

Applicability of Next-generation Sequencing Techniques in Assessing Non-alcoholic Fatty Liver Disease: A Comprehensive Review

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ABSTRACT

Non-alcoholic Fatty Liver Disease (NAFLD) is a burgeoning global health concern, with a spectrum of severity ranging from simple steatosis to Non-alcoholic Steatohepatitis (NASH). While liver biopsy remains the gold standard for diagnosis, the need for non invasive alternatives has spurred interest in Next-generation Sequencing (NGS) technologies. NGS technologies enable the simultaneous sequencing of millions of Deoxyribonucleic Acid (DNA) or Ribonucleic Acid (RNA) fragments, providing a high-throughput approach to analyse genetic variations, gene expression and epigenetic modifications. A comprehensive literature search was conducted to identify studies investigating the use of NGS in NAFLD. Data extraction focused on NGS techniques, study design, key findings and clinical implications. NGS has demonstrated potential in unravelling the complex genetic and molecular underpinnings of NAFLD. The identification of genetic variants, epigenetic modifications and non coding RNA alterations has advanced our understanding of disease pathogenesis. Moreover, NGS-based approaches have shown promise in differentiating NAFLD subtypes and predicting disease progression. However, challenges related to data analysis, standardisation and clinical translation persist. NGS offers a promising avenue for improving NAFLD diagnosis, prognosis and management. While significant advancements have been made, further research is needed to fully realise its clinical potential.

Keywords: Diagnosis, Epigenetics, Genetics, Gene expression, Non invasive, Omics, Pathogenesis, Surveillance

INTRODUCTION

Non-alcoholic Fatty Liver Disease has seen an alarming surge and is a cause of global health concerns [1]. Schaffner first coined the term NAFLD in 1986 [2]. It is a pathological condition characterised by increased fat deposition in liver cells that is neither attributable to alcohol consumption [3] nor to viral hepatitis [4]. The excessive accumulation of fat in hepatocytes results in increased intracellular fat vacuoles, which impairs mitochondrial beta-oxidation and triggers endoplasmic reticulum stress, oxidative stress and hepatocyte apoptosis [3].

NAFLD encompasses a range of conditions, from simple steatosis to NASH, which can progress to cirrhosis, Hepatocellular Carcinoma (HCC), and ultimately, liver failure [4]. Biopsy specimens should be categorised as either NAFL (steatosis), NAFL with inflammation, or NASH (steatosis with lobular and portal inflammation and hepatocellular ballooning) according to the 2018 NAFLD guidelines published by the American Association for the Study of Liver Diseases (AASLD) [5]. The NAFLD Activity Score (NAS) was introduced in 2005 by the NASH Clinical Research Network (CRN) as a standardised tool for assessing histological changes in NAFLD during clinical trials. The entire range of NAFLD, including simple steatosis, can be evaluated using this score [6].

The score, which ranges from 0 to 8, is determined by summing the scores of its individual components for hepatocellular ballooning (0-2), lobular inflammation (0-3) and steatosis (0-3). The primary purpose of the NAS is not to diagnose NASH, but rather to assess histological changes over time [6]. Given that NAS scores of 5 or greater are often linked to a diagnosis of NASH, and scores of 2 or lower are generally considered 'not NASH', few studies [7-9] have focused on the specific threshold values of the NAS, specifically NAS ≥ 5 , as a surrogate for the histological diagnosis of NASH. However, NAS should not be used as a definitive diagnostic or classification tool in a clinical setting, and careful interpretation is recommended. Early detection of NAFLD is important, particularly for identifying individuals who may have silent progressive fatty liver disease. The

current diagnostic approach for NAFLD involves a combination of radiographic, biochemical and clinical testing. The differences between NGS and traditional diagnostic methods for NAFLD has been outlined in [Table/Fig-1]. Although liver biopsy remains the gold standard for diagnosing NAFLD, its intrusiveness and high cost make it an unfeasible option for many patients. Consequently, physicians at all levels of care need access to sensitive and specific diagnostic tests [10].

Features	NGS	Traditional methods
Invasiveness	Non invasive	Invasive (liver biopsy)
Sensitivity	High sensitivity for detecting genetic and molecular markers	Lower sensitivity for early-stage disease
Specificity	High specificity for identifying specific genetic variants	Moderate specificity, can be influenced by other liver diseases
Cost	High initial cost but decreasing with technological advancements	Lower initial cost but may require additional tests

[Table/Fig-1]: Comparison of NGS and traditional diagnostic methods for NAFLD.

Through the combination of different sequencing chemistries, sequencing matrices and bioinformatics technology, NGS has emerged as a revolutionary technological advancement in interrogating the nucleotide order of a given fragment through DNA sequencing [11]. This massively parallel or deep sequencing technology offers several advantages, including increased sequence output per run, simultaneous sequencing of multiple target regions, clonal sequencing of individual DNA molecules, sample multiplexing, improved diagnostic sensitivity, streamlined workflows and reduced sequencing costs per base [12]. Since its introduction, NGS has led to a dramatic increase in the availability of genomic data [12].

The NGS workflow consists of three major steps: first, template preparation (also known as sample preparation); next, sequencing; and finally, imaging. Bioinformatic analysis of NGS data typically involves two main phases: primary analysis, which includes alignment, variant identification and annotation; and secondary analysis, which focuses on gene prioritisation and predicting the

pathogenicity of mutations. The main objective is to discover the driver mutations associated with a particular disease or phenotype [13]. Targeted gene panels are an effective NGS strategy for the rapid and accurate verification of clinical suspicion, while Whole Exome Sequencing (WES) is particularly useful for filtering variants, especially in cases with unclear clinical information [14].

The present review was aimed to comprehensively examine the current literature on the use of NGS techniques in the diagnosis and surveillance of NAFLD, evaluating the diagnostic accuracy, sensitivity and specificity of NGS-based approaches in the detection and monitoring of NAFLD. It seeks to identify key genetic variants, molecular alterations, and dysregulated pathways associated with NAFLD through NGS technologies. Additionally, it will assess the feasibility and utility of NGS-based liquid biopsies for NAFLD surveillance and treatment response monitoring, and finally, highlight the potential benefits, limitations and future directions in the utilisation of NGS techniques for NAFLD management.

LITERATURE SEARCH

The review began with a literature search to identify relevant articles, studies and databases on the use of NGS techniques in NAFLD diagnosis and surveillance. The search strategy utilised electronic databases such as PubMed, Scopus, Google Scholar and Web of Science, along with manual searches of reference lists from identified studies.

Following the literature review, studies were selected based on predefined inclusion and exclusion criteria. The inclusion criteria encompassed studies that utilised NGS techniques for the detection, characterisation and monitoring of NAFLD in human subjects. Exclusion criteria included studies not published in peer-reviewed journals, studies not written in English, studies that used only animal models or in-vitro studies, and studies that focused on Alcoholic Fatty Liver Disease (AFLD) or other liver diseases without specific mention of NAFLD.

After selecting the relevant studies, data extraction was performed to collect key information, such as the NGS platforms used, the sample types, the specific genes or genetic regions targeted, and the data analysis methods applied.

Furthermore, a qualitative synthesis of the extracted data was conducted to identify emerging themes and patterns related to the utility of NGS in NAFLD diagnosis and surveillance.

DISCUSSION

Complexities of NAFLD

The NAFLD can progress from hepatic steatosis to life-threatening secondary diseases. It has become imperative to identify non invasive biomarkers. Several Non Invasive Tests (NITs), such as serum and genetic biomarkers, as well as, imaging modalities, are under investigation as potential alternatives for diagnosing NAFLD and NASH [15]. Novel biomarkers are being uncovered using omics approaches, including lipidomics, metabolomics and RNA molecule profiling [16]. Over the past decade, knowledge about the genetic component of NAFLD has grown exponentially [17].

Genetic Determinants

Recently, the heritable component of NAFLD has gained credence due to findings from epidemiological, familial aggregation and twin studies. These findings strongly indicate the potential of genetic mapping strategies for identifying genes with therapeutic value. In early genetic studies of non autoimmune familial diseases, candidate gene approaches were utilised. However, with the advent of NGS and high-throughput genotyping arrays, more reliable and objective methods for genetic mapping studies, such as Genome-wide Association Studies (GWAS) and Exome-wide Association Studies (EWAS), have become possible.

The GWAS has effectively identified genetic loci linked to the risk of various complex diseases and traits within the exonic (protein-coding) regions of the genome, using common variants discovered through genotyping. While EWAS primarily examines variations in the exonic (protein-coding) regions of the genome, recent research employing Whole Exome Sequencing (WES) can identify exonic variants, thanks to the declining cost of NGS [18].

Han SK et al., reviewed the genetic determinants that play a central role in NAFLD development [2]. Typically, Patatin-like Phospholipase domain-containing Protein 3 (PNPLA3) and Transmembrane 6 Superfamily member 2 (TM6SF2) nucleotide polymorphisms initiate and facilitate the progression of the disease [19]. Moreover, homozygous carriers of p.148M mutations face a 12-fold increased risk of developing HCC, implying the potential for monogenic inheritance. Hispanics have a higher prevalence of these variants compared to non Hispanic whites and African Americans [20].

The rs738409 (G) allele of PNPLA3 is linked to greater liver fat content, necroinflammatory scores, and a higher risk of developing fibrosis. Asians with lean NAFLD who do not have metabolic syndrome exhibit a higher prevalence of the PNPLA3 rs738409 (G) allele, similar to Caucasian populations with NAFLD. Additionally, patients with cryptogenic cirrhosis show a similar prevalence of PNPLA3 rs738409 genotypes as those with NASH, regardless of the presence of type 2 diabetes mellitus and obesity. The expression of the PNPLA3 allele can also be affected by additional factors, including lifestyle, viral infections and alcohol consumption.

The rs58542926 allele of TM6SF2 is a genetic variant associated with NASH. The TM6SF2 E16K variant is linked to a higher risk of progressive NASH, although recent findings suggest it may lower the risk of cardiovascular disease. Genetic risk factors for liver fibrosis include variants in the TM6SF2 and MBOAT7 genes [21].

Additionally, the enzyme Hydroxysteroid 17 β Dehydrogenase 13 (HSD17B13), which is part of a larger family of enzymes primarily associated with sex hormone metabolism, is identified as a novel liver-specific lipid droplet-related protein in both mice and humans in relation to NAFLD. The overexpression of HSD17B13 in the liver leads to increased levels of lipid accumulation, signifying its contribution to the advancement of NAFLD. A loss-of-function variant of HSD17B13 has been found to decrease the risk of developing chronic liver diseases and the progression from steatosis to steatohepatitis, highlighting its potential therapeutic importance [22]. Genes associated with carbohydrate metabolism, insulin signaling pathways, inflammatory pathways, oxidative stress and fibrogenesis are likely to contribute further to the advancement and progression of NAFLD/NASH [23]. Sveinbjornsson G et al., identified 18 independent sequence variants at 17 loci in the combined GWAS [Table/Fig-2] [24].

Closest gene	rs. no.	OR, NAFL
PNPLA3	rs738409	1.47
TM6SF2	rs58542926	1.39
TM6SF2	rs187429064	1.42
APOE	rs429358	0.81
TRIB1	rs28601761	0.89
GCKR	rs1260326	1.14
GPAM	rs2792751	1.09
COBLL1	rs13389219	0.94
PNPLA2	rs140201358	1.16
TMC4	rs641738	1.07
MTARC1	rs2642438	0.89
TOR1B	rs7029757	0.92
APOH	rs1801689	1.11
ADH1B	rs1229984	0.85

MTTP	rs11944752	0.92
GUSB	rs6955582	0.95
HFE	rs1800562	1.10
ERLIN1	rs2862954	0.93

Table/Fig-2: GWAS with NAFLD [24].

Pelusi S evaluated the role of NGS technology, particularly Whole Exome Sequencing (WES), in the diagnosis and clinical management of cryptogenic liver disease, as well as, in stratifying the risk of NAFLD progression to cirrhosis and HCC [13]. The study proposed that genetic risk scores, based on a detailed assessment of genetic risk factors using WES, could be used to stratify the risk of liver-related complications, guide HCC surveillance, and select appropriate pharmacological treatments.

It has been consistently observed that a fraction of chronic liver disease is identified at an advanced stage, and in one-third of cases, the aetiology remains unknown (cryptogenic cirrhosis). Genetic testing using the WES approach provides a valuable tool for the timely diagnosis of complicated cases in which conventional diagnostic work-ups, despite being extensive, have been inconclusive. The study revealed that genetic testing could benefit at least 30% of cryptogenic cirrhosis cases by allowing the identification of previously unidentified genetic disorders, facilitating family screening, and potentially enabling therapeutic interventions [13].

Expression of Non Coding RNA in NAFLD

Di Mauro S et al., presented novel high-throughput data on the expression of non coding RNA in the serum of NAFLD patients, laying the groundwork for the identification of novel biomarkers that could aid in the identification of NAFLD patients, distinguish between NASH and NAFL, and stage fibrosis [17]. However, these biomarkers require validation in larger and more diverse patient cohorts before their clinical implementation. The genetic markers implicated in various stages of NAFLD has been shown in [Table/Fig-3a,b] [2,13].

NAFL	NASH	Fibrosis
Patatin-like phospholipase	Many genes involved in	TM6SF2 and
Domain-containing protein 3 (PNPLA3) and Transmembrane 6 Superfamily member 2, (TM6SF2) nucleotide polymorphisms [2]	Carbohydrate metabolism, insulin signaling pathways, inflammatory pathways, oxidative stress and fibrogenesis trigger the development and progression of NAFLD/NASH- e.g., GCKR, APOB, LPIN1, UCP2, and IFNL4 [2].	MBOAT7 [2]

Table/Fig-3a: Genetic markers implicated in various stages of NAFLD [2].

NAFL	NASH	Fibrosis
miR-122, miR-192, miR-16	miR-122, miR-192, miR-16	miR-122, miR-192, miR-16
miR-21, miR-27b, miR-197, miR-34a, miR-375, miR-451, miR-1290, miR-885	miR-21, miR-27b, miR-197, miR-34s, miR-375, miR-30c, miR-22, lncRNA LexXis, lncRNA RP11-128N14.5 [13]	miR-21, miR-27b
miR-181d, miR-99a, miR-146b, miR-29, miR-1296, miR-132, miR-135, miR-19a, miR-19b, miR-125, miR-223, lncRNA ARSR [13]		miR-197, miR-30c, lncRNA APTR, lncRNA RP11-128N14.5. lncRNA TGFb2/TGFb2-OT1, lncRNA GA55 [13]

Table/Fig-3b: Deregulated non coding RNA pattern (specific miRNAs and lncRNAs) implicated in NAFLD [13].

Application of Plasma Protein in NAFLD

Another multiomics study investigating the diagnosis and monitoring of complications in NAFLD patients developed models using plasma proteins that were more effective than models trained on liver enzymes and Genetic Risk Scores (GRSs) in differentiating between

NAFL and cirrhosis. Thus, plasma protein levels may serve as a non invasive tool for diagnosing and monitoring the disease, whereas GRSs are linked with a lifetime risk of disease [25].

Thrombospondin 2 (THBS2) levels were found to be elevated in people with cirrhosis compared to individuals with NAFL and the general population. In contrast, ACY1 levels were found to be increased in persons with NAFL compared to the general population. Intrahepatic THBS2 expression is known to be involved in fibrosis in individuals with NAFLD [26]. The association of IGFBP2 and IGFBP7 with cirrhosis and NAFL is recognised to be linked with the development of NASH. Both proteins bind to Insulin-like Growth Factors (IGFs), modifying their accessibility. Since IGFs are produced in the liver, increased levels of IGFBP2 and IGFBP7 may reflect disturbances in the IGF system triggered by liver injury. While an etiological role has been suggested for IGFBP7, it may also play a role in hepatic fibrogenesis and act as a tumour suppressor in HCC [27]. Additionally, Sex Hormone Binding Globulin (SHBG) levels are found to be elevated in cirrhosis compared to NAFL, consistent with previous reports of a positive correlation with advanced fibrosis in NASH. There are conflicting epidemiological studies focusing on whether NAFLD is associated with increased or decreased levels of SHBG. In line with this, many NAFL variants are linked to SHBG plasma levels with unpredictable directions of effect, relative to their impact on hepatic fat content [28].

The present study is limited by a lack of data that could enable further detailed phenotype stratification, particularly regarding histological data for disease staging. Additionally, information related to other potential causes of liver disease, such as alcohol consumption, is restricted. However, the present study approach is consistent with the current recommendation to avoid basing the diagnosis of liver disease exclusively on the elimination of other diseases, such as Alcoholic Liver Disease (ALD). Consequently, plasma proteomics has the potential to stage NAFLD [29,30].

Proteo-transcriptomic Connections

Regarding proteo-transcriptomic connections associated with progressive NAFLD, while CFHR4 is exclusively expressed in a healthy liver, ADAMTS2, AKR1B10 and TREM2 appear to be involved in the progression of liver diseases and NAFLD. Single-cell RNA sequencing has demonstrated that TREM2-positive macrophages are related to hepatic portal fibrosis, while ADAMTS2 exhibits zonal activation of hepatic stellate cells. Soluble ADAMTS2 seems to be a good biomarker for assessing significant and advanced fibrosis in patients with NAFLD, while circulating TREM2 levels have been shown to stratify patients with NASH.

Soluble levels of TREM2 appear to be associated with the recruitment and expansion of TREM2-positive macrophages in the fibrotic areas of the liver, in response to the resolution of steatohepatitis [30]. Employing a high-throughput RNA sequencing approach in a cohort of 206 NAFLD biopsies to understand the pathogenesis of disease progression revealed that changes in the transcription of the epithelial markers AKR1B10 and GDF15 can also lead to altered circulating concentrations of these proteins, serving as putative biomarkers for fibrosing steatohepatitis [31].

Immunohistochemical staining was performed on a series of 30 NAFLD biopsies. AKR1B10 positivity was more prevalent in advanced NAFLD and was observed in ballooned hepatocytes, as well as, in hepatocytes adjacent to necroinflammatory foci and in periportal/periseptal areas [32]. However, the association between this protein and hepatic micro RNA (mRNA) was observed only in the European White cohort. This highlights the complexity of the different liver cell populations and suggests that circulating proteins associated with hepatic mRNA could be used to assess patients at risk for NASH [31].

Dysregulation of Epigenetic and Epitranscriptomic Mechanism in NAFLD

The NGS was quickly adopted by the epigenetics community. With advancements in methodology, it is now possible to profile the mammalian methylome in small numbers of cells with high coverage and single-base resolution [33].

Herranz JM et al., conducted a comprehensive analysis of the expression of epigenetic and epitranscriptomic genes in a total of 903 liver tissue samples from patients with normal livers, obesity, Non-alcoholic Fatty Liver (NAFL) and NASH, representing the stages of NAFLD progression. Major differences in their expression patterns across NAFL and NASH patients were observed relative to normal liver samples. Out of the 379 samples examined, 108 epigenetic effectors and 40 epitranscriptomic genes showed differential expression. Changes in expression evident in both NAFL and NASH stages included epigenetic genes like DNMT1, SIRT1 and ZBTB33, as well as, epitranscriptomic genes like IGFBP1. HAT1, which codes for a histone acetyl and succinyl transferase and was also induced in HCC with protumourigenic outcomes, and SMYD2, a histone methyltransferase, were associated with poor prognosis in HCCs. CBX1 and MPHOSPH8, epigenetic readers that bind methylated lysine residues, were significantly upregulated in NASH tissues. Both CBX1 and the genes coding for epitranscriptomic readers (YTHDF3 and YTHDC2) and writers (RNMT, METTL5, TRNMT10C, and PUS7L) have been linked to carcinogenesis, including the progression to HCC for CBX1. Furthermore, these epitranscriptomic genes were upregulated in NASH tissues and were associated with hepatocarcinogenesis [34].

Although NAFLD has a lower incidence of HCC compared to other chronic liver diseases, the global prevalence of NAFLD suggests that NAFLD-HCC cases are expected to increase more rapidly in the future [35]. To address the limitations of screening and surveillance and to identify early HCC, particularly in non cirrhotic patients, several biomarkers and risk scores are being proposed. The association between NAFLD and a genetic component of susceptibility indicates a genetic contribution to disease risk [36]. As mentioned earlier, the genetic polymorphisms in the PNPLA3 C>G, TM6SF2 C>T, MBOAT7 C>T and GCKR C>T genes predispose individuals to the progression of NAFLD and advanced HCC. Meanwhile, the rs72613567 HSD17B13 TA variant tends to impede hepatic fibrosis and HCC tumourigenesis, leading to the proposal of a Polygenic Risk Score (PRS) to identify the risk of HCC in NAFLD patients [37].

In addition, the evaluation of circulating tumour DNA (ctDNA), non coding RNAs (ncRNAs) and circular RNAs (circRNAs) using liquid biopsies is a promising approach, although it is not yet part of routine practice. Combining multiple biomarkers could provide more valuable and accurate information for the diagnosis of HCC compared to a single biomarker [35]. In the context of liver disease, genetic research by Whole Exome Sequencing (WES) [13] can be a useful tool for risk stratification and allows for real-time monitoring of dynamic molecular changes, enabling personalised treatment adjustments and precision medicine approaches targeted at the disease's underlying cause [13].

The integration of NGS technology into NAFLD research has opened up new possibilities for diagnosis and treatment. The present research highlights the potential of NGS to transform our understanding of NAFLD pathogenesis, leading to more precise and personalised management strategies. Challenges and opportunities in NGS for NAFLD research has been shown in [Table/Fig-3a,b]. Central to this potential is NGS's capacity to unravel the intricate genetic underpinnings of NAFLD [2]. The identification of genetic variants linked to disease susceptibility, progression and treatment response offers great potential. While studies have emphasised the importance of genes like PNPLA3 and TM6SF2, the genetic architecture of

NAFLD is complex, involving multiple genes with varying effects. GWAS and EWAS have been instrumental in identifying these loci, but their translational impact is still evolving [24].

The NGS has enabled a comprehensive exploration of the epigenomic landscape in NAFLD, going beyond genetics. The complex interplay between DNA methylation, histone modifications and non coding RNAs has been revealed as a critical determinant of disease phenotype. Studies investigating the differential expression of mRNAs and long non coding RNAs have yielded promising results, suggesting their potential as diagnostic and prognostic biomarkers [17]. However, it is necessary to further investigate the functional significance of these epigenetic alterations and their precise role in the pathogenesis of NAFLD.

The integration of multiomics approaches, encompassing genomics, transcriptomics, proteomics and metabolomics, offers the potential to provide a holistic perspective of NAFLD. By integrating this data, researchers can identify novel biomarkers, uncover complex molecular pathways, and gain insights into disease heterogeneity. Sveinbjornsson G et al., demonstrated the effectiveness of plasma proteomics in distinguishing NAFL from cirrhosis [24], but further validation in larger and more diverse cohorts is essential.

Despite significant advancements, challenges remain in translating NGS findings into clinical practice [Table/Fig-4]. The complexity of NGS data analysis, combined with the need for robust bioinformatics pipelines, poses a significant obstacle. Additionally, interpreting genetic and molecular findings in the context of individual patient variability is complex. Large-scale prospective studies are necessary to establish the clinical utility of NGS-based biomarkers and to develop effective strategies for integrating NGS into routine clinical care.

Challenges	Opportunity
Data analysis complexity	Advancements in bioinformatics tools and algorithms
Standardisation protocols of NGS	Improved comparability of studies
High cost of NGS	Decreasing costs with technological advancements
Limited clinical implementation	Development of clinical guidelines and reimbursement models

[Table/Fig-4]: Challenges and opportunities in NGS for NAFLD research.

FUTURE RESEARCH POINTS

Future research on NAFLD should focus on several key areas to advance our understanding of the disease and its clinical management. Large-scale prospective studies are needed to evaluate the clinical utility of NGS-based biomarkers in predicting disease progression and treatment response. Standardised analytical pipelines and bioinformatics tools must be developed to streamline NGS data analysis and interpretation. A deeper understanding of the interplay between genetic, epigenetic and environmental factors in NAFLD pathogenesis is essential. Longitudinal studies should investigate the dynamic changes in the NAFLD molecular landscape over time. NGS-based research can identify novel therapeutic targets, leading to the development of personalised treatment strategies. Finally, fostering collaboration among researchers, clinicians and industry will accelerate the clinical translation of NGS findings.

CONCLUSION(S)

While liver biopsy remains the gold standard for NAFLD diagnosis, its limitations have driven the search for non invasive alternatives. NGS technology offers promising potential by providing insights into the genetic, epigenetic and molecular underpinnings of the disease. Despite challenges in data analysis and clinical translation, NGS has the potential to revolutionise NAFLD diagnosis, prognosis and treatment through the identification of novel biomarkers and personalised medicine strategies.

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