to a family of secreted proteins that work as carriers, mostly carrying small lipophilic molecules. Recently, additional roles for these proteins have been characterized, e.g. regulation of cell division, cell survival, cell differentiation and cell-cell adhesion [137]. The members of this family can share as low as 20% in amino-acid similarity, although they show a common secondary and tertiary structural domain, known as "lipocalin fold". The lipocalin fold is made of an antiparallel beta barrel structure, comprising eight beta sheets, linked by hydrogen bonds, and giving rise to a cup-shaped cavity that binds to specific ligands. The beta sheets are connected to one another by seven short loops (L1–L7), with loop L1 forming a lid-like structure that closes the ligand-binding pocket. The differences in the primary structure allows to bind a wide variety of ligands by the members of this protein family. Lipocalins share three regions with sequence and structural conservation, known as SCRs (structurally conserved regions), that are used to classify these proteins into two groups: the kernel and the outlier lipocalins. While the kernel lipocalins have all three SCRs, the outliers have only one or two, but never all three SCRs [137].

NGAL

NGAL, also known as neutrophil glucosaminidase-associated lipocalin, 24p3, oncogene 24p3, p25, migration stimulating factor inhibitor (MSFI), human

neutrophil lipocalin (HNL), α1-microglobulin related protein, siderocalin, or uterocalin, is a 198 amino acid long glycoprotein encoded by the LCN2 gene, located at 9q34.11. The NGAL gene is made up of seven exons that encode for at least five isoforms. The three-dimensional structure of human NGAL contains an N-terminal 3_{10}-helix, followed by eight antiparallel beta strands, an alpha helix and a C-terminal beta strand. The beta strands form a barrel like structure. A negatively charged patch is present in a pit-like region at the floor of the barrel, near to an unpaired cysteine residue, that forms a disulfide bond with the gelatinase MMP-9. The cavity in NGAL, differently from other lipocalins, is significantly polar and big enough to accommodate macromolecular ligands, including proteins [137]. In particular, NGAL interacts with bacterial proteins, called siderophores, that bind to circulating and intracellular free iron and are essential for the survival of many microorganisms. The ability of NGAL to act as a siderophore-binding protein mediates its physiologic role as a key player of the innate immune system. Indeed, NGAL is normally synthesized as a component of the late granules of neutrophils. In particular, it is located in the azurophilic granules, where it co-localized with myeloperoxidase [138].

In the mouse, Lcn2 is strongly expressed in the bone marrow, and at a lower level, in the spleen, lung and granulocytes; while no expression has been found in the liver, heart, kidney, small bowel or thymus. Interestingly, Lcn2 mRNA

expression in adult mice progressively declines with advancing age, particularly in the liver, kidney and the spleen, disappearing by the time the mice are about 2.5 months old [139]. In accordance to the data on mouse Lcn2, higher protein expression of NGAL is described in The Human Protein Database in adult bone marrow and uterine cervix, followed by bronchus nasopharynx, spleen and stomach, and then in salivary glands and skin [137].

NGAL in cancer

Increasing body of data support the notion that NGAL exerts a key role in several cancer types, including breast [140–142], colorectal [132,133], endometrial [145], esophageal [146–148], gastric [149], liver [150,151], lung [152–154], ovarian [155], pancreatic [156,157], prostate [158,159], renal [160] and thyroid cancer [161].

The dysregulated expression of NGAL observed in human tumors suggests that it may be a valuable clinical marker and molecular target for treatment in cancer.

High NGAL levels have indeed been found in the urine and plasma of patients with different cancers, including brain, breast, colon, ovarian, pancreatic and prostate cancer [162]. Also, NGAL expression was associated with a worse prognosis and overall survival in lung adenocarcinoma [154], in endometrial cancer [145] and in colorectal cancer (CRC) [143] patients. It should however

be noted that, although serum NGAL levels are augmented in patients with CRC cancer [144,163], it may not represent a suitable diagnostic biomarker [139], but it may instead be useful in metastatic patients [163,164].

On the other hand, in a few cases, such as pancreatic and thyroid cancer, NGAL may be associated with a better outcome [156,161]. Along the same lines, one study has shown that NGAL is reduced in primary and metastatic oral cancer samples [165].

Hence, it is of the utmost importance to discriminate the physiological role of NGAL in normal human tissue and to understand its pathological role in the initiation and progression of tumors.

In a recent meta-analysis by Roli and colleagues, it has been shown that high NGAL levels in biological fluids, such as serum and urine, are useful to predict disease-free survival in patients with CRC and breast cancer, while its prognostic and diagnostic accuracy remains debatable in pancreatic, thyroid, liver, lung, esophageal, oral, and kidney tumors [166].

The elevated levels of NGAL in most cancer types seems to be determined by multiple factors. The nuclear factor-kappaB (NF-κB) signaling pathway, which is usually activated in cancer, modulates the transcription of NGAL and, along with the MAPK pathway, may contribute to the overexpression of NGAL [137]. Moreover, several stimuli from the tumor microenvironment, such as hypoxia and inflammatory cytokines [167], and epigenetic

modifications, might be important in sustaining NGAL expression by tumor cells.

Overexpression of NGAL was demonstrated to promote migration, invasion and lung metastasis in preclinical *in vitro* and *in vivo* studies. Indeed, the invasiveness of some cancer cells is reduced upon inhibition of NGAL expression and it is increased following NGAL over-expression [168].

It has also been shown that radiation and chemotherapy may increase NGAL expression [169]. However, the role of NGAL in chemoresistance needs to be better deciphered. Moreover, elevated levels of NGAL have been associated to radiation resistance, in oral cancer and lung cancer. This observation suggests the possibility of using NGAL as a potential biomarker for the prediction of radioresistance [152].

NGAL also forms a 46 kDa disulfide-linked homodimer a 130 kDa heterodimer bound to the inactive form of the matrix metalloproteinase-9 (proMMP-9). The latter complex has been described as marker of disease in several solid tumors [170–175], since it is associated with the aggressiveness of gastric [176], anaplastic thyroid [174], breast [170], kidney [173], and oral cancer cells [175]. Also, the NGAL complex has been proposed for its potential use be useful in the assessment of tumor stage in endometrial cancer [177]. Finally, the NGAL-MMP-9 complex can be detected in the urine of many cancer patients and seems to be correlated with metastasis [178]. However,

another report by Muñoz et al. has shown that no differences in NGAL/MMP-9 complex activities (gelatin substrate) in men with detected prostate cancer [179].

NGAL in prostate cancer

A growing body of evidences suggests that NGAL could be a novel promising diagnostic and prognostic biomarker and therapeutic target for prostate cancer.

Rahimi et al. have demonstrated that knock out of NGAL, in the invasive prostate cancer cell line PC3, was associated with reduced cell proliferation and cell migration and with enhanced cell sensitivity to the combination therapy with cisplatin (CDDP) [180].

Muşlu et al. have investigated the possibility to use serum NGAL as a biomarker for prostate cancer diagnosis. In particular, they compared serum PSA and NGAL levels in prostate cancer patients and in benign prostatic hyperplasia patients. They found that serum NGAL concentrations have higher specificity than serum PSA levels as a screening test to distinguish prostate cancer from benign prostatic hyperplasia [181].

Moreover, in castration-resistant prostate cancer, NGAL promoted cell proliferation by modulating the androgen receptor (AR) transcriptional activity [149].

We have previously shown that NGAL and MMP-9 and NGAL/MMP-9 complexes are detectable in the urine of prostate cancer patients. Moreover, we have evaluated the effects of p53, NF-κB and the AR on NGAL expression in four prostate cancer cell lines (LNCaP, 22Rv-1, DU145 and PC3 cells). We found that NGAL is expressed at higher levels in AR negative prostate cancer cells, either expressing the mutant or lacking p53 (DU145 and PC3) as compared to the AR positive prostate cancer cells (LNCaP and 22Rv-1) that instead express wild type (WT) p53. While NGAL expression was reduced following the introduction of WT-p53 in PC3 cells, it was increased after the introduction of dominant negative (DN) p53 or a retroviral construct expressing NF-κB into LNCaP cells. Furthermore, NGAL expression promotes the ability of prostate cancer cells to form colonies in soft agar. These data suggest that p53 and NF-κB are involved in the modulation of NGAL expression through negative and positive mechanisms, respectively (Figure 1) [178].

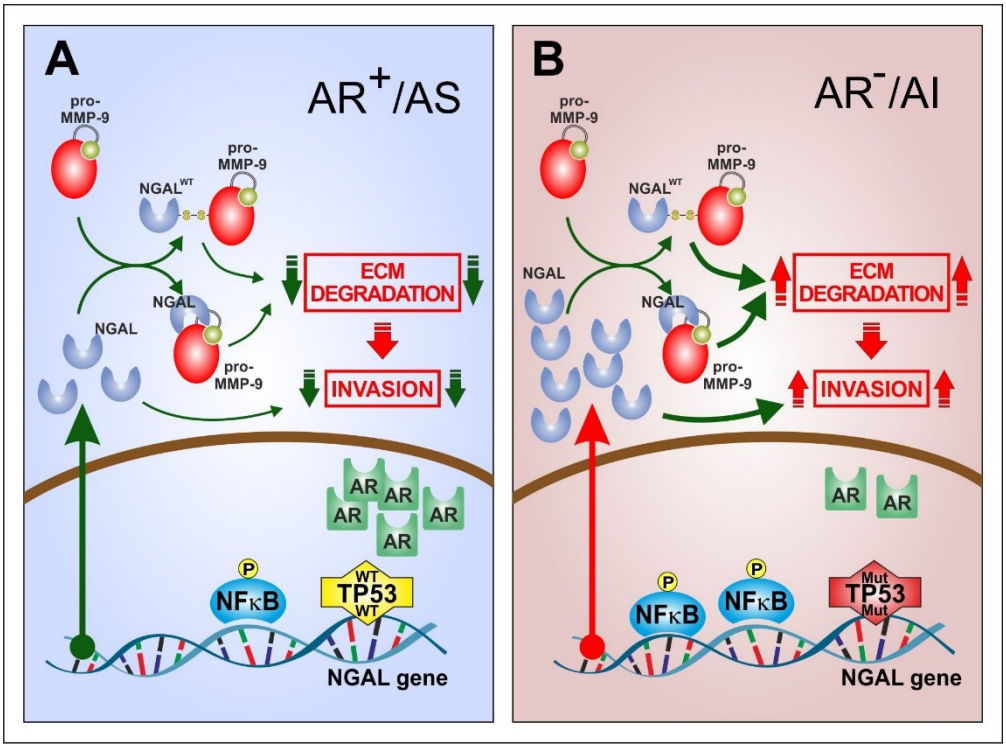

Figure 1. Effects of AR, p53 and NF-κB on NGAL expression.

Finally, another study has shown that NGAL promotes EMT in the prostate cancer cell line, 22RV-1, via activation of ERK signaling that, in turn, leads to increased SLUG activation [182].

AIM OF THE STUDY

It is known that NGAL positively regulates the activity of MMP-9 through a protein-protein interaction, thus resulting in the formation of the NGAL/MMP-9 complex.

MMP-9 belongs to the family of endoproteinases with iron-, zinc-, and calcium-dependent activity. Metalloproteinases are involved in the degradation of the extracellular matrix (ECM) and basement membrane, promoting cancer cells infiltration and metastatization.

It has been found that, by binding NGAL, MMP-9 is protected from degradation. This complex is stabilized by a disulfide bond between the Cysteine 107 (Cys107) of NGAL and one of the available cysteines of MMP-9. The NGAL/MMP-9 complex is detectable in the plasma and urine of patients with several types of tumors, such as gliomas, cholangiocarcinoma, breast cancer and prostate cancer [170,173,183–185].

Our previous study [178] has shown that the TP53 mutation and NF-kB over-expression lead to NGAL over-expression in advanced prostate cancer cells. In particular, while NGAL over-expression in prostate cancer cells, that express low NGAL basal levels, enhanced tumor cell growth, NGAL silencing in cells that over-express NGAL, resulted in a lower growth rate.

Since NGAL is involved in prostate cancer growth and progression, targeting the interaction between NGAL and MMP-9 may result in a novel approach to reduce growth, invasion and aggressiveness of prostate and many other cancer types. These results prompted us to investigate the functional role of NGAL in cancer and in particular the interaction mechanisms between NGAL and MMP-

9, which are responsible for the enhancement of MMP-9 proteolytic activity and may be involved in the aggressiveness of cancer cells.

In the present study, we have investigated the functional effects of the NGALC107G mutation, which affect the stability of the NGAL/MMP-9 complex, in A375 cells, as they represent a good model of invasive cells, characterized by high transient transfection rate. The results from this study set the basis for the future investigation of therapeutic strategies targeting either NGAL, MMP-9, or their interaction, in order to ameliorate the clinical course of cancer patients, and to increase their life expectancy, possibly by improving the success rate of concomitant Standard of Care treatments and overcoming drug resistance.

MATERIALS AND METHODS

Cell Cultures

A375 melanoma cell line was used as a good model of invasive cells and suitable for double transfection protocol. These cells were obtained from the American Type Culture Collection (ATCC - Rockville, MD, USA). Cells were cultured in RPMI-1640 medium supplemented with L-glutamine (2 mmol/L), penicillin (100 IU), streptomycin (100 µg/ml) and 10% fetal bovine serum (FBS) (all provided from Corning Incorporated, NY, USA) and grown in humidified incubator (5% CO_2) at 37°C. The different transfected clones were

seeded in triplicate in 100 mm cell-culture dishes (Thermo Fisher Scientific Inc., Waltham, MA, USA) at a density of 1 x 10^{6} cells until reaching an 80% confluence. From each cell culture, cell supernatants were collected and centrifuged at 1200 rpm for 5 minutes in order to remove cell debris. In parallel, after one wash with DPBS 1X (Corning Incorporated, NY, USA), cells were collected by scraping and cell pellets were obtained by centrifugation at 1200 rpm for 5 minutes. All the obtained samples were stored at -80°C until use.

NGAL and MMP-9 cell transfections

Plasmid constructs and modification of NGAL Cystein 107

In order to evaluate the role of NGAL wild-type and NGAL mutated (p.C107G $NGAL^{C107G}$) in activating MMP-9, the A375 cell line was transfected with 3 different plasmid vectors in different combinations to induce the over-expression of MMP-9, $NGAL^{WT}$ and $NGAL^{C107G}$, as showed in Figure 2.

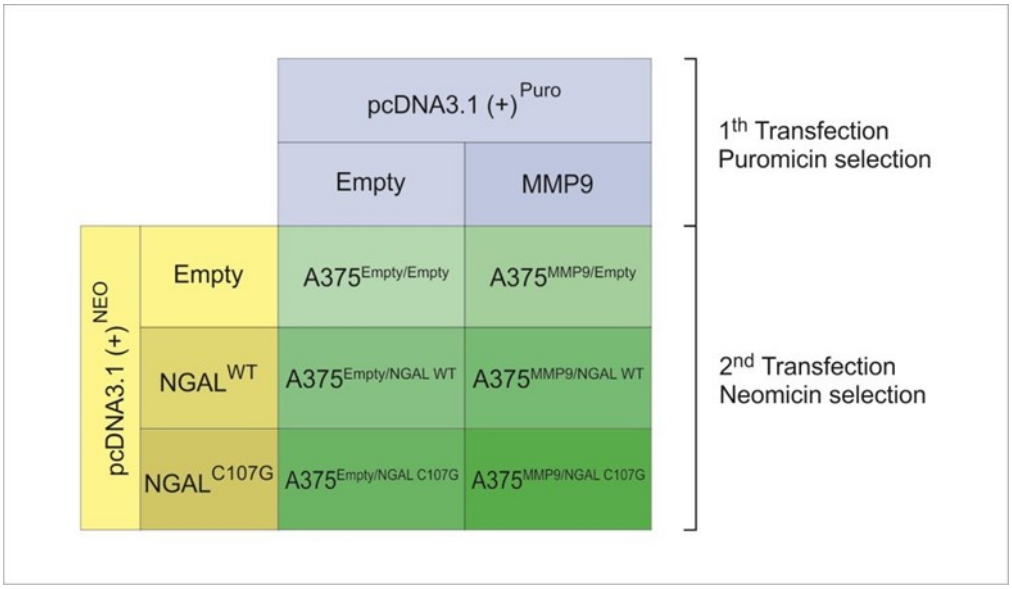

Figure 2. Double transfection protocol.

The NGAL^{C107G} variant was obtained by introducing with PCR the 319 T>G point mutation in the TGC codon (Cystein) in order to obtain the GGC codon (Glycin).

For the realization of MMP-9 over-expressing plasmid, the MMP-9 coding sequence was obtained by PCR using the MMP-9 cDNA of normal fibroblast. The coding sequence was amplified by using Phusion DNA Polymerases (Thermo Fisher, Waltham, Massachusetts, USA) and the specific primers containing EcoRI restriction sites (Table 3). The MMP-9 PCR product was subsequently cloned in the pcDNA3.1 (+) PURO plasmid vector, by using EcoRI restriction enzyme. The cloned sequence was verified through Sanger sequencing.

The NGAL^{WT} over-expressing plasmid was kindly provided by Prof. James A. McCubrey of the East Carolina University (North Carolina, USA).

The mutant NGAL^{C107G} variant was obtained through site-specific mutagenesis via PCR performed on pcDNA3.1 NGAL^{WT} plasmid using mutated oligonucleotides (Table 3). The mutated amplicon obtained, containing at the 5' and 3' ends the restriction sites for XbaI, was subsequently cloned into the pcDNA3.1 (+) NEO vector.

MMP-9 CDS		
MMP-9 CDS FW	5'-gtaggaattc**ATG**AGCCTCTGGCAGCCCCTGGT-3'	98°C for 30 s, followed by forty cycles of 98°C for 10 s, 72°C for 32 s and 72°C for 10 min
MMP-9 CDS Rev	5'-catcgaattc**CTA**GTCCTCAGGGCACTGCAGGA-3'	
puroR cloning		
Puro XmaI Fw	5'-agctcccgggATGACCGAGTACAAGCCCACGGT-3'	98°C for 30 sec,

Puro BstBI Rev	5’- tcatttcgaaTCAGGCACCGGGCTTGCGGGTCA-3’	followed by forty cycles of 98°C for 10 s, 72°C for 15 s and 72°C for 10 min
NGAL site specific mutation		
NGAL GLY_CYS FW	5’-GTTCCAGGTGGCCAGCCCGGCGAG-3’	98°C for 30 s, followed by forty cycles of 98°C for 10 s, 72°C for 15 s and 72°C for 10 min
NGAL GLY_CYS Rev	5’-CTCGCCGGGCTGGCCACCTGGAAC-3’	
MMP-9 expression		
MMP-9 FW	5’-GAACCAATCTCACCGACAGG-3’	

MMP-9 Rev	5’-CCACAACTCGTCATCGTCG-3’	94°C for 10 min, followed by forty cycles of 94°C for 15 s, 64°C for 40 s and 72°C for 1 min.
PGK1 expression		
PGK-1 FW	5’-TTAAAGGGAAGCGGGTCGTT-3’	94°C for 10 min, followed by forty cycles of 94°C for 15 s, 64°C for 40 s and 72°C for 1 min.
PGK-1 Rev	5’-CAGGCATGGGCACACCAT-3’	

Table 3. Primers and PCR thermal conditions.

Cell transfections with plasmid vectors

The aforementioned plasmid vectors were used for the transfection of cells by using Lipofectamine 2000 (Thermo Fisher, Waltham, Massachusetts, USA) following the manufacturer's instructions. Briefly, cells were seeded in 6-wells culture plates at the density of 2.5 x 10^{5} cells in serum-free medium. After reaching a confluence of 70-80% the cells were transfected with the mixture of Lipofectamine 2000 (50 µL) and plasmid DNA adequately diluted in OptiMEM I medium (50 µL), previously obtained and incubated for 5 minutes at room temperature. After the addition of the transfection mix, the cells were incubated at 37 °C in a CO_2 incubator for 24 hours, after which clonal selection was made with puromycin (1 µg/mL) and neomycin (600 µg/mL). After 6 days of selection, the cells were removed and re-seeded to evaluate the efficiency of transfection by Western blot.

MTT Assay

The 3-(4,5-dimetiltiazol-2-yl)-2,5-diphenyltetrazolium (MTT, Sigma-Aldrich) assay was used to evaluate cell proliferation at 24 hours, 48 hours and 72 hours time intervals, for cell clones transfected with the different over-expressing plasmid vectors $NGAL^{WT/C107G}$ and/or MMP-9. The cells were seeded in 96 multiwell plates at a concentration of 3000 cells/well. After 24, 48, 72 hours, a solution of RPMI 1640 + MTT 1:10 (0.5 ug/mL) was added to each well. The plate was incubated at 37 °C for 4 hours. After this period, insoluble formazan

crystals were dissolved by adding 100 µL of a stop C solution consisting of isopropanol and HCl (50 mL + 167 µL) into each well, pipetting up and down vigorously. The absorbance of each well was measured at 620 nm using the Tecan Sunrise ELISA plate reader to obtain the values relative to the optical density of the wells (OD). Cell viability was then expressed as a percentage of the growth time between the sowing of the cells and the proliferation rate.

Western Blot

To assess the efficacy of cell transfections the supernatant and cell protein levels of MMP-9 and NGAL were detected by using Western Blot, as described in a previous study [178]. Briefly, proteins were extracted by using the lysis buffer nonidet-P40 (NP40) (150 mM NaCl, 1.0% NP40, pH 8.0 50 mM Tris) plus proteases and phosphatases inhibitors (Roche Diagnostics, Indianapolis, IN). After centrifugation, supernatant and cell proteins were dosed through Quick StartTM Bradford 1X Dye Reagent (Biorad, Hercules, California, USA). Optical density (OD) was measured by Tecan ELISA plate reader (Tecan, Männedorf, Switzerland) at 465 nm and 595 nm.

Subsequently, 30 µg of protein were separated by using Mini Protean TGX precast 4–15% gels (cat. n. 4561083 - Bio-Rad Laboratories, Inc., Hercules, California, USA) at 125V for 1 hour and then transferred in a nitrocellulose membrane with the Trans Blot Turbo system (Bio-Rad Laboratories, Inc., Hercules, California, USA). The nitrocellulose membrane was subsequently

incubated for one hour with skimmed milk at 5% in TBS-T (0.1% Tween 20, 20 mM Tris-HCl pH 7.6, 137 mM NaCl). Then the membrane was incubated overnight with the primary polyclonal antibodies NGAL (H 130) (sc50350 - Santa Cruz, Dallas, Texas, USA) and MMP-9 Antibody (# 3852 - Cell Signaling, Danvers, Massachusetts, USA) diluted 1:1000. After three washes in TBS-T the membrane was incubated for one hour at room temperature with a secondary anti-rabbit antibody, conjugated with HRP horseradish peroxidase, diluted 1:10000 in 5% skimmed-milk solution in TBS-T. Finally, the detection step was carried out using the Clarity Western ECL Substrate (cat. No. 1705060 - Bio-Rad Laboratories, Inc., Hercules, California, USA) and the images were acquired through Chemidoc Touch Imaging System (Bio-Rad Laboratories, Inc., Hercules, California, USA) and analyzed by the software ImageJ (National Institutes of Health, Bethesda, MD, USA). All Western blot experiments were performed in triplicate.

Real-Time PCR

Total RNA was extracted from cell pellets using the PureLink RNA minikit (Ambion/Life Technologies, Carlsbad, California, USA) following the manufacturer's protocol. For each sample, 1 μg of total RNA was retro-transcribed using the SuperScript™ III Reverse Transcriptase kit (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). Real-Time PCR was performed using the AB 7300 system (Applied Biosystems, Foster City,

California, USA). The amplification was performed using Fast SYBR Green Master Mix (Applied Biosystems, Foster City, California, USA) based on the following conditions: initial denaturation at 95 °C for 10 minutes, denaturation at 95 °C for 15 seconds elongation/annealing at 60 °C for 1 minute (40 cycles). The relative expression of the MMP-9 transcript was performed using the $2^{-\Delta\Delta Ct}$ method. The following primers were used to detect the transcript of MMP-9: Forward 5'-GAACCAATCTCACCGACAGG-3', Reverse 5'-CCACAACTCGTCATCGTCG-3'.

For the normalization of the data the housekeeping gene phosphoglycerate kinase 1 (PGK1) was used as internal control; the primer sequences for PGK1 were as follows: Forward 5'TTAAAGGGAAGCGGGTCGTT-3';

Reverse 5'CAGGCATGGGCACACCAT-3'.

Invasion/migration assay

The invasive activity of cells transfected with the different plasmids were evaluated using a polycarbonate membrane with an 8-µm pore size (Corning, MA, USA) to detect cell migration. Before the seeding of transfected cells, the membrane was coated with ECM gel from Engelbreth-Holm-Swarm murine sarcoma (cat. no. E6909 - Sigma-Aldrich, Saint Louis, Missouri, USA) diluted 1:10 in serum-free RPMI 1640. After 24 hours of ECM polymerization, cells (2.5×10^{4}) were seeded with 100 µl of serum-free medium into the upper

chamber previously placed to the lower chambers of a 24-well culture plate containing 600 μl of RPMI-1640 that contained 10% FBS, defined chemo-attractive medium. After 18 hours, the cells that invaded the polycarbonate membranes were fixed with 3.7% paraformaldehyde in PBS for 2 minutes. After two washes in PBS, the cells were further fixed in methanol 100% for 20 minutes at room temperature in order to permeabilize the cell membrane for the subsequently staining. After the incubation and two PBS washes, the cells were stained with GIEMSEA diluted 1:20 in ddH2O for 15 minutes at room temperature. After the staining, the membranes were washed again and gently scraped with a cotton swab to remove the residual ECM gel. The number of migrated cells were counted by using NIKON ECLIPSE Ts2 FL microscope.

Statistical Analysis

The statistical significance of the mRNA and protein levels of NGAL, MMP-9 and NGAL/MMP-9 complex was assessed by using two-tailed Student's *t*-test. Student's *t*-test was used also for the statistical analyses of proliferation assay and invasion assay. The results were considered statistically significant when $p \leq 0.05$ (two-tailed).

RESULTS

After the transfection of cells with the three different plasmid vectors, MMP-9, NGAL^{WT} and NGAL^{C107G} (Figure 2), the NGAL and MMP-9 over-expression in transfected clones was confirmed through Real-Time PCR and Western Blot analyses. In particular, the supernatant levels of MMP-9 were significantly increased in MMP-9/NGAL^{Empty}, MMP-9/NGAL^{WT} and MMP-9/NGAL^{C107G} cells. In detail, the levels of MMP-9 were higher in MMP-9/NGAL^{WT} and MMP-9/NGAL^{C107G} clones compared to MMP-9/NGAL^{Empty} cells (Figures 3A and 3B). However, no differences in MMP-9 protein levels were observed between the NGAL wild-type and mutated clones.

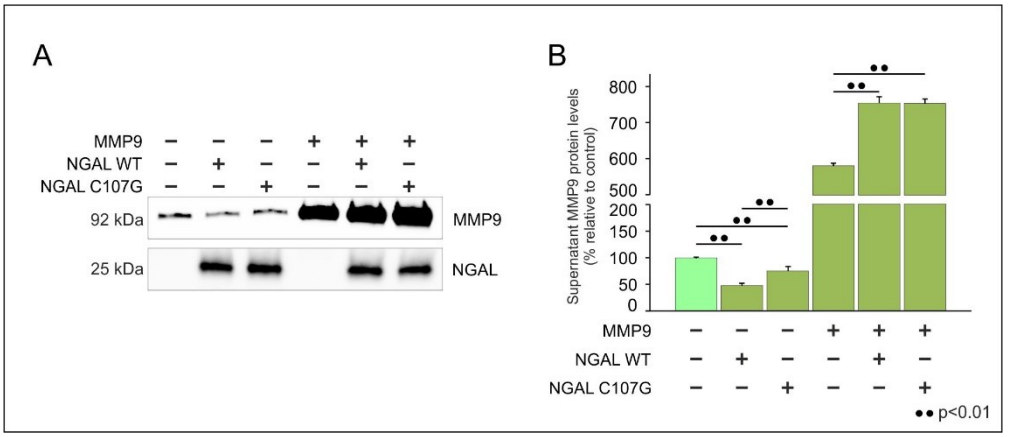

Figure 3. Protein levels of NGAL and MMP-9 in transfected cell clones.

Conversely, Real Time PCR revealed that no differences in the MMP-9 gene expression were observed comparing all the MMP-9 transfected clones (Figure 4).

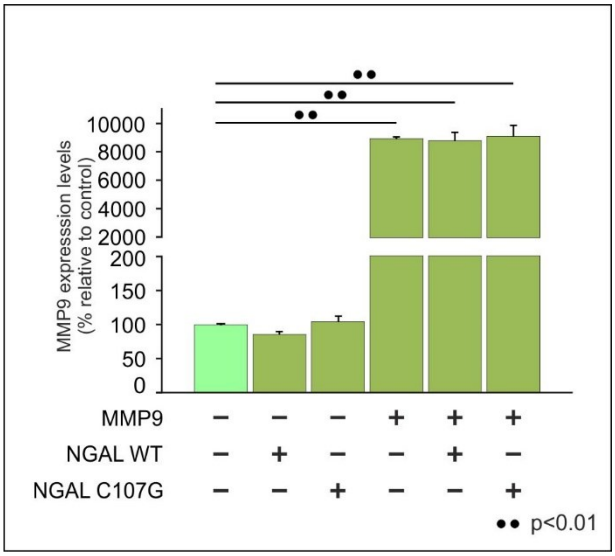

Figure 4. Gene expression of NGAL and MMP-9 in transfected cell clones.

Figure 5 reports the proliferation rates of the six transfected clones, showing that the MMP-9 over-expressing clones had less proliferation rate compared to control (Figure 5).

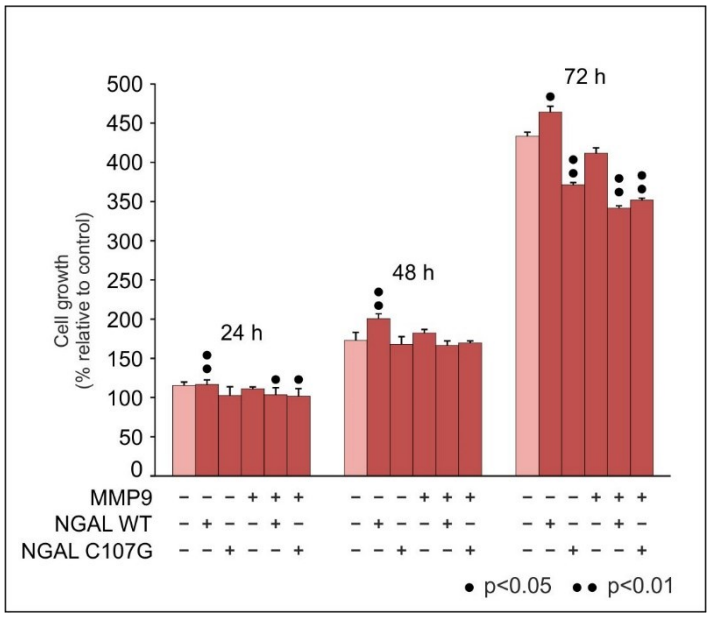

Figure 5. Proliferation rates of the transfected clones.

Finally, the invasiveness of the cell clones transfected with MMP-9 and NGAL has been evaluated through the invasion assay as described in the Materials and Methods section.

The assay has shown a statistically significant increase of cell invasiveness for all transfected clones compared to the MMP-9^{Empty}/$NGAL^{Empty}$ control, highlighting an higher invasiveness level in all MMP-9 transfected clones (MMP-9/$NGAL^{Empty}$, MMP-9/$NGAL^{WT}$ and MMP-9/$NGAL^{C107G}$) compared to the negative ones ($p>0.05$) (Figure 6).

Moreover, it has been observed that NGAL wild type cells (MMP-9^{Empty}/$NGAL^{WT\ and}$ MMP-9/$NGAL^{WT}$) had higher invasiveness than NGAL mutated cells (MMP-9^{Empty}/$NGAL^{C107G}$ and MMP-9/$NGAL^{C107G}$), independently from the MMP-9 over-expression.

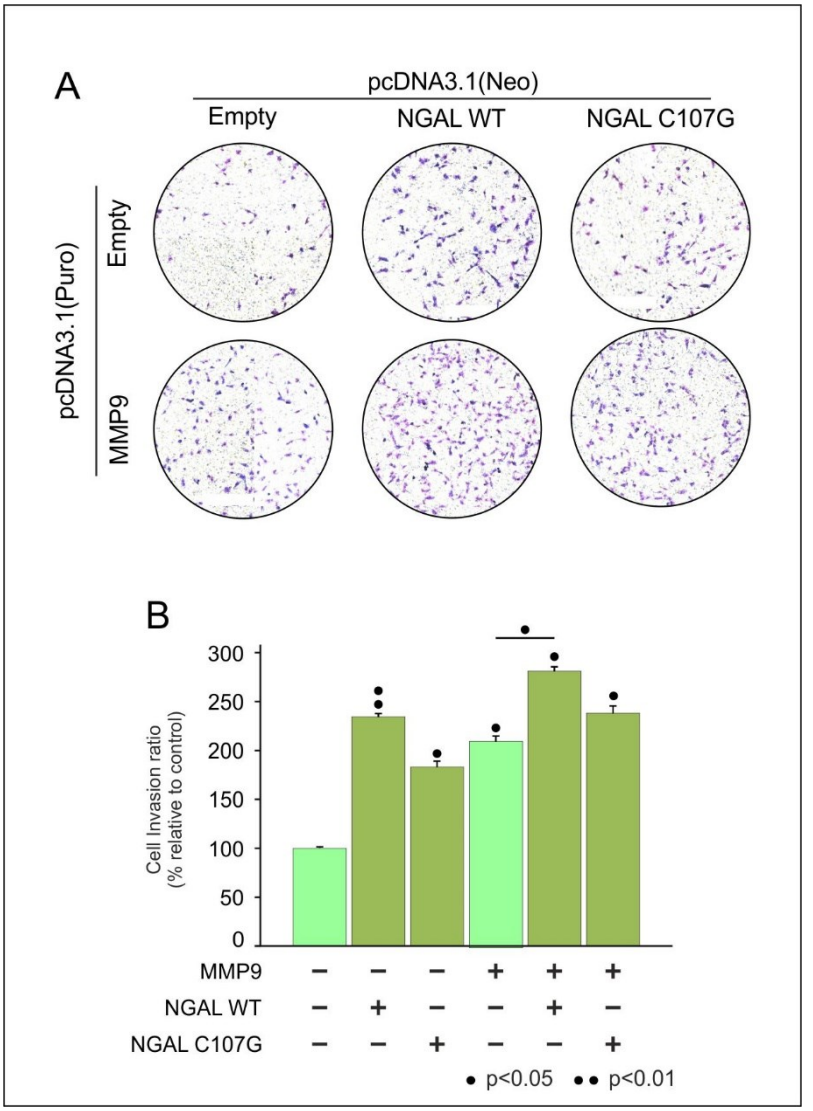

Figure 6. Invasion assay of cells transfected with NGAL and MMP-9.

DISCUSSION

The degradation of the ECM, the infiltration of the surrounding tissue and the dissemination of cancer cells through blood and lymphatic vessels are fundamental steps in the metastatic process [186–188]. The acquisition of cancer cells invasive phenotype is the result of the alteration of several proteins, among which matrix metalloproteinases play a pivotal role [189,190]. In particular, several studies showed that the over-expression of MMP-9 is frequently associated with high invasive and metastatic power in several cancers [191–193], leading to a worse prognosis for the patients [194]. Therefore, there is an urgent need to understand the mechanisms responsible of MMP-9 over-expression in order to develop new therapeutic strategies with MMP-9 selective inhibitors aimed to reduce cancer aggressiveness.

Along this line of research, a recent study has shown that the blockade of active MMP-9, through a monoclonal antibody that targets the active site of gelatinases, named SDS3, suppressed metastatic growth in the lungs in an animal model of breast cancer. In particular, the inhibition of MMP-9 reduced migration, invasion, and colony formation of breast cancer cells and enhanced immune response by promoting $CD8^+$ T cell infiltration and activation [195].

Although some mechanisms of regulation of MMP-9 have been well described (e.g. gene promoter polymorphism, epigenetic events, transcription factors

activation), some post-transductional mechanisms of regulation mediated by other proteins have not been completely clarified [1,196–198].

In particular, it has been showed that NGAL, when over-expressed in solid tumours, is able to improve the proteolytic activity of MMP-9 favouring the activating cleavage of pro-MMP-9 and preventing its degradation in the ECM through the formation of the NGAL/MMP-9 complex [168].

Therefore, it is evident that NGAL plays a fundamental role in regulating the degradative activity of MMP-9 and consequently in the degradation of the ECM and in the tumour spreading.

In the present study, the interaction mechanisms between MMP-9 and NGAL were investigated in order to determine if NGAL cysteine domains' modifications may reduce the activation of MMP-9 and in turn reduce tumour invasiveness.

For this purpose, the A375 cell line was chosen as good model of tumour invasiveness and it was transfected with different plasmids containing the MMP-9 and NGAL coding sequences (CDS). Furthermore, cells were also transfected with the CDS of a mutated form of NGAL obtained by site-specific mutation inducing the substitution of cysteine 107 with glycine to test the hypothesis that the substitution of a cysteine residue of NGAL may destabilize the formation of the NGAL/MMP-9 complex due to the lack of an intramolecular disulphide bond.

As expected, after cell transfection, the Real Time PCR and the Western blot analyses performed on the culture medium of the different transfected clones revealed that the MMP-9 transcription and protein levels were increased in all the MMP-9 transfected clones. Interestingly, higher MMP-9 protein levels were observed in MMP-9/NGAL^{WT} and MMP-9/NGAL^{C107G} clones compared to the MMP-9/NGAL^{Empty} clones. However, the over-expression of NGAL^{WT} and NGAL^{C107G} did not affect the transcriptional levels of MMP-9.

We have also demonstrated that all the MMP-9 transfected clones were more invasive than the negative ones. The most interesting data regarded the over-expression of the wild-type and mutated forms of NGAL. Indeed, the invasion assay has shown also that the over-expression of either NGAL^{WT} or NGAL^{C107G} is sufficient to enhance the invasive behavior of NGAL-transfected cells as compared to the controls (MMP-9^{Empty}/NGAL^{Empty} and MMP-9/NGAL^{Empty}). However, the invasiveness of the MMP-9^{Empty}/NGAL^{C107G} and MMP-9/NGAL^{C107G} clones was reduced compared to that of MMP-9^{Empty}/NGAL^{WT} cells and MMP-9/NGAL^{WT} cells (Figure 6).

Taken together, these results suggest that NGAL over-expression enhances the invasion of cells through the modulation of the MMP-9 activity and other molecular mechanisms directly mediated by NGAL and that the NGAL^{C107G} mutation result in a weak reduction of the invasiveness compared to the wild type form of NGAL.

Considering that the inhibition of NGAL, MMP-9 or the complex NGAL/MMP-9 may result in decreased cancer cell growth and invasiveness, the possible use of tailored-targeted therapeutic strategies in metastatic patients seems to be promising.

Further studies are warranted in order to better characterize and target the interaction mechanisms between NGAL and MMP-9 and to test NGAL-MMP9-targeted cancer therapy, both in preclinical models and in clinical trials.

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