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# ”Molecular predictors of clinical outcome in differentiated thyroid cancer: prognostic significance of germline polymorphisms of VEGF-A, VEGFR-2, and PDGFR-α”

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**LIST OF PUBLICATIONS**

This dissertation is based upon the following publications:

1. **Marotta V,** Guerra A, Sapio MR, Campanile E, Motta M, Fenzi G, Rossi G, Vitale M. Are RET/PTC rearrangements in benign thyroid nodules of biological significance? Thyroid. 2010 Oct;20(10):1191-2. PubMed PMID: 20860421.
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6. Guerra A, Sapio MR, **Marotta V**, Campanile E, Rossi S, Forno I, Fugazzola L, Budillon A, Moccia T, Fenzi G, Vitale M. The primary occurrence of BRAF(V600E) is a rare clonal event in papillary thyroid carcinoma. J Clin Endocrinol Metab. 2012 Feb;97(2):517-24. Epub 2011 Dec 14. PubMed PMID: 22170714.
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10. **Marotta V**, Franzese MD, Del Prete M, Chiofalo MG, Ramundo V, Esposito R, Marciello F, Pezzullo L, Carratù A, Vitale M, Colao A, Faggiano A. Targeted therapy with kinase inhibitors in aggressive endocrine tumors. Expert Opin Pharmacother. 2013 Jun;14(9):1187-203. doi: 10.1517/14656566.2013.796931. Review. PubMed PMID: 23675883.
11. **Marotta V,** Ramundo V, Camera L, Del Prete M, Fonti R, Esposito R, Palmieri G, Salvatore M, Vitale M, Colao A, Faggiano A. Sorafenib in advanced iodine-refractory differentiated thyroid cancer: efficacy, safety and exploratory analysis of role of serum thyroglobulin and FDG-PET. Clin Endocrinol (Oxf). 2013 May;78(5):760-7. doi: 10.1111/cen.12057.
12. **Marotta V**, Di Somma C, Rubino M, Sciammarella C, Modica R, Camera L, Del Prete M, Marciello F, Ramundo V, Circelli L, Buonomano P, Colao A, Faggiano A. Second-line sunitinib as a feasible approach for iodine-refractory differentiated thyroid cancer after the failure of first-line sorafenib. Endocrine. 2015 Aug;49(3):854-8. doi: 10.1007/s12020-014-0448-y. Epub 2014 Oct 11. PubMed PMID: 25305056.
13. **Marotta V**, Colao A, Faggiano A. Complete disappearance of liver metastases in a patient with iodine-refractory differentiated thyroid cancer subjected to sorafenib re-challenge. Endocrine. 2015 Dec;50(3):824-5. doi: 10.1007/s12020-015-0570-5. Epub 2015 Mar 10.
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**LIST OF ABBREVIATIONS USED**

| | |
|---|---|
| VEGF-A | Vascular-endothelial growth factor-A |
| VEGFR | Vascular-endothelial growth factor receptor |
| PDGF | Platelet-derived growth factor |
| PDGFR | Platelet-derived growth factor receptor |

All other abbreviations have been specified within the text.

**Abstract**

Angiogenesis is crucial for cancer progression and its efficiency may affect disease evolution, and therefore clinical outcome. Given that cancer-related vessel formation relies on the host angiogenic machinery, individual genetic variability affecting physiological angiogenesis may impact on cancer prognosis.

Function of angiogenesis-regulating genes may be affected from single nucleotide polymorphisms (SNPs) through the modulation of gene-expression.

Prognostic stratification of differentiated thyroid cancer (DTC) is still suboptimal as no effective tools are available for identifying patients with persistent/recurrent disease after thyroid ablation.

Our objective was to evaluate germline SNPs of VEGF-A, VEGFR-2, and PDGFR-α, as prognostic markers of clinical outcome in DTC.

Multicenter retrospective study including consecutive DTC patients subjected to post-surgical follow-up. Eight angiogenesis-related SNPs were included in the analysis: -2578 C>A (rs699947), -460 T>C (rs833061), +405 G>C (rs2010963), and +936 C>T (rs3025039) for the VEGF-A gene; +1192 C>T (rs2305948) and +1719 T>A (rs1870377) for the VEGFR-2 gene; -1309 G>A (rs6554162) and -635 G>T (rs1800810) for the PDGFR-α gene. Genotyping was performed by means of TaqMan protocol. Prognostic outcome was categorized as persistent structural disease, recurrent structural disease, and no evidence of disease at last follow-up. Genotypes were analyzed as three-group categorical variable and according to the dominant and recessive model. Haplotype analysis was performed by means of the Haploview software. Positive (PPV) and negative (NPV) predictive values were calculated for identified genetic markers.

Overall, 249 patients were included. No statistically significant results for any of the included SNPs were found at analysis of the overall population. Stratified analysis demonstrated that minor homozygous genotypes of VEGF-A -2578 C>A and -460 T>C (AA and CC, respectively) conferred protection

against recurrent structural disease in AJCC/UICC stage I-II and ATA low-intermediate risk patients (p=0.035 with RR 0.17 and p=0.031 with RR 0.16, respectively). Haplotype analysis of VEGF-A SNPs identified 3 common haplotypes: the $^{-2578}$C, $^{-460}$T, $^{+405}$C (CTC); the $^{-2578}$A, $^{-460}$C, $^{+405}$G (ACG); the $^{-2578}$C, $^{-460}$T, $^{+405}$G (CTG). ACG and CTG haplotypes were associated with the rate of structural recurrent disease in AJCC/UICC stage I-II (p=0.05 with OR 0.22 and 0.005 with OR 2.6, respectively) and ATA low-intermediate risk patients (p=0.036 with OR 0.51 and 0.039 with OR 1.93, respectively), exerting protective and deleterious effect, respectively. Analysis of combined-SNPs genotype found that the ACG homozygous genotype (ACG+/+) offered a protective effect against structural recurrence in both stage I-II (p=0.018, RR 0.2) and ATA low-intermediate (p=0.035, RR 0.17) risk patients, whereas the CTG homozygous genotype (CTG+/+) was significantly associated to higher rate of structural recurrence in stage I-II (p=0.018, RR=3.55), and was slightly deleterious also in ATA low-intermediate risk (p=0.079, RR=2.59) subjects. The ACG+/+ genotype retained its prognostic effect in ATA low-intermediate risk patients after adjustment for tumour size and multifocality. Both ACG+/+ and CTG+/+ genotypes showed high NPV, but only CTG+/+ revealed acceptable PPV for structural recurrent disease (42.8% and 33.3% in stage I-II and ATA low-intermediate risk patients, respectively).

Analysis of germline VEGF-A SNPs may refine risk stratification of DTC with “early” disease by providing stable and easily accessible prognostic markers.

The validation of these markers may facilitate clinical decision-making, which is still challenging regarding several therapeutic aspects.

The relevance of VEGF-A genetic variability in this group of DTC may provide *rationale* for considering VEGF-A targeted therapies as a possible tool for the treatment of subjects harbouring the disease recurrence risk genotype.

# 1. Background

## 1.1 Prediction of clinical outcome in differentiated thyroid cancer

Thyroid cancer represents not only the most common endocrine malignancy, but its incidence is progressively increasing over time in several Western countries, including Italy (Albores-Saavedra et al. 2007; Dal Maso et al. 2011; Davies et al. 2014). Differentiated thyroid cancer (DTC), including papillary and follicular histotypes, arises from epithelial follicular cells (Schlumberger 1998). It accounts for the vast majority (90%) of thyroid malignancies (Sherman 2003) and can be virtually considered responsible for the entire increase of thyroid cancer incidence (Siegel et al. 2014), thus representing a relevant problem for public health. Despite the raising morbidity, mortality rate of thyroid cancer was stable during last decades (Davies et al. 2014). Indeed, prognosis of patients affected with DTC is typically favourable with a 10-years disease-related survival of 85% (Eustatia-Rutten et al. 2006). This is due to both the intrinsic indolent behaviour of the disease (Schlumberger 1998) and the efficacy of initial treatment, consisting in total/near-total thyroidectomy and, in selected cases, radioactive iodine (RAI), followed by suppression of thyroid-stimulating hormone (TSH) (Haugen et al. 2016). The low mortality rate of DTC makes difficult to perform prognostic studies having as primary endpoint overall survival because of the long follow-up needed to achieve a significant group of dead patients. By contrast, the persistence of structural disease after initial treatment or the development of recurrences after complete remission have been reported in about 25-30% of patients (Castagna et al. 2011; Pitoia et al. 2013; Tuttle et al. 2010b; Vaisman et al. 2012), and are strictly related to disease-specific survival (Brown et al. 2011; Mazzaferri et al. 1994; Tuttle et al. 2010a). Thus, the rate of persistent/recurrent disease or the disease-free status (if performing survival analyses) are considered as more feasible outcomes to be analyzed and therefore used as primary endpoints in the majority of prognostic studies of DTC. Given that the AJCC/UICC

(American Joint Commettee on Cancer/Union for International Cancer Control) system was able to predict mortality but not persistence/recurrence (Baek et al. 2010; Orlov et al. 2009; Tuttle et al. 2010b; Vaisman et al. 2012), a great effort has been done in the last decade to build novel staging systems specifically dedicated to the prediction of persistent/recurrent disease. Particularly, each of the major societies dealing with thyroid diseases (ATA [American Thyroid Association], ETA [European Thyroid Association], and LATS [Latin American Thyroid Society]) has validated a categorical classification identifying subgroups with different risk of persistent/recurrent disease (Pacini et al. 2006; Pitoia et al. 2013; Pitoia et al. 2009). Nevertheless, the long-term risk stratification obtained by the mentioned systems is still suboptimal as all of them showed a proportion of variance explained, a statistical measure of how well a staging system can predict the outcome of interest (Schemper et al. 1996), less than 30% (Momesso et al. 2014). In order to refine the risk estimate of persistent/recurrent disease, recent guidelines from the ATA have introduced a personalized non-categorical model based on the concept of "continuum of risk", where further variables were added in order to perfectly fit individual clinico-pathological features and provide a quantitative determination of the risk (Haugen et al. 2016). Thus, the identification and characterization of novel prognosticators of persistent/recurrent disease, including molecular markers, is crucial for empowering this model.

### 1.2 Molecular prognostication of differentiated thyroid cancer

Although molecular prognostication of DTC, namely the understanding of the possible relationship between those genetic alterations with demonstrated pathogenetic role and the clinical outcome, has been widely studied in last several years, it still represents an evolving field. Indeed, any molecular marker has a well-defined role in the risk-stratification of DTC, and the introduction of molecular prognosis into "real-life" clinical practice is still far to be performed. Historically, RET rearrangements and BRAF mutation, genetic alterations

specifically occurring in PTC, have represented the mainstays of molecular research about thyroid carcinogenesis, being the most studied and characterized molecular abnormalities in this field. Thus, we will firstly focalize current role of these molecular markers as prognosticators in DTC. Afterwards, we will discuss about emerging molecular markers, specifically focusing on TP53 and TERT promoter mutations and the co-occurrence of driver mutations. Finally, we will provide some insights about the possible future role of non-tissutal molecular markers.

### 1.2.1 RET rearrangements

RET/PTC are a group of chimeric oncogenes (with RET/PTC 1 and 3 variants being the most frequent) generated by the fusion of the catalytic domain of the tyrosine kinase receptor *RET* to the 5' terminal region of heterologous genes (Santoro et al. 2006). RET/PTC is an exclusive occurrence of the thyroid gland (Nikiforova et al. 2000), and its pathogenetic role in DTC has been deeply described (Jhiang et al. 1998; Powell et al. 1998; Santoro et al. 1993; Tallini et al. 1998). In last years, the introduction of highly sensitive techniques allowed the detection of non-clonal mutational events, namely the presence of RET rearrangements in a small proportion of tumour cells, or even in one single cell. This significantly changed some of pre-existing notions about RET/PTC, allowing the detection of the rearrangements also in benign thyroid diseases (Marotta et al. 2011a). Particularly, we searched for RET/PTC 1 and 3 by using a high sensitive method, namely Southern Blot on RT-PCR products. We detected the rearrangements in 36% of PTC, a higher percentage as compared with what previously found by using less sensitive techniques (Jhiang et al. 1998; Santoro et al. 1992), and in a relevant portion (13.3%) of thyroid nodules with benign histology (Guerra et al. 2011).

The biological significance of non-clonal occurrence of RET/PTC in both malignant and benign nodules is still a challenging issue (Marotta et al. 2010a). We tried to provide some insights about this aspect by comparing clinical

evolution of benign nodules with or without non-clonal RET/PTC occurrence, showing that the presence of RET rearrangements, even as non-clonal, was associated with more rapid volume increase (Marotta et al. 2010b; Sapio et al. 2011). This suggests that non-clonal RET/PTC may be considered as a biologically relevant event.

By the prognostic sight, some evidence suggested that RET/PTC 1 was associated with more favourable behaviour of PTC (Nikiforov 2004). Furthermore, PTC harbouring RET rearrangements, particularly RET/PTC 1, had a very low probability of progression to poorly differentiated and anaplastic carcinomas, as compared with those carrying BRAF and RAS mutations (Mayr et al. 1997; Soares et al. 1998; Tallini et al. 1998). Despite these data, the strict dependence from the sensitivity of the detection method and the biological difference between clonal and non-clonal mutation, which needs to be further defined, strongly hampered analysis and validation of RET/PTC in the prognostic setting. Furthermore, pre-clinical studies identified RET/PTC as a weak tumour-initiating factor and suggested that secondary genetic or epigenetic changes were required for full neoplastic transformation (Powell et al. 1998; Wang et al. 2003). This makes unlikely the driver role of RET rearrangements in tumour progression and therefore its impact on prognosis. Owing this set of data, RET/PTC has no current role in the prognostic stratification of PTC.

### 1.2.2 $BRAF^{V600E}$

The T1799A transverse point mutation of the proto-oncogene BRAF, resulting in the valine-to-glutamate (V600E) amino-acidic substitution, is nearly the only BRAF mutation found in thyroid cancer, with a very few exceptions for the K601E and A598V missense mutations, the AKAP9/BRAF recombination, the 1799-1801 deletion and the 1799-1816 insertion (Ciampi et al. 2005; Hou et al. 2007b; Santarpia et al. 2009; Xing et al. 2005). $BRAF^{V600E}$ represents the most common genetic alterations in PTC (approximately 45% of

cases) (Marotta et al. 2011b; Xing 2005), and its pathogenetic role has been widely proved by pre-clinical studies (Knauf et al. 2005; Liu et al. 2007).

Unlikely RET rearrangements, several authors reported a clear association of $BRAF^{V600E}$ with molecular features suggestive of biological and clinical aggressiveness. Particularly, the mutation was associated with decreased or absent expression of thyroid iodide-handling genes (the sodium-iodide symporter, the TSH receptor, the pendrin gene [SLC26A4], the tireoperossidase, and the thyroglobulin) (Durante et al. 2007; Xing 2007), whom expression was demonstrated to be strictly dependent from that of $BRAF^{V600E}$ (Chakravarty et al. 2011; Liu et al. 2010). Furthermore, *BRAF* mutation was associated with overexpression of many tumour-promoting factors, such as VEGF-A and c-MET (Xing, 2007). By the clinico-pathological sight, $BRAF^{V600E}$ was associated with the tall cell variant of PTC, which represents the most aggressive histological subtype (Ghossein et al. 2007; Milione et al. 2010). Despite still controversial, the majority of studies also reported the association of mutated BRAF with several other clinico-pathological features having a negative prognostic impact, such as lymph node metastases, extra-thyroidal extension and advanced disease stage (Frasca et al. 2008; Kebebew et al. 2007; Lee et al. 2007a; Wang et al. 2008; Xing et al. 2005).

Owing this body of evidence, $BRAF^{V600E}$ has been considered the best candidate as molecular prognosticator of PTC and several prognostic studies have been dedicated to assess its relationship with clinical outcome. After a wide series of single-center studies showing controversial results, 2 large multicenter cohorts have been recently analyzed for assessing the impact of BRAF mutation on mortality and recurrence, respectively. The first paper including 1849 patients showed the association of mutated BRAF with increased disease specific mortality at univariate analysis (Xing et al. 2013a). More importantly, the second one including 2099 patients demonstrated an independent association between *BRAF* mutation and recurrent disease both in

the overall PTC population and after stratification for histotypes (classic and follicular variant) (Xing et al. 2015).

Despite the unequivocal association with disease recurrence, clinical application of $BRAF^{V600E}$ as prognostic marker is hampered by its low specificity. Indeed, analysis from the largest meta-analysis available to date (2167 patients) showed acceptable sensitivity (65%), but poor specificity for the prediction of recurrent disease with a positive predictive value (PPV) of only 25% (Tufano et al. 2012). Thus, current role of mutated BRAF for the risk stratification of PTC is limited, as it is unlikely to be used in isolation, but only in a multivariable context, combined with other prognostic features. To date, the 2015 ATA guidelines do not suggest the routinary determination of BRAF status, but consider $BRAF^{V600E}$ as an information to be included (if present) for the risk estimate of recurrent disease in ATA low-risk patients according to the "continuum of risk" model (Haugen et al. 2016).

Our recent study about the clonality of BRAF mutation in PTC (Guerra et al. 2012b) has opened a burning issue among researchers dealing with thyroid carcinogenesis because of its possible impact on both the biological role and the clinical implications of $BRAF^{V600E}$. We searched for BRAF mutation in PTC surgical samples by means of pyrosequencing, a sequencing-by-synthesis method that measures the incorporation of each of the four nucleotides at each template position in an automated process involving a pyrosequencer device (Ronaghi et al. 1998). As demonstrated by many studies from our and other research groups (Guerra et al. 2014; Jo et al. 2006), pyrosequencing showed higher sensitivity in detecting mutated BRAF as compared with dideoxy sequencing. More importantly, pyrosequencing allowed the careful quantification of the percentage of mutated alleles and therefore of the portion of tumour cells harbouring $BRAF^{V600E}$. Among 41 BRAF-mutated PTC, only 4 cases (about 10%) were consistent with a clonal mutation showing a percentage of mutated alleles of nearly 50%. By contrast, in the majority of PTC (27, about 65%) $BRAF^{V600E}$ alleles were in the range of 25 to 5.1%,

which was consistent with a subclonal mutational event. Our results were exactly replicated by a subsequent study from another Italian research group (Gandolfi et al. 2013). In order to support these findings and to assess the clinical implications of performing a quantitative analysis of BRAF mutation, we planned a prognostic study with inclusion of 168 patients, demonstrating that the percentage of mutated alleles significantly impacted on the risk of recurrence (Guerra et al. 2012a). Particularly, BRAF-mutated tumours with more than 30% mutated alleles showed lower disease-free survival, as compared with those harbouring less than 30%. The clonality of BRAF mutation in PTC is still an hot point of current research on thyroid cancer. Recently, de Biase and colleagues (de Biase et al. 2014a) have assessed the percentage of BRAF-mutated alleles in a PTC series by means of modern and more accurate techniques, such as the allele-specific locked nucleic acid PCR and 454 next-generation sequencing (de Biase et al. 2014b; Morandi et al. 2012). They confirmed the heterogeneity of the mutation, demonstrating that $BRAF^{V600E}$ was a clonal event in less than 50% of cases. By contrast, the recently published study about genomic sequencing of PTC from The Cancer Genome Atlas (Agrawal et al. 2014), performed by means of the most innovative next-generation sequencing techniques, applied a dedicate software (ABSOLUTE package (Carter et al. 2012)) to calculate cancer cell fraction of the previously identified driver mutations with inclusion of $BRAF^{V600E}$, finding that the majority of tumour cells harboured the mutation. Thus, authors concluded that mutations of founder genes were always clonal events. Despite this result, we consider the issue about the clonality of $BRAF^{V600E}$ still opened. The same study from the Cancer Genome Atlas found a wide variation in the pattern of gene expression within the cohort of BRAF-mutated tumours, meaning that PTCs with BRAF mutation include a *spectrum* of tumours having different biology and clinics. It is our hypothesis that this could be explained by the heterogeneity of the mutation.

Although further studies are mandatory for a better definition of this issue, quantitative determination of $BRAF^{V600E}$ and therefore categorization of mutated tumours basing on the percentage of mutated alleles (or cells bearing the mutation) may dramatically change not only the knowledge of the biological role of the oncogene, but also its clinico-pathological implications including the application as molecular prognosticator. Indeed, larger prognostic studies may allow to test and identify a cut-off of percentage of BRAF-mutated alleles, with the aim of improving specificity and therefore PPV for disease recurrence, which represents the main limit of qualitative $BRAF^{V600E}$ determination in the prognostic setting.

### 1.2.3 Emerging molecular markers: TP53, TERT promoter, and co-occurring driver mutations

Despite limited in its clinical application, $BRAF^{V600E}$ is the more powerful molecular prognosticator of DTC. Unfortunately, other genetic abnormalities historically associated to DTC (including RAS point mutations and PAX8/PPARγ rearrangement, which are mostly detected in FTC (Xing 2013)) have failed to demonstrate enough prognostic impact and are not currently considered as feasible prognosticators (Nikiforova et al. 2009; Xing et al. 2013b). In recent years, mutations of other 2 genes, the tumour suppressor TP53 and the promoter of the catalytic subunit of the telomerase TERT, are gaining growing relevance in this field.

Although typically considered as a marker of tumour dedifferenziation and detected in a wide portion of poorly differentiated and anaplastic thyroid cancer (Donghi et al. 1993; Fagin et al. 1993), recent mutational analysis by means of next-generation sequencing techniques has identified TP53 mutations also in a low percentage of DTC, namely 3.5% of PTC and 11% of oncocytic FTC (Nikiforova et al. 2013). Despite the limited samples size, authors reported a more aggressive clinical behaviour for this little subgroup of TP53-mutated tumours.

In last years, mutations of the TERT promoter have represented the focus of a relevant part of translational cancer research (Huang et al. 2013). Recently, the mutations 1295228 C>T, termed C228T, and 1295250 C>T, termed C250T, leading to an increase of translational activity and therefore to telomerase activation and immortalization of cancer cells, were detected in follicular cell-derived thyroid cancers. As for TP53, TERT-promoter mutations were more frequent in less differentiated tumours, but involved also a significant portion of DTC (7-22% of PTC and 14-17% of FTC) (Landa et al. 2013; Melo et al. 2014). Importantly, the mutations revealed strong prognostic impact, being identified as independent risk factor for persistent disease, distant metastases, and disease-specific mortality (Melo et al. 2014).

Basing on the reported data, both TP53 and TERT promoter mutations are promising tools in the field of molecular prognostication of DTC. Although further studies are needed mainly to confirm the negative prognostic impact of TP53, it seems that these markers may identify a small subgroup of tumours having a highly aggressive behaviour. Thus, mutations of TP53 and TERT promoter may have higher specificity and PPV for persistence/recurrence, as compared with $BRAF^{V600E}$. Furthermore, both the markers seems to be associated with BRAF-mutation (Landa et al. 2013; Liu et al. 2013; Melo et al. 2014), thus suggesting a possible synergistic interplay. Thus, co-occurrence of TP53 and TERT promoter may in part explain the wide biological and clinical variance characterizing BRAF-mutated PTC (Agrawal et al. 2014), and may therefore be used for identifying those BRAF-mutated tumour with worst outcome (Xing et al. 2014). To conclude, larger and dedicated studies are needed to assess the actual accuracy of TP53 and TERT promoter mutations as predictors of clinical outcome among DTC-patients, thus allowing their introduction into clinical practice.

The co-existence of driver mutations within the same tumour was previously considered as typical of less differentiated and more biologically aggressive forms of follicular-derived thyroid cancer, namely poorly and anaplastic

histotypes (Garcia-Rostan et al. 2005; Hou et al. 2007a; Liu et al. 2008). Recently, the next generation sequencing analysis performed by Nikiforova et al. (Nikiforova et al. 2013) have represented a breakthrough, reporting the co-occurrence of drivers mutations also in a small portion, namely 4%, of DTC (Nikiforova et al. 2013). Even more importantly, this mutational status was associated to an aggressive clinical evolution and the presence of distant metastases. This led the latest ATA guidelines (Haugen et al. 2016) to consider the combination of mutations involving multiple founder genes as an independent genetic signature of aggressiveness, which allows the identification of a small subgroup of tumours with extremely aggressive behaviour. Within this model, $BRAF^{V600E}$ is considered as the main "actor" whereas T*ERT* promoter and TP53, but also PIK3CA, AKT1, or RET/PTC mutations are considered as co-occurring events. The proposal of this multi-genetic approach of risk estimate has the aim to overcome the limit that any genetic alteration known to be associated with DTC has, taken alone, enough specificity for identifying persisting/recurring patients. Nevertheless, this model is still at a preliminary level and further studies are needed to validate it.

### 1.2.4 Non-tissutal prognosticators

To date, molecular analysis of DTC, and therefore molecular prognostication, is based exclusively on tissue markers. This represents a limit as tumour tissue, including surgical samples but also fine-needle ago-biopsy specimens, is not always available. Furthermore, a different mutational status may occur in metastatic sites as compared with primary tumour, thus hampering tissue-based molecular characterization. Therefore, the identification of non-tissutal markers may facilitate and empower molecular prognostication of DTC.

Given that $BRAF^{V600E}$ is the more frequent somatic mutation and the main prognosticator of DTC, several authors searched for the mutation in circulating free DNA (Marotta et al. 2011b). Firstly, Chuang et al. analyzed serum of a small series of patients, demonstrating that 60% of cases who were positive for

$BRAF^{V600E}$ in primary tumours also had detectable circulating BRAF mutation (Chuang et al. 2010). Afterwards, Cradic et al. investigated whether BRAF mutation could be detected in the blood of patients with residual or metastatic disease, finding the mutation in 21% of cases (Cradic et al. 2009). By contrast, recent data failed to detect circulating $BRAF^{V600E}$ in 94 serum samples from patients with PTC harbouring the mutation at the somatic level by using a quantitative PCR method (Kwak et al. 2013). This discrepancy could be related to the use of assay reagents with inadequate sensitivity and/or not optimized for plasma samples in addition to uncontrolled pre-analytical steps. Recently, research by Pupilli et al. further empowered the use of circulating BRAF mutation as biomarkers in DTC (Pupilli et al. 2013). Authors demonstrated that the percentage of $BRAF^{V600E}$ detected in the serum increased progressively across cytological categories, being higher in patients with histologically confirmed thyroid cancer compared to those with benign histology. Furthermore, analysis of the mutation before and after treatment clearly indicates an association between the mutation and the presence of active disease. Thus, BRAF mutation detected on circulating DNA represents a promising tool to be specifically analyzed also for the prognostic setting.

Molecular prognostication of DTC may be further improved by the application of microRNAs (miRNAs), which are short (about 19–22 nucleotides), non-coding RNA sequences having relevant role in cancer development and progression through their regulatory activity on gene expression at both the transcriptional and post-transcriptional level (Calin et al. 2002; Ma et al. 2007). To date, several miRNAs have been found to be deregulated in PTC (He et al. 2005; Nikiforova et al. 2008; Pallante et al. 2006; Tetzlaff et al. 2007). Particularly, miR-146b, miR-221, and miR-222, have been identified as the most deregulated, showing increases of 11- to 19-fold. Therefore, many authors have focused the association of miRNAs, particularly those previously mentioned, with the clinical outcome. Firstly, Gao et al. analyzed miRNAs expression in three subpopulations of PTC cell lines with

increased lymph node metastatic potency compared with the control subpopulations (Gao et al. 2010). MiR-146b, miR-221, and miR-222 were confirmed to be overexpressed in PTC tissue, compared with normal thyroid tissue, and also were associated with high-risk features such as extra-thyroidal extension, lymph node metastasis, distant metastasis, recurrence, and $BRAF^{V600E}$ mutation. Afterwards, studies from Chou et al. showed that BRAF-mutated PTC had higher miR-146b expression as compared with those not carrying the oncogene (Chou et al. 2010). Furthermore, they performed a follow-up study demonstrating poorer overall survival among patients with high levels of miR-146b (Chou et al. 2013). Two research groups found an association between miR-146b and miR-222 overexpression and distant metastasis, recurrence and BRAF expression (Lee et al. 2013; Yip et al. 2011). Indeed, Zhou et al. found that overexpression of miR-221 was associated with extra-thyroidal extension, lymph node metastasis, advanced disease stages, and BRAF mutation (Zhou et al. 2012). In all mentioned studies, prognostic impact of miRNAs was based on the evaluation of the expression on tumour tissue. Nevertheless, tumour-derived miRNAs are also released into the bloodstream (Mitchell et al. 2008), where they can be detected and therefore used as circulating biomarkers. Although reliability and accuracy of circulating miRNAs as tumour markers is limited by the possible discordant distribution between tissue and the bloodstream (Garcia et al. 2008; Heegaard et al. 2012), they are considered promising diagnostic and prognostic tools in various types of cancers, such as lung, stomach, and ovary neoplasms (Cheng et al. 2011; Kroh et al. 2010; Shen et al. 2011; Tsujiura et al. 2010). Role of circulating miRNAs as biomarkers of thyroid cancer is still under evaluation. Besides performing miRNAs evaluation on tumour tissues, the previously mentioned study by Lee et al. demonstrated that PTC-related miRNAs can be measured in plasma (Lee et al. 2013). Importantly, authors reported that miR-222 and miR-146b were overexpressed in plasma from patients with PTC compared with plasma from healthy individuals and that circulating levels significantly

decreased after surgery. This suggests a close relationship between circulating miRNAs and active disease. To date, studies specifically assessing feasibility of circulating miRNAs in the prognostic setting are missing, so their introduction into clinical practice is still far from reality.

## 1.3 Angiogenesis and cancer

Angiogenesis is a physiological process consisting in growth and development of new blood vessels from pre-existing vasculature (Norrby 2006). It is involved in several aspects of human physiology, such as embryogenesis, tissue growth and development, inflammation, wound healing and placental development (Carmeliet 2003).

Nevertheless, angiogenesis plays a role also in many pathological processes, including cancer (Folkman 1995). Indeed, an adequate supply of oxygen, metabolites and an effective way to remove waste products are required for neoplastic tissues, similarly to normal ones (Papetti et al. 2002). These requirements depend not only from the tumour type, but widely vary basing on the course of tumour progression (Hlatky et al. 2002). Particularly, formation of new vasculature is considered crucial for tumour maintenance and progression hesitating to metastatic disease, rather than initial neoplastic transformation (Hanahan et al. 2000). This is confirmed by the observation that vascularity is associated with aggressive behaviour and poor prognosis in different types of cancer (Jubb et al. 2004), thus leading recent anti-cancer research to focus on the development and subsequent introduction into clinical practice of a set of anti-angiogenic molecules, which are typically indicated in those patients with advanced disease stage and/or experiencing escape from conventional therapies (Bridges et al. 2011; Welti et al. 2013). Importantly, anti-angiogenic treatment is gaining relevant role in advanced forms of endocrine tumours, including DTC, which are poorly responsive to conventional anti-cancer treatments, such as cytotoxic agents and radiotherapy (Marotta et al. 2013).

### 1.3.1 Molecular mechanisms of angiogenesis in cancer

Angiogenesis is a complex process regulated by multiple agents, being the VEGF-A and its downstream system the main one, and by the interaction of several cellular types, being the endothelial-cell the main one (Carmeliet et al. 2011). Indeed, activation of new blood vessels development results from the balance of a wide range of molecules, which are strictly interactive and may act as angiogenic "activators" or "inhibitors" (Carmeliet 2000). These factors typically act by binding tyrosine-kinase receptors, thus activating their downstream molecular cascades (Carmeliet et al. 2011). Activators of endothelial-cell proliferation and migration, termed angiogenic factors, include mainly soluble proteins such as vascular endothelial growth factors (VEGFs), fibroblast growth factors (FGFs), platelet-derived growth factor (PDGF), placental-growth factor (PlGF), and epidermal growth factor (EGF) (Ferrara et al. 1989; Folkman 1995), but also molecules of different nature, such as lysophosphatic acid (Hu et al. 2001). Among angiogenic inhibitors, the first factor to be identified was thrombospondin-1 (Volpert et al. 1995). Afterwards, a class of proteins named 'statins', derived from larger molecules not affecting angiogenesis, (including angiostatin (O'Reilly 1997), endostatin (O'Reilly et al. 1997), tumstatin (Maeshima et al. 2001), and canstatin (Kamphaus et al. 2000)) has been identified.

During adult life, balance between positive and negative regulators of angiogenesis is settled to induce a quiescence state, which is typical of adult vasculature, with the exception of female reproductive organs, physiologically growing organs and injured tissues (Carmeliet 2000). By contrast, the occurrence of neoplastic transformation induces an alteration of this balance, which is usually defined "angiogenic switch", thus leading to abnormal angiogenesis activation (Dvorak 1986). Tumour angiogenesis mainly mimics mechanisms of the physiological counterpart. Indeed, tumours use this host-mediated process for allowing its maintenance and progression. The *primum movens* is represented by the release of angiogenic factors, which stimulate

tyrosine-kinase receptors on endothelial cells of pre-existing blood vessels. Despite the complexity of the process, VEGF-A is clearly the leading molecule (Fassnacht et al. 2009; Nagy et al. 2007). Indeed, high levels of VEGFA expression alone are capable of initiating angiogenesis in a quiescent vasculature (Pettersson et al. 2000). Importantly, angiogenic factors are released both from tumour cells and from host cells, including endothelial- or other myeloid- or stromal- cells (Lee et al. 2007b; Stockmann et al. 2008). The earliest stages of angiogenesis are characterized by vasodilatation and increased vascular permeability, which induce extra-vasation of plasma proteins and constitution of a provisional matrix. Simultaneously, endothelial cells release proteolytic enzymes that allow the degradation of the basement membrane and the migration towards the neo-formed matrix (Folkman 1995). This is accompanied by the loosening of perycite covering, which is mainly related to angiopoietin 2 and its tyrosine-kinase receptor TIE-2 (Holash et al. 1999). While migrating, endothelial cells also proliferate, with the development of a migration column leaded by the so-called tip cells. Therefore, endothelial cells gradually adhere each other, forming a lumen. This lumen eventually thickens, and, finally, additional pericytes are recruited to form a basal lamina surrounding the endothelial cells and completing the development of a new blood vessel (Bergers et al. 2003a). In normal angiogenesis, pericyte associations reduce endothelial-cell proliferation by decreasing their dependence from VEGF-A (Benjamin et al. 1998; Hirschi et al. 1996), thus providing a stop signal to angiogenesis. By contrast, the association of perycites to vessels is abnormal in tumours, and this may in part explain the fact that neoplastic vasculature will never achieve a quiescence phase and will be constantly growing (Benjamin et al. 1999; Benjamin et al. 1998). This confers to tumour vessels distinctive features, as compared with the normal counterpart, including morphological characteristics (irregular shape, dilatation, tortuosity and the frequent presence of dead ends) and organization (chaotic without clear distinction between arterioles, venules, and capillaries)

(Benjamin et al. 1999; Morikawa et al. 2002). These structural abnormalities also generate functional impairments, with occurrence of haemorrhage and/or thrombosis within the tumour mass.

While angiogenesis is the most investigated, other mechanisms of tumour vascularisation have been observed in cancer. Firstly, endothelial progenitor cells, which can either reside in the vascular wall or migrate from bone marrow in response to chemo-attractants released by tumour cells, can differentiate into endothelial cells and contribute to vessel formation (Rafii et al. 2002). Although several angiogenic factors, including VEGF-A and PlGF, have been shown to stimulate this process (Hattori et al. 2002), the entity of endothelial-precursor-cell incorporation seems to be limited and also dependent from the nature of the tumour. However, in some model systems, tumours are mostly reliant on this mechanism (Lyden et al. 2001). Other mechanisms of neoplastic vascularization include: vascular mimicry, a process where cancer cells replace endothelial cells by lining the neo-vessels; vessel cooption, whereby tumour cells arise near to (or migrates toward) a pre-existing blood vessel; the occurrence of chromosomal abnormalities in putative cancer stems cells allowing them to differentiate into endothelial cells. To date, clinical relevance of these mechanisms remains unclear (Kirschmann et al. 2012; Ricci-Vitiani et al. 2010; Wang et al. 2010), but this redundancy in tumour vessels formation has to be kept in mind when planning VEGF-A specific anti-angiogenic therapies.

### 1.3.2 The VEGF-system and its role in physiological and tumour angiogenesis

The VEGF family is composed by few members with non redundant biological activity, including VEGF-A, B, C, D, E, and PlGF (Ferrara et al. 2003; Neufeld et al. 1999). Among them, VEGF-A is the main actor and, more importantly, has a predominant role in the regulation of angiogenesis. It is a highly conserved, disulfide-bonded homodimeric glycoprotein of 45 kDa,

discovered basing on its ability to increase vascular permeability (Senger et al. 1983). Analysis of crystal structure revealed that the two chains composing VEGF-A are arranged anti-parallel, with receptor binding sites at either end (Muller et al. 1997). The human VEGF-A gene is located on the short arm of chromosome 6 and consists of eight exons separed by seven introns (Houck et al. 1991; Tischer et al. 1991). A promoter of 2.36 kbp has been described in the human gene and harbours several consensus-binding sites for transcriptional factors, including AP1, AP2 and Sp1, which are strictly regulated by growth factors, cytokines, hormones, tumour suppressor genes and oncogenes (Buteau-Lozano et al. 2002; Pages et al. 2005). An alternative promoter was located within the 5'UTR (1038 bp), 633 nucleotides downstream of the main starting site (Akiri et al. 1998), and controls transcriptional start at two alternative initiation codons. Another crucial region for the VEGF-A physiology is the 3'UTR (1881 bp), which is the main mediator of VEGF-A mRNA stability through the binding of the ARE-binding proteins (such as AUF1 and tristetraprolin) and many miRNAs (miR-20 a/b, miR-106 a/b, miR-17-5p, miR-16 and miR-15b) (DeMaria et al. 1996; Lei et al. 2009; Stoecklin et al. 2003). In vitro, VEGF-A promotes growth and allows survival of vascular endothelial cells derived from arteries, veins and lymphatics (Alon et al. 1995; Benjamin et al. 1999; Ferrara et al. 2003; Ferrara et al. 2004; Gerber et al. 1998). Furthermore, it also demonstrated to promote monocyte chemotaxis (Clauss et al. 1990) and to stimulate haematopoiesis through the induction of colony formation by mature subsets of granulocyte-macrophage progenitor cells (Broxmeyer et al. 1995). Although VEGF-A mainly acts as paracrine mediator, an autocrine action has been described in the survival of both endothelial cells and hematopoietic stem cells (Gerber et al. 2002; Lee et al. 2007b). Importantly, VEGF-A is subjected to alternative splicing, resulting in the generation of different isoforms including polypeptides of 206, 189, 165, 145, and 121 amino acids (Kowanetz et al. 2006). Among them, VEGF-$A_{165}$ represents the predominant isoform in both normal and pathological

angiogenesis and is therefore the most studied molecule (Ferrara et al. 1992). An additional variant, termed 165b, firstly detected in normal kidneys, acts as endogenous inhibitor of VEGF-$A_{165}$ and is therefore anti-angiogenic (Woolard et al. 2004). Despite having apparently similar biological activity *in vitro*, VEGF-A splice variants significantly differ in their binding to heparin (VEGF-A was originally purified on heparin affinity columns (Senger et al. 1983)), and therefore to cells and matrices (Grunstein et al. 2000; Maes et al. 2002; Park et al. 1993; Yu et al. 2002). This affects bioavailability of the molecules and therefore their function in vivo. Indeed, the formation of new blood vessels requires both long- and short-range guidance cues for directing endothelial cells migration (Eichmann et al. 2005; Ruhrberg 2003). VEGF-$A_{165}$ binds proteoglycans and other negatively charged matrices (Ferrara et al. 1992) and is both soluble and matrix bound, thus supplying both types of cues. VEGF-$A_{189}$ binds heparin more strongly than VEGF-$A_{165}$, whereas VEGF-$A_{121}$ is acidic, does not bind heparin, and diffuses freely in tissues. Thus, VEGF-$A_{121}$ predominantly mediates the long range- and $VEGFA_{189}$ the short range-guidance, being deficient in the other part of the process. This explains why mice expressing only the VEGF-$A_{164}$ isoform (consider that murine isoforms are one amino acid shorter) develop normally, whereas those expressing only VEGF-$A_{120}$ or VEGF-$A_{188}$ develop severe vascular abnormalities (Carmeliet 2003; Ruhrberg 2003). In addition, VEGF-A in vivo activity is also controlled by extra-cellular proteolysis. Particularly, proteases such as plasmin, which cleaves the C-terminal portion of bound VEGF-A, are required to generate a biologically active peptide (Park et al. 1993; Roth et al. 2006).

VEGF-A and the other members of its family act by binding three tyrosine-kinase receptors: VEGFR-1 (Flt-1), VEGFR-2 (KDR), and VEGFR-3. Structurally, they consist of seven immunoglobulin-like domains in the extracellular part, a single transmembrane region and a consensus tyrosine kinase sequence that is interrupted by a kinase-insert domain (Shibuya et al. 1990; Terman et al. 1991). The major effect of VEGF-A on angiogenesis is

obtained by signalling through VEGFR-2 (Ferrara 2009; Nagy et al. 2007). Indeed, VEGF-A mutants selectively binding to VEGFR-2 fully maintain their capability to stimulate mitoses of endothelial cells and to enhance vascular permeability, whereas mutants specific for VEGFR-1 lose both activities (Takahashi et al. 1999). Binding of VEGF-A induces VEGFR-2 dimerization and auto-phosphorylation, which is followed by phosphorylation of numerous downstream proteins, including protein kinase C, phospholipase C-γ, and phosphatidylinositol 3-kinase. Another component of the VEGF-A pathway is the serine/threonine protein kinase Akt, which regulates endothelial cell survival, migration, and tube formation (Sun et al. 2005). Other molecules involved in the VEGF-A downstream system are mammalian target of rapamycin (mTOR) and endothelial nitric oxide synthase, whom biological effect is to enhance vascular permeability (Fukumura et al. 2001a; Sun et al. 2005). Recently, a role for G proteins was also reported within the VEGF-A-induced molecular cascade (Mukhopadhyay et al. 2004). Importantly, recent findings indicate that biological activity of VEGFR-2 activation depends on its sub-cellular localization. Particularly, the induction of arterial morphogenesis requires that VEGFR-2 is located in intracellular compartments (Lanahan et al. 2010). Despite showing high affinity with VEGF-A, biological activity and therefore the role in angiogenesis of VEGFR-1 is limited. Indeed, VEGFR-1 can be considered as a kinase-impaired receptor, meaning that its activation through VEGF-A binding leads to a weak kinase activity, not sufficient to obtain significant biological effects (Rahimi 2006). However, recent evidence reported a more complex and ambiguous role for this receptor, which can act by different mechanisms (ligand trapping, receptor homo- and hetero-dimerization) and can both stimulate or inhibit angiogenesis. In contrast to VEGFR- 1 and VEGFR-2, VEGFR-3 is not activated by VEGF-A but only by VEGF-C and VEGF-D. In adults, it is mainly involved in the regulation of lymphangiogenesis, and its expression is predominantly restricted to lymphatic endothelial cells (Alitalo et al. 2005). The system also includes co-receptors,

whom role is to facilitate VEGFRs activation (Ferrara et al. 2004). Among them, the most important are neuropilins (NRPs), including NRP-1 and NRP-2, which not only enhance the activity of VEGFR-2, but can also stimulate angiogenesis in an independent way (Neufeld et al. 1999). Particularly, NRP-1 has been shown to stimulate the migration, but not the proliferation, of cultured endothelial cells (Mukhopadhyay et al. 2004).

Physiologically, VEGF-A is expressed at low levels in the majority of normal adult tissues, with the exception of renal glomerular podocytes, adrenal cortex, breast, lung, and also macrophages and cardiac myocytes, where the expression is typically high (Berse et al. 1992; Brown et al. 1995; Maharaj et al. 2006). The main inductor of VEGF-A expression is hypoxia, which acts by stimulating both gene transcription and mRNA stabilization (Claffey et al. 1996; Levy et al. 1997). As for other oxygen sensitive proteins, crucial role in VEGF-A transcription is played by hypoxia-inducible factor (HIF), a heterodimeric protein transcription factor. Normally, one HIF-1 peptide, HIF-1α, is rapidly degraded under normoxic conditions through the ubiquitin pathway. By contrast, hypoxia stabilizes HIF-1α, thus allowing its dimerization with HIF-1β. The complex binds to and activates a hypoxia-responsive element in the VEGF-A promoter.

Besides being crucial in physiological angiogenesis, role of VEGF-A is considered predominant also in tumour angiogenesis. Indeed, VEGF-A mRNA up-regulation has been detected in many human tumours by means of *in situ* hybridation (Dvorak et al. 1995; Ferrara et al. 1997). Furthermore, different types of anti-VEGF treatments, such as VEGF-A and VEGFR-2 monoclonal antibodies, small-molecule inhibitors of VEGFR signaling, and antisense oligonucleotides, have showed in vivo inhibition of cell-lines from many tumours (Ferrara et al. 1997; Kim et al. 1993). VEGF-A up-regulation in cancer may be related to several mechanisms, also depending on the tumour type. Hypoxic regulation of VEGF-A has been demonstrated in several tumours (Semenza 2002). However, VEGF-A expression is also increased by

low pH, another hallmark of tumours, and this happens by a HIF-1 independent way (Fukumura et al. 2001b). Given that many other tumours show high expression of VEGF-A under normoxic conditions, it is conceivable that other mechanisms are involved. One of them is the action of oncogenes and tumor suppressor genes (Mukhopadhyay et al. 2004; Rak et al. 2004). Indeed, these genes act not only by stimulating tumour growth and survival, but also by inducing VEGF-A expression and thereby angiogenesis. Another possible mechanism is the production (by tumour or host cells) of growth factors (including EGF, TGF-α, TGF-β, FGF, and PDGF) or inflammatory cytokines (such as IL-1α and IL-6), which have demonstrated to up-regulate VEGF-A expression (Ferrara et al. 1997; Neufeld et al. 1999).

### 1.3.3 The PDGF-system and its role in physiological and tumour angiogenesis

Like VEGFs, members of the PDGF family are dimers of disulfide-linked polypeptide chains (Heldin et al. 1999). Particularly, PDGFs are composed by the combination of four structurally related single polypeptide units, which constitute five homo- or hetero- dimers: PDGF-AA, PDGF-BB, PDGF-AB, PDGF-CC, and PDGF-DD (Andrae et al. 2008; Heldin et al. 1999; Hoch et al. 2003). The A-, B-, C-, and D- chains are encoded from different genes, localized on the chromosomes 7p22, 22q13, 4q31, and 11q22, respectively, and their expression is independently regulated (Betsholtz et al. 2001; Dijkmans et al. 2002; Heldin 1992; LaRochelle et al. 2001; Uutela et al. 2001). Whereas the AA, BB, and AB isoforms are already active when secreted, PDGF-CC and PDGF-DD need to be activated through the cleavage of the CUB domain, performed by plasmin or tissue-plasminogen activator (Bergsten et al. 2001; LaRochelle et al. 2001).

PDGF isoforms act through activation of two structurally related cell surface tyrosine-kinase receptors, namely PDGFR-α and PDGFR-β. Coding genes of

PDGFR-α and PDGFR-β are localized on chromosomes 4q12 and 5q33, respectively. Both receptors are composed from five extracellular immunoglobulin-like domains, a transmembrane domain, a juxtamembrane domain, splitted kinase domains, a kinase insert domain, and a cytoplasmic tail. Each chain of the PDGF dimer interacts with one receptor subunit, thus inducing dimerization. Particularly, PDGFRs may constitute three different dimers, namely αα, αβ, and ββ. This makes possible multiple PDGF–PDGFR combinations, which allow the PDGF system to exert several and complex biological functions. *In vivo* studies of associations between PDGFs and these dimeric receptors have showed as follows: PDGF-AA exclusively act via the αα dimer; PDGF-AB and –CC can activate either the αα and the αβ dimers; PDGF-DD activates only the ββ dimer, but the possible stimulation of the αβ dimer is under study; PDGF-BB is the only isoform showing activity on all three dimers (Bergsten et al. 2001; Claesson-Welsh 1994; Claesson-Welsh et al. 1988; Gilbertson et al. 2001; LaRochelle et al. 2001; Li et al. 2000; Matsui et al. 1989).

Following ligand binding and dimerization, PDGFRs undergo auto-phosphorylation, thus activating an intra-cellular molecular cascade which includes: phospholipase C-γ, the G-protein Ras, the phosphatidylinositol 3-kinase, the growth-factor receptor-bound protein 2, Syp (tyrosine-specific phosphatase), Src homology and collagen protein, Crk (a group of adaptor proteins) and Src, a family of non-receptor tyrosine kinases (Claesson-Welsh 1994). This signaling system culminates in the activation of many factors which regulate gene expression, including mitogen activated protein kinase family members (ERKs, JNKs), and focal adhesion kinase (FAK, a mediator of integrin signaling pathway) among others. This leads to the expression of a panel of immediate-early-response genes involved in the regulation of cell cycle, cell migration, and transformation.

A still discussed point about the physiology of the PDGF system is the differential activity of the two PDGFR subunits. Studies on NIH3T3 clones

demonstrated that the inhibition of PDGFR-α signaling was associated with the enhancement of PDGF-BB(which acts through the activation of all three receptor dimers)-mediated phenotypic transformation, suggesting that PDGFR-α antagonizes PDGFR-β-induced transformation (Yu et al. 2000). Thus, signal from PDGFR-α is thought to regulate PDGF-pathway leading to cell transformation with both positive and negative activity, while PDGFR-β mainly stimulate this process. PDGFR-α-mediated simultaneous activation of both positive and negative signaling has also been demonstrated in cell migration and chemotaxis (Koyama et al. 1994; Yokote et al. 1996). As speculated by the majority of authors, this was likely related to the fact that PDGFR-α, but not PDGFR-β, may activate stress activated protein kinase-1/c-Jun NH2-terminal kinase-1 (SAPK1/JNK-1), which in turn antagonizes PDGF-mediated positive signals. It is conceivable that PDGFR-α-mediated agonistic and antagonistic activities for cell growth and motility represent a fine molecular mechanism for the modulation of PDGF signal basing on genetic background of the cells and additional extracellular factors. PDGFs, especially AA and BB, stimulate proliferation (by acting on G0/G1 transition (Pledger et al. 1981; Stiles et al. 1979)) and/or act as chemotactic agents of mesenchymal cells, including fibroblast, vascular smooth muscle cells, glial cells, macrophages and chondroncytes (Deuel 1987; Heldin 1992). Importantly, PDGFs are able to amplify mitogenic signal by stimulating PDGF itself and other growth factors expression (Clemmons et al. 1981; Paulsson et al. 1987). They are is also involved in the production of collagen (Canalis 1981; Narayanan et al. 1983), fibronectin (Blatti et al. 1988), proteoglycan (Schonherr et al. 1991), hyaluronic acid (Heldin 1992), and collagenase (Chua et al. 1985). These findings are consistent with a relevant role for PDGF system in connective tissue homeostasis. Whereas *in vitro* studies revealed that PDGF induced similar set of molecular events and cellular responses in cells expressing PDGFR-α or PDGFR-β (Rosenkranz et al. 1999), there was a remarkable difference in the phenotype of knock-out mice where genetic

deletion of PDGFR-α or PDGFR-β resulted in early embryonic lethality through different mechanisms (Soriano 1994, 1997). Indeed, PDGFR-α was mainly involved in formation of central nervous system and organogenesis (Lindahl et al. 1997), while PDGFR-β was essential for development of supportive cells in the vasculature failure. Particularly, pericyte migration to the new blood vessels was impaired in PDGFR-β-deficient mice, thus leading to abnormal blood vessel formation and defective cardiovascular system development (Leveen et al. 1994; Soriano 1994). This indicates a more active role for PDGFR-β signal in angiogenesis, as compared with that from PDGFR-α. It has been demonstrated that endothelial tip cell, located at the leading front of angiogenic vessels, release PDGF-BB to chemoattract pericytes specifically harbouring the PDGFR-β receptor (Gaengel et al. 2009; Hellberg et al. 2010). Furthermore, PDGF-BB also allows pericyte recruitment to tumour vessels indirectly, by stimulating VEGF-A expression in the endothelium (Guo et al. 2003). This activity is crucial for maturation and stabilization of neo-formed vessels, as endothelial cells stop proliferating and achieve a quiescence state only when covered by mural cells (Gerhardt et al. 2003; Guarani et al. 2011). Thus, an abnormal pericytes covering induces an uncontrolled endothelial cell growth (Hellstrom et al. 2001), which is typical of tumour angiogenesis. Indeed, the overexpression of PDGF-BB in mice paradoxically inhibits tumour growth by promoting pericyte recruitment and inducing endothelial cell growth arrest (McCarty et al. 2007). Interestingly, a recent study has shown that VEGF-A negatively regulates pericyte function and vessel maturation, and this happens through the inhibition of the PDGF system (Greenberg et al. 2008).

In tumour angiogenesis, activation of the PDGF system mainly relies on the production of PDGF-BB by neoplastic cells. Through this mechanism, tumour cells recruit pericytes not only by the direct action on PDGFR-β expressing endothelial cells, but also through the overexpression of stromal-cell-derived factor-1α. Furthermore, pericytes can also arise from perivascular PDGFR-β+ pericyte progenitors, recruited from the bone marrow (Song et al. 2005). Up-

regulation of PDGFs and PDGFRs has been detected in various cancers, including glioma, Kaposi's sarcoma, prostate cancer, and pancreatic cancer (Heinrich et al. 2003; Hermanson et al. 1992; McCarty et al. 2007; Sitaras et al. 1988). Particularly, PDGFR-α is mainly expressed in tumour cells, whereas PDGFR-β is expressed in stromal and peri-vascular cells (Hellstrom et al. 1999; Hermanson et al. 1992; Soriano 1994). However, genetic alterations specifically involving the PDGF system and inducing its activation have been found in different tumours (Simon et al. 1997; Sirvent et al. 2003). Co-expression of ligands and receptors in malignant cells suggests the existence of an autocrine loop for the PDGF system in the stimulation of tumour cells growth and motility (Heldin et al. 1987). Particularly, PDGFs, namely the AA and BB isoforms (already active before secretion), may induce cellular transformation not only by extra-cellular, but also by an intra-cellular autocrine mechanism, which is based on the interaction with PDGFRs in the endoplasmic reticulum where they are subjected to phosphorylation (Bejcek et al. 1989; Keating et al. 1988). In animal tumour models, autocrine activation of PDGF signaling promotes breast cancer metastasis (Jechlinger et al. 2006). Furthermore, autocrine and paracrine activation of the PDGF system may induce epithelial–mesenchymal transition, a characteristic feature of cancer invasion and metastasis, in several cancer types, including breast cancer, prostate cancer, and mesothelioma (Bierie et al. 2006; Jechlinger et al. 2006; Kong et al. 2008; Patel et al. 2010). The role of PDGF system in tumour progression is further confirmed by the fact that PDGFRs inhibition reduces growth by determining pericyte detachment and therefore vessel regression (Bergers et al. 2003b). Similarly to VEGF system, this suggest a more active role of PDGFs signaling in promoting tumour progression, rather than initial neoplastic transformation. Indeed, expression of PDGF-BB in PDGFR-negative tumours leads to hypervascularization and accelerated tumour growth rates (Nissen et al. 2007; Xue et al. 2012). In a glioma model, tumour-cell-derived PDGF-BB stimulates migration of PDGFR-β expressing endothelial

cells, accelerating tumour progression (Guo et al. 2003). PDGF-BB also stimulates peri- and intra-tumoral lymphangiogenesis by a direct action on lymphatic endothelial cells, thus favouring occurrence of metastases in sentinel lymph nodes (Bruyere et al. 2008; Cao et al. 2004). However, the involvement of PDGF system in both physiological and pathological angiogenesis is highly complex, being characterized by strict interactions with other angiogenic factors such VEGF-A, angiopoietins, and FGFs (Nissen et al. 2007). This may in part explain the dual role of PDGF system in tumour evolution. In primary tumours activation of PDGF system has a protective action, as it allows neo-vessels stabilization and limits tumour cells intra-vasation, thus antagonizing neoplastic spread (Gerhardt et al. 2008). In this early phase of tumour evolution PDGF blockage can paradoxically promote malignancy. By contrast, activation of PDGFRs sustains tumour progression in a more advanced phase of the disease, once micro-metastatic sites have been already developed.

## 1.4 Genetics of angiogenesis and cancer

Similarly to the majority of biological processes, angiogenesis presents a wide variability between individuals. The major factor affecting this variability is genetic background. Indeed, a large cohort study of 478 individuals demonstrated that genetics accounts for almost 80% of circulating VEGF-A variability, whereas environmental factors determined only 20% (Pantsulaia et al. 2004). Furthermore, studies of nuclear families revealed significant correlations between circulating VEGF-A in all pairs of relatives, excluding spouses (Berrahmoune et al. 2007).

Given the complexity of angiogenesis, study of the underlying genetic basis, the so-called “angio-genome”, is challenging. According to the current evidence, various types of genetic variability may affect angiogenesis, and this happens mainly through the modulation of gene expression (Rogers et al. 2012). Thus, a myriad of genetic and molecular modifications, potentially

regulating gene expression, may be involved, including genetic mutations or single nucleotide polymorphismsb(SNPs) (McCarthy et al. 2008), translocations, copy number variations (Beckmann et al. 2007), epigenetic changes (DNA methylation and histone modifications) (Schones et al. 2008), and miRNAs (Couzin 2008).

Although several models have been developed in animals, no assays are available for quantifying angiogenic-response in humans. Thus, knowledge about human "angio-genome" mainly relies on candidate gene studies, focusing on whether genetic variability of genes already known as associated with angiogenesis can affect angiogenesis-dependent diseases. Particularly, several studies assessing the relationship of angiogenic SNPs with susceptibility and aggressiveness of cancer have been performed, with heterogeneous results mainly depending on the tumour type (Rogers et al. 2012). The main weakness of this approach is that no novel genes can be found, so it is possible that genetic features strongly affecting angiogenesis have not been identified yet. Currently, these studies are mainly performed by testing for single SNPs.

SNPs are inherited germline genetic variants that are commonly found in the human genome (Frazer et al. 2007). As compared with mutations, SNPs often involve the substitution of a single nucleotide base, but are more frequent, with a minor allele frequency of 1% or more (Efferth et al. 2005). SNPs can be found in any part of the human genome, including regulatory regions of genes (promoter or 3´-untranslated regions), within intronic or exonic sequences and within inter-genic regions. SNPs within a DNA sequence distinguish into three subgroups characterized by their location in potentially coding (cSNP), regulatory (rSNPs) or splicing (sSNP) regions of the human genome. The majority of SNPs are clinically silent without any functional implication on the final gene product. Although belonging to a specific gene, SNPs may also be able to regulate other genes, either on the same chromosome or other different chromosomes. Due to the variations in genetic recombination of different

regions of chromosomes, these single variants can often be inherited together as a linkage disequilibrium (LD) block, which is defined ‘haplotype’.

### 1.4.1 VEGF-A SNPs and cancer

The human VEGF-A is a highly polymorphic gene, with hundreds of SNPs disseminated through the various regions already registered in the National Center for Biotechnology Information Databank (NCBI) SNP database (http://www.ncbi.nlm.nih.gov). The majority of SNPs are functionally neutral, and those showing association with diseases are believed to modulate VEGF-A function by affecting its expression (Je et al. 2009; Renner et al. 2000). Indeed, large-scale studies observed that disease-associated SNPs were frequently located in non-coding genomic regions (rSNPs), particularly the promoter, the 5’UTR, and the 3’UTR, which are significantly involved in transcriptional and post-transcriptional gene modulation (Arcondeguy et al. 2013).

Out of 24 SNPs indentified within the promoter and 5’UTR regions, about one-third showed correlation with severity of diseases (Metzger et al. 2015). The main hypothesis is that these SNPs may affect VEGF-A function through the elimination or creation of transcription-factors binding sites (TFBS), thus being able to induce strong modifications in gene expression (Ponomarenko et al. 2002; Stenson et al. 2009; Wray 2007). For example, the promoter SNP -2578 C>A (rs699947) has been associated to severity of many diseases, both benign (such as atherosclerosis and rheumatoid arthritis) and malignant (such as breast and lung cancer) (Chen et al. 2012; Howell et al. 2005; Jin et al. 2005; Kammerer et al. 2010). The minor homozygous genotype AA is associated with decreased serum levels of VEGF-A, thus indicating that the SNP affects VEGF-A function by acting on its expression (Shahbazi et al. 2002). Importantly, it has been reported that the C-allele of the -2578 C>A offered the binding for the dimer HIF1α/β, which represents the main mediator of hypoxia-inducted VEGF-A production, whereas the A-allele eliminates the site (Buroker et al. 2013). Thus, the absence of any binding for HIF1α/β may

explain the significant reduction of VEGF-A expression in subjects carrying the AA-genotype, and therefore the association with disease. Similarly, the 5'UTR SNP +405 G>C (rs2010963) is associated to severity of several diseases (Awata et al. 2002; Jin et al. 2005), and its GG-genotype, which represents the common homozygous variant, is associated with the highest VEGF-A production (Watson et al. 2000). Regarding its impact on TFBS, this SNP is located within a potential myeloid zinc finger protein binding site and may affect the binding specificity (Watson et al. 2000). Particularly, a reduction of the binding specificity has been associated to the minor C-allele, which may explain its association with a loss in VEGF-A production (Jin et al. 2005). Despite their impact on TFBS is still unclear, other SNPs of the promoter/5'UTR affecting gene function are: the -460 T>C (rs833061), located in the promoter, where a lowered VEGF-A production for carriers of the common T-allele was reported (Hansen et al. 2010b); the -1154 G>A (rs1570360), located in the promoter, where a reduced VEGF-A production was reported for the common G-allele.

Regarding SNPs located in the 3'UTR region, it is conceivable that they can affect VEGF-A function by acting at the post-transcriptional level, through the modification mRNA stability. Among the 3′UTR SNPs, the only one showing an association with diseases and with VEGF-A production is the +936 C>T (rs3025039), where the minor allele T was associated with significantly lower VEGF-A levels (Krippl et al. 2003; Renner et al. 2000).

In recent years, growing interest to the assessment of VEGF-A SNPs as feasible markers of prognosis in cancer has been observed, particularly for the epidemiologically prevalent tumours. Results from different studies were sometimes controversial, basing on the tumour type, the geographic area, and also whether germline or somatic SNPs were subjected to analysis. In colorectal cancer, the SNP +936 C>T revealed the major prognostic significance. Of 3 large studies, 2 showed that patients carrying the T allele had improved outcome (Dassoulas et al. 2009; Lurje et al. 2008), whereas 1

showed worsened outcome related to T allele (Kim et al. 2008b). Importantly, the papers reporting the protective effect of the T allele assessed germline SNPs, whereas the one showing worsened prognosis performed the analysis at the somatic level. There was more consistency with the +405 G>C, where the cited papers from both Dassoulas et al. (Dassoulas et al. 2009) and Kim et al. (Kim et al. 2008b) showed improved outcome in patients carrying the C allele, despite analysis was performed at the germline and somatic level, respectively. By contrast, other 2 studies found no correlation between this SNP and prognosis (Hansen et al. 2011; Lurje et al. 2008). The study by Dassoulas (Dassoulas et al. 2009) also found an association between the A allele of -2578 C>A and improved OS but this finding was not confirmed by three other studies (Hansen et al. 2011; Kim et al. 2008b; Vidaurreta et al. 2010). Among the 5 most important breast cancer studies, SNPs -7 C>T and +405 G>C were found to be slightly associated with OS (Koutras et al. 2015). Of 4 papers about genitourinary cancers (Kawai et al. 2007; Kim et al. 2005; Li et al. 2005; Mucci et al. 2009), only one reported significant results, namely the association between the A allele of -2578 C>A and improved OS in renal cell carcinoma (Kawai et al. 2007). Nevertheless, another study in bladder cancer did not confirm this association (Stenson et al. 2009). Out of 8 gynaecological cancer studies (Amano et al. 2008; Goode et al. 2010; Hefler et al. 2007; Lose et al. 2010; Polterauer et al. 2007), a considerable relationship between the C allele of the SNP +405 G>C and poor prognosis was found in two papers (Amano et al. 2008; Lose et al. 2010). Furthermore, the -460 T>C was found to be associated with OS in some studies (Goode et al. 2010; Lose et al. 2010). Among the 4 lung cancer studies, the C allele of +405 G>C, the C allele of -460T>C and the G allele of -1154G>A were found to be associated with improved OS in separate studies (Guan et al. 2010; Heist et al. 2008). The five most commonly evaluated VEGF SNPs (+405 G>C, -460 T>C, -1154 G>A, -2578 C>A and +936 C>T) were also included in a pooled meta-analysis, and a strong correlation between the C allele of the SNP +405 G>C and improved

OS was observed (hazard ratio: 0.79; 95% CI: 0.67–0.94; p = 0.007) (Eng et al. 2012).

No studies are available about the association between VEGF-A SNPs and clinical outcome of thyroid cancer. Nevertheless, a single study by Hsiao et al., making the analysis at the germline level, found that the A allele of -2578 C>A was associated with increased risk of developing DTC and to the presence of lymph node metastases among men (Hsiao et al. 2007).

### 1.4.2 VEGFR-2 SNPs and cancer

Genetic variability may affect the biological function of VEGFR-2. Particularly, several SNPs have been identified in both coding and regulary regions of the VEGFR-2 gene, with potential impact on protein function. Among others, the rSNP -604 C>T (rs2071559), and the nonsynonimous cSNPs 1192 C>T (rs2305948) and 1719 T>A (rs1870377) have been mostly characterized regarding their impact on VEGFR-2 function.

The -604 C>T is located in the promoter, and research found that genetic variants of this SNP suppress transcriptional activity, thus leading to down-regulation of expression level of VEGFR-2 (Wang et al. 2007). This is likely due to alteration of the binding site for transcriptional factor E2F (Wang et al. 2007). Furthermore, Galan et al. found that the -604 C>T, particularly the C-allele, exerts inhibition of VEGF-A signal and was associated with increased risk of age-related macular degeneration (Garcia-Closas et al. 2007).

SNPs 1192 C>T and 1719 T>A are located in exon 7 and 11, respectively, which correspond to the third and fifth NH2-terminal immunoglobulin-like domains within the extracellular region (Leppanen et al. 2010). C>T variant of rs2305948 leads to change of amino acid at residues 297V>I and similar change at residues 472H>Q happened with T>A variant of rs1870377. Functional research discovered that the exchange of these residues decreases the binding efficiency to VEGF-A (Wang et al. 2007; Zhang et al. 2009).

As compared with VEGF-A SNPs, fewer and non conclusive data are available about possible impact of VEGFR-2 SNPs on cancer prognosis. In colorectal cancer, the -604 CC genotype was associated with increased microvessel density and decreased survival, whereas the 1192 CC genotype was associated with decreased microvessel density and increased survival (Hansen et al. 2010a). Nevertheless, studies on breast and lung cancer did not reveal any significant association. Interesting but not conclusive data about a possible correlation of VEGFR-2 SNPs with clinical outcome of glioblastoma have been also reported (Sjostrom et al. 2011).

No studies are available about the association between VEGFR-2 SNPs and thyroid cancer.

### 1.4.3 PDGFRs SNPs and cancer

Although up-regulation of the PDGFs and their receptors has been reported for many cancers (as aforementioned), the knowledge about association between SNPs relying on these genes and cancer is still at a preliminary level (Cao 2013). Nevertheless, some data about association of SNPs of PDGFR-α and PDGFR-β with disease are available. Particularly, all identified SNPs are located in the promoter regions, thus indicating a possible impact on gene expression. Wu et al. reported that the promoter SNP -635 G>T (rs1800810) of PDGFR-α was associated with the severity and allergic status of childhood asthma (Wu et al. 2006). Kim et al. reported the association of 3 promoter SNPs of PDGFR-β (rs3756314, rs3756312, and rs3756311) with schizophrenia (Kim et al. 2008a). Besides these benign conditions, De Bustos et al. revealed the association of a specific promoter haplotype of PDGFR-α with the occurrence of primitive neuroectodermal tumors and ependymomas (De Bustos et al. 2005).

Our interest in involving the PDGF system in the present study about angiogenic SNPs and thyroid cancer follows the findings recently reported by Kim et al. (Kim et al. 2012). Indeed, authors found that two promoter SNPs,

namely the aforementioned -635 G>T and the -1309 G>A (rs6554162) of PDGFR-α, were associated with PTC susceptibility. Particularly, frequencies of the A allele of -1309 G>A and the T allele of -635 G>T, therefore the minor variants, were decreased in the PTC group, thus suggesting a protective effect. This association was also confirmed through haplotype analysis based on the identification of the GG and AT blocks. Importantly, analysis was performed only at the somatic level.

Recently, polymorphic sites within the PDGFR-α promoter have been deeply characterized (Joosten et al. 2001), and was clearly demonstrated that they may affect the transcriptional regulation of the gene. Particularly, authors identified 5 promoter haplotypes, displaying wide difference in their ability to induce reporter gene expression in human U2-OS osteosarcoma cells. To date, transcription factors involved in this haplotype specific PDGFR-α promoter regulation remain unknown.

## 2. Aims of the study

General objectives of the study were: a) to find out novel and easily available molecular markers that could improve prognostic stratification, and therefore clinical management of patients affected with DTC; b) to speculate about the biological role of angiogenesis in a “simple” cancer model such as DTC, where treatment strategy is almost similar in all patients, independently from initial pathological features.

Main aim was to evaluate germline SNPs of VEGF-A, VEGFR-2, and PDGFR-α, as prognostic markers of clinical outcome in DTC.

# 3. Materials and Methods

## 3.1 Patients and samples

We performed a multicenter retrospective study involving 4 neighbour centers from Naples: University Federico II; *INT Pascale*; Second University of Naples; *Cardarelli* hospital. The study was approved by the Ethics Committees of each included center and informed content was obtained from each patient before the enrolment. Inclusion criteria were: a) histological diagnosis of DTC at local pathological review; b) diagnosis and follow-up entirely performed at a single institution; c) availability of clinico-pathological data d) at least 18 months of follow-up after surgery. Exclusion criterium: patients younger than 18 years. Blood samples were obtained from consecutive DTC patients afferent to the involved centers from October 2013 to October 2015. At the time of enrolment, all patients were subjected to clinical/biochemical/instrumental follow-up after receiving the conventional treatment approach (total thyroidectomy with/without RAI, followed by TSH suppression). Clinico-pathological data had been prospectively collected according to recommendations in each center. All files were reviewed by a single investigator (the candidate Vincenzo Marotta). Data recorded included: gender (male/female); age at diagnosis (years); histology (histotype and variants); primary tumour size (cm); multifocality (yes/no); extra-thyroidal extension (yes/no); concomitant Hashimoto's thyroiditis (yes/no); lymph node status (Nx/N0/N1); distant metastasis (yes/no); AJCC/UICC stage (I/II/III/IVa-b-c); ATA group risk (low/intermediate/high). Data about clinical outcome were obtained by consulting the files and, if necessary, by interviewing the attending physician or the patient himself. Follow-up data were last updated in December 2015 for all included patients.

### 3.2 Polymorphisms

Overall, 8 angiogenesis-related SNPs were included in the analysis: -2578 C>A (rs699947), -460 T>C (rs833061), +405 G>C (rs2010963), and +936 C>T (rs3025039) for the VEGF-A gene; +1192 C>T (rs2305948) and +1719 T>A (rs1870377) for the VEGFR-2 gene; -1309 G>A (rs6554162) and -635 G>T (rs1800810) for the PDGFR-α gene. The selection of this specific set of angiogenic SNPs was performed basing on the following criteria: a) previous documentation and characterization; b) preceded publications attesting the possible impact on protein function; c) previous data about the impact on cancer prognosis; d) previous data about the relationship with DTC. Current knowledge about each of these issues has been already discussed in the *Background* section.

### 3.3 DNA extraction and genotyping

DNA was extracted and purified from peripheral blood according to the manufacturer protocol using a QIAamp tissue kit (Qiagen, Hilden, Germany). DNA concentration was determined by means of NanoDrop® (Wilmington, DE) ND-1000 Spectrophotometer and samples were diluted to 10 ng/μl. SNP genotyping was carried out according to the TaqMan® genotyping protocol (Applied biosystems StepOnePlus$^{TM}$) with 20-ng DNA template. The TaqMan SNP genotyping is an advanced, validated, and widely used technology having high throughput and low running costs (Borodina et al. 2004; Giles et al. 2004; Hampe et al. 2001). It requires forward and reverse PCR primers, and two differently labeled TaqMan minor groove binder (MGB) probes. Briefly, the bi-allelic SNP is located in the middle third of the probe. Each allele-specific MGB probe is labeled with a fluorescent reporter dye (either a FAM or a VIC reporter molecule) and is attached with a fluorescence quencher. When the MGB probe is intact, the reporter dye is quenched. During PCR, the 5'-nuclease activity of Taq DNA polymerase cleaves the reporter dye (FAM or VIC) from an MGB probe that is completely hybridized to the DNA

strand. Once separated from the quencher, the reporter dye fluoresces. By contrast, if a single point mismatch is present between the probe and the target DNA strand because of a SNP, the binding of the probe to DNA is destabilized during PCR, and this prevents the probe cleavage to happen efficiently, thus letting the fluorescent reporter dye remain quenched. Therefore, an increase in either FAM or VIC dye fluorescence indicates homozygosity for FAM- or VIC specific alleles, whereas an increase in the fluorescence of both dyes indicates heterozygosity. By means of this method, genotypes of multiple samples can be rapidly generated, thus allowing the simultaneous analysis of a SNP in many patients. For each analyzed SNP existing and established TaqMan® genotyping assays were used. A positive control, previously verified by sequencing, was used for confirming homozygous genotypes. We used a 96-well plate. For overall quality assurance, 10% of analyzed samples were randomly selected and analysis was repeated in triplicates. Genotype concordance was ≥99%.

### 3.4 Clinical management during follow-up

Despite being a multicenter study, involved centers were neighbours and strictly interacting. Thus, clinical management was homogeneous between different institutions. All patients with tumours ≥1cm were treated by means of total thyroidectomy, whereas DTC <1cm (microcarcinomas) were subjected to near-total thyroidectomy. Lymphadenectomy was performed in case of clinically involved lymph nodes with therapeutic intent (central and/or lateral dissection) and in patients with T3/T4 primary tumour (lesions >4 cm and/or with extra-thyroidal extension) without evident lymph node involvement with prophylactic intent (central compartment). Post-surgery RAI ablation was performed in all patients with the exception of unifocal microcarcinomas (pT1a according to the AJCC/UICC classification). Preparation and treatment procedures were in accordance with dedicated guidelines from the Society for

Nuclear Medicine and Molecular Imaging and the European Association of Nuclear Medicine (Luster et al. 2008; Silberstein et al. 2012). Particularly, I-131 was administered by means of thyroid-hormone withdrawal (thus achieving TSH level ≥30 mU/ml) 5-12 months after surgery. Administered activity ranged from 50 to 163 mCi, basing on disease characteristics and patient age. Importantly, all RAI treatments were performed at 2 Nuclear Medicine referral centers (University Federico II and *INT Pascale*), and this further ensures homogeneity in treatment procedures. After thyroid ablation, patients were subjected to TSH suppressive therapy. During follow-up, patients were subjected to clinical (neck palpation), biochemical (thyroglobulin [Tg] Tg and Tg-antibodies [AbTg] levels) and instrumental (neck ultrasonography [US]) examinations every 6 months. Twelve-eighteen months after surgery patients were subjected to recombinant human TSH stimulation test to attest remission from disease. During follow-up, patients showing measurable basal (under TSH suppression) or stimulated Tg, suspicious neck US findings, or both were advised to morphological or functional imaging or both, including computed tomography or 18-fluorodeoxyglucose positron emission tomography. All ultrasonographically suspicious nodules ≥1 cm in diameter underwent fine-needle aspiration with measurement of Tg in the aspirate.

### 3.5 Definitions of clinical outcome

Patients were classified as having no evidence of disease (NED) if at the time of final follow-up the suppressed Tg was <1 ng/ml, AbTg were negative, neck US did not present suspicious finding, and there were no pathological findings on any other study performed for clinically indicated reasons, such as I-131 whole-body scan, radiography, computed tomography, 18-fluorodeoxyglucose positron emission tomography, or biopsy. Patients showing measurable basal/stimulated Tg and/or raising AbTg after thyroid ablation until last follow-up and did not present any structural evidence of disease were classified as

having biochemical persistent disease. Patients showing structural evidence of disease, independently from Tg and AbTg levels, after thyroid ablation until last follow-up were classified as having structural persistent disease. Patients achieving remission, defined as a period of NED after conventional therapeutic approach, who develop a new biochemical (measurable basal/stimulated Tg and/or raising AbTg) or structural evidence of disease were classified as having recurrent disease (biochemical or structural). Dates of recurrences were carefully recorded in order to calculate the disease-free survival (DFS), defined as the length of time after achieving NED in which the patient was without any evidence of disease.

### 3.6 Statistical analysis

For all statistical analyses, SPSS version 20.0 for Windows (SPSS Inc., Chicago, IL) was used. Chi-square test was applied for assessing Hardy-Weinberg equilibrium. The deviation of the SNPs Hardy-Weinberg equilibrium was established at $p>0.05$. Relationship of each SNP with clinical outcome and with clinico-pathological features at diagnosis was assessed by considering the genotype as a three-group categorical variable in accordance to the reference model (homozygous common variant versus heterozygous versus homozygous minor variant) and by grouping in accordance to the dominant (homozygous common variant versus heterozygous + homozygous minor variant) and recessive (homozygous common variant + heterozygous versus homozygous minor variant) models. In case of minor homozygous genotype frequency ≤10%, analyses were performed exclusively by means of dominant model. Group comparisons of categorical variables were performed by means of chi-square test. ANOVA T-test was used to compare continuous variables between genotypes. Results were reported as number and percentage of genotypes within each group for categorical variable and as median[range] for each genotype for continuous variable. Relative risk (RR) with 95% confidence

interval (CI) was calculated for each variable. Survival analyses were performed according to the Kaplan–Meier method, and the log-rank test was used to test for differences between groups. Estimate of allelic frequencies and haplotype analysis were performed by means of the Haploview software. The degree of LD was expressed by Lewontin coefficient (D') and by coefficient of correlation of $r^2$. Both parameters estimate the non-random association of alleles at two loci. Association of haplotype frequencies with clinical outcome was performed by means of Haploview, and odds ratios (OR) with 95% CI were provided by the software. Given that biochemical persistences/recurrences hesitate in structural disease only in 20% of cases (Haugen et al. 2016), we decided to exclude these conditions from the outcome analysis. Therefore, we considered as prognostic endpoints: persistent structural disease, recurrent structural disease, NED at last follow-up, and DFS when performing survival analysis. Accuracy of genotypes as prognostic markers was assessed according to Galen (Galen 1982), by considering true positive (TP), true negative (TN), false positive (FP), and false negative (FN) results. The PPV was TP/(TP+FP) and the negative predictive value (NPV) was TN/(FN+TN). The 95% CI of all these estimates was also evaluated. Binary logistic regression analysis was applied for adjusting genotypes with significant association with clinical outcome at univariate analysis for selected factors having demonstrated prognostic impact. In multivariate analysis, OR with 95% CI were reported. All tests were two sided, and p-values of less than 0.05 were used for considering an association of statistical significance.

# 4. Results

## 4.1 Study cohort

Overall, 249 patients were included in the study. Demography, pathological features, staging according to the AJCC/UICC system, risk stratification according to the ATA guidelines, and clinical outcome are summarized in Table 1. Briefly, study population included 46 males (18%) and 203 females (82%). Median age at the time of diagnosis was 43 years (range 15-74). Mean and median follow-up were 67±54 months and 3.75 years (45 months), respectively.

As expected, the vast majority of patients (90%) were affected with PTC, being the classic variant the most represented subgroup. Forty-one (16%) patients were considered as having aggressive histology, with inclusion of 3 tall cell, 10 diffuse sclerosing, and 4 solid PTC variants, and 24 FTC. Remarkable features of our study population were the inclusion of a relevant percentage of microcarcinomas (35%) and the low portion of patients showing distant metastases at diagnosis (2%). This resulted in lower portion of subjects with advanced staging (10% for stage III and 8% for stage IV) and having high risk of recurrence (4%), as compared with recent large cohort studies about DTC (Castagna et al. 2011; Pitoia et al. 2013; Tuttle et al. 2010b; Vaisman et al. 2012).

After thyroid ablation, 7 patients (3%) showed persistent structural disease. Of them, 4 subjects (57%) had distant metastases at the time of diagnosis. Recurrent disease was observed in 42 subjects (17%) achieving a period of NED. Importantly, 35 of them (14%) showed structurally confirmed recurrence, whereas isolated biochemical recurrence (measurable basal/stimulated Tg and/or raising AbTg) without any structural correlate was observed in 7 patients (3%). However, at the time of last follow-up 65% of patients were classified as NED.

**Table 1. Demographic, clinico-pathological and prognostic features of the study population of patients with DTC ( n=249).**

| | |
|---|---|
| **Median age (years) at diagnosis [range]** | 43 [15-74] |
| **Sex-ratio (males/females)** | 46/203 |
| **Median follow-up (months) after diagnosis [range]** | 37 [12-281] |
| **Histology, number (%)** | |
| **Papillary** | 225 (90) |
| **Classic variant** | 116 (47) |
| **Follicular variant** | 50 (20) |
| **Warthin-like variant** | 17 (7) |
| **Hurtle-cells variant** | 7 (3) |
| **Tall-cell variant** | 3 (1) |
| **Diffuse sclerosing variant** | 10 (4) |
| **Solid variant** | 4 (2) |
| **Unknown papillary variant** | 18 (7) |
| **Follicular** | 24 (10) |
| **Median primary tumor size, cm [range]** | 1.2 [0.1-8.5] |
| **Microcarcinoma, number (%)** | 88 (35) |
| **Multifocality, number (%)** | |
| **Yes** | 60 (24) |
| **No** | 173 (69) |
| **Unknown** | 16 (6) |

| | |
|---|---|
| **Extra-thyroidal extension, number (%)** | |
| **Yes** | 63 (25) |
| **No** | 162 (65) |
| **Unknown** | 24 (10) |
| **Concomitant autoimmune thyroiditis, number (%)** | |
| **Yes** | 85 (34) |
| **No** | 116 (47) |
| **Unknown** | 48 (19) |
| **Lymph node metastasis, number (%)** | |
| **N0** | 68 (27) |
| **N1** | 68 (27) |
| **Nx** | 113 (45) |
| **Distant metastasis, number (%)** | |
| **Yes** | 6 (2) |
| **No** | 239 (96) |
| **Unknown** | 4 (2) |

| | |
|---|---|
| **Radiometabolic treatment, number (%)** | |
| **Yes** | 190 (76) |
| **No** | 49 (20) |
| **Uncertain** | 10 (4) |
| **I-131 ablation dose mCi, median [range]** | 100 (42-163) |
| **I-131 cumulative dose mCi, median [range]** | 141 (42-964.5) |
| **AJCC/UICC Stage, number (%)** | |
| **I** | 189 (76) |
| **II** | 8 (3) |
| **III** | 25 (10) |
| **IV** | 20 (8) |
| **IVa** | 14 (6) |
| **IVb** | 0 (0) |
| **IVc** | 6 (2) |
| **Uncertain** | 7 (3) |
| **ATA initial risk classification, number (%)** | |
| **Low** | 113 (45) |
| **Intermediate** | 118 (47) |
| **High** | 9 (4) |
| **Uncertain** | 9 (4) |

| | |
|---|---|
| **Clinical status at last follow-up , number (%)** | |
| **Persistent structural disease** | 7 (3) |
| **Recurrent disease** | 42 (17) |
| **Biochemical** | 7 (3) |
| **Structural** | 35 (14) |
| **NED** | 161 (65) |
| **Uncertain** | 3 (1) |

**AJCC/UICC:American Joint Commettee on Cancer/Union for International Cancer Control; ATA:American Thyroid Association.**

### 4.2 Polymorphisms: alleles and genotypes frequencies and Hardy-Weinberg equilibrium

Genotyping of the 8 selected SNPs was successfully performed in all patients. Alleles and genotypes frequencies, as well as results from Hardy-Weinberg equilibrium test are reported in Table 2 (alleles frequencies reported as minor allele frequency [MAF]). Allele frequencies were consistent with those reported in the NCBI SNP database for the Caucasian population. Furthermore, they were highly similar to those detected in a cohort of healthy control subjects from the same geographic area (n=143, data not shown). Genotype frequencies conformed to Hardy-Weinberg equilibrium for all SNPs ($p>0.05$). Five SNPs (+936 C>T for VEGF-A; +1192 C>T and +1719 T>A for VEGFR-2; -1309 G>A and -635 G>T for PDGFR-α) showed minor homozygous genotype frequency less than 10% and were therefore analyzed basing on the dominant model (as previously specified).

**Table 2. Distribution of genotypes, MAF and results from the Hardy-Weinberg equilibrium test for the analyzed SNPs.**

| SNPs | Genotype n (%) | MAF | Hardy-Weinberg equilibrium p-value |
|---|---|---|---|
| -2578 C>A (rs699947) | CC 92 (37); CA 113 (45); AA 44 (18) | 0.404 | 0.420 |
| -460 T>C (rs833061) | TT 91 (37) TC 113 (45) CC 45 (18) | 0.408 | 0.394 |
| +405 G>C (rs2010963) | GG 86 (35) GC 123 (49) CC 40 (16) | 0.408 | 0.844 |
| +936 C>T (rs3025039) | CC 190 (76) CT 52 (21) TT 7 (3) | 0.133 | 0.232 |
| +1192 C>T (rs2305948) | CC 193 (78) CT 55 (22) TT 1 (0.5) | 0.114 | 0.273 |
| +1719 T>A (rs1870377) | TT 154 (62) TA 79 (32) AA 16 (6) | 0.223 | 0.24 |
| -1309 G>A (rs6554162) | GG 142 (57) GA 94 (38) AA 13 (5) | 0.241 | 0.774 |
| -635 G>T (rs1800810) | GG 174 (70) GT 70 (28) TT 5 (2) | 0.161 | 0.709 |

**MAF=Minor allele frequency**

### 4.3 Association of clinico-pathological factors with clinical outcome

In order to attest the consistency of our study population and the accuracy of data collection and follow-up assessment, thus facilitating the interpretation of translational analysis, we firstly evaluated the relationship between clinico-pathological factors and clinical outcome.

Results from this analysis are reported in Table 3. Our findings were almost conformant to what expected basing on the majority of studies about clinical prognostication of DTC (Baek et al. 2010; Ghossein et al. 2014; Jukkola et al.

2004; Simpson et al. 1987): microcarcinoma (which we used as categorical variable to assess the prognostic impact of tumour size) ($p<0.0001$, RR=0.16, 95% CI 0.05-0.51), multifocality ($p=0.001$, RR=3.01, 95% CI 1.53-5.93), extra-thyroidal extension ($p<0.0001$, RR=3.71, 95% CI 1.81-7.61), and lymph node metastases ($p=0.004$, RR=3.5, 95% CI 1.37-8.94) were associated with recurrent structural disease among patients achieving NED after thyroid ablation; microcarcinoma ($p<0.0001$, RR=1.48, 95% CI 1.25-1.76), multifocality ($p<0.0001$, RR=0.56, 95% CI 0.41-0.76) extra-thyroidal extension ($p<0.0001$, RR=0.58, 95% CI 0.43-0.76), lymph node metastases ($p<0.0001$, RR=0.51, 95% CI 0.37-0.72), and distant metastasis ($p=0.001$, RR 0.21 95% CI 0.03-1-3) were associated with NED at last follow-up; age at diagnosis (≥ 45 years $p=0.027$, RR=7.4, 95% CI 0.9-60.7), unfavourable histology (involving aggressive variants of PTC and FTC) ($p=0.005$, RR=6.26, 95% CI 1.45-26.91), multifocality ($p=0.006$, RR=11.33, 95% CI 1,29-99.4), extra-thyroidal extension ($p=0.032$, RR=5.16, 95% CI 0.97-27.46), and distant metastases ($p<0.0001$, RR=52.54, 95% CI 14.89-184.64) were associated with persistent structural disease.

As expected, ATA classification was able to predict all analyzed clinical outcomes: recurrent structural disease: $p<0.0001$; intermediate risk RR=7.72, 95% CI 2.39-24.91; high risk RR=27.75, 95% CI 7.93-96.99; NED: $p<0.0001$; intermediate risk RR=0.6, 95% CI 0.5-0.73; high risk RR=0.35, 95% CI 0.26-0.47; persistent structural disease: intermediate risk RR=1.91, 95% CI 0.17-20.82; high risk RR=56, 95% CI 7.06-444.05.

**Table 3. Relationship between clinico-pathological features of DTC at diagnosis and clinical outcome.**

| | Persistent structural disease, N(%) | | | | Recurrent structural disease, N(%) | | | | NED, N(%) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Yes | No | Relative risk(95%CI) | p-value | Yes | No | Relative risk (95%CI) | p-value | Yes | No | Relative risk (95%CI) | p-value |
| **Gender** | | | | | | | | | | | | |
| **Male** | 1(2.2) | 43(93.5) | 0.76(0.09-6.19) | 0.801 | 7(15.2) | 36(78.3) | 1.14(0.53-2.43) | 0.738 | 31(67.4) | 13(28.3) | 1.09(0.88-1.36) | 0.441 |
| **Female** | 6(3) | 196(96.6) | 1(reference) | | 28(13.8) | 168(82.8) | 1(reference) | | 130(64) | 72(35.5) | 1(reference) | |
| **Age at diagnosis** | | | | | | | | | | | | |
| **<45yrs** | 1(0.7) | 135(97.8) | 1(reference) | **0.027** | 21(15.2) | 114(82.6) | 1(reference) | 0.65 | 87(63) | 49(35.5) | 1(reference) | 0.588 |
| **≥45 yrs** | 6(5.4) | 104(93.7) | 7.4(0.9-60.7) | | 14(12.6) | 90(81.1) | 0.86(0.46-1.61) | | 74(66.7) | 36(32.4) | 1.05(0.87-1.26) | |
| **Histology*** | | | | | | | | | | | | |
| **Favourable** | 3(1.6) | 185(97.4) | 1(reference) | **0.005** | 22(11.6) | 163(85.8) | 1(reference) | 0.178 | 127(67.6) | 61(32.4) | 1 (reference) | 0.13 |
| **Unfavourable** | 4(9.8) | 36(87.8) | 6.26(1.45-26.91) | | 8(19.5) | 28(68.3) | 1.08(0.57-2.04) | | 22(55) | 18(45) | 0.81(0.60-1.09) | |
| **Tumor size**** | | | | | | | | | | | | |
| **Microcarcinoma** | 1(1.1) | 87(98.9) | 0.29(0.03-2.44) | 0.229 | 3(3.4) | 84(95.5) | 0.16(0.05-0.51) | **<0.0001** | 73(83) | 15(17) | 1.48(1.25-1.76) | **<0.0001** |
| **Macrocarcinoma** | 6(3.7) | 152(94.4) | 1(reference) | | 32(19.9) | 120(74.5) | 1(reference) | | 88(54.7) | 70(43.5) | 1(reference) | |
| **Multifocality** | | | | | | | | | | | | |
| **Yes** | 4(6.7) | 56(93.3) | 11.33(1,29-99.4) | **0.006** | 14(23.3) | 42(70) | 3.01(1.53-5.93) | **0.001** | 26(43.3) | 34(56.7) | 0.56(0.41-0.76) | **<0.0001** |
| **No** | 1(0.6) | 169(97.7) | 1(reference) | | 14(8.1) | 155(89.6) | 1(reference) | | 130(75.1) | 40(23.1) | 1(reference) | |
| **Extra-thyroidal extension** | | | | | | | | | | | | |
| **Yes** | 4(6.3) | 58(92.1) | 5.16(0.97-27.46) | **0.032** | 15(23.8) | 43(68.3) | 3.71(1.81-7.61) | **<0.0001** | 28(44.4) | 34(54) | 0.58(0.43-0.76) | **<0.0001** |
| **No** | 2(1.2) | 158(97.6) | 1(reference) | | 11(6.8) | 147(90.7) | 1(reference) | | 124(76.5) | 36(22.1) | 1(reference) | |
| **Concomitant thyroiditis** | | | | | | | | | | | | |
| **Yes** | 0(0) | 85(73.3) | Not assessable | 0.132 | 6(7.1) | 79(92.9) | 0.49(0.2-1.19) | 0.106 | 64(75.3) | 21(24.7) | 1.17(0.97-1.41) | 0.09 |
| **No** | 3(2.6) | 111(95.7) | 1(reference) | | 16(13.8) | 95(81.9) | 1(reference) | | 73(85.9) | 41(35.3) | 1(reference) | |
| **LN metastasis***** | | | | | | | | | | | | |
| **N0** | 1(1.5) | 67(98.5) | 1(reference) | 0.551 | 5(7.4) | 62(91.2) | 1(reference) | **0.004** | 51(75) | 17(25) | 1(reference) | **<0.0001** |
| **N1** | 2(2.9) | 65(95.6) | 2.03(0.18-21.85) | | 17(25) | 48(70.6) | 3.5(1.37-8.94) | | 26(38.2) | 41(70.6) | 0.51(0.37-0.72) | |
| **Distant metastasis** | | | | | | | | | | | | |
| **Yes** | 4(66.7) | 2(33.3) | 52.44(14.89-184.64) | **<0.0001** | 1(16.7) | 1(16.7) | 3-64(0.87-15.1) | 0.142 | 0(0) | 6(100) | 0.21(0.03-1-3) | **0.001** |
| **No** | 3(1.3) | 233(97.5) | 1(reference) | | 32(13.4) | 201(84.1) | 1(reference) | | 159(66.5) | 77(32.2) | 1(reference) | |
| **AJCC/UICC stage** | | | | | | | | | | | | |
| **I-II** | 3(1.5) | 191(97) | 1(reference) | **0.008** | 22(11.2) | 169(85.8) | 1(reference) | 0.075 | 137(69.5) | 57(28.9) | 1(reference) | **0.002** |
| **III-IV** | 4(8.9) | 41(91.1) | 5.74(1.33-24.78) | | 9(20) | 32(71.1) | 1.90(0.94-3.83) | | 21(46.7) | 24(53.3) | 0.66(0.47-0.91) | |
| **ATA group** | | | | | | | | | | | | |
| **Low** | 1(0.9) | 111(98.2) | 1(reference) | **<0.0001** | 3(2.7) | 108(95.6) | 1(reference) | **<0.0001** | 96(85) | 16(14.2) | 1(reference) | **<0.0001** |
| **Intermediate** | 2(1.7) | 115(97.5) | 1.91(0.17-20.82) | | 24(20.3) | 91(77.1) | 7.72(2.39-24.91) | | 61(51.7) | 56(47.5) | 0.6(0.5-0.73) | |
| **High** | 4(44.4) | 4(44.4) | 56(7.06-444.05) | | 3(33.3) | 1(11.1) | 27.75(7.93-96.99) | | 0(0) | 8(100) | 0.35(0.26-0.47) | |

**N:number; CI: confidence interval; NED: no evidence of disease; LN: Lymph node; AJCC/UICC: American Joint Commettee on Cancer/Union for International Cancer Control; ATA: American Thyroid Association. *Favourable histology includes classic, follicular, Warthin-like, and Hurtle-cells variants of papillary thyroid cancer; unfavourable histology includes tall-cell, diffuse sclerosing, and solid variants of papillary thyroid cancer and follicular thyroid cancer; **Categorization in micro- and macro- carcinoma was used for the analysis. ***Patients not subjected to cervical lymphadenectomy (Nx) have been excluded from the analysis.**

### 4.4 Association of genotypes with clinico-pathological features

This analysis was aimed to identify correlations between SNP genotypes and clinico-pathological features, thus providing initial indications about possible prognostic impact of included SNPs.

Results are reported in Table 4. Schematically, the following associations were detected:

- VEGF-A -2578 C>A (rs699947):
    1. Analysis by means of dominant model revealed an association with primary tumour size (p=0.024). Particularly, the minor A allele was associated with higher tumour size.
    2. Analysis by means of recessive model revealed a strong association with the presence of distant metastases at diagnosis (p=0.002). Particularly, the minor homozygous genotype AA was associated to the presence of metastases.
- VEGF-A -460 T>C (rs833061):
    1. Analysis by means of dominant model revealed an association with primary tumour size (p=0.023). Particularly, the minor C allele was associated with higher tumour size.
    2. Analysis by means of recessive model revealed a strong association with the presence of distant metastases at diagnosis (p=0.002). Particularly, the minor homozygous genotype CC was associated to the presence of metastases.

Due to the association with distant metastases, minor homozygous genotypes of VEGF-A -2578 C>A and VEGF-A -460 T>C (AA and CC, respectively) were also associated with the ATA high risk group.

- VEGF-A +936 C>T (rs3025039):
    1. Analysis, performed exclusively by means of dominant model, revealed an association with aggressive histology (p=0.027). Particularly, the minor T was the risk allele.

2. An association with Hashimoto's thyroiditis was also detected (p=0.039). Particularly, the minor T allele was more frequent among patients with concomitant autoimmune thyroiditis.

- VEGFR-2 +1192 C>T (rs2305948):
  1. Analysis, performed exclusively by means of dominant model, revealed an association with gender (p=0.027). Particularly, the minor T allele was more frequent in females.
- PDGFR-α -1309 G>A (rs6554162):

1. Analysis, performed exclusively by means of dominant model, revealed an association with primary tumour size (p=0.02). Particularly, the minor A allele was associated to higher volume.

No significant associations were found for VEGF-A +405 G>C (rs2010963), VEGFR-2 +1719 T>A (rs1870377), and PDGFR-α -635 G>T (rs1800810).

| | Gender, n(%) | | | Age at diagnosis (yrs), median[range] | | Histology,** n(%) | | | Tumor size (cm), median[range] | | Multifocality, n(%) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Male** | **Female** | **p-value** | | **p-value** | **Favourable** | **Unfavourable** | **p-value** | | **p-value** | **Yes** | **No** | **p-value** |
| **VEGF-A rs699947** | | | 0.209 | | 0.067 | | | 0.762 | | 0.071 | | | 0.699 |
| **CC=92** | 19(20.7) | 73(79.3) | | 40 [15-65] | | 17(18.5) | 68(73.9) | | 0.9[0.3-5.5] | | 24(26.1) | 63(68.5) | |
| **CA=113** | 23(20.4) | 90(79.7) | | 43[17-72] | | 18(15.9) | 88(77.9) | | 1.3[0.1-8.5] | | 24(21.2) | 80(70.8) | |
| **AA=44** | 4(9) | 40(90.9) | | 45[22-74] | | 6(13.7) | 34(85) | | 1.3[0.2-7] | | 12(27.3) | 30(68.2) | |
| **Dominant model (CC vs. CA+AA)** | | | 0.498 | | 0.053 | | | 0.494 | | **0.024** | | | 0.621 |
| **Recessive model (CC+CA vs. AA)** | | | 0.077 | | 0.059 | | | 0.617 | | 0.235 | | | 0.644 |
| **VEGF-A rs833061** | | | 0.186 | | 0.097 | | | 0.715 | | 0.072 | | | 0.857 |
| **TT=91** | 19(20.9) | 72(79.1) | | 40 [15-65] | | 17(18.7) | 67(73.6) | | 0.9[0.3-5.5] | | 23(25.3) | 63(69.2) | |
| **TC=113** | 23(20.4) | 90(79.6) | | 43[17-72] | | 18(15.9) | 88(77.9) | | 1.3[0.1-8.5] | | 25(22.1) | 79(69.9) | |
| **CC=45** | 4(8.9) | 41(91.1) | | 45[22-74] | | 6(13.3) | 35(77.8) | | 1.25[0.2-7] | | 12(26.7) | 31(68.9) | |
| **Dominant model (TT vs. TC+CC)** | | | 0.458 | 0.086 | | | | 0.454 | | **0.023** | | | 0.791 |
| **Recessive model (TT+TC vs. CC)** | | | 0.067 | 0.067 | | | | 0.565 | | 0.271 | | | 0.720 |
| **VEGF-A rs2010963** | | | 0.129 | | 0.745 | | | 0.556 | | 0.109 | | | 0.867 |
| **GG (n=86)** | 11(12.8) | 75(87.2) | | 43.5[15-74] | | 69(80.2) | 12(14) | | 1.4[0.2-7] | | 23(26.7) | 60(69.8) | |
| **GC (n=123)** | 24(19.5) | 99(80.5) | | 43[17-72] | | 89(72.4) | 23(18.7) | | 1.2[0.1-8.5] | | 28(22.8) | 84(68.3) | |
| **CC (n=40)** | 11(27.5) | 29(72.5) | | 40.5[24-62] | | 32(80) | 6(15) | | 0.8[0.3-5.5] | | 9(22.5) | 29(72.5) | |
| **Dominant model (GG vs. GC+CC)** | | | 0.093 | | 0.871 | | | 0.391 | | 0.210 | | | 0.611 |
| **Recessive model (GG+GC vs. CC)** | | | 0.108 | | 0.445 | | | 0.729 | | 0.055 | | | 0.750 |
| **VEGF-A rs3025039*** | | | 0.265 | | 0.429 | | | **0.027** | | 0.55 | | | 0.499 |
| **CC =190** | 38(20) | 52(27.4) | | 43[15-74] | | 37(19.5) | 141(74.2) | | 1.2[0.1-8.5] | | 48(25.3) | 131(68.9) | |
| **CT+TT=59** | 8(13.6) | 51(86.4) | | 40[21-71] | | 4(6.8) | 49(83.1) | | 1[0.3-7] | | 12(20.3) | 42(71.2) | |
| **VEGF-R2 rs2305948*** | | | **0.027** | | 0.723 | | | 0.662 | | 0.656 | | | 0.157 |
| **CC =193** | 30(15.5) | 163(84.5) | | 43[15-74] | | 33(17.1) | 147(76.2) | | 1.25[0.2-7] | | 51(26.4) | 132(68.4) | |
| **CT+TT=56** | 16(28.6) | 40(71.4) | | 42[24-62] | | 8(14.3) | 43(76.8) | | 1[0.1-8.5] | | 9(16.1) | 41(73.2) | |
| **VEGF-R2 rs1870377*** | | | 0.853 | | 0.445 | | | 0.456 | | 0.197 | | | 0.257 |
| **TT=154** | 29(18.8) | 125(81.2) | | 42.5[15-74] | | 28(18.2) | 118(76.6) | | 1.1[0.1-8.5] | | 42(27.3) | 107(69.5) | |
| **TA+AA=95** | 17(17.9) | 78(82.1) | | 43[19-72] | | 13(13.7) | 72(75.8) | | 1.3[0.2-7] | | 18(18.9) | 66(69.5) | |
| **PDGFR-α rs6554162*** | | | 0.084 | | 0.903 | | | 0.930 | | **0.02** | | | 0.544 |
| **GG=142** | 21(14.8) | 121(85.2) | | 42 [19-72] | | 23(16.2) | 108(76.1) | | 1.1[0.1-6.2] | | 36(25.4) | 96(67.6) | |
| **GA+AA=107** | 25(23.4) | 82(76.6) | | 43[15-74] | | 18(16.8) | 82(76.6) | | 1.3[0.2-8.5] | | 24(22.4) | 77(72) | |
| **PDGFR-α rs1800812*** | | | 0.140 | | 0.304 | | | 0.829 | | 0.124 | | | 0.927 |
| **GG=174** | 28(16.1) | 146(83.9) | | 42 [15-72] | | 28(16.1) | 133(76.4) | | 1.2[0.1-8.5] | | 42(24.1) | 120(69) | |
| **GT+TT=75** | 18(24) | 57(76) | | 43[17-74] | | 13(17.3) | 57(76) | | 1.2[0.2-7] | | 18(24) | 53(70.7) | |

**Table 4. Relationship between VEGF-A, VEGFR-2, and PDGFR-α polymorphi ms and clinico-pathological features of DTC at diagnosis.**

| | Extra-thyroidal extension, n(%) | | | Concomitant thyroiditis, n(%) | | | LN metastases, n(%) | | | | Distant metastases, n(%) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Yes | No | p-value | Yes | No | p-value | Nx | N0 | N1 | p-value | Yes | No | p-value |
| **VEGF-A rs699947** | | | 0.687 | | | 0.967 | | | | 0.618 | | | **0.004** |
| **CC=92** | 21(22.8) | 64(69.6) | | 30(32.6) | 43(46.7) | | 39(42.4) | 26(28.3) | 27(29.3) | | 2(2.2) | 89(96.7) | |
| **CA=113** | 30(26.5) | 69(61.1) | | 40(35.4) | 53(46.9) | | 52(46) | 28(24.8) | 33(29.2) | | 0(0) | 110(97.3) | |
| **AA=44** | 12(27.3) | 29(65.9) | | 15(34.1) | 20(45.5) | | 22(50) | 14(31.8) | 8(18.2) | | 4(9.1) | 40(90.9) | |
| **Dominant model (CC vs. CA+AA)** | | | 0.391 | | | 0.796 | | | | 0.757 | | | 0.845 |
| **Recessive model (CC+CA vs. AA)** | | | 0.841 | | | 0.940 | | | | 0.320 | | | **0.002** |
| **VEGF-A rs833061** | | | 0.725 | | | 0.901 | | | | 0.515 | | | **0.005** |
| **TT=91** | 21(23.1) | 63(69.2) | | 29(31.9) | 43(47.3) | | 39(42.9) | 26(28.6) | 26(28.6) | | 2(2.2) | 88(96.7) | |
| **TC=113** | 30(26.5) | 69(61.1) | | 40(35.4) | 53(46.9) | | 52(46) | 27(23.9) | 34(30.1) | | 0(0) | 110(97.3) | |
| **CC=45** | 12(26.7) | 30(66.7) | | 16(35.6) | 20(44.4) | | 22(48.9) | 15(33.3) | 8(17.8) | | 4(8.9) | 41(91.1) | |
| **Dominant model (TT vs. TC+CC)** | | | 0.439 | | | 0.666 | | | | 0.832 | | | 0.861 |
| **Recessive model (TT+TC vs. CC)** | | | 0.927 | | | 0.773 | | | | 0.258 | | | **0.002** |
| **VEGF-A rs2010963** | | | 0.416 | | | 0.286 | | | | 0.817 | | | 0.218 |
| **GG (n=86)** | 26(30.2) | 54(62.8) | | 28(32.6) | 43(50) | | 41(47.7) | 20(23.3) | 25(29.1) | | 4(4.7) | 82(95.3) | |
| **GC (n=123)** | 29(23.6) | 78(63.4) | | 38(30.9) | 57(46.3) | | 56(45.6) | 36(29.3) | 31(25.2) | | 2(1.6) | 117(95.1) | |
| **CC (n=40)** | 8(20) | 30(75) | | 19(47.5) | 16(40) | | 16(40) | 12(30) | 12(30) | | 0(0) | 40(100) | |
| **Dominant model (GG vs. GC+CC)** | | | 0.264 | | | 0.545 | | | | 0.579 | | | 0.101 |
| **Recessive model (GG+GC vs. CC)** | | | 0.295 | | | 0.114 | | | | 0.757 | | | 0.273 |
| **VEGF-A rs3025039*** | | | 0.798 | | | **0.039** | | | | 0.480 | | | 0.683 |
| **CC =190** | 48(25.3) | 126(66.3) | | 59(31.1) | 95(50) | | 86(45.3) | 55(28.9) | 49(25.8) | | 5(2.6) | 182(95.8) | |
| **CT+TT=59** | 15(25.4) | 36(61) | | 26(44.1) | 21(35.6) | | 27(45.8) | 13(22) | 19(32.2) | | 1(1.7) | 57(96.6) | |
| **VEGF-R2 rs2305948*** | | | 0.839 | | | 0.789 | | | | 0.821 | | | 0.094 |
| **CC =193** | 49(25.4) | 128(66.3) | | 68(35.2) | 91 (47.2) | | 88(45.6) | 54(28) | 51(26.4) | | 3(1.6) | 188(97.4) | |
| **CT+TT=56** | 14(25) | 34(60.7) | | 17(30.4) | 25(44.6) | | 25(44.6) | 14(25) | 17(30.4) | | 3(5.4) | 51(91.1) | |
| **VEGF-R2 rs1870377*** | | | 0.348 | | | 0.436 | | | | 0.559 | | | 0.285 |
| **TT=154** | 37(24) | 106(68.8) | | 58(37.7) | 73(47.4) | | 66(42.9) | 45(29.2) | 43(27.9) | | 5(3.2) | 148(96.1) | |
| **TA+AA=95** | 26(27.4) | 56(58.9) | | 27(28.4) | 43(45.3) | | 47(49.5) | 23(24.2) | 25(26.3) | | 1(1.1) | 91(95.8) | |
| **PDGFR-α rs6554162*** | | | 0.801 | | | 0.494 | | | | 0.646 | | | 0.633 |
| **GG=142** | 35(24.6) | 93(65.5) | | 51(35.9) | 64(45.1) | | 62(43.7) | 42(29.6) | 38(26.8) | | 4(2.8) | 136(95.8) | |
| **GA+AA=107** | 28(26.2) | 69(64.5) | | 34(31.8) | 52(48.6) | | 51(47.7) | 26(24.3) | 30(28) | | 2(1.9) | 103(96.3) | |
| **PDGFR-α rs1800812*** | | | 0.370 | | | 0.988 | | | | 0.847 | | | 0.477 |
| **GG=174** | 47(27) | 111(63.8) | | 60(34.5) | 82(47.1) | | 77(44.3) | 49(28.2) | 48(27.6) | | 5(2.9) | 167(96) | |
| **GT+TT=75** | 16(21.3) | 51(68) | | 25(33.3) | 34(45.3) | | 36(48) | 19(25.3) | 20(26.7) | | 1(1.3) | 72(75.8) | |

| | AJCC/UICC stage, N(%) | | | ATA group, N(%) | | |
|---|---|---|---|---|---|---|
| | I-II | III-IV | p-value | Low-intermediate | High | p-value |
| **VEGF-A rs699947** | | | | | | |
| **CC=92** | 76(82.6) | 15(16.3) | 0.682 | 88(95.7) | 2(2.2) | **0.013** |
| **CA=113** | 87(77) | 20(17.7) | | 104(92) | 2(1.8) | |
| **AA=44** | 34(77.3) | 10(22.7) | | 39(88.6) | 5(11.4) | |
| **Dominant model (CC vs. CA+AA)** | | | 0.512 | | | 0.335 |
| **Recessive model (CC+CA vs. AA)** | | | 0.436 | | | **0.003** |
| **VEGF-A rs833061** | | | 0.736 | | | **0.016** |
| **TT=91** | 75(82.4) | 15(16.5) | | 87(95.6) | 2(2.2) | |
| **TC=113** | 87(77) | 20(17.7) | | 104(92) | 2(1.8) | |
| **CC=45** | 35(77.8) | 10(22.2) | | 40(88.9) | 5(11.1) | |
| **Dominant model (TT vs. TC+CC)** | | | 0.553 | | | 0.347 |
| **Recessive model (TT+TC vs. CC)** | | | 0.488 | | | **0.004** |
| **VEGF-A rs2010963** | | | 0.265 | | | 0.109 |
| **GG (n=86)** | 67(77.9) | 19(22.1) | | 80(93) | 6(7) | |
| **GC (n=123)** | 94(76.4) | 22(17.9) | | 111(90.2) | 3(2.4) | |
| **CC (n=40)** | 36(90) | 4(10) | | 40(100) | 0(0) | |
| **Dominant model (GG vs. GC+CC)** | | | 0.299 | | | 0.051 |
| **Recessive model (GG+GC vs. CC)** | | | 0.126 | | | 0.171 |
| **VEGF-A rs3025039*** | | | 0.534 | | | 0.470 |
| **CC =190** | 153(80.5) | 33(17.4) | | 178(93.7) | 6(3.2) | |
| **CT+TT=59** | 44(74.6) | 12(20.3) | | 53(89.8) | 3(5.1) | |
| **VEGF-R2 rs2305948*** | | | 0.060 | | | 0.108 |
| **CC =193** | 157(81.3) | 30(15.5) | | 181(93.8) | 5(2.6) | |
| **CT+TT=56** | 40(71.4) | 15(26.8) | | 50(89.3) | 4(7.1) | |
| **VEGF-R2 rs1870377*** | | | 0.382 | | | |
| **TT=154** | 122(79.2) | 31(20.1) | | 145(94.2) | 8(5.2) | 0.110 |
| **TA+AA=95** | 75(78.9) | 14(14.7) | | 86(90.5) | 1(1.1) | |
| **PDGFR-α rs6554162*** | | | 0.777 | | | 0.193 |
| **GG=142** | 114(80.3) | 25(17.6) | | 129(90.8) | 7(4.9) | |
| **GA+AA=107** | 83(77.6) | 20(18.7) | | 102(95.3) | 2(1.9) | |
| **PDGFR-α rs1800812*** | | | 0.888 | | | 0.199 |
| **GG=174** | 138(79.3) | 32(18.4) | | 159(91.4) | 8(4.6) | |
| **GT+TT=75** | 59(78.7) | 13(17.3) | | 72(96) | 1(1.3) | |

**N:number; LN: Lymph node; Nx: patients not subjected to cervical lymphadenectomy; AJCC/UICC: American Joint Commettee on Cancer/Union for International Cancer Control; ATA: American Thyroid Association. *Polymorphisms analyzed exclusively by means of dominant model because of a Minor Homozygous Genotype frequency ≤10%. **Favourable histology includes classic, follicular, Warthin-like, and Hurtle-cells variants of papillary thyroid cancer; unfavourable histology includes tall-cell, diffuse sclerosing, and solid variants of papillary thyroid cancer and follicular thyroid cancer.**

### 4.5 Association of genotypes with clinical outcome

Results from this analysis are reported in Table 5. No statistically significant associations were found between any of the included SNP and clinical outcome ($p>0.05$).

Nevertheless, significant trends were observed for the VEGF-A SNPs -2578 C>A and -460 T>C. Indeed, minor homozygous genotypes of both SNPs (AA and CC, respectively) were slightly associated with persistent structural disease ($p=0.066$ and 0.073, respectively) and with recurrent structural disease ($p=0.066$ and 0.059, respectively). Surprisingly, the prognostic impact of the 2 genotypes was opposite if considering structural persistence or recurrence as clinical outcome. Regarding the former, RRs were 3.64 (95% CI 0.84-15.68) for the AA genotype of -2578 C>A and 3.54 (95% CI 0.82-15.25) for the CC genotype of -460 T>C, meaning that genotypes were associated with higher likelihood to have persistent structural disease and had therefore negative prognostic impact. By contrast, analysis of recurrent structural disease found that RRs were 0.31(95% CI 0.07-1.24) for the AA genotype of -2578 C>A and 0.30(95% CI 0.07-1.2) for the CC genotype of -460 T>C, indicating that genotypes were associated with a reduced risk of developing recurrences after a period of NED and were therefore protective.

**Table 5. Relationship between VEGF-A, VEGFR-2, and PDGFR-α polymorphisms and clinical outcome.**

| | Persistent structural disease, N(%) | | | | Recurrent structural disease, N(%) | | | | NED, N(%) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Yes | No | Relative risk(95%CI) | p-value | Yes | No | Relative risk (95%CI) | p-value | Yes | No | Relative risk (95%CI) | p-value |
| **VEGF-A rs699947** | | | | | | | | | | | | |
| **CC=92** | 3(3.3) | 89(96.7) | 1(reference) | 0.110 | 14(15.2) | 75(81.5) | 1(reference) | 0.178 | 61(66.3) | 31(33.7) | 1(reference) | 0.971 |
| **CA=113** | 1(0.9) | 111(98.2) | 0.27(0.29-2.58) | | 19(16.8) | 92(81.4) | 1.08(0.57-2.04) | | 73(64.6) | 39(34.51) | 0.98(0.8-1.19) | |
| **AA=44** | 3(6.8) | 39(88.6) | 2.19(0.46-10.4) | | 2(4.5) | 37(84.1) | 0.32(0.07-1.36) | | 27(61.4) | 15(34.1) | 0.97(0.74-1.26) | |
| **Dominant model (CC‡ vs. CA+AA)** | | | 0.79(0.18-3.48) | 0.277 | | | 0.89(0.47-1.66) | 0.715 | | | 0.97(0.81-1.18) | 0.827 |
| **Recessive model (CC+CA‡ vs. AA)** | | | 3.64(0.84-15.68) | 0.066 | | | 0.31(0.07-1.24) | 0.066 | | | 0.97(0.76-1.25) | 0.862 |
| **VEGF-A rs833061** | | | | | | | | | | | | |
| **TT=91** | 3(3.3) | 88(96.7) | 1(reference) | 0.118 | 14(15.4) | 74(81.3) | 1(reference) | 0.163 | 61(67) | 30(33) | 1(reference) | 0.918 |
| **TC=113** | 1(0.9) | 111(98.2) | 0.27(0.29-2.56) | | 19(16.8) | 92(81.4) | 1.07(0.57-2.02) | | 72(63.7) | 40(35.4) | 0.95(0.78-1.17) | |
| **CC=45** | 3(6.7) | 40(88.9) | 2.11(0.44-10.05) | | 2(4.4) | 38(84.4) | 0.31(0.07-1.31) | | 28(62.2) | 15(33.3) | 0.97(0.74-1.26) | |
| **Dominant model (TT‡ vs. TC+CC)** | | | 0.78(0.17-3.42) | 0.744 | | | 0.87(0.46-1.63) | 0.673 | | | 0.96(0.8-1-15) | 0.689 |
| **Recessive model (TT+TC‡ vs. CC)** | | | 3.54(0.82-15.25) | 0.073 | | | 0.30(0.07-1.2) | 0.059 | | | 0.99(0.78-1.26) | 0.96 |
| **VEGF-A rs2010963** | | | | | | | | | | | | |
| **GG (n=86)** | 4(4.7) | 80(93) | 1(reference) | 0.308 | 9(10.5) | 71(82.6) | 1(reference) | 0.242 | 53(61.6) | 31(36) | 1(reference) | 0.106 |
| **GC (n=123)** | 3(2.4) | 119(96.7) | 0.51(0.11-2.24) | | 22(17.9) | 97(78.9) | 1.64(0.79-3.38) | | 76(61.8) | 46(37.4) | 0.98(0.79-1.22) | |
| **CC (n=40)** | 0(0) | 40(100) | Not assessable | | 4(10) | 36(90) | 0.88(0.29-2.7) | | 32(80) | 8(20) | 1.26(1.01-1.58) | |
| **Dominant model (GG‡ vs. GC+CC)** | | | 0.38(0.89-1.69) | 0.193 | | | 1.45(0.71-2.95) | 0.292 | | | 1.05(0.86-1.28) | 0.576 |
| **Recessive model (GG+GC‡ vs. CC)** | | | Not assessable | 0.237 | | | 0.64(0.24-1.71) | 0.363 | | | 1.27(1.05-1.54) | 0.055 |
| **VEGF-A rs3025039*** | | | | | | | | | | | | |
| **CC =190** | 5(2.6) | 182(95.8) | 1(reference) | 0.773 | 26(13.7) | 156(82.1) | 1(reference) | 0.779 | 127(66.8) | 60(31.6) | 1(reference) | 0.147 |
| **CT+TT=59** | 2(3.4) | 57(96.6) | 1.26(0.25-6.36) | | 9(15.3) | 48(81.4) | 1.1(0.55-2.21) | | 34(57.6) | 25(42.4) | 0.84(0.66-1.07) | |
| **VEGF-R2 rs2305948*** | | | | | | | | | | | | |
| **CC =193** | 4(2.07) | 186(96.4) | 1(reference) | 0.198 | 25(13) | 161(83.4) | 1(reference) | 0.324 | 127(65.8) | 63(32.6) | 1(reference) | 0.397 |
| **CT+TT=56** | 3(5.4) | 53(94.6) | 2.54(0.58-11.03) | | 10(17.9) | 43(76.8) | 1.4(0.72-2.73) | | 34(60.7) | 22(39.3) | 0.9(0.71-1.14) | |
| **VEGF-R2 rs1870377*** | | | | | | | | | | | | |
| **TT=154** | 4(2.6) | 148(96.1) | 1(reference) | 0.797 | 20(13) | 128(83.1) | 1(reference) | 0.528 | 100(64.9) | 52(33.8) | 1(reference) | 0.886 |
| **TA+AA=95** | 3(3.2) | 91(95.8) | 1.21(0.27-5.29) | | 15(15.8) | 76(80) | 1.22(0.65-2.25) | | 61(64.2) | 33(34.7) | 0.98(0.81-1.19) | |
| **PDGFR-α rs6554162*** | | | | | | | | | | | | |
| **GG=142** | 5(3.5) | 135(95.1) | 1(reference) | 0.431 | 17(12) | 118(83.1) | 1(reference) | 0.307 | 90(63.4) | 50(35.2) | 1(reference) | 0.66 |
| **GA+AA=107** | 2(1.9) | 104(97.2) | 0.52(0.1-2.67) | | 18(16.8) | 86(80.4) | 1.37(0.74-2.53) | | 71(66.4) | 35(32.7) | 1.04(0.86-1.25) | |
| **PDGFR-α rs1800812*** | | | | | | | | | | | | |
| **GG=174** | 6(3.4) | 166(95.4) | 1(reference) | 0.355 | 21(12.1) | 145(83.3) | 1(reference) | 0.189 | 112(64.4) | 60(34.5) | 1(reference) | 0.868 |
| **GT+TT=75** | 1(1.3) | 73(97.3) | 0.38(0.47-1-16) | | 14(18.7) | 59(78.7) | 1.51(0.81-2.81) | | 49(65.3) | 25(33.3) | 1.01(0.83-1.23) | |

**N:number; CI: confidence interval; NED: no evidence of disease. ‡Subgroup considered as reference for the assessment of the relative risk. *Polymorphisms analyzed exclusively by means of dominant model because of a Minor Homozygous Genotype frequency ≤10%.**

## 4.6 Association of VEGF-A SNPs with clinical outcome after stratification for AJCC/UICC stage and ATA risk group

The controversial finding of an opposite prognostic impact of VEGF-A -2578 C>A and -460 T>C depending on the considered clinical endpoint required a more in depth analysis of the actual prognostic value of these SNPs.

However, we have previously showed that patients harbouring the AA genotype of -2578 C>A and CC genotype of -460 T>C were more commonly metastatic at diagnosis, as compared with other genotypes, and that the presence of metastases at diagnosis was the most powerful clinical predictor of persistent structural disease in our series (see paragraph 4.3). Therefore, association with metastases may act as confounding factor generating the correlation of the highlighted genotypes with persistent structural disease as clinical outcome. Indeed, the majority of patients with the AA and CC genotypes (for VEGF-A -2578 C>A and -460 T>C, respectively) having persistent structural disease as clinical outcome were metastatic at diagnosis (see Figure 1.). By contrast, analysis of recurrent structural disease revealed lower rates of recidivisms for the highlighted genotypes, and among these few recurring cases about a half showed metastatic disease at diagnosis (Figure 1.). These observations led us to hypothesize that more exhaustive information about VEGF-A -2578 C>A and -460 T>C prognostic value could be obtained after discriminating between “early” and “advanced” disease. Therefore, we decided to re-assess prognostic impact of VEGF-A SNPs by stratifying for two major classification system, namely AJCC/UICC (I-II versus III-IV) and ATA risk group (low-intermediate versus high risk).

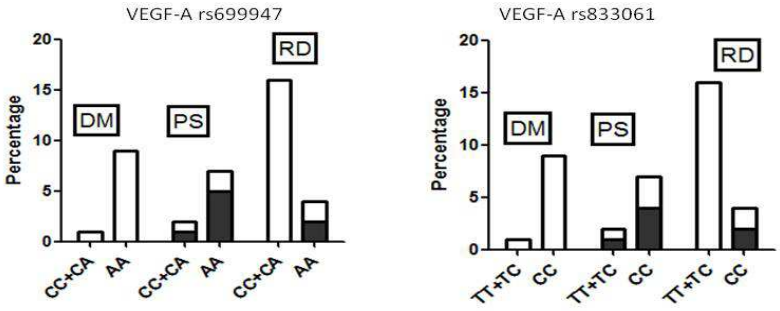


Figure 1. Percentage of patients with DM=Distant metastasis at diagnosis; PS=Persistent structural disease; RD=Recurrent structural disease according to genotypes (recessive model applied) for the SNPs VEGF-A rs699947 and VEGF-A rs833061. Grey areas indicate the portion of patients with PS and RD having distant metastasis at diagnosis.

### 4.6.1 VEGF-A SNPs and prognosis of early disease: stage I-II and ATA low-intermediate risk patients.

This analysis was focused on a) stage I-II subjects (according to the AJCC/UICC system) including intra-thyroidal tumours equal or less than 4 cm in size; b) ATA low-intermediate risk patients (according to the ATA guidelines) including a wider range of patients, namely subjects with completely resected tumour, without gross extra-thyroidal extension (pT4a-b), and without metastatic disease. Given the inclusion of patients with non advanced disease with low likelihood to have persistent disease after conventional therapeutic, we decided to assess the rate of recurrent structural disease as single clinical endpoint.

Results are reported in Table 6. Analysis of stage I-II patients included 197 out of 249 subjects. The AA and CC genotypes of -2578 C>A and -460 T>C were both associated with reduced risk of recurrent structural disease (p=0.018

and 0.016, respectively), showing RRs of 0.2 (95% CI 0.02-1.42) and 0.19 (95% CI 0.02-1.38), respectively. Analysis of ATA low-intermediate risk subjects involved 231 out of 249 patients. The AA and CC genotypes of -2578 C>A and -460 T>C were both associated with recurrent structural disease (p=0.035 and 0.031, respectively), demonstrating to exert protective action with RRs of 0.17 (95% CI 0.02-1.22) and 0.16 (95% CI 0.02-1.18), respectively.

Prognostic impact of minor homozygous genotypes of -2578 C>A and -460 T>C was further demonstrated by means of survival analysis, being DFS the endpoint (see Figure 2.). Indeed both the AA and CC genotype, for the SNPs -2578 C>A and -460 T>C respectively, were associated with longer DFS, as compared with other genotype subgroups (common homozygous and heterozygous). This association was confirmed in both stage I-II and ATA low-intermediate risk patients. Here we report median DFS and p-values from the survival analysis analysis.

1) Stage I-II patients: a) -2578 C>A: median DFS 34 months for the AA genotype versus 30.6 months for the CC+CA genotypes; p=0.017; b)-460 T>C: median DFS 36 months for the CC genotype versus 30.3 months for the TT+TC genotypes; p=0.014.
2) ATA low-intermediate risk patients: a) -2578 C>A: median DFS 37 months for the AA genotype versus 31.4 months for the CC+CA genotypes; p=0.03; b)-460 T>C: median DFS 38 months for the CC genotype versus 31.4 months for the TT+TC genotypes; p=0.026.

**Table 6. Relationship between VEGF-A polymorphisms and recurrent disease in AJCC/UICC stage I-II and ATA low-intermediate risk DTC patients.**

| | AJCC/UICC stage I-II, N(%) | | | | ATA classification low-intermediate risk, N(%) | | | |
|---|---|---|---|---|---|---|---|---|
| | Recurrence | No recurrence | Relative risk(95%CI) | p-value | Yes | No | Relative risk (95%CI) | p-value |
| **VEGF-A rs699947** | | | | | | | | |
| **CC=92** | 11(12) | 65(70.7) | 1(reference) | 0.058 | 14(15.2) | 75(81.5) | 1(reference) | 0.108 |
| **CA=113** | 15(13.3) | 76(67.3) | 1.13(0.55-2.33) | | 17(15) | 92(81.4) | 0.99(0.51-1.89) | |
| **AA=44** | 0(0) | 31(70.4) | 0.2(0.02-1.42) | | 1(2.3) | 36(81.8) | 0.17(0.02-1.26) | |
| **Dominant model (CCł vs. CA+AA)** | | | 0.84(0.41-1.75) | 0.659 | | | 0.78(0.41-1.49) | 0.461 |
| **Recessive model (CC+CAł vs. AA)** | | | 0.2(0.02-1.42) | **0.018** | | | 0.17(0.02-1.22) | **0.035** |
| **VEGF-A rs833061** | | | | | | | | |
| **TT=91** | 11(12.1) | 64(70.3) | 1(reference) | 0.053 | 14(15.4) | 74(81.3) | 1(reference) | 0.098 |
| **TC=113** | 15(13.3) | 76(67.3) | 1.12(0.55-2-29) | | 17(15) | 92(81.4) | 0.98(0.51-1.87) | |
| **CC=45** | 0(0) | 32(71.1) | 0.19(0.02-1.38) | | 1(2.2) | 37(82.2) | 0.16(0.02-1.21) | |
| **Dominant model (TTł vs. TC+CC)** | | | 0.83(0.40-1.71) | 0.617 | | | 0.77(0.4-1.46) | 0.637 |
| **Recessive model (TT+TCł vs. CC)** | | | 0.19(0.02-1.38) | **0.016** | | | 0.16(0.02-1.18) | **0.031** |
| **VEGF-A rs2010963** | | | | | | | | |
| **GG (n=86)** | 7(8.1) | 56(65.1) | 1(reference) | 0.417 | 7(8.1) | 70(81.4) | 1(reference) | 0.170 |
| **GC (n=123)** | 16(13) | 83(67.5) | 1.45(0.63-3.33) | | 21(17.1) | 97(78.9) | 1.95(0.87-4.38) | |
| **CC (n=40)** | 3(7.5) | 33(82.5) | 0.75(0.20-2.72) | | 4(10) | 36(90) | 1.1(0.34-3.53) | |
| **Dominant model (GGł vs. GC+CC)** | | | 1.26(0.56-2.85) | 0.565 | | | 1.74(0.78-3.84) | 0.158 |
| **Recessive model (GG+GCł vs. CC)** | | | 0.58(0.18-1.84) | 0.346 | | | 0.69(0.25-1.87) | 0.46 |
| **VEGF-A rs3025039*** | | | | | | | | |
| **CC =190** | 19(10) | 133(70) | 1(reference) | 0.633 | 25(13.2) | 155(81.6) | 1(reference) | 0.826 |
| **CT+TT=59** | 7(11.9) | 39(66.1) | 1.26(0.25-6.36) | | 7(11.9) | 48(81.4) | 0.91(0.41-2) | |

**N:number; CI:confidence interval; inter AJCC/UICC: American Joint Commettee on Cancer/Union for International Cancer Control; ATA: American Thyroid Association. ŀSubgroup considered as reference for the assessment of the relative risk. *Polymorphisms analyzed exclusively by means of dominant model because of a Minor Homozygous Genotype frequency≤10%.**

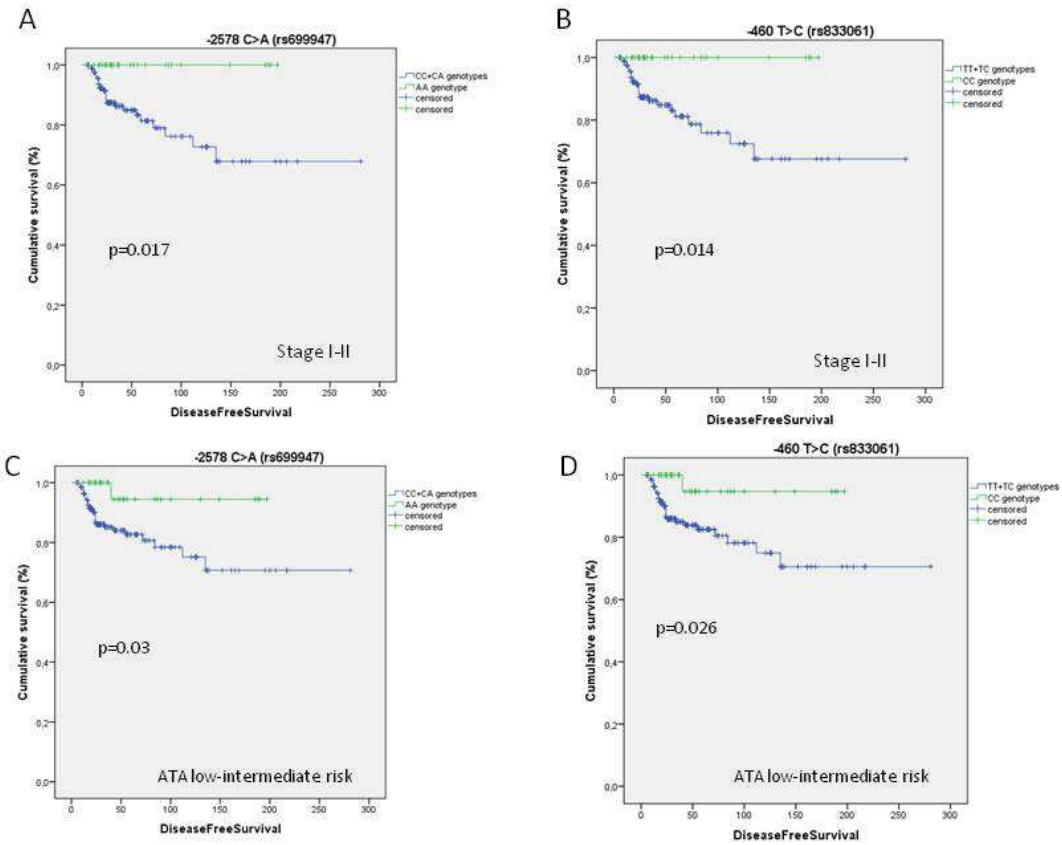


Figure 2. Kaplan-Meier analysis of disease-free survival according to SNPs VEGF-A -2578 C>A and -460 T>C. Analysis was performed in stage I-II (A and B for -2578 C>A and -460 T>C, respectively) and ATA low-intermediate risk (C and D for SNPs -2578 C>A and -460 T>C, respectively) DTC patients. ATA: American Thyroid Association.

### 4.6.2 VEGF-A SNPs and prognosis of advanced disease: stage III-IV patients.

Given the low number of patients classified as ATA high risk (9 subjects, 4%), this analysis was exclusively performed on stage III-IV patients, including tumours with any extra-thyroidal extension and/or more than 4 cm in size. Persistent structural disease, recurrent structural disease, and NED were considered as clinical endpoints. Only 45 out of 249 patients were included, and this represents a limit of this analysis.

Results are reported in Table 7. No statistically significant associations were found ($p>0.05$). Nevertheless, a significant trend was observed for the association between the AA and CC genotypes of -2578 C>A and -460 T>C and NED ($p=0.065$ for both genotypes). Particularly, RRs of showing NED at last follow-up were 0.38 for both genotypes, indicating lower likelihood of being disease-free at final follow-up and therefore a negative prognostic impact.

**Table 7. Relationship between VEGF-A polymorphisms and clinical outcome in AJCC/UICC stage III-IV DTC patients.**

| | Persistent structural disease, N(%) | | | | Recurrent structural disease, N(%) | | | | NED, N(%) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Yes | No | Relative risk(95%CI) | p-value | Yes | No | Relative risk (95%CI) | p-value | Yes | No | Relative risk (95%CI) | p-value |
| **VEGF-A rs699947** | | | | | | | | | | | | |
| **CC=92** | 2(2.2) | 14(15.2) | 1(reference) | 0.09 | 3(3.3) | 11(12) | 1(reference) | 0.809 | 8(8.7) | 8(8.7) | 1(reference) | 0.177 |
| **CA=113** | 0(0) | 26(23) | Not assessable | | 8(7.1) | 18(15.9) | 1.43(0.45-4.56) | | 14(12.4) | 12(10.6) | 1.07(0.58-1.97) | |
| **AA=44** | 2(4.5) | 8(18.2) | 1.6(0.26-9.61) | | 2(4.5) | 6(13.6) | 1.16(0.24-5.57) | | 2 (4.5) | 8(18.2) | 0.4(0.1-1.51) | |
| **Dominant model (CC‡ vs. CA+AA)** | | | 0.44(0.06-2.88) | 0.386 | | | 1.37(0.44-4.25) | 0.572 | | | 0.88(0.48-1.63) | 0.711 |
| **Recessive model (CC+CA‡ vs. AA)** | | | 4.2(0.67-26.3) | 0.104 | | | 0.9(0.24-3.34) | 0.885 | | | 0.38(0.1-1.36) | 0.065 |
| **VEGF-A rs833061** | | | | | | | | | | | | |
| **TT=91** | 2(2.2) | 14(15.4) | 1(reference) | 0.09 | 3(3.3) | 11(12.1) | 1(reference) | 0.809 | 8(8.8) | 8(8.8) | 1(reference) | 0.177 |
| **TC=113** | 0(0) | 26(23) | Not assessable | | 8(7.1) | 18(15.9) | 1.43(0.45-4.56) | | 14(12.4) | 12(10.6) | 1.07(0.58-1.97) | |
| **CC=45** | 2(4.4) | 8(17.8) | 1.6(0.26-9.61) | | 2(4.4) | 6(13.3) | 1.16(0.24-5.57) | | 2(4.4) | 8(17.8) | 0.4(0.1-1.51) | |
| **Dominant model (TT‡ vs. TC+CC)** | | | 0.44(0.06-2.88) | 0.386 | | | 1.37(0.44-4.25) | 0.572 | | | 0.88(0.48-1.63) | 0.711 |
| **Recessive model (TT+TC‡ vs. CC)** | | | 4.2(0.67-26.3) | 0.104 | | | 0.9(0.24-3.34) | 0.885 | | | 0.38(0.1-1.36) | 0.065 |
| **VEGF-A rs2010963** | | | | | | | | | | | | |
| **GG (n=86)** | 2(2.3) | 17(19.8) | 1(reference) | 0.75 | 2(2.3) | 15(17.4) | 1(reference) | 0.184 | 9(10.5) | 10(11.6) | 1(reference) | 0.973 |
| **GC (n=123)** | 2(1.6) | 27(22) | 0.65(0.1-4.26) | | 10(8.1) | 17(13.8) | 3.14(0.78-12.66) | | 13(10.6) | 16(13) | 0.94(0.50-1.76) | |
| **CC (n=40)** | 0(0) | 4 (10) | Not assessable | | 1(2.5) | 3(7.5) | 2.12(0.25-18.04) | | 2(5) | 2(5) | 1.05(0.35-3.13) | |
| **Dominant model (GG‡ vs. GC+CC)** | | | 0.57(0.08-3.76) | 0.561 | | | 3.01(0.75-12.05) | 0.077 | | | 0.96(0.52-1.75) | 0.894 |
| **Recessive model (GG+GC‡ vs. CC)** | | | Not assessable | 0.548 | | | 0.91(0.15-5.35) | 0.922 | | | 1.09(0.39-3.04) | 0.872 |
| **VEGF-A rs3025039*** | | | | | | | | | | | | |
| **CC =190** | 3(1.6) | 34(17.9) | 1(reference) | 0.860 | 10(5.3) | 24(12.6) | 1(reference) | 0.572 | 19(10) | 18(9.5) | 1(reference) | 0.238 |
| **CT+TT=59** | 1(1.7) | 14(23.7) | 0.82(0.93-7.29) | | 3(5.1) | 11(18.6) | 0.72(0.23-2.25) | | 5(8.5) | 10(17) | 0.64(0.29-1.41) | |

**N:number; AJCC/UICC: American Joint Commettee on Cancer/Union for International Cancer Control; ATA: American Thyroid Association. ‡Subgroup considered as reference for the assessment of the relative risk. *Polymorphisms analyzed exclusively by means of dominant model because of a Minor Homozygous Genotype frequency≤10%.**

### 4.7 Haplotype analysis of VEGF-A SNPs and association with clinical outcome in stage I-II and ATA low-intermediate risk patients.

Given that 2 VEGF-A SNPs (-2578 C>A and -460 T>C) showed association with recurrent structural disease in stage I-II and ATA low-intermediate risk patients, we performed haplotype analysis of the 4 included SNPs of the VEGF-A gene, and subsequently assessed haplotypes relationship with structural recidivisms. This aimed to identify a possible combined prognostic effect of VEGF-A SNPs.

Analysis of LD in the overall study population revealed a strong association between the 3 neighbour *loci* -2578 C>A (rs699947), -460 T>C (rs833061), and +405 G>C (rs2010963), as reported in Table 8. Particularly, the prognostic relevant SNPs -2578 C>A and -460 T>C showed complete LD (D'=1 and $r^2$=0.98).

**Table 8. Linkage disequilibrium between the 4 VEGF-A SNPs (-2578 C>A, -460 T>C, +405 G>C, and +936 C>T) assessed by means of Lewontin coefficient (D') and coefficient of correlation of** $r^2$.

| SNPs | SNPs | D' | R2 |
|---|---|---|---|
| -2578 C>A (rs699947) | 460 T>C (rs833061) | **1,00** | **0,98** |
| -2578 C>A (rs699947) | +405 G>C (rs2010963) | **1,00** | **0,47** |
| -2578 C>A (rs699947) | +936 C>T (rs3025039) | 0,28 | 0,02 |
| -460 T>C (rs833061) | +405 G>C (rs2010963) | **0,99** | **0,46** |
| -460 T>C (rs833061) | +936 C>T (rs3025039) | 0,28 | 0,02 |
| +405 G>C (rs2010963) | +936 C>T (rs3025039) | 0,17 | 0,00 |

Three common haplotypes with frequency above 10% were defined by means of Haploview program based on population frequencies of the SNPs (Figure 3).

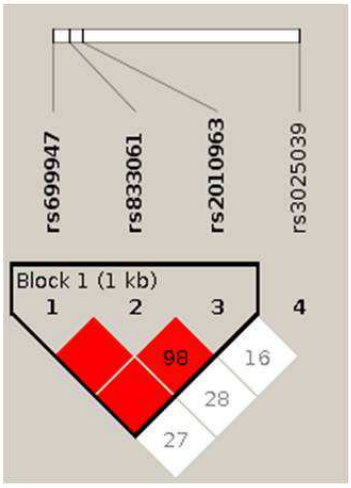


Figure 3. Identification of 3 common haplotypes among the 4 included VEGF-A SNPs: $^{-2578}$C, $^{-460}$T, $^{+405}$C, named CTC; the $^{-2578}$A, $^{-460}$C, $^{+405}$G, named ACG; the $^{-2578}$C, $^{-460}$T, $^{+405}$G, named CTG. Analysis performed by means of the Haploview software.

These include: the $^{-2578}$C, $^{-460}$T, $^{+405}$C (named CTC) haplotype; the $^{-2578}$A, $^{-460}$C, $^{+405}$G (named ACG) haplotype; the $^{-2578}$C, $^{-460}$T, $^{+405}$G (named CTG) haplotype. Haplotypes frequencies were similar if considering the overall study cohort, stage I-II, and ATA low-intermediate risk patients (Table 9.). However, estimated frequencies for each haplotype were consistent with those reported for other Caucasian populations (Zhai et al. 2008).

Comparison of estimated haplotypes frequencies between patients with and without recurrent structural disease (results reported in Table 10.) revealed significant prognostic effect for ACG and CTG haplotypes in both stage I-II (p=0.05 and 0.005, respectively) and ATA low-intermediate risk patients (p=0.036 and 0.039, respectively). Particularly, the ACG haplotype confers protection (stage I-II: 25% and 40.2% for recurring and non recurring patients, respectively, OR=0.22 [95% CI 0.11-0.46]; ATA low-intermediate: 25.9% and 40.7% for recurring and non recurring patients, respectively, OR=0.51 [95% CI 0.27-0.97]), whereas the CTG confers risk for structural recurrence (stage I-II: 34.1% and 16.6% for recurring and non recurring patients, respectively, OR=2.6 [95% CI 1.31-5.17]; ATA low-intermediate: 29.6% and 17.8% for recurring and non recurring patients, respectively, OR=1.93 [95% CI 1.02-3.67]). This was consistent with results from the genotype analysis reporting a negative prognostic impact for the AA and CC genotypes of -2578 C>A and -460 T>C.

**Table 9. Common VEGF-A haplotypes and frequencies in overall study cohort, AJCC/UICC stage I-II, and ATA low-intermediate risk patients. All p-values>0.05.**

| Haplotype | Frequency % in overall study cohort | Frequency % in AJCC/UICC stage I-II | Frequency % in ATA low-intermediate |
|---|---|---|---|
| CTC | 40.4 | 41.6 | 40.9 |
| ACG | 40.4 | 39.3 | 39.4 |
| CTG | 18.9 | 18.5 | 19.3 |

**AJCC/UICC: American Joint Commettee on Cancer/Union for International Cancer Control; ATA: American Thyroid Association.**

**Table 10. Common VEGF-A haplotypes and association with recurrent structural disease in stage I-II and ATA low-intermediate risk patients.**

| Haplotype | Frequency % recurrent/non recurrent patients AJCC/UICC stage I-II | p-value | OR(95% CI) | Frequency % recurrent/non recurrent patients ATA low-intermediate | p-value | OR(95% CI) |
|---|---|---|---|---|---|---|
| CTC | 40.9/42.6 | 0.831 | 0.93(0.49 1.77) | 44.4/41 | 0.239 | 1.15 (0.65-2.04) |
| ACG | 25/40.2 | **0.05** | 0.22 (0.11-0.46) | 25.9/40.7 | **0.036** | 0.51 (0.27-0.97) |
| CTG | 34.1/16.6 | **0.005** | 2.60 (1.31-5.17) | 29.6/17.8 | **0.039** | 1.93 (1.02-3.67) |

**AJCC/UICC: American Joint Commettee on Cancer/Union for International Cancer Control; ATA: American Thyroid Association; OR: Odds ratio; CI: Confidence interval.**

## 4.8 Combined genotype analysis in stage I-II and ATA low-intermediate risk patients.

Haplotype analysis, using the Haploview software, relies on estimates of frequencies and determines only the likelihood of the haplotipic phase for each individual. Thus, this kind of analysis is useful for identifying prognostic effects related to SNPs combination, but cannot estimate individual haplotype. To overcome this limit, we tried to identify combined genotypes having prognostic effect. Basing on results from the genotype (single SNP) and haplotype analysis, we decided to assess prognostic impact (namely the association with recurrent structural disease) of the combination of the SNPs -2578 C>A, -460 T>C, and +405 G>C in a recessive model.

Results are reported in Table 11. As expected, the ACG homozygous genotype (ACG+/+) offered a protective effect against structural recurrence in both stage I-II (p=0.018, RR 0.2, 95% CI 0.02-1.42) and ATA low-intermediate (p=0.035, RR 0.17, 95% 0.02-1.22) risk patients. Importantly, ACG+/+ showed p-values and RRs exactly consistent with those demonstrated

by analysis of single SNPs (namely the -2578 C>A and -460 T>C) and therefore no additional prognostic information was provided by analysis of combined genotypes. By contrast, the CTG homozygous genotype (CTG+/+) was significantly associated to higher rate of structural recurrence in stage I-II (p=0.018, RR=3.55, 95% CI 1.39-9.08), and was slightly deleterious also in ATA low-intermediate risk subjects (p=0.079, RR=2.59, 95% 0.97-6.95), where the absence of statistical significance was likely due to the low number of CTG subjects (9 out of 231). The identification of the CTG+/+ genotype as deleterious prognostic marker represented, indeed, an improvement as compared with single SNP analysis, where no negative prognostic markers were found. Survival analysis, having DFS as primary endpoint, further confirmed the prognostic role of ACG+/+ and CTG+/+ genotypes (Figure 4.).

**Table 11. Relationship between combined genotypes CTC+/+, ACG+/+, CTG+/+ and recurrent structural disease in stage I-II and ATA low-intermediate risk patients.**

| Genotype | AJCC/UICC Stage I-II | | ATA low-intermediate risk | |
|---|---|---|---|---|
| | p-value | RR(95% CI) | p-value | RR(95% CI) |
| CTC+/+ | 0.346 | 0.58(0.18-1.84) | 0.464 | 0.69(0.25-1.87) |
| ACG+/+ | **0.018** | 0.2(0.02-1.42) | **0.035** | 0.17(0.02-1.22) |
| CTG+/+ | **0.018** | 3.55(1.39-9.08) | 0.079 | 2.59(0.97-6.95) |

**AJCC/UICC: American Joint Commettee on Cancer/Union for International Cancer Control; ATA: American Thyroid Association; RR: Relative risk; CI: Confidence interval.**

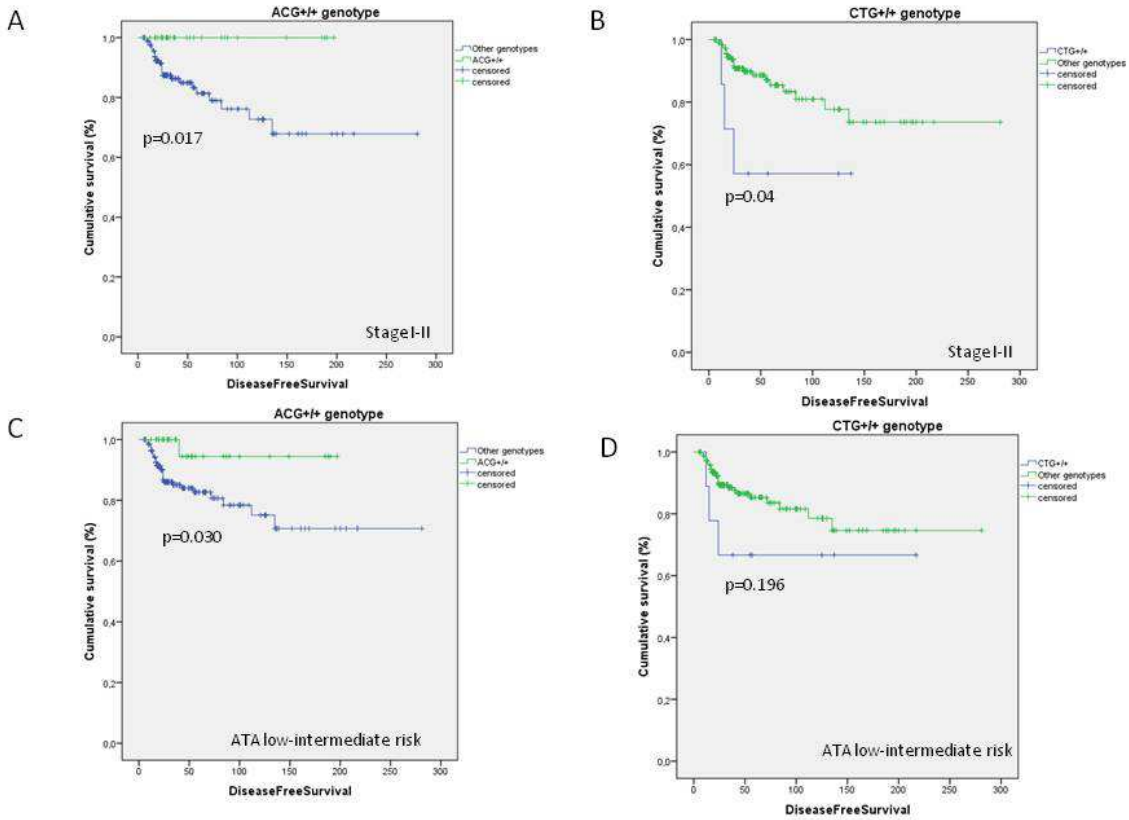


Figure 4. Kaplan-Meier analysis of disease-free survival according to genotypes ACG+/+ and CTG+/+. Analysis was performed in stage I-II (A and B for ACG+/+ and CTG+/+, respectively) and ATA low-intermediate risk (C and D for ACG+/+ and CTG+/+, respectively) DTC patients. ATA: American Thyroid Association.

### 4.9 PPV and NPV of the ACG+/+ and CTG+/+ genotypes for disease recurrence in stage I-II and ATA low-intermediate risk DTC patients.

We evaluated the accuracy of the ACG+/+ and CTG+/+ genotypes as prognostic markers in DTC, by determining PPV and NPV for the development of structural recurrences after the achievement of NED following conventional therapeutic approach. Analysis was performed in both stage I-II and ATA low-intermediate risk patients.

Results are shown in Table 12. The 2 genotypes showed remarkable NPV in both analyzed subgroups. Particularly, NPV of ACG+/+ was 84.4% (95% CI 78.03-89.57) and 84.3% (95% CI 78.52-89.11) in stage I-II and ATA low-intermediate risk patients, respectively; NPV of CTG+/+ was 87.9% (95% CI 82.48-92.21) and 87.2% (95% CI 82.09-91.24) in stage I-II and ATA low-

intermediate risk patients, respectively. Nevertheless, given that the major mission of prognostic stratification of DTC is to identify the subgroup of patients who will develop recurrences, our attention was mainly focused on PPV. As expected, PPV of ACG+/+ genotype, which has been previously associated to low risk of recurring, was null for stage I-II (0%, 95% CI 0-11.22) and very low for low-intermediate risk (2.7%, 95% CI 0.07-14.16) patients. By contrast, the CTG+/+ genotype, which has demonstrated association with occurrence of structural recidivism, showed acceptable PPV, namely 42.8% (95% CI 9.9-81.59) in stage I-II and 33.3% (95% CI 7.40-70.07) in ATA low-intermediate risk subjects.

**Table 12. Assessment of PPV (positive predictive value) and NPV (negative predictive value) for the ACG+/+ and CTG+/+ genotypes among stage I-II and ATA low-intermediate risk DTC patients.**

| | AJCC/UICC Stage I-II, N=198 | | | | ATA low-intermediate risk, N=226 | | | |
|---|---|---|---|---|---|---|---|---|
| **Genotype** | **PPV%** | **95%CI** | **NPV%** | **95%CI** | **PPV%** | **95%CI** | **NPV%** | **95%CI** |
| **ACG+/+** | 0 | 0-11.22 | 84.4 | 78.03-89.57 | 2.7 | 0.07-14.16 | 84.3 | 78.52-89.11 |
| **CTG+/+** | 42.8 | 9.9-81.59 | 87.9 | 82.48-92.21 | 33.3 | 7.40-70.07 | 87.2 | 82.09-91.24 |

**N: Number; CI: Confidence interval; AJCC/UICC: American Joint Commettee on Cancer/Union for International Cancer Control; ATA: American Thyroid Association.**

### 4.10 Multivariate analysis in ATA low-intermediate risk patients

Given that the ATA classification is the only system with demonstrated ability to predict persistent/recurrent disease, which represents the primary endpoint of this study, we decided to perform a multivariate analysis in the subgroup of ATA low-intermediate risk patients. Particularly, we decided to adjust the ACG+/+ genotype, the only demonstrating statistically significant association with recurrent structural disease, for two pathological features

representing well-known prognostic factors in the phase of "early" disease: tumour size (assessed through the categorical variable microcarcinoma versus macrocarcinoma) and multifocality.

Results from this analysis are reported in Table 13. All the factors involved in the analysis, namely ACG+/+ genotype, tumour size, and multifocality, revealed to be independent prognostic factors of recurrent structural disease (p=0.048, 0.008, 0.003, respectively). Particularly, the ACG+/+ genotype retained its protective prognostic significance after adjustment, showing adjusted OR of 0.12 (95% CI 0.01-0.98).

**Table 13. Model of multivariate analysis with inclusion of ACG+/+ VEGF-A haplotype, tumour size (microcarcinoma vs macrocarcinoma) and multifocality in ATA (America Thyroid Association) low-intermediate risk patients.**

| ATA low-intermediate risk | Univariate analysis | | | | Multivariate analysis | |
|---|---|---|---|---|---|---|
| | Recurrence | No recurrence | OR (95%CI) | p-value | Adjusted OR (95%CI) | Adjusted p-value |
| **VEGF-A genotype** | | | | | | |
| **AC G+/+** | 1(2.7) | 36(97.3) | 0.15(0.02-1.13) | **0.035** | 0.12(0.01-0.98) | **0.048** |
| **Other haplotypes** | 31(15.7) | 167(84.3) | 1(reference) | | | |
| **Tumor size** | | | | | | |
| **Microcarcinoma** | 3(3.4) | 84(96.6) | 0.14(0.04-0.49) | **<0.0001** | 0.18(0.05-0.64) | **0.008** |
| **Macrocarcinoma** | 29(19.6) | 19(12.8) | | | | |
| **Multifocality** | | | | | | |
| **Yes** | 11(12.1) | 64(70.3) | 3.97(1.68-9.34) | **0.001** | 3.9(1.59-9.57) | **0.003** |
| **No** | 15(13.3) | 76(67.3) | 1(reference) | | | |

**AJCC/UICC: American Joint Commettee on Cancer/Union for International Cancer Control; ATA: American Thyroid Association; OR: Odds ratio; CI: Confidence interval.**

## 5. Discussion

Angiogenesis is considered a hallmark of cancer, being more heavily involved in neoplastic progression rather than disease development (Hanahan et al. 2000). It is therefore conceivable that the efficiency of angiogenic process may significantly affect cancer evolution, and therefore clinical outcome. Particularly, a less efficient angiogenic process is expected to exert protective action against cancer progression, thus determining better prognosis. Importantly, cancer-related neo-vessels formation relies on the use of the host angiogenic machinery, thus being strictly dependant from those factors affecting physiological angiogenesis (Carmeliet et al. 2011; Dvorak 1986). It has been ascertained that efficiency of human angiogenesis, defined as the ability to respond to angiogenic *stimuli*, including those derived from cancer cells, mostly depends from individual genetic background, rather than environmental factors (Berrahmoune et al. 2007; Pantsulaia et al. 2004). Importantly, the impact of genetic variability on angiogenesis is mainly exerted through the modulation of gene expression (Rogers et al. 2012). In humans, the absence of tools for quantifying angiogenic response makes not feasible the direct identification of those hereditary traits involved in the modulation of angiogenesis. Therefore, information about the so-called “angio-genome” is still partial in humans and mainly relies on studies about the association of previously identified candidate genes with angiogenesis-related diseases, including cancer.

SNPs are the major source of human genome variability (Frazer et al. 2007). Despite being functionally neutral in the majority of cases, they may affect gene expression mainly through the elimination or creation of TFBS. Importantly, genes with recognized role in the angiogenic process, are usually highly polymorphic. These observations make feasible a role for SNPs in affecting human angiogenesis (Rogers et al. 2012). Therefore, a wide number of association studies assessing the relationship between selected SNPs of angiogenic-related genes, namely those with characterized or suspected

functional effects, with phenotype, and therefore prognosis, of different forms of cancer have been performed. The majority of these studies were focused on the VEGF-A gene, whom product is the leading molecule in the modulation of angiogenesis (Nagy et al. 2007), and SNPs located in the promoter, the 5'UTR, and the 3'UTR regions, with demonstrated or suspected impact on transcriptional and post-transcriptional gene modulation, were the most studied (Arcondeguy et al. 2013).

Results were highly heterogeneous and, sometimes, controversial (Eng et al. 2012), with prognostic effect of some SNPs being demonstrated by some authors and rebutted by others. Even, opposite prognostic impact was sometimes demonstrated, with different risk alleles reported for a single SNP. Discrepancies between studies may be only in part explained by means of different ethnicity, as controversial results were also reported within the same population (Heist et al. 2008; Masago et al. 2009). Thus, other variables should be taken into consideration. Firstly, it seemed that VEGF-A SNPs-related prognostic effects were highly tumour specific. It is conceivable that each cancer represents an independent model with its own molecular and biological features, and this may imply different biological and therefore prognostic relevance for angiogenesis. However, some studies demonstrated prognostic value for VEGF-A SNPs only in specific stages of the same tumour (Lurje et al. 2008). Indeed, role of VEGF-A may be different according to disease stage, due to possible modifications in the balance between pro- and anti- angiogenic molecules, and particularly to the production of pro-angiogenic factors other than VEGF-A, which is typical of advanced tumours (Carmeliet et al. 2011). Another relevant variable is the possible interaction between therapeutic strategies and VEGF-A genotypes. For example, Guan et al. (Guan et al. 2010) found beneficial effect for the C-allele of the VEGF-A -460 T>C in locally advanced non small cell lung cancer. Given that the C-allele was associated to increased VEGF-A production, and was therefore expected to worsen the prognosis, and that a previous report effectively showed deleterious effect for

the allele (Masago et al. 2009), authors explained their controversial finding by the fact that the majority of patients had been treated with radiotherapy, thus suggesting a possible favourable interaction treatment-genotype. Therefore, the performance of different therapeutic approaches may at least partially induce discrepancies of VEGF-A SNPs prognostic impact between different tumour types and, within the same tumour type, between different stages, due to both a direct impact on prognosis and to possible interaction with intrinsic features of the angiogenic machinery. To date, only few studies involved the VEGFR-2 gene, which codifies for the major mediator of VEGF-A effects on angiogenesis (Ferrara 2009), whereas no studies have tested yet (to the best of our knowledge) the possible association of PDGFRs SNPs with cancer prognosis (Rogers et al. 2012). Therefore, conclusive data about possible prognostic information deriving from SNPs of these genes are still missing.

Besides the possible prognostic implications, understanding the actual role of angiogenesis in each tumour type is mandatory due to the development and, in many cases, approval of anti-angiogenic drugs for the treatment of different forms of cancer (Bridges et al. 2011; Welti et al. 2013). Therefore, the characterization of the underlying molecular mechanisms, namely the identification of the specific role of each angiogenic molecule, and, even more importantly, the phase of disease evolution where angiogenesis exerts the major influence, would strongly allow the optimization of anti-angiogenic treatment strategies.

By the clinical sight, DTC can be defined as a “simple” cancer model. Independently from disease stage at diagnosis, conventional therapeutic approach is almost similar in all patients, being based on surgery with/without RAI, followed by TSH suppressive therapy (Haugen et al. 2016). Afterwards, no additional treatments are performed until the development of recurrent disease, which is considered the endpoint of the majority of prognostic studies about DTC. This homogeneity in patients management makes the assessment of clinical outcome less dependent from the interference of differential

treatment strategies, as compared with other tumours. Thus, DTC represents a feasible model allowing a better comprehension of the actual impact and underlying mechanisms of angiogenesis on tumour phenotype, and therefore prognosis.

As a proof of this concept, consistent results showing relevant role of angiogenesis in DTC have been reported by the totality of studies focusing this issue. Indeed, several studies performed in last 15-20 years have assessed the expression of VEGF-A on tumour tissues from DTC, both by protein detection through immunoistochemistry and by mRNA detection through RT-PCR. All of them demonstrated not only VEGF-A overexpression in tumour tissue, as compared with the normal counterpart, but also a clear association with aggressive pathological features, including lymph node and distant metastases, and worsened clinical outcome, namely higher rates of recurrent disease (Bunone et al. 1999; Kilicarslan et al. 2003; Klein et al. 2001; Lennard et al. 2001; Salajegheh et al. 2013). Importantly, some studies also reported VEGFR-2 overexpression, but its association with clinical outcome has not been proved (Bunone et al. 1999). Although data are still preliminary and far to be conclusive, some studies have suggested a possible role for the PDGF-system in DTC. In 2006, Chen et al. (Chen et al. 2006) showed that mRNA and protein expression of PDGF-AA and PDGFR-α was increased in thyroid carcinoma cell lines compared to benign tissues from thyroid nodular hyperplasia. More recently, higher expression of PDGFR-α has been demonstrated in PTC harbouring lymph node metastases, as compared with those tumours without lymph node involvement (Zhang et al. 2012). Furthermore, a recent study by Cong et al. (Cong et al. 2015), based on gene expression profiling of DTC samples obtained from The Cancer Genome Atlas, demonstrated association of PDGFR-α expression and aggressive clinico-pathological features.

To date, studies about the relationship between angiogenic SNPs and DTC are few and poorly conclusive. Particularly, some authors have assessed the possible association with disease susceptibility and pathological features, but

no reports have been published about the association with clinical outcome. The only study analyzing association between germline VEGF-A SNPs and DTC was proposed by Hsiao et al. in 2007 (Hsiao et al. 2007). This was a case-control analysis including SNPs -2578 C>A, +405 G>C, and +936 C>T. Results were poorly conclusive, as statistically significant findings were found only in men, where the A-allele of -2578 C>A was associated to increased risk of developing PTC. Furthermore, an association of the allele with the risk of lymph node metastases was also reported. More recently, Salajegheh et al. (Salajegheh et al. 2011) performed an association study between 3 VEGF-A SNPs (-141 A>C, +405 G>C, and +936 C>T) and pathological features of DTC, finding that the C-allele of the SNP -141 was associated to lymph node metastases, whereas the G-allele of the SNP +405 and the CC genotype of the SNP +936 were more common in advanced stages. Despite interesting, these data were not conformant to our analysis because genotyping was performed at the somatic level. Importantly, authors found no relationship between VEGF-A mRNA expression and SNPs, and this confirms the fact that DTC-related angiogenesis is mainly related to host and not to tumour genetic characteristics, thus empowering our study approach. To date, no studies have been performed about the possible association of VEGFR-2 SNPs and DTC. By contrast, Kim et al. (Kim et al. 2012) have recently published a paper about the association of PDGFRs SNPs and DTC. Authors performed a case-control study, finding that two PDGFR-α SNPs located in the promoter, the -635 G>T and the -1309 G>A, were associated with the risk of developing PTC. Despite performed at the somatic level, this analysis further empowers the thesis of a possible involvement of the PDGF-system in DTC. Given this body of evidence, we decided to include in our analysis not only SNPs from the VEGF-system, encompassing the two main factors of angiogenesis modulation VEGF-A and VEGFR-2 (Ferrara 2009; Nagy et al. 2007), but also from the PDGF-system.

As already specified in the *Materials and Methods* section, SNPs were selected basing on previous characterization of the functional impact, on

previous data about prognostic impact on cancer and, if available, on preceded publications focusing their relationship with DTC. Regarding the VEGF-A gene, we included 4 well-characterized SNPs (2578 C>A, -460 T>C, +405 G>C, and +936 C>T), encompassing all regions involved in the regulation of gene expression (namely the promoter, the 5'UTR, and the 3'UTR). All of them showed enough evidence of affecting gene expression (functional impact of each included VEGF-A SNP has been described in the paragraph *1.4.1 VEGF-A SNPs and cancer*). Furthermore, each included VEGF-A SNP had already demonstrated prognostic impact in other tumour types (associations of included VEGF-A SNPs with prognosis of other tumour types has been reported in the paragraph *1.4.1 VEGF-A SNPs and cancer*). Regarding the VEGFR-2 gene, data about the prognostic impact of related SNPs on cancer are still poor. Given that data about the correlation of VEGFR-2 overexpression and prognosis of DTC are still lacking, we chose to focus our analysis on the 2 nonsynonimous cSNPs, namely 1192 C>T and 1719 T>A, located in the extra-cellular domain of the receptor, which are involved in the modulation of the binding affinity to VEGF-A. Regarding the PDGF-system, data about the functional effects related to the SNPs are still unclear and, as already discussed, studies about prognostic impact on cancer are missing. Basing on the previously cited studies (Chen et al. 2006; Cong et al. 2015; Zhang et al. 2012), which reported that higher PDGFR-α expression was a hallmark of PTC and, also, was associated with aggressive disease features, and on the Kim's study, finding that the PDGFR-α promoter SNPs -635 G>T and the -1309 G>A affected susceptibility to develop PTC (although analysis was performed at the somatic and not at the germline level) (Kim et al. 2012), we decided to include these 2 SNPs in the analysis.

Correlations between genotypes of the selected SNPs and clinical outcomes (categorized as persistent structural disease, recurrent structural disease, and NED, as specified in the *Materials and Methods* section) in the overall study population showed no statistically significant results. Nevertheless, analysis

according to recessive model revealed trends of association between the minor homozygous genotypes of the VEGF-A SNPs -2578 C>A and -460 T>C (AA and CC, respectively) and 2 prognostic endpoints: persistent structural disease and recurrent structural disease. Surprisingly, prognostic effect related to these genotypes was opposite basing on the considered clinical endpoint. Despite not achieving statistical significance, they conferred protection against structural recurrences among DTC patients achieving NED after thyroid ablation, but were more frequent, as compared with the other genotypes (common homozygous and heterozygous), among patients showing persistence of structural disease after treatment.

It is important to note that the prognostic endpoints recurrent and persistent structural disease defines two different sets of DTC patients. The former is typical of patients, representing the vast majority, who can be defined as having an "early" disease and easily achieve remission after treatment. The latter, indeed, typically involves that low portion of DTC patients having "advanced" disease, particularly those with metastatic spread (Tuttle et al. 2010b; Vaisman et al. 2012). Our study population was consistent with these concepts, as the presence of distant metastasis at diagnosis represented the strongest clinical predictor of persisting structural disease, but was not associated to structural recurrences. Importantly, analysis of genotypes association with clinico-pathological factors revealed that the minor homozygous genotypes of VEGF-A -2578 C>A and -460 T>C were strongly associated with the presence of distant metastases at diagnosis. Despite being based on few cases of metastatic patients (6 subjects), this association complicates data interpretation, as the proportion of risk of persistent structural disease that is attributable to the highlighted genotypes versus that attributable to the presence of distant metastases is difficult to be determined.

We thought that more consistent and exhaustive results about prognostic impact of VEGF-A SNPs could be derived from the separate assessment of the 2 described clinical scenarios, namely "early" and "advanced" DTC. Indeed,

previous studies of other tumour types have already showed different prognostic significance of VEGF-A SNPs according to disease stage (Lurje et al. 2008). This could be related both to the use of different treatment strategies, which is not the case of DTC where first-line treatment is almost similar independently from initial staging, and also, more interestingly, to intrinsic modifications of the angiogenic machinery, namely of the balance between angiogenic regulating factors, that may occur through different phases of the pathological process. Therefore, we performed a stratified analysis trying to discriminate between "early" and "advanced" DTC patients. Given that a clear distinction between "early" and "advanced" disease in DTC has not been codified yet, we applied the 2 mostly used stratification systems, namely the AJCC/UICC and the ATA (Momesso et al. 2014). Particularly, we considered as "early" disease 2 DTC subgroups: AJCC/UICC stage I-II subjects, involving intra-thyroidal tumours equal or less than 4 cm in size, and ATA low-intermediate risk patients, including patients without gross extra-thyroidal extension (pT4a-b) and without metastatic disease. We found that both AA and CC genotypes of VEGF-A -2578 C>A and -460 T>C were associated with significantly lower rate of structural recurrence, and therefore exerted protective action against the development of structural recidivisms in both subgroups. These findings were further confirmed by analysis of DFS, which was significantly higher among patients with the highlighted genotypes, as compared with others.

Given the extremely low number of subjects classified as ATA high risk, which did not allow to perform any statistical analysis, assessment of "advanced" disease was only based on AJCC/UICC stage III-IV patients, including tumours with any extra-thyroidal extension and/or more than 4 cm in size. Analysis showed no statistically significant prognostic impact for the AA and CC genotypes of VEGF-A -2578 C>A and -460 T>C. Nevertheless, a trend was observed with the likelihood of being NED at last follow-up, which is a critical endpoint for patients presenting advanced stages at diagnosis.

Particularly, the presence of the highlighted genotypes was associated to lower risk of being disease-free at last follow-up, thus suggesting a possible deleterious prognostic impact. However, conclusive information about "advanced" disease is hampered by the low number of subjects, as our study cohort mainly included patients with early stages. A higher number of patients with "advanced" DTC, particularly those being metastatic at diagnosis, should be analyzed for a more careful evaluation of VEGF-A -2578 C>A and -460 T>C prognostic impact.

Given that statistically significant impact on prognosis was demonstrated for 2 VEGF-A SNPs only in stage I-II and ATA low-intermediate risk patients, we performed more in depth analyses of VEGF-A related SNPs in these specific subgroups of patients. In order to verify possible empowerment of prognostic information related to SNPs combination and to obtain more exhaustive information about the underlying biology, we performed haplotype analysis and assessed haplotypes association with the rate of recurrent structural disease. The Haploview software, providing estimates of haplotype frequencies, identified 3 common haplotypes involving the SNPs -2578 C>A, -460 T>C, and +405 G>C, namely CTC, ACG, and CTG. Of them, ACG and CTG showed association with prognosis in both stage I-II and ATA low-intermediate risk patients. As expected from genotype analysis, ACG conferred protection against structural disease recurrence, whereas CTG was associated to higher risk of recurring.

Given that the SNP +405 G>C provides its common G-allele to both the protective and deleterious haplotype, a relevant biological role determining an actual prognostic impact for this SNP has to be excluded. Conclusive information about the actual biological relevance, and therefore prognostic impact, for SNPs -2578 C>A and -460 T>C, is not possible by means of this kind of study, which is based on a SNP-candidate approach. Particularly, gene-throughout association studies are needed to exclude that other SNPs or genetic markers, in LD with those reported in the present analysis, may be

associated with prognosis, and *in vitro* and *in vivo* studies are required to confirm differential biological effects for polymorphic variants of the highlighted SNPs. Basing on our results, we can only perform some biological speculations. We have already reported available data about impact of analyzed SNPs on VEGF-A function (see paragraph *1.4.1 VEGF-A SNPs and cancer*). Consistently with the protective effect shown in our analysis, the AA genotype of -2578 C>A has been associated with decreased serum levels of VEGF-A, and therefore with reduced gene expression (Shahbazi et al. 2002). By contrast, a lowered VEGF-A production was reported for the common T-allele of -460 T>C (Hansen et al. 2010b), which was part of the deleterious haplotype, and this was not conformant to our results. Therefore, among the 2 prognostic relevant SNPs detected in our study, which are in complete LD, the -2578 C>A is that with higher likelihood to play an actual biological role in DTC-related angiogenesis and to affect prognosis. Importantly, elimination/creation of TFBS related to this SNP has been carefully described (Metzger et al. 2015), thus allowing to discuss about possible biological differences between polymorphic variants that could explain the role in DTC. As previously reported, the AA genotype is associated to the loss of any binding site for the dimer HIF1α/β, which represents the main mediator of hypoxia-inducted VEGF-A production (Buroker et al. 2013). By the biological sight, this produces a dramatic change as VEGF-A expression, being the main regulator of the angiogenic process, becomes independent from HIF1-mediated hypoxia. Notably, HIF1-α overexpression has been associated to molecular and morphological changes leading to disease progression (such as the epithelial-mesenchymal transition) and to aggressive pathological features (including advanced stage and lymph node metastases) in DTC, and this suggests a relevant role for HIF1-mediated hypoxia in disease progression of such tumour type (Wang et al. 2014; Yang et al. 2015). This is consistent with the protective role demonstrated for the AA genotype, where VEGF-A expression, and therefore angiogenesis, related to HIF1-mediated hypoxia is hampered by

the absence of binding sites within the promoter. Furthermore, the C-allele is associated to the presence of a TFBS specific for E2F1, which is a key regulator of cell cycle progression mediating proliferative stimulation from almost all growth factors (Ertosun et al. 2016). Importantly, the previously mentioned study by Cong et al. (Cong et al. 2015) reported overexpression of this transcription factor in PTC. Therefore, the presence of the C-allele determines the exposition of the binding site for E2F1, which may in turn amplify VEGF-A induction following proliferative *stimuli*, including those generated from cancer cells. These observations, despite preliminary and speculative, may provide some *rationale* for the prognostic significance of SNP -2578 C>A emerged by our analysis.

Another hot point of our study is the differential prognostic significance of the identified genetic markers in patients with advanced disease stage, where an absent or, even, pejorative prognostic impact has been reported (although results from this set of patients has not been considered conclusive). The main hypothesis explaining this issue is that in advanced disease, especially in metastatic patients, up-regulation of pro-angiogenic molecules other than VEGF-A has been demonstrated, involving fibroblast growth factors, ephrins, angiopoietins, and interleukins (Bergers et al. 2008). This cancels the leading role of VEGF-A in the modulation of angiogenesis, and may therefore explain the loss of prognostic significance of those factors affecting its function, including genetic variability related to SNPs. Considering (even if it remains just a speculation) the -2578 C>A as the biologically relevant marker, another possible hypothesis may derive from the fact that the A-allele is in LD with a 18-bp insertion at position -2549, which harbours at least twelve additional TFBS (Brogan et al. 1999; Schneider et al. 2009), and is suspected to enhance VEGF-A expression (Supic et al. 2012). It is therefore conceivable that modifications of gene expression occurring in advanced disease may lead to the production of a different set of transcription factors, which may enhance VEGF-A expression through the binding to this A-allele related insertion. This

may explain the possible deleterious impact of the AA genotype of -2578 C>A in advanced DTC.

Besides the biological speculations, primary aim of the study was to test the selected set of angiogenic SNPs as feasible prognostic markers in DTC, and to verify if they can improve current prognostic approach. Main aim of prognostic stratification of DTC is to identify that low, but not negligible, portion of patients (about 25-30%), who will experience persistent/recurrent disease (Castagna et al. 2011; Pitoia et al. 2013; Tuttle et al. 2010b; Vaisman et al. 2012). Although dedicated categorical classification systems, mainly based on clinico-pathological factors, have been recently proposed by the major societies dealing with thyroid diseases (Pacini et al. 2006; Pitoia et al. 2013; Pitoia et al. 2009), PPV for the identification of persisting/recurring patients is still far to be optimal (Castagna et al. 2011). Therefore, the ATA has recently introduced the "continuum of risk" model, which is an individualized non-categorical approach for persistence/recurrence risk estimate including a wider range of variables (Haugen et al. 2016). Despite the deep characterization of molecular alterations related to DTC, and particularly to PTC (Xing 2013), molecular prognostication has only a marginal role in prognostic definition. Indeed, the most powerful and best characterized marker, the oncogene $BRAF^{V600E}$, showed poor specificity, and therefore limited PPV, for the prediction of persistence/recurrence. Thus, mutated BRAF does not represent a significant addition to current prognostic systems and its determination is not routinely recommended from the latest 2015 ATA guidelines (Haugen et al. 2016). Given this body of evidence, searching for novel molecular prognosticators with high specificity and PPV for persistent/recurrent disease is the major objective of this research field. Furthermore, molecular prognostication of DTC is exclusively based on tissue markers, but accessibility to tumour samples is not always feasible. Therefore, providing non-tissutal prognostic markers, easily available independently from tissue retrieval, would represent a relevant advantage.

Given that Haploview program just calculates the likelihood of the haplotipic phase of each individual for allowing inferential analyses, it cannot provide molecular markers useful for the characterization of individual risk in clinical practice. Therefore, basing on information obtained by single SNP and haplotype analysis, we constructed "risk" genotypes by combining VEGF-A SNPs -2578 C>A, -460 T>C, and +405 G>C (which were in LD) according to a recessive model (given that minor homozygous variants of 2 of these SNPs had revealed significant prognostic value), and assessed their prognostic impact on the occurrence of recurrent structural disease in stage I-II and ATA low-intermediate risk DTC patients. As expected, ACG+/+ genotype conferred protection against structural recurrence in both subgroups, whereas the CTG+/+ conferred significantly higher risk of structural recurrence in stage I-II and was deleterious also in ATA low-intermediate risk subjects, where we attributed the lack of statistical significance to the low number of subjects harbouring the genotype. To further reinforce these data, we proposed a model of multivariate regression analysis focusing on the subgroup of ATA low-intermediate risk patients. Indeed, ATA classification is specifically based on prediction of disease recurrence, which represents the endpoint of our study. Given that we have found prognostic role and speculated about biological impact of identified genetic markers in DTC patients with "early" disease, we decided to adjust prognostic impact of the genotype ACG+/+, the only achieving statistical significance, for the 2 main prognostic features of "early" DTC, namely tumour size and multifocality (Ito et al. 2012; Mazzaferri 2007; Roti et al. 2008). Notably, the marker retained its protective effect after adjustment, and this partially attests the independent prognostic role of VEGF-A genetic variability. Indeed, this result is limited by the fact that restricting analysis only to patients with low risk according to ATA was not feasible given the too low number of patients harbouring the risk genotype.

In order to assess prognostic accuracy and compare these markers to the current set of variables available for DTC prognostication, we calculated PPV

and NPV for recurrent structural disease. As expected from the association with reduced risk of recurrence, ACG+/+ genotype showed considerable NPV but extremely poor PPV. Therefore, this marker is not useful for selecting those patients with significant risk of recurrence. However, it may be included within the set of variables considered for quantifying the risk estimate of recurrence according to the "continuum of risk" model. More relevantly, CTG+/+ genotype displayed not only considerable NPV, but also acceptable PPV, which was 42.8% and 33.3% in stage I-II and ATA low-intermediate risk subjects, respectively. According to our analysis (which needs to be confirmed and possibly refined by further studies) and to the best of our knowledge, CTG+/+ genotype represents the most powerful molecular marker allowing the identification of those patients affected with "early" DTC (stage I-II and ATA low-intermediate risk are considered under this definition) who will develop structural recurrence. Indeed, the BRAF mutation, currently considered as the best molecular prognosticator in this field, showed PPV of only 25% in the largest meta-analysis available to date (Tufano et al. 2012). Despite being the highest reported for a molecular marker (according to our knowledge), PPV of CTG+/+ genotype for disease recurrence is acceptable but still limited. Therefore, future studies about its association with $BRAF^{V600E}$ as well as other molecular features having prognostic relevance (i.e. mutations of p53 and TERT promoter) are mandatory for verifying possible correlation and prognostic empowerment from markers combination.

Importantly, clinical management of many subgroups of patients included in the heterogeneous group of "early" DTC presents several controversies (McLeod et al. 2013). Particularly, the absence of randomized controlled trials makes challenging several therapeutic aspects, including the extent of surgery (Barney et al. 2011; Bilimoria et al. 2007; Haigh et al. 2005; Mendelsohn et al. 2010), prophylactic central node dissection (Perrino et al. 2009; Popadich et al. 2011; Shan et al. 2012; Zetoune et al. 2010), and RAI-treatment (Sacks et al. 2010; Sawka et al. 2008). Therefore, an improvement in the capability of

predicting disease recurrence represents a relevant breakthrough, as it may significantly optimize clinical decision-making. Furthermore, the selection of a subgroup of patients with higher risk of disease recurrence by means of genetic features affecting VEGF-A activity, may provide the *rationale* for testing new therapeutic strategies based on the introduction of treatments specifically targeting the VEGF-A, such as the neutralizing antibody bevacizumab. To date, anti-angiogenic treatment of DTC exclusively relies on the use of tyrosine-kinase inhibitors, which are multimodal drugs exerting anticancer activity by means of both anti-proliferative and anti-angiogenic function (Smith et al. 2004). Up to now, these compounds, being sorafenib and lenvatinib those approved by the US *Food and Drug Administration*, have been exclusively used in the uncommon setting of RAI-refractory macro-metastatic patients showing morphological disease progression (Marotta et al. 2012; Marotta et al. 2015). Therefore, they are not part of the conventional therapeutic approach. Nevertheless, the use of VEGF-A blockage, mainly by means of bevacizumab, has been tested and, in some case, introduced into clinical practice in the adjuvant setting of several tumour models (Jain et al. 2006). The validation of CTG+/+ genotype as predictor of significant risk of recurrence among DTC patients with an initially "early" disease may justify the planning of randomized clinical trial assessing the impact of bevacizumab, administered as adjuvant treatment after thyroidectomy, on the rate of disease recurrence.

## 6. Conclusions

This is the first study assessing possible prognostic impact of a set of germline SNPs of angiogenesis-related genes, namely VEGF-A, VEGFR-2, and PDGFR-α, on clinical outcome of a large cohort of DTC patients.

We found that analysis of germline VEGF-A SNPs may provide stable and easily accessible prognostic markers in the setting of "early" DTC, including patients with AJCC/UICC stage I-II and ATA low-intermediate risk of disease recurrence. Particularly, single-SNP, haplotypes, and combined-SNPs analyses, led to the identification of 2 molecular markers with possible role in prognostic stratification of DTC. These include the ACG homozygous genotype, termed ACG+/+, and the CTG homozygous genotype, termed CTG+/+, of the SNPs -2578 C>A (rs699947), -460 T>C (rs833061), and +405 G>C (rs2010963). Both these markers showed significant association with the rate of structural recurrences, with the ACG+/+ being the protective genotype and the CTG+/+ conferring higher risk of recidivism. Of them, the CTG+/+ may more relevantly impact on clinical practice, as it showed the highest PPV for disease recurrence reported to date for any molecular prognosticator, thus improving the capability to discriminate "early" DTC patients who will develop recurrences after thyroid ablation, which represents the main aim of prognostic definition of DTC. The validation of this marker and its combination with other genetic features may facilitate decision-making of these patients, which is still challenging regarding several therapeutic aspects. Importantly, the relevance of VEGF-A genetic variability, affecting gene function, in the early phase of the disease may provide *rationale* for introducing VEGF-A targeted therapy in this setting.

Data about prognostic impact of VEGF-A SNPs in "advanced" disease were partial and not conclusive, given that study cohort mainly included patients with "early" disease. Nevertheless, single-SNP analysis suggested absent or, even, deleterious prognostic value for SNPs -2578 C>A and -460 T>C, which were, indeed, protective in "early" DTC. It is conceivable that this discrepancy

was related to the loss of VEGF-A dependency of the cancer-related angiogenic process and/or to modified production of transcription factors affecting VEGF-A expression, which may occur in advanced tumours.

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## 9. SUMMARY OF ORIGINAL PAPERS

**Marotta V,** Guerra A, Sapio MR, Campanile E, Motta M, Fenzi G, Rossi G, Vitale M. Are RET/PTC rearrangements in benign thyroid nodules of biological significance? Thyroid. 2010 Oct;20(10):1191-2. PubMed PMID: 20860421.

**Marotta V,** Guerra A, Sapio MR, Campanile E, Motta M, Fenzi G, Rossi G, Vitale M. Growing thyroid nodules with benign histology and RET rearrangement. Endocr J. 2010;57(12):1081-7. Epub 2010 Oct 30. PubMed PMID: 21048359.

Guerra A, Sapio MR, **Marotta V**, Campanile E, Moretti MI, Deandrea M, Motta M, Limone PP, Fenzi G, Rossi G, Vitale M. Prevalence of RET/PTC rearrangement in benign and malignant thyroid nodules and its clinical application. Endocr J. 2011;58(1):31-8. Epub 2010 Dec 14. PubMed PMID: 21173509.

Sapio MR, Guerra A, **Marotta V**, Campanile E, Formisano R, Deandrea M, Motta M, Limone PP, Fenzi G, Rossi G, Vitale M. High growth rate of benign thyroid nodules bearing RET/PTC rearrangements. J Clin Endocrinol Metab. 2011 Jun;96(6):E916-9. Epub 2011 Mar 16. PubMed PMID: 21411555.

**Marotta V,** Guerra A, Sapio MR, Vitale M. RET/PTC rearrangement in benign and malignant thyroid diseases: a clinical standpoint. Eur J Endocrinol. 2011 Oct;165(4):499-507. Epub 2011 Jul 12. Review. PubMed PMID: 21750045.

This group of papers focus the possible biological difference between clonal and non-clonal RET rearrangements, trying to make light on the related clinical implications. Our study dealing with this aspect culminated in a review paper published on the “European Journal of Endocrinology”, where current role of RET/PTC in thyroid diseases, both malignant and benign, is described.

Guerra A, Sapio MR, **Marotta V**, Campanile E, Rossi S, Forno I, Fugazzola L, Budillon A, Moccia T, Fenzi G, Vitale M. The primary occurrence of BRAF(V600E) is a rare clonal event in papillary thyroid carcinoma. J Clin Endocrinol Metab. 2012 Feb;97(2):517-24. Epub 2011 Dec 14. PubMed PMID: 22170714.

Guerra A, Fugazzola L, **Marotta V**, Cirillo M, Rossi S, Cirello V, Forno I, Moccia T, Budillon A, Vitale M. A high percentage of BRAFV600E alleles in papillary thyroid carcinoma predicts a poorer outcome. J Clin Endocrinol Metab. 2012 Jul;97(7):2333-40. Epub 2012 Apr 16. PubMed PMID: 22508706.

Guerra A, Di Stasi V, Zeppa P, Faggiano A, **Marotta V**, Vitale M. BRAF (V600E) assessment by pyrosequencing in fine needle aspirates of thyroid nodules with concurrent Hashimoto's thyroiditis is a reliable assay. Endocrine. 2014 Mar;45(2):249-55. doi: 10.1007/s12020-013-9994-y. Epub 2013 Jun 18.

**Marotta V**, Guerra A, Zatelli MC, Uberti ED, Stasi VD, Faggiano A, Colao A,Vitale M. BRAF mutation positive papillary thyroid carcinoma is less advanced when Hashimoto's thyroiditis lymphocytic infiltration is present. Clin Endocrinol (Oxf). 2013 Nov;79(5):733-8. doi: 10.1111/cen.12194. Epub 2013 Apr 1.

These papers are focused on biology and prognostic implications of the BRAF mutation in PTC. The first 2 articles aimed to determine, by means of the quantitative technique named pyrosequencing (whom accuracy was verified by our group even in cytology samples from DTC patients with concurrent Hashimoto's thyroiditis), if the BRAF mutation occurs clonally, as expected from its recognized pathogenetic and prognostic role, or may also be a subclonal event. This aspect is still discussed in literature. We demonstrated clonality only in a subgroup of patients. We also attested significance of this biological finding demonstrating that the percentage of BRAF-mutated alleles correlates with clinical outcome. The fourth paper, indeed, analyzed the interaction between $BRAF^{V600E}$ and concurrent Hashimoto's thyroiditis in affecting clinical outcome of PTC, given that they exert significant but opposite prognostic influence.

**Marotta V**, Franzese MD, Del Prete M, Chiofalo MG, Ramundo V, Esposito R, Marciello F, Pezzullo L, Carratù A, Vitale M, Colao A, Faggiano A. Targeted therapy with kinase inhibitors in aggressive endocrine tumors. Expert Opin Pharmacother. 2013 Jun;14(9):1187-203. doi: 10.1517/14656566.2013.796931. Review. PubMed PMID: 23675883.

**Marotta V,** Ramundo V, Camera L, Del Prete M, Fonti R, Esposito R, Palmieri G, Salvatore M, Vitale M, Colao A, Faggiano A. Sorafenib in advanced iodine-refractory differentiated thyroid cancer: efficacy, safety and exploratory analysis of role of serum thyroglobulin and FDG-PET. Clin Endocrinol (Oxf). 2013 May;78(5):760-7. doi: 10.1111/cen.12057.

**Marotta V**, Di Somma C, Rubino M, Sciammarella C, Modica R, Camera L, Del Prete M, Marciello F, Ramundo V, Circelli L, Buonomano P, Colao A, Faggiano A. Second-line sunitinib as a feasible approach for iodine-refractory differentiated thyroid cancer after the failure of first-line sorafenib. Endocrine. 2015 Aug;49(3):854-8. doi: 10.1007/s12020-014-0448-y. Epub 2014 Oct 11. PubMed PMID: 25305056.

**Marotta V**, Colao A, Faggiano A. Complete disappearance of liver metastases in a patient with iodine-refractory differentiated thyroid cancer subjected to sorafenib re-challenge. Endocrine. 2015 Dec;50(3):824-5. doi: 10.1007/s12020-015-0570-5. Epub 2015 Mar 10.

**Marotta V**, Sciammarella C, Vitale M, Colao A, Faggiano A. The evolving field of kinase inhibitors in thyroid cancer. Crit Rev Oncol Hematol. 2015 Jan;93(1):60-73. doi: 10.1016/j.critrevonc.2014.08.007. Epub 2014 Sep 16. Review. PubMed PMID: 25240824

This is a series of clinical papers dealing with current role of anti-angiogenic treatment in endocrine tumours, and specifically in DTC. This is consistent with the submitted thesis, whom objective is also to provide new information about the biological role and the underlying mechanisms of angiogenesis in DTC. Particularly, we provided innovative insights about treatment strategies, based on the use of tyrosine-kinase inhibitors, to be applied in the setting of DTC refractory to radiometabolic treatment. Our research culminated in a review paper published on “Critical Review in Oncology/Hematology”, which is the official journal of the “European Society of Oncology”.