Article

Influence of vitamin D in advanced non-small cell lung cancer patients treated with nivolumab

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Abstract: Nivolumab is one of most used monoclonal antibody for advanced non-small cell lung cancer treatment; presence of its anti-antibody is considered a negative prognostic factor. Vitamin D (VD) modulates expression of genes involved in drug metabolism/elimination and immune system regulation and its deficiency is frequent in these patients. To date no data have been reported about nivolumab and VD relationship. Aim of this study was to quantify plasma 25-hydroxyVD (25-VD) and 1,25-VD, nivolumab and its anti-antibody before starting treatment (baseline) and at 15, 45 and 60 days of therapy.VD-pathway associated gene single nucleotide polymorphisms (SNPs) were also evaluated. Molecules were quantified through enzyme-linked immunosorbent assay, SNPs through real-time PCR. Forty-five patients are enrolled: median nivolumab concentrations were 12.5ug/mL, 22.3ug/mL and 27.1ug/mL at 15, 45 and 60 days respectively. No anti-nivolumab antibodies were found. Correlations were observed between nivolumab concentrations and 25-VD levels. Nivolumab concentrations were affected by VD-pathway related gene SNPs. *VDBP* AC/CC genotype and baseline 25-VD <10 ng/mL predicted nivolumab concentrations <18.7ug/mL cut-off value at 15 days, associated with tumor progression. This is the first study showing VD markers predictors of nivolumab concentrations in real-life context of non-small cell lung cancer treatment.

Keywords: monoclonal antibody; NSCLC; immune-therapy; ELISA; pharmacokinetics; pharmacogenetics

1. Introduction

Immunotherapy represents most revolutionary treatment for solid cancers nowadays. To date, several types of immunotherapy are available, including monoclonal antibodies, non-specific immunotherapies, oncolytic virus therapy, T-cell therapy and cancer vaccines. The evolution of immune checkpoint inhibitors, as anticancer treatment options, represents one of the most successful approach in cancer drug research in the past few years[1]. Check point inhibitors antibodies, such as anti- programmed cell death protein 1 (PD-1) and its ligand (PD-L1), are new drugs acting as tumor suppressing factor, since they are able to modulate the interaction between immune cell and tumor cell[2]. These therapies proved to be safe and effective option in advanced non-small cell lung cancer (NSCLC) and can be recommended selectively[3].

Nivolumab, a monoclonal antibody, binds to the immune-modulating PD-1, blocking ligand interaction and downstream signaling pathways. The result is a positive regulation of T-cell function resulting in an antitumor effect[4]. In 2015 this drug was approved by FDA for treatment of patients with advanced squamous and non-squamous NSCLC with progression or after platinum-based chemotherapy (second-line therapy)[5]. In a randomized trial 272 patients treated with nivolumab had an overall survival of 3.2 months longer than those on docetaxel[2].

In a conference abstract, authors measured nivolumab plasma concentrations in patients and suggested that partial responders had higher nivolumab mean trough concentrations (27.4 ug/mL) compared to subjects with tumor progression (18.7 ug/mL)[6].

PD-1 inhibitors typically cause fewer and less severe treatment-related adverse events (AEs) compared with conventional chemotherapy compounds, although immune-related AEs can occur requiring monitoring and specialized management to prevent serious complications[7]. Moreover, immunogenicity, in terms of presence of nivolumab's anti-antibodies, is considered a negative prognostic factor[8]. Immunogenicity and immune checkpoints in general are regulated by different factors such as vitamin D (VD)[9]: reported studies show that VD controls different pathways related to innate and adaptive immunity regulating the expression of many genes involved in drug metabolism/elimination through its receptor (VDR). Moreover, in another study, single nucleotide polymorphisms (SNPs) in genes involved in VD pathway could affect VD kinetics and, consequently, its action. Polymorphisms present near genes involved in cholesterol production, hydroxylation, and VD transport are able to predict who could have risk of VD insufficiency, as suggested by Wang *et al.* [10]. Genetic variations near DHCR7 (4p12 (overall p=1.9x10(-109) for rs2282679, in GC); 11q12 (p=2.1x10(-27) for rs12785878), near CYP2R1 (11p15 (p=3.3x10(-20) for rs10741657) and near CYP24A1 (20q13) are genome-wide significant in that population. Furthermore, participants with a score obtained combining the three variants in the highest quartile are at increased risk of 25-VD levels lower than 75 nmol/L or than 50 nmol/L, compared with those in the lowest quartile.

Since VD deficiency is frequent in lung cancer patients[11] and no data on nivolumab and its relationship with VD are currently available, aim of this study was to quantify 25-hydroxyVD (25-VD), 1,25-hydroxyVD (1,25-VD), nivolumab and its anti-antibody levels in patients' plasma at different timings, also considering their influence in predicting the cut-off value (18.7 ug/mL), associated with tumor progression.

2. Results

2.1 Patients characteristics

Baseline (BL) characteristics for 45 included patients are reported in Table 1. Thirty-one (69) were male, age was 73 years and body mass index (BMI) was 23.4 Kg/m^2.

Table 1. Baseline characteristics of study population

Characteristics	n (%), median (IQR)	
n	45	
Age (years)	73 (65-79.5)	
Male sex	31 (69)	
BMI (Kg/m^2)	23.4 (20.1-26.4)	
Caucasian	45 (100)	
NSCLC type	Adenocarcinoma	34 (52.3)
	Squamous cell carcinoma	9 (13.8)
	Poorly differentiated carcinoma	1 (1.5)
	Large-cell neuroendocrine carcinoma	1 (1.5)
Concomitant drugs	Cardiovascular	24 (36.9)
	Diabetes	4 (6.2)
	Opioids	9 (13.8)
	Protease inhibitors	20 (30.8)
	Corticosteroid	12 (18.5)
	Vitamin D	2 (3.1)
Pre-treatment drugs	Cisplatine	24 (53.3)
	Docetaxel	10 (22.2)
	Carboplatine	24 (53.3)
	Gemcitabine	12 (26.7)
	Gefitinib	2 (4.4)
	Pemetrexed	30 (66.7)
	Afatinib	1 (2.2)
	Osimertinib	1 (2.2)
	Erlotinib	20 (44.4)
	Vinorelbine	10 (22.2)
	Paclitaxel	3 (6.7)
	Bevacizumab	3 (6.7)
	Etoposide	4 (8.9)
	Zoledronic acid	1 (2.2)
	Bavicizumab	1 (2.2)
	Farletuzumab	1 (2.2)
	Radiotherapy	1 (2.2)

2.2. Nivolumab and vitamin D concentrations

Median nivolumab concentrations were 12.5 ug/mL (9.5-17.1 ug/mL), 22.3 ug/mL (IQR:18.30-34.88 ug/mL) and 27.1 ug/mL (IQR:17.4-39.4 ug/mL) respectively at 15, 45 and 60 days (figure 1). No anti-nivolumab antibodies were detected.

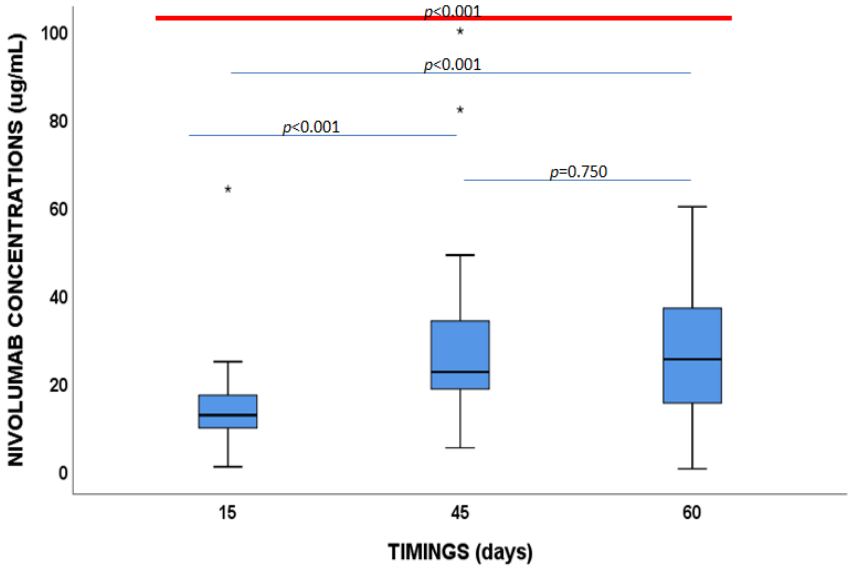
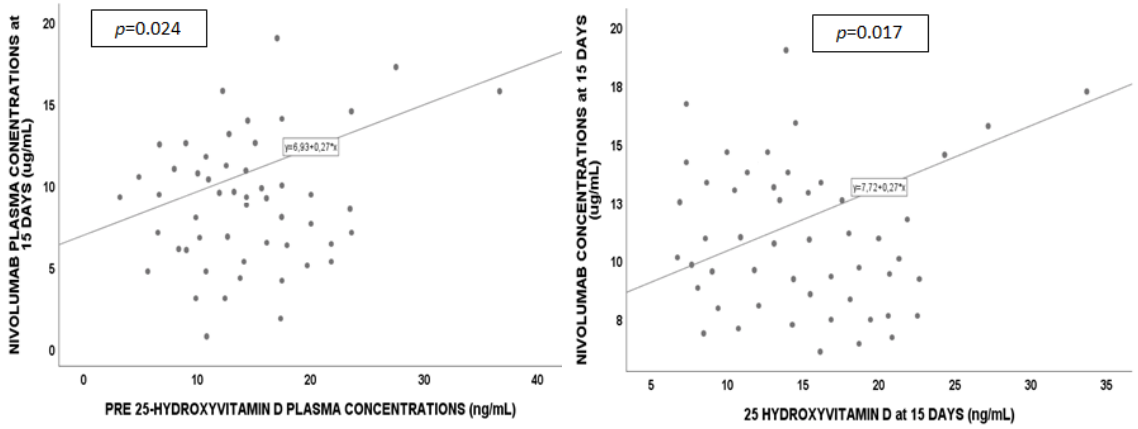


Figure 1. Nivolumab plasma concentrations at 15, 45 and 60 days.

25-VD concentration was 12.8 ng/mL (10.1-16.6 ng/mL), 13.6 ng/mL (10.9-16.1 ng/mL), 11.8 ng/mL (10.1-18.9 ng/mL) and 12.9 ng/mL (10.8-17.0 ng/mL) at BL, 15, 45 and 60 days respectively.

1,25-VD value was 33.7 pg/mL (23.4-40.6 ng/mL), 34.7 ng/mL (22.3-45.4 ng/mL), 28.5 ng/mL (20.7-41.5 ng/mL) and 35.7 ng/mL (IQR:19.2-49.0 ng/mL) respectively at BL, 15, 45 and 60 days.

Correlations (figure 2) were observed between nivolumab concentrations at 15 days and BL 25-VD levels (p=0.024, Pearson's coefficient (PC) 0.451) and at 15 days (p=0.017, PC=0.542); nivolumab exposure at 60 days was correlated with 25-VD at BL (p=0.001, PC=0.730), at 15 (p<0.001, PC=0.858), 45 (p=0.001, PC=0.779) and 60 days (p<0.001, PC=0.900). Furthermore, in a sub-group, patients were stratified according to 25-VD deficiency: BL 25-VD levels < 10 ng/mL were associated with lower nivolumab concentrations at 15 (p=0.103, a trend without statistical significance), 45 (p=0.018) and 60 days (p=0.021); 15 days 25-VD < 10 ng/mL with 15 (p=0.019), 45 (p=0.019) and 60 days (p=0.028) nivolumab lower concentrations; finally, 60 days 25-VD < 10 ng/mL with 60 days lower nivolumab levels (p=0.030). No correlation was observed for 1,25-VD and nivolumab exposure.



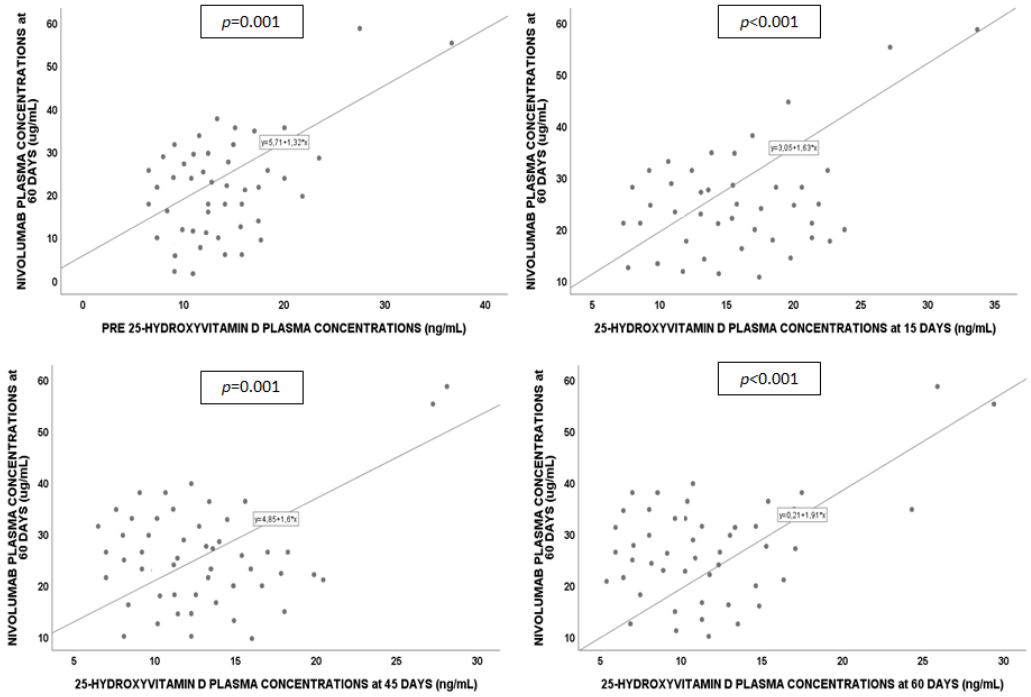


Figure 2. Nivolumab and 25-hydroxyvitamin D correlations at different timings.

2.3. Pharmacogenetics

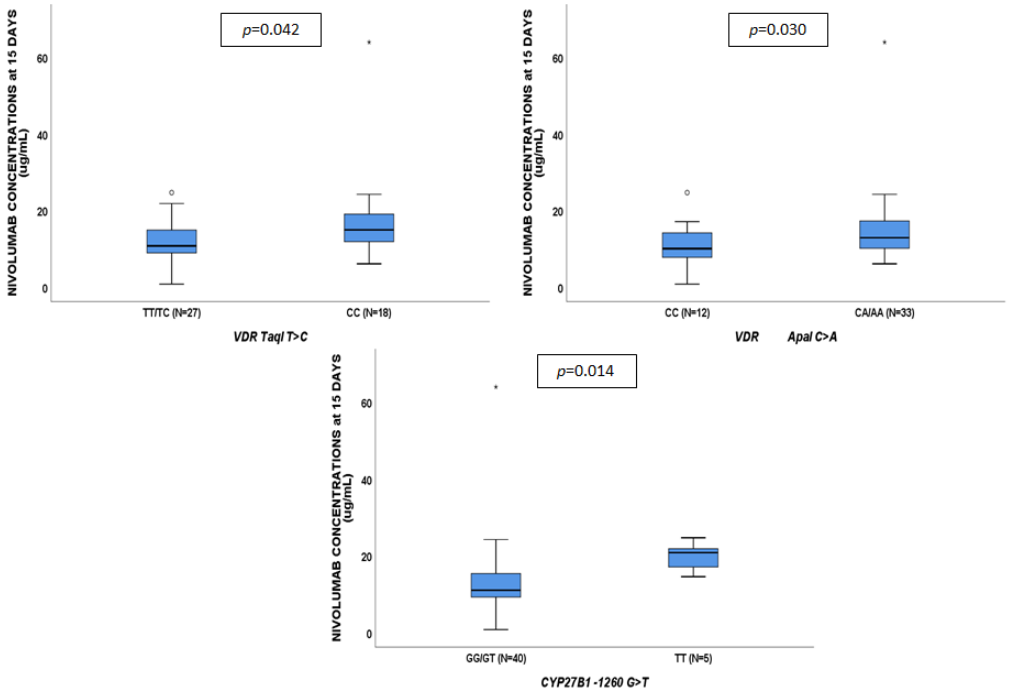
Variant genotype frequencies (%) were calculated and reported in Table 2.

Table 2. Variant allele frequencies.

SNP	% HOMOZIGOUS WILD TYPE	% HETEROZYGOUS	% HOMOZYGOUS MUTANT
CYP27B1 +2838 C>T	*20 CC*	*2.2 CT*	*77.8 TT*
CYP27B1 -1260 G>T	*73.3 CC*	*15.6 CT*	*11.1 TT*
CYP24A1 rs2248359 T>C	*42.2 TT*	*40 TC*	*17.8 CC*
CYP24A1 rs927650 C>T	*33.3 CC*	*22.2 CT*	*44.5 TT*
CYP24A1 rs2585428 A>G	*31.1 AA*	*28.9 AC*	*40 CC*
VDR Cdx2 A>G	*17.8 AA*	*13.3 AG*	*68.9 GG*
VDR TaqI T>C	*33.3 TT*	*26.7 TC*	*40 CC*
VDR FokI T>C	*11.1 TT*	*42.2 TC*	*46.7 CC*
VDR BsmI G>A	*42.2 GG*	*57.8 GA*	-
VDR ApaI C>A	*26.7 CC*	*28.9 CA*	*44.4 AA*
VDBP rs7041 T>G	*6.7 TT*	*62.2 TG*	*31.1 GG*

No genetic variants showed to affect VD concentrations.

Nivolumab plasma concentrations at 15 days (figure 3) were associated with *VDR* TaqI CC (p=0.042), ApaI CA/AA (p=0.030) and *CYP27B1*-1260 TT (p=0.014). Nivolumab exposure at 45 days (figure 4) were influenced by *VDR* Cdx2 AG/GG (p=0.019), *VDBP* rs7041 AC/CC (p=0.035) and *CYP27B1*-1260 TT (p=0.028); nivolumab exposure at 60 days (figure 5) was affected by *VDR* Cdx2 AG/GG (p=0.022) and TaqI TC/CC (p=0.021).



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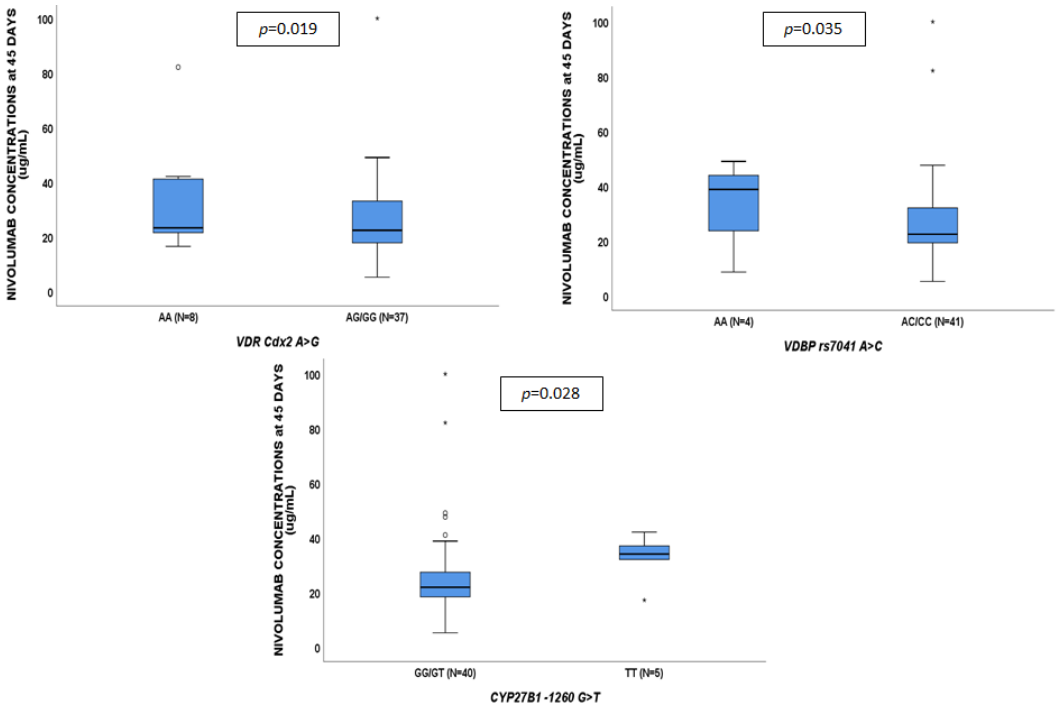
129 **Figure 3.** Gene variants' influence on nivolumab plasma concentrations at 15 days.



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131 **Figure 4.** Gene variants' influence on nivolumab plasma concentrations at 45 days.

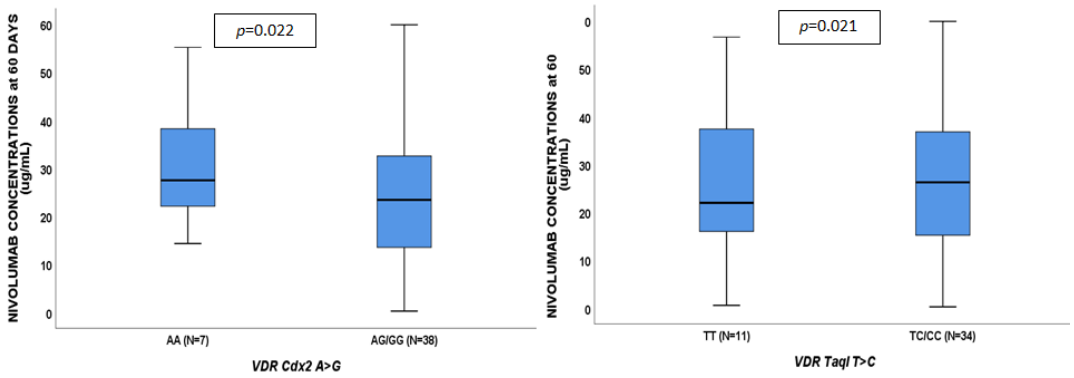

Figure 5. Gene variants' influence on nivolumab plasma concentrations at 60 days.

2.4. Regression analysis

Logistic regression analysis was performed to evaluate factors (demographic, clinical, pharmacological or genetic ones) were able to predict nivolumab concentrations < 18.7 ug/mL at 15 days (table 3). According to Bonferroni test, $p<0.003$ was considered the adjusted p-value, but no factors reached this value in univariate analysis. In a multivariate model, *VDBP* (GC) AC/CC genotype and BL 25-VD resulted predictors of this cut-off value, associated with tumor progression (figure 6).

Table 3. Logistic regression analyses: factors able to predict nivolumab concentrations < 18.7 ug/mL at 15 days of therapy. Bold represents statistically significant values. NC: all the factors belong to a single group, thus statistics could show p-values and OR.

	NIVOLUMAB CONCENTRATIONS ≤ 18.7 ug/mL			
	UNIVARIATE		**MULTIVARIATE**	
	***p* VALUE**	**OR (95% IC)**	***p* VALUE**	**OR (95% IC)**
BMI < 25 Kg/m2	0.766	1.270 (0.392-6.112)		
Age > 60 years	0.939	0.970 (0.091-9.145)		
Gender (male)	0.213	2.260 (0.692-12.419)		
DRUG DOSAGE < 200 mg	0.945	1.056 (0.099-4.867)		
***VDBP* (GC) AC/CC**	0.059	11.667 (0.909-149.700)	**0.049**	**10.667 (0.830-137.145)**
***CYP24A1* 3999 CC**	NC			
VDR TaqI TC/CC	0.164	3.077 (0.632-14.976)		
CYP27B1 -1260 GG	0.148	3.250 (0.658-16.040)		
PRE 25-HYDROXYVITAMIN D	NC		**NC**	
PRE 1,25-HYDROXYVITAMIN D	0.124	3.840 (0.692-21.312)		
Adenocarcinoma NSCLC type	NC			
Squamous cell carcinoma	NC			
Cisplatine pre-treatment	0.093	4.442 (0.852-24.853)		
Carboplatine pre-treatment	0.190	0.300 (0.051-1.854)		
Pemetrexed pre-treatment	NC			

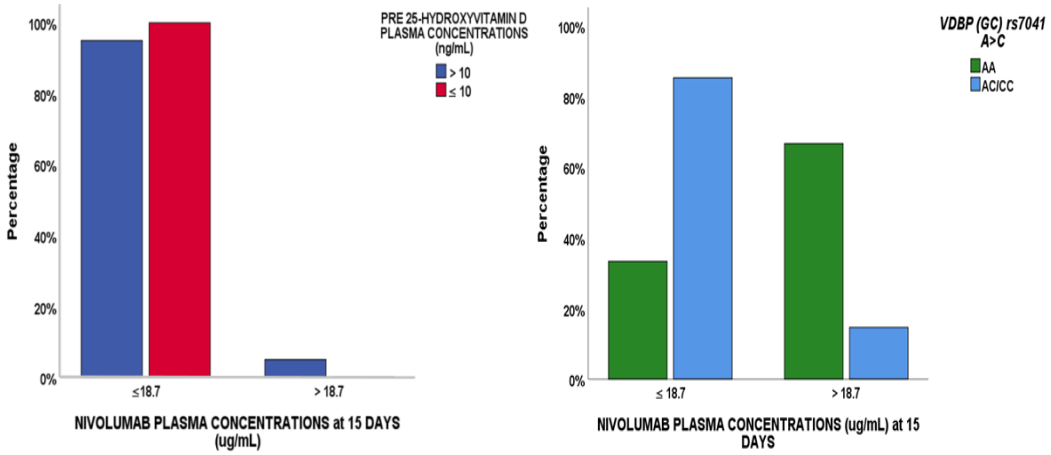

Figure 6. *VDBP* rs7041 SNP and pre-25 hydroxyvitamin D levels predictors of the nivolumab cut-off value of 18.7 ug/mL at 15 days, associated with tumor progression.

3. Discussion

Nivolumab represents an active treatment strategy with the potential of long-term disease control[12]. Unfortunately, reliable efficacy biomarkers are lacking, thus nivolumab has not been considered to be cost-effective in several national health systems[13].[14].

However, a meta-analysis[3] about immune checkpoint inhibitors and chemotherapy in the treatment of advanced NSCLC, showed significant advantages in terms of overall survival, progression free survival and overall response rate, compared with conventional chemotherapy in patients with advanced disease.

VD is able to regulate the immune system. Its synthesis begins by action of the ultraviolet light in the contest of skin tissue; cholecalciferol is hydroxylated to calcifediol (25-VD) in liver through cytochrome P-450 (CYP, 27A1, 2R1); in kidney calcitriol (1,25-VD, the active form) is synthesized through CYP27B1 and transported in bloodstream through vitamin D binding protein (VDBP). Inactivation of 25-VD to calcitroic acid (24,25-VD) is carried on by CYP24A1. VD deficiency is frequently observed in cancer patients: Bochen *et al.* suggested that VD serum levels were significantly lower in head and neck cancer patients compared to controls, particularly in patients with lymphatic metastasis[15]. Different studies show that lower 25-VD serum level is associated with several negative outcomes in lung cancer. Feng *et al.* analyzed seventeen studies in a meta-analysis: statistically significant relationship between 25-VD and lung cancer risk and mortality, but not with overall lung cancer survival were observed. In addition, they suggested differences between male and female and in Caucasian and Asian, in terms of cancer risk.

In the current study, 25-VD influences nivolumab concentrations, but not 1,25-VD. Here, we only evaluate nivolumab and VD concentrations and not the effect on the immune cells: VD deficiency could have a relapse in terms of immune system, which is directly related to this treatment. In fact, in another study, and not in the current, a relationship between immune cells and 25-VD and not with 1,25-VD exists, as shown for regulatory T cell function in multiple sclerosis affected patients[16]. The information about the VD influence on immune system lacks in this study and this limitation will be the aim of further studies, as considered by our group.

Furthermore, 1,25-VD is present with a concentration 1000 times lower than 25-VD in blood: such low 1,25-VD concentrations could be more difficult to measure compared to 25-VD levels. Finally, absence of statistical significance could be due to the small sample size.

Furthermore, in the current study, nivolumab plasma levels in real-life context of NSCLC were described at different timings and, in addition, the role of 25-VD concentrations and *VDBP* rs7041 A>C SNP in predicting concentrations lower than 18.7 ug/mL (cut-off value associated with tumor progression as shown by Stijn *et al.*[6]) is suggested.

Various *VDBP* genetic variants are known; the two most common polymorphisms, 1296 A>C (rs7041, Glu432Asp) and 1307 C>A (rs4588, Thr436Lys) are localized in exon 11 and they are in

complete linkage disequilibrium[17]. Circulating VDBP seems to be not influenced by rs7041 SNP, however, considering 1296/1307 diplotype, there is a slight transport increase in AC/CA, compared to AA/CA. Probably, lysine to threonine substitution at position 436 eliminates an O-glycosylation site from the molecule and the loss of glycosylation influences VDBP half-life. Moreover, glutamine to asparagine change in 432 position, affects the extent of O-glycosylation at the 436. It is not known how changes in VDBP molecule modify its serum concentration, but the described substitutions could result in altered rates of transcription, changes in mRNA stability or in a self-clearance of the protein[18]. In a recent study on Caucasian women, AA genotype was related to higher breast cancer risk, compared to healthy controls[19].

Controversial studies are present in literature concerning VDBP rs7041 influence on VD levels: Lafi *et al.* show that genotypes containing the variant allele of rs7041 (TT, TG) are associated with lower 25-VD concentrations than the GG genotype, whereas Daffara *et al.* did not find an association in coronary heart disease affected patients and suggest that 25-VD levels, but not VDBP genetic status, independently predicted the presence of coronary lesions at angiography[20, 21]. Also in the current study, an association between *VDBP* genetic variant and VD levels has been evidenced, although a *border-line* influence (p=0.049) is present with nivolumab cut-off value, but the best predictor factor remains 25-VD < 10 ng/mL, as showed in the regression. It is important to understand which is the relationship: is the VD associated with poorer outcome or it could be an underlying condition? In our opinion, VD deficiency could be able to affect the outcome, since it is involved in the regulation of the immune system; furthermore, in deficient individuals before starting therapy, the situation could be more difficult to manage and complications could be more severe (for example concerning cachexia).

Schmid *et al*. showed immunotherapy efficacy was dependent on the metastatic location[22]. For these reasons, it is very important to understand which biomarkers could predict patients with higher probability to have tumor progression.

Our study would recommend to clinicians to evaluate 25-VD levels and *VDBP* rs7041 genotype, before starting therapy, and to quantify nivolumab concentrations at 15 days, to eventually consider a drug dosage modification or VD supplementing, reducing the risk of tumor progression. It is important to highlight that these analyses are preliminary and have several limitations: they are conducted on few individuals (only 45 patients), only one cohort is analyzed and *VDBP* SNP has a *border-line* influence (p=0.049).

4. Materials and methods

Patients treated with nivolumab affected by advanced NSCLC treated within the Italian Nivolumab Expanded Access Program (NCT02475382) and enrolled in a mono-institutional translational research study at the Lung Cancer Unit of the Ospedale San Martino (Genova, Italy) approved by the Local Ethics Committee (registry number: P.R. 191REG2015). Patients were eligible if they met the following criteria: *i)* cytologically or histologically confirmed advanced/metastatic NSCLC, *ii)* progression after at least one line of platinum-based chemotherapy, *iii)* Eastern Cooperative Oncology Group Performance Status (ECOG-PS)= 0-2, *iv)* no previous treatment with immune checkpoint inhibitors, *iv)* any brain metastasis had to be treated and clinically stable for at least 14 days before starting nivolumab, v) no treatment with corticosteroids at a dose higher than 10 mg/day of prednisone or equivalent. Eligible patients receive nivolumab at 3 mg/kg every 14 days, with assessment by computed tomography scan (CT-scan) every 8 weeks. Nivolumab was administered until onset of unacceptable toxicities, patient's refusal, death or up to 96 weeks from the start of treatment; treatment beyond tumor progression was allowed based on Investigators' judgment as long as clinical benefit is perceived.

Values of 25-VD and 1,25-VD were evaluated at BL and at 15, 45 and 60 days after starting therapy, with enzyme linked immunosorbent assay technique (DRG DIAGNOSTIC, Marburg, Germany) and with LIAISON® XL (DiaSorin, Saluggia, Italy), respectively. Nivolumab and its anti-antibody are quantified with validated ELISA kits (Matrix Biotek, Ankara, Turkey).

Whole blood was withdrawn in EDTA tubes, genomic DNA was isolated from blood samples (MagnaPure Compact, Roche, Monza, Italy) and genotypes were assessed through a real-time polymerase chain reaction allelic discrimination system (LightCycler 480, Roche, Monza, Italy). Investigated gene SNPs were: *CYP27B1* (encoding cytochrome 27B1 enzyme responsible for VD active metabolite 1,25-VD production) rs4646536 (+2838) C>T and rs10877012 (-1260) G>T, *VDR* (encoding VD receptor) rs7975232 (ApaI) C>A, rs731236 (TaqI) T>C, rs10735810 (FokI) T>C, rs11568820 (Cdx2) A>G and rs1544410 (BsmI) G>A, *CYP24A1* (encoding cytochrome 27B1 enzyme responsible for VD inactive metabolite 24,25-dyhydroxyvitamin D (24,25-VD) production) rs2248359 (3999) T>C, rs927650 (22776) C>T and rs2585428 (8620) A>G and finally *GC* (encoding VD transporter, VDBP) rs7041 A>C.

All variables were tested for normality through the Shapiro-Wilk test. Normal variables were described as average and standard deviation, non-normal ones as median values and interquartile range (IQR) and categorical ones as numbers and percentages. Allele frequencies were tested for Hardy-Weinberg equilibrium. Kruskal-Wallis and Mann-Whitney tests were adopted for differences in continuous variables between genetic groups, considering statistical significance with a two-sided p-value < 0.05. Stepwise multivariate logistic regression analysis was performed including variables with a *p*-value below 0.2 at univariate analysis to evaluate factors are able to predict nivolumab levels < 18.7 ug/mL at 15 days. Bonferroni correction has been performed, since an adjustment made to *p* values is needed when several dependent or independent statistical tests are being performed simultaneously on a single data set[23].

Tests are performed with IBM SPSS Statistics 25.0 for Windows (Chicago, Illinois, USA).

5. Conclusions

In conclusion, this is the first study showing an association between VD-related biomarkers and nivolumab plasma concentrations.

In the current study, for the first time, VD deficiency seems to result in altered nivolumab clearance, as shown by different associations. Furthermore, another interesting information to highlight from these analyses is that the reduction in VD concentrations is not through antibodies.

In future, aims will be to analyze VD deficiency effect on the immune system, for example evaluating the immunologic profile according to VD-related biomarkers or PD-1 or PD-L1 levels and their genetic variants.

These are preliminary and limited analyses, but further studies in larger and different cohorts are needed to clarify these aspects and to improve the knowledge in the field of monoclonal antibodies treatment used in NSCLC.

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Other authors have no conflict.

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