

Triple Negative Myeloproliferative Neoplasms - Sometimes Driver Mutations Stay Low-Key in Plain Sight

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Abstract

Triple Negative Myeloproliferative Neoplasms (TN MPNs) have recently emerged as a separate molecular entity, being characterized by the absence of mutations in the 3 driver genes that represent the hallmarks of classical BCR-ABL1 negative myeloproliferative neoplasms (MPNs): JAK2, calreticulin (CALR) and thrombopoietin receptor (MPL/TpoR). It seems that this entity has a different clinical outcome than other MPN categories although pathologic activation of JAK/STAT signaling is also at the core of the disease.

A deeper exploration of the genetic background of TN MPNs reveals a heterogeneous molecular profile that consists either of canonical MPN driver mutations with very low allele burden or detected only at the level of platelet ARN, non-canonical MPL and JAK2 mutations of either somatic or germ-line origin, or also mutations in other non-MPN-driver genes. Polyclonal hematopoiesis is present in some cases suggesting that those are more likely benign disorders of platelet production, like hereditary thrombocytosis, than MPNs.

A mutational analysis beyond the routine molecular investigations is often necessary to unravel the "low-key" drivers of the TN MPNs.

Keywords: Myeloproliferative Neoplasms (MPNs), Triple - Negative MPNs (TN MPNs), secondary acute myeloid leukemia (sAML), JAK/STAT signaling

1. Introduction

Myeloproliferative Neoplasms (MPNs) are a group of chronic disorders that are able to progress to secondary acute myeloid leukemia (sAML), which make them a good model for stepwise oncogenesis.

The most prevalent MPNs are the BCR-ABL1 negative group, particularly Polycythemia Vera (PV), Essential Thrombocytosis (ET) and Primary Myelofibrosis (PMF). Together they are significantly more prevalent than chronic myeloid leukemia. In 2005, the

most dominant driver mutation (*JAK2 V617F*) was identified and the importance of JAK/STAT signaling pathway was clearly demonstrated (JAMES & al. [1]; KRALOVICS & al. [2]; BAXTER & al. [3]; LEVINE & al. [4]). This led to a major effort of diagnosis, a change in the WHO criteria of diagnosis and in the development of JAK2 inhibitors for treatment (ARBER & al. [5]). In 2006 and 2013, mutations in the other two major drivers were discovered, namely thrombopoietin receptor (*MPL/TpoR*) mutations at W515 (STAERK & al. [6]; PIKMAN & al. [7]; PARDANANI & al. [8]; PECQUET & al. [9] and mutations in the calreticulin (*CALR*) gene KLAMPFL & al. [10]; NANGALIA & al. [11]). Importantly, this group of disorders can be associated with different gene alterations which include loss-of-function mutations in epigenetic regulators, like *TET2*, *EZH2*, *ASXL1* and *DNMT3A* (KLAMPFL & al. [10]; NANGALIA & al. [11]; SCOTT & al. [12]; CARBUCCIA & al. [13]; ERNST & al. [14]; VANNUCCHI & al. [15]; CAZZOLA & al. [16]; MAMBET & al. [17]). However, it remains incompletely understood how these mutations in epigenetic regulators interact and influence disease initiation, clonal evolution, and blast crisis.

In terms of pathogenesis, it is now well established that most (> 85%) of MPNs are characterized by somatic driver mutations, apparently mutually exclusive, which lead directly or indirectly to constitutive activation of JAK2 / STAT signaling (Figure 1). *JAK2 V617F* mutation in exon 14 was identified in over 95% of patients with PV and 50 – 60% of patients with PMF and ET, *JAK2* mutations in exon 12 in about 3% of *JAK2 V617F* negative PV (SCOTT & al. [12]; SCOTT [18]), *CALR* gene mutations were present in 20% of ET and 25% of PMF and was reported only in two *JAK2 V617F*-negative PV cases (BROSEUS & al. [19]) and thrombopoietin receptor *MPL W515L/K/A/R* mutations or rare S505N mutations were found in 3% of ET and 7% of PMF (TEFFERI & al. [20]; VAINCHENKER & al. [21]; TEFFERI & al. [22]).

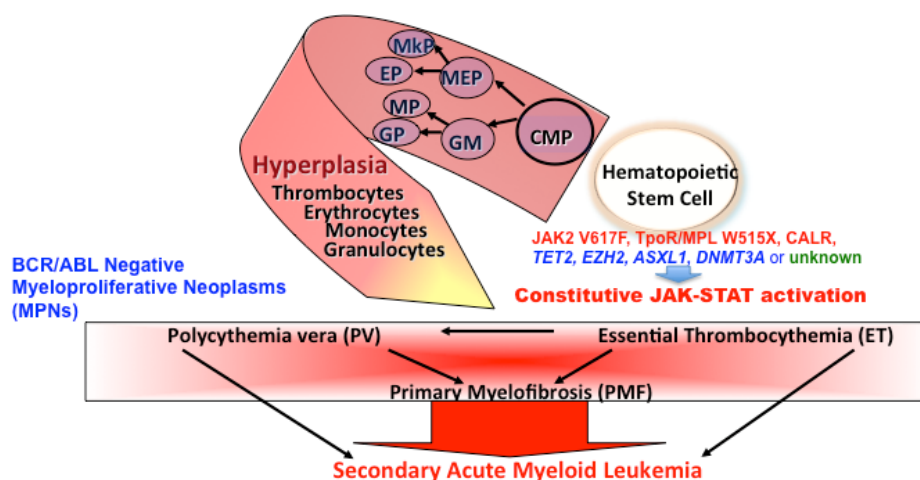


Figure 1. Classic Myeloproliferative Neoplasms (MPNs), particularly, Polycythemia Vera (PV), Essential Thrombocytosis (ET) and Primary Myelofibrosis (PMF) are clonal disorders arising after molecular alterations, apparently mutually exclusive, which lead directly or indirectly to constitutive activation of JAK2 / STAT signaling and may evolve to secondary Acute Myeloid Leukemia.

In 5 – 10% of ET or PMF, evidence of these three particular driver mutations could not be recognized, although JAK / STAT signaling was activated and the term of triple-negative was applied to those MPN cases (RAMPAL & al. [23]; SILVENNOINEN & al. [24]; RUMI & al. [25]). Actually, it seems that certain rare MPNs are even more complex involving several other genes beyond the 3 drivers (RUMI & al. [25]), or atypical mutations in *JAK2* and *TpoR/MPL* genes (MILOSEVIC FEENSTRA & al. [26]; CABAGNOLS & al. [27]). Using deep sequencing technologies and / or going to RNA level in cells that are directly involved in pathogenesis of these disorders, several reports revealed the presence of the canonical driver

mutations at low allele frequency or after mRNA isolation exclusively from the platelets, indicating that TN MPNs are a heterogeneous group of diseases, with some exhibiting non-penetrant driver mutations, others being either non-clonal or not meeting the criteria of MPNs (MILOSEVIC FEENSTRA & al. [26]; CABAGNOLS & al. [27]; ANGONA & al. [28]).

This review summarizes current advances on TN MPNs emphasizing the importance of the diagnostic accuracy and new prospective biomarkers potentially targetable in future selective therapies.

2. Triple Negative Myeloproliferative Neoplasm prognosis

Somatic activating *JAK2*, *CALR*, and *MPL/TpoR* driver mutations, as hallmarks of clonal disease, are currently included in the major diagnostic criteria of classical BCR-ABL1 negative MPNs that have been recently revised by World Health Organization (WHO) (ARBER & al. [5]). However, for classifying MPNs into ET, PV or PMF a proper integration of hematological abnormalities, bone marrow histological findings and molecular analysis results is required (BARBUI & al. [29]). The distinction between these three clinical entities is particularly important for prognosis assessment, as survival rate is significantly reduced in PMF and to a lesser degree in PV, while in “true” ET it seems to be similar to that of European individuals, matched for age and sex (HULTCRANTZ & al. [30]; BARBUI & al. [31]). In addition, discrimination between prefibrotic PMF – an early stage of myelofibrosis that manifests predominantly with high platelet levels – and ET has also prognostic relevance (BARBUI & al. [31]).

On the other hand, a negative result for a driver mutation does not rule out the MPN diagnosis, TN cases being reported in up to 20% of ET and 5 – 10% of PMF (RUMI & al. [25]; TEFFERI & al. [32]). According to WHO recommendations, in order to get an evidence of disease clonality, the molecular investigations must be expanded to mutations in genes involved in epigenetic regulation and mRNA splicing that are not restricted to classical MPNs (ARBER & al. [5]; RUMI & al. [25]).

Although required for diagnostic purpose, MPN driver mutations or other clonal markers are not added to the current prognostic scoring systems, except for the *JAK2 V617F* mutation. These prognostic models are used in clinical practice to evaluate the overall survival rate and the risk of MPN complications, namely thrombotic events, leukemic transformation, secondary myelofibrosis (in case of PV or ET) or conversion to a polycythemic phenotype (in case of ET) (PASSAMONTI & al. [33]; PASSAMONTI & al. [34]; BARBUI & al. [35]; BARBUI & al. [36]).

By taking into account the somatic mutations in MPN driver genes as well as in non-MPN-driver myeloid genes the prognostic assessment of each MPN nosologic entity would be considerably improved and new disease subgroups with impact on clinical outcome might be delineated (RUMI & al. [25]). From this perspective, TN ET patients represent a low-risk category, exhibiting slow disease progression unlikely to be complicated by vascular events, while those with *JAK2 V617F* mutation may undergo polycythemic transformation and have a higher incidence of arterial and venous thrombosis than carriers of *CALR* mutations (BERTOZZI & al. [37]; RUMI & al. [38]).

On the contrary, several studies have reported that TN PMF is a high-risk molecular category with an increased rate of leukemic transformation (TEFFERI & al. [22]; RUMI & al. [39]; TEFFERI & al. [40]). It was shown also that TN PMF patients exhibited the lowest survival rate (3.2 years) when compared to patients harboring mutations in *JAK2* (9.2 years), *MPL* (9.1 years) and *CALR* genes (17.7 years) (RUMI & al. [39]). Morphologically, TN PMF displays bone marrow hypercellularity and multilineage dysplasia resulting in pancytopenia, resembling myelodysplastic syndrome with bone marrow fibrosis (VAINCHENKER & al. [41]). Identification of this particular form of PMF is very important - this includes the study of peripheral blood and bone marrow morphology to assess abnormalities of peripheral blood

cells and hematopoietic precursors, bone marrow biopsy to evaluate marrow cellularity and fibrosis, and molecular biology approaches. Sometimes, if it is possible, to demonstrate clonal disease it is recommended to test the most common mutations associated with this pathology: *ASXL1*, *EZH2*, *TET2*, *IDH1/IDH2*, *SRSF2*, *SF3B1*. It is also important to rule out the presence of BCR-ABL transcript (VAINCHENKER & al. [42]).

3. Canonical MPN driver mutations discovered at low allele frequency or at the mRNA level

PV cases negative for *JAK2 V617F* and exon 12 mutations are very rarely encountered in clinical practice (SCOTT & al. [12]). Such situations require a careful diagnostic reevaluation. Sometimes, a small hematopoietic clone harboring exon 12 *JAK2* mutations (around residue K539) that can be missed by conventional sequencing techniques performed on granulocytic DNA might be responsible for the PV phenotype (RUMI & al. [25]; VAINCHENKER & al. [42]) and can be detected in EPO-independent erythroid colonies (SCOTT & al. [12]).

An interesting fact is that in PV many patients progress from heterozygous to homozygous *JAK2 V617F*. This is also the case for PV patients that had previously been in an ET phase. In contrast, ET patients remain strictly heterozygous for *JAK2 V617F*. The reasons behind this major difference remain unclear. One possibility is that the hematopoietic stem and progenitor cells that give rise to ET might exhibit high sensitivity to the anti-proliferative effects of TpoR signaling at high *JAK2 V617F* levels. In model cell lines, it has been shown that Tpo can induce also negative signaling, with a stop in proliferation and senescence (PECQUET & al. [43]; BESANCENOT & al. [44]). Isolation of the different subsets of MPN stem cells in ET and PV would allow investigation by RNA-seq and chromatin state, which might reveal specific mechanisms that would limit and select out homozygous *JAK2 V617F* in ET. Noteworthy, in PV even when overall the mutation appears heterozygous, cells with homozygous mutations can be detected early (GODFREY & al. [45]), which is not the case in ET.

ET is the chronic myeloproliferative disorder with the best prognosis, characterized by megakaryocyte hyperplasia and thrombocytosis. The genetic cause of this disorder is represented by mutations in *JAK2* (50 – 60%), *CALR* (15 – 30%) and *TpoR/MPL* (1 – 5%) genes (CAZZOLA & al. [16]). Most of the studies, which reported such percentages, were performed using DNA obtained from isolated granulocytes or from whole blood. Other studies which analyzed the platelet RNA reported *JAK2 V617F* detection in only 10% of ET patients (TOYAMA & al. [46]; TOYAMA & al. [47]; CAMPBELL & al. [48]). In contrast, another study observed a higher *JAK2 V617F* allele burden in platelets when compared with granulocytes in ET patients (BELLOSILLO & al. [49]). Even if the data appear contradictory, it is considered that 10 – 30% of all ET patients are wild type for *JAK2*, *CALR* and *TpoR/MPL*.

One recent study published in 2016 by X. CABAGNOLS *et al.* (CABAGNOLS & al. [27]) on triple-negative ET patients identified two new mutations on *MPL*, *MPL S204P* and *MPL Y591N*, using whole-exome sequencing (WES) followed by next-generation sequencing (NGS) targeted on *JAK2* and *MPL*. Functional studies on *MPL S204P* and *MPL Y591N* revealed that they are weak gain-of-function mutants that lead to a partial spontaneous activation of *MPL* signaling (CABAGNOLS & al. [27]). Other mutations were found in 2 *MPL S204P*-positive samples: in epigenetic regulators that modulate Polycomb Repressor Complex 2, such as *EZH2* and in *ASXL1*, one of the most frequently mutated genes in malignant myeloid diseases (GELSI-BOYER & al. [50]), or in *SRSF2*, one of the RNA splicing machinery genes, that has been previously identified in a substantial proportion of patients with myelodysplastic syndrome (MDS) (WU & al. [51]). Additionally, they found in three patients, known as triple-negative ET, previously described *MPL* mutations (*MPL W515R* and *MPL S505N*). This surprising result led to NGS studies targeting all *MPL* and *JAK2* exons. Strikingly, in one patient the *JAK2 V617F* mutation was detected, and in another the *MPL S204P* mutation was identified. The authors concluded that these mutations

were not previously seen in WES because of the low allelic burden for *JAK2* (< 5%) and a missing coverage of the assay for *MPL* S204P position.

Another aspect revealed by the study was the presence of polyclonal hematopoiesis in granulocytes in 7 triple-negative ET patients with no mutation detected by WES. However, the WES technique has a sensitivity of around 10% for variant allele frequency (VAF) so it seems that polyclonal triple-negative ET can have multiple mutations with low VAF in granulocytes (VAINCHENKER & al. [42]). This can be similar with the results obtained from ANGONA A. *et al.* (ANGONA & al. [28]) that observed a higher *JAK2* V617F VAF in platelets RNA when compared with granulocytes DNA in ET patients. Therefore, it will be important in the future to directly perform WES or whole-genome sequencing on megakaryocytes or to sequence platelet RNA.

4. Canonical signaling culprits giving different outputs

Somatic mutations in *CALR* gene were detected in most patients with ET or PMF with unmutated *JAK2* or *MPL*, ~ 20 – 25% of cases presents *CALR* exon 9 indels: *CALRdel52* (type 1 mutant) and *CALRins5* (type 2 mutant) being the most frequent variants. By generating a novel C-terminus of the mutant protein in which the negatively charged amino acids are replaced by neutral and positively charged amino acids, these indels give *CALR* mutant – MPN distinctive clinical features and were classified as: (i) type 1-like; (ii) type 2-like; and (iii) other types (RUMI & al. [38]; RUMI & al. [39]; PIETRA & al. [52]). The two major subtypes of mutant *CALR* have different effects on calcium signals in cultured megakaryocytes (Mks). The larger ER-dependent Ca^{2+} release is correlated with the impairment of the ER Ca^{2+} -storage ability caused by the loss of the ER Ca^{2+} -binding residues observed in Mks from type 1 *CALR* mutation patients (PIETRA & al. [52]).

The mechanism by which *CALR* mutations induce MPNs was elucidated and is represented by pathologic activation of TpoR signaling intracellularly and at the cell-surface by the *CALR* mutants. The N-glycosylation of the distal N-terminus of TpoR was required for activation as well as the lectin binding domain of *CALR* mutants and the novel sequence at the C-terminus (CHACHOUA & al. [53]). While both del52 and ins5 mutants activate TpoR, a stronger signaling via the JAK-STAT pathway is observed with del52.

Studies conducted by retroviral mouse modeling, showed that progression of marked thrombocytosis to a condition similar to myelofibrosis is rapidly developed on *CALRdel52* (type 1 mutant) expressing mice, while mice with *CALRins5* (type 2 mutant) had a mild ET phenotype with low susceptibility to disease progression (MARTY & al. [54]). Further studies on patients with ET and PMF confirmed the process observed on mice, marking differences between *CALR*-mutant subtypes 1 and 2. Type 2-like *CALR* mutations are mainly associated with an ET phenotype, low risk of thrombosis and indolent clinical course, while type 1-like mutations are mainly associated with myelofibrotic progression (CABAGNOLS & al. [55]). *CALR*-mutant MPNs are all associated with activated *JAK2* signaling and marked thrombocytosis which validates the JAK-STAT pathway activation via TpoR (PIETRA & al. [52]). Patients with *CALR*-mutated ET, despite having high platelet counts, have a lower risk of thrombosis than patients with *JAK 2*-mutant ET. PMF patients with different types of mutations in *CALR* were not significantly clinically different and have better survival than patients with *JAK2*-mutant or *MPL*-mutant PMF.

5. Deeper search reveals new potential markers

The above-cited examples of WES and targeted NGS on TN MPNs illustrate the concept that deeper searches will identify causative mutations in MPNs where the classical driver mutations cannot be detected (MILOSEVIC FEENSTRA & al. [26]; CABAGNOLS & al. [27]).

This approach led to detection of non-canonical *MPL/TpoR* and *JAK2* mutations (outside exon 10, and respectively exon 14 and 12) in about 19% of TN ET and PMF. Using paired DNA isolated from purified granulocytes (as tumoral / clonal cells) and DNA isolated from T cells (which are usually not mutated given the long half-life of T cells), the somatic or germline origin of the mutations could be established. The somatic *MPL* mutations were located either in the extracellular domain, in the exons 3, 4 and 5 (T119I, S204F, S204P, and E230G) or in the intracellular domain of the receptor, at the level of exon 12 (Y591D, Y591N). Germline mutations in *MPL* were represented by V285E and R321W and involved exon 6. Functional studies proved that all *MPL* genetic lesions were gain-of-function mutations able to induce TPO-independent *JAK2*-*STAT5* signaling, although less effectively than the *MPL* W515K driver mutation. Apart from atypical *MPL* mutations, several mutations in *JAK2* were detected – 4 of them proved to be germline (G335D, G571S, V625F, N1108S) and 1 of them of unknown origin (F556V). All of them were associated with the ET phenotype. Only V625F and F556V mutants were able to increase *JAK2*-*STAT5* signaling and confer hypersensitivity to TPO in functional assays, while N1108S was hypothesized to be a gain-of-function mutation (MILOSEVIC FEENSTRA & al. [26]; CABAGNOLS & al. [27]).

MPL S204F/P mutations were also reported by a different group of researchers together with a new *MPL* variant (P222S) that was not analyzed in functional assays. Their study included TN ET patients and mutational analysis was performed in granulocytic DNA by targeted NGS. Additionally, mutations in other genes like *TET2*, *CBL*, *SF3B1*, *SH2B3* (*LNK*) were detected in certain patients, indicating that TN ET is characterized by a wide spectrum of genetic abnormalities (ANGONA & al. [28]).

The driver mutations have clearly enhanced diagnostic efficiency and, somatic mutations profile, discovered by the next generation sequencing technique, have allowed better stratification of patients and newly discovered markers will be able to improve personalized treatment.

6. Triple-Negative MPNs – not a homogenous entity

As it was previously shown, TN MPNs have a complex molecular profile, including either somatic or germline mutations in both MPN and non-MPN drivers, suggesting that this category of MPNs encompasses different conditions rather than being a homogenous entity (MILOSEVIC FEENSTRA & al. [26]).

Moreover, according to X-inactivation-based clonality assays performed on granulocytes a significant number of TN MPN patients associate with a polyclonal hematopoiesis, supporting the idea of disease heterogeneity. This fact has impact on therapeutic approach, as those cases may not benefit from cytoreductive medication (RUMI & al. [25]).

However, the absence of recurrent mutations in some cases of TN MPNs does not rule out the existence of disease drivers as WES cannot detect molecular anomalies like fusion oncogenes or mutations in regulatory sequences. More refined techniques, such as RNA sequencing and whole-genome sequencing, would be appropriate for this purpose.

7. Some Triple-Negative MPNs might not be MPN after all

The fact that some TN ET patients harbor germline non-canonical *MPL* or *JAK2* mutations raises the possibility that these cases might actually represent hereditary thrombocytosis (HT) (RUMI & al. [25]; VAINCHENKER & al. [42]).

HT is an early-onset autosomal-dominant transmitted disorder that mimics ET in its clinical presentation but, in contrast to ET, it is characterized by polyclonal hematopoiesis and thus considered a nonmalignant disease (MARTY & al. [56]). HT can be misdiagnosed as ET especially if the family history is absent (CABAGNOLS & al. [27]) and unnecessary cytoreductive treatment may be initiated (RUMI & al. [25]). An interesting example of HT is induced by germline mutation in *JAK2* V617I (MEAD & al. [57]). This mutation had been identified as a weak-activating mutation in a saturation mutagenesis study (DUSA & al. [58]). The *JAK2* V617I, like several other such germ-line mutations in *JAK2* induce weak signaling, amplifying

megakaryocytes but not erythroid or granulocytic compartments (MEAD & al. [57]) and mainly via STAT1 inducing non-clonal thrombocytosis (MARTY & al. [56]; MEAD & al. [57]).

In PMF patients, 5 to 10% are TN, but as in the case of TN ET, it is essential to distinguish between possible causes of myelofibrosis. In the case of TN PMF might be in fact a secondary myelofibrosis caused by other diseases like a diffuse large B cell lymphoma (DLBCL) with bone marrow involvement mimicking TN PMF (KUMAGAI & al. [59]) or myelodysplastic syndrome associated with bone marrow fibrosis (RUMI & al. [25]). Some cases of TN PMF, due to the prominent dysplastic features, might be probably more appropriately considered as myelodysplastic syndromes accompanied by myelofibrosis than classical MPNs (VAINCHENKER & al. [42]).

When TN status is established, the clonal nature of the disease should be proven and abnormalities in *ASXL1*, *EZH2*, *TET2*, *IDH1 / IDH2*, *SRSF2*, *SF3B1* should be investigated as well. These are “non MPN-restricted” acquired mutations, because of their implication in other myeloid malignancies VAINCHENKER & al. [42].

8. Conclusions and perspectives

MPNs moved from diseases without known molecular cause to entities that are covered to an extent of 85 – 90% with respect the phenotypic driver mutation. The phenotype of the disease faithfully reflects the driving mutation, with *CALR* and *MPL/TpoR* mutations driving megakaryocyte pathologic expansion and differentiation defects (ET and MF), while *JAK2* V617F driving all three diseases, PV, ET and MF. Starting with the rare exon 12 *JAK2* mutations in isolated erythrocytosis (PV), it has been observed that certain mutations do not penetrate in peripheral granulocytes, and remain confined to specific progenitors, yet they give phenotype. This can occur also in occasional patients for the canonical driving mutations, where for example mutations can be detected in platelet mRNA but not in total blood or granulocytes. An opposite example is that of *CALR* mutations that give high allele burdens at young ages and penetration early in the disease. Certain triple negative patients carry either non-penetrant clonal canonical mutations, or atypical *JAK2* and *MPL/TpoR* mutations, that can be non-clonal. Interestingly, typical MPN driver mutations were found at low allele frequency or only at the mRNA level by means of techniques that are not usually employed in current practice. Also, non-canonical MPN mutations could be detected after full sequencing of *MPL* and *JAK2* genes that is not routinely done at diagnosis. In this perspective, it is necessary to define and implement new strategies for molecular diagnosis that would recognize the drivers that are not obvious at the first sight.

A new era needs to start in MPNs whereby whole genome sequencing coupled to RNA sequencing and investigation of chromatin state will elucidate the role of additional epigenetic mutations in disease establishment and progression.

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